Oenological Tannins : characteristics, properties and functionalities. Impact on wine quality.

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TANINS ŒNOLOGIQUES : CARACTÉRISTIQUES, PROPRIÉTÉS ET FONCTIONNALITÉS

Impact sur la qualité des vins

Sous la direction de Pierre-Louis TEISSEDRE et de Fernando ZAMORA

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A ma famille,

Pour leur présence et leur soutien,

Avec tout mon amour.

«Todos tus sueños pueden hacerse realidad si tienes el coraje de perseguirlos»

Walt Disney

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Substantial summary in French

Les composés phénoliques, ou polyphénols, sont des molécules supports des principales propriétés organoleptiques des vins. Ils sont largement présents dans le vin et se retrouvent sous forme flavonoïde dans les tanins, les anthocyanes ou les flavones. Ils peuvent néanmoins être présents sous une forme non flavonoïde, tels que les acides phénoliques et les stilbènes. Ces composés phénoliques sont extraits des parties solides du raisin (pellicules et pépins), au cours de la fermentation et de la macération. Les acides phénoliques sont constitués d'acides benzoïques, d'acides hydroxycinnamiques et d'esters d'hydroxycinnamates. Les stilbènes sont constitués de deux cycles aromatiques reliés par une double liaison. Les anthocyanes quant à elles, sont les pigments rouges responsables de la couleur des vins rouges, tandis que les flavones sont la matière colorante donnant la couleur aux vins blancs.
Le terme "tanins" désigne une substance d'origine organique que l'on trouve dans la plupart des plantes. On peut alors parler indifféremment de tanins végétaux, de polyphénols ou même de composés phénoliques. Les tanins du vin proviennent (principalement pour les cépages rouges) de la pellicule (90%), des pépins (5-8%) et de la rafle (3%), conférant aux vins leur astringence et leur amertume. De plus, par leur propriété antioxydante, ils protègent le vin au cours de son vieillissement. Enfin, leur action antiseptique peut contribuer à l'annihilation de certaines bactéries considérées comme nuisibles. Les tanins peuvent être définis comme la colonne vertébrale des vins rouges en raison de leur contribution à son succès. La biosynthèse des tanins, leurs origines botaniques, leurs concentrations et leurs compositions sont donc, d'une grande importance pour aider à la compréhension de l'astringence, de la sensation en bouche et de la couleur attribuées aux vins rouges.

Les tanins œnologiques, utilisés principalement pour la clarification et le collage des vins blancs, trouvent aujourd'hui leur intérêt en vinification. Ceux-ci sont fortement recommandés par les vinificateurs et font désormais partie intégrante de leur boîte à outils. Sur le marché, de nombreux tanins exogènes sont disponibles puisque leurs diverses origines, types, structures, compositions chimiques et propriétés offrent un large éventail de choix. La qualité finale du tanin œnologique dépendra ensuite de son origine botanique, du processus d'obtention, du type de solvant d'extraction et, enfin, du temps d'extraction. Cette qualité finale, cependant, sera conditionnée par la richesse en polyphénols totaux et plus précisément par la typologie et la concentration en principe actif (tanins). Les tanins œnologiques peuvent présenter de nombreux intérêts, bien que certains soient encore discutés, tels que la stabilisation de la couleur rouge des vins, la structure du corps du vin, l'activité anti-laccase mais également la protection des vins contre l'oxydation.

La méthode actuelle de l'OIV, qui consiste à mesurer la richesse en tanins des tanins œnologiques commerciaux, pose un réel problème puisque les résultats obtenus sont très élevés et parfois même supérieurs à 100%, ce qui semble absurde. En outre, même s'ils sont utilisés à de nombreuses fins, les tanins œnologiques ne sont autorisés jusqu'en 2016 que pour la clarification et le collage des moûts et des vins.

L'objectif principal de cette thèse est donc, de faire la lumière sur les tanins œnologiques, un nouvel outil couramment utilisé en vinification, mais présentant un manque de connaissances élevé quant à leur composition et à leurs possibles propriétés. L'objectif final étant la modification du codex œnologique international afin d'ajouter et de légaliser d'autres utilisations des tanins œnologiques.

Par conséquent, ce manuscrit propose d'une part de développer une méthode afin de déterminer rapidement la richesse tannique des tanins œnologiques, et d'autre part, le développement d'une méthode plus fine permettant la caractérisation des principaux composés qui les constituent. Une fois la caractérisation et la classification des différents tanins œnologiques réalisées, trois des principales propriétés qui leur sont supposément attribuées, seront étudiées. Ces propriétés concernent leur capacité à protéger les moûts et les vins de l'oxydation (capacité antioxydante et habilité à consommer directement l'oxygène), leur capacité à stabiliser la couleur des vins (copigmentation), et enfin leur capacité antioxidasique (protection des moûts et des vins vis-à-vis de *Botrytis cinerea*).

Ainsi, le **chapitre 3** propose une étude exhaustive (36 tanins œnologiques) afin de déterminer la richesse en tanins des tanins œnologiques, ainsi que de caractériser et quantifier les différents composés constitutifs de chacun d'entre eux. Les résultats obtenus démontrent que parmi les différentes méthodes utilisées pour caractériser les trente-six tanins œnologiques, la précipitation à la méthyl-cellulose semble être une très bonne méthode pour connaître rapidement la richesse en tanins des différents extraits. De plus, nous avons pu démontrer l'absence de différence significative, entre des tanins œnologiques de différentes origines botaniques, mais présentant une structure chimique similaire. En conséquence, il est possible de proposer une classification des tanins œnologiques en quatre grandes familles, que sont les ellagitanins (chêne et châtaignier), les gallotanins (tara et noix de galle), les procyanidines/prodelphinidines (raisin, pépins de raisin et pellicules de raisin) et les profisétinidines/prodelphinidines (quebracho et acacia). Les ellagitanins et les gallotanins appartenant à la grande famille des tanins hydrolysables, tandis que les procyanidines/prodelphinidines et les profisétinidines/prodelphinidines, appartiennent à la grande famille des tanins condensés.

Le **chapitre 4** décrit l'étude de la capacité des tanins œnologiques à protéger les moûts et les vins contre l'oxydation. Pour cela, une première partie propose d'évaluer la capacité antioxydante des tanins œnologiques à travers cinq différentes méthodes se basant sur différents mécanismes d'action

(ABTS, CUPRAC, FRAP, DPPH et ORAC). Les résultats obtenus démontrent que les tanins œnologiques possèdent une capacité antioxydante, et suggèrent de combiner ces différentes méthodes afin de mimer au mieux les différents mécanismes/réactions pouvant se produire dans les moûts et les vins. De plus, l'analyse en composante principale des données, montre que les tanins condensés peuvent être séparés des tanins hydrolysables, ces derniers présentant une capacité antioxydante plus élevée. Dans une seconde partie, une nouvelle méthode se basant sur une approche non invasive par luminescence (OCR) est proposée afin de mesurer directement l'oxygène consommé par les tanins œnologiques. Les résultats obtenus avec la nouvelle approche (OCR) permettent de distinguer à l'intérieur des tanins hydrolysables, les gallotanins des ellagitanins, ces derniers étant les plus efficaces. Cette étude nous permet donc de conclure que les ellagitanins sont les plus efficaces des différents tanins œnologiques, suivis par ordre décroissant des tanins condensés et enfin des gallotanins en termes de protection du vin contre l'oxydation chimique.

La stabilisation de la couleur des vins, au cours de leur vieillissement, présente une grande importance notamment, pour le consommateur. La capacité des tanins œnologiques à protéger la couleur des vins et à la stabiliser dans le temps a donc été étudiée dans le **chapitre 5**. Une première expérience a alors été menée avec les trente-six tanins œnologiques, soulignant l'importance de leur origine botanique puisqu'ils ne présentent pas les mêmes capacités. En effet, les gallotanins apparaissent comme les plus efficaces, suivis des ellagitanins, pour stabiliser la couleur des vins rouges. Afin de compléter cette première étude, et de suivre l'évolution du comportement des tanins œnologiques, une seconde expérience a été réalisée à différents jours (1, 7, 14 et 21 jours). Quelques modifications intéressantes ont été notées, puisque les ellagitanins, qui semblaient présenter une grande capacité à stabiliser la couleur des vins au cours de la première expérience, ont vu leur indice de copigmentation diminuer avec le temps. Une troisième expérience a ensuite été réalisée afin de déterminer l'influence des conditions intrinsèques des vins, telles que le pH et la teneur en éthanol. Les résultats montrent qu'avec l'augmentation du pH des vins et leur concentration en éthanol, l'efficacité des tanins œnologiques diminue quelle que soit leur origine botanique. Néanmoins, indépendamment des conditions, les gallotannins restent les plus efficaces. Cette étude nous permet donc de conclure, que les gallotanins apparaissent comme le candidat à utiliser en priorité, leur effet restant constant dans le temps et avec la plus grande efficacité quelles que soient les conditions œnologiques (évolution du pH et de la teneur en éthanol).

En ce qui concerne la capacité antioxidasique des tanins œnologiques, diverses expériences ont été conduites dans le **chapitre 6**, puisque le manque de connaissances est criant et que le problème reste économiquement important pour les viticulteurs.

Tout d'abord, la supplémentation en tanins œnologiques sur vins blancs, rosés et rouges botrytisés a été réalisée afin d'évaluer leur impact sur l'activité résiduelle de la laccase, la couleur et les aspects organoleptiques. La supplémentation avec tous les tanins œnologiques atténue réellement l'effet négatif sur la couleur dû à la présence de laccase. Cet effet étant d'autant plus remarquable sur les vins rosés puisqu'ils permettent de protéger les anthocyanes de l'oxydation. Sur les vins rouges, les résultats ne sont pas aussi clairs, mais s'expliquent par le fait que le taux de botrytisation est très élevé (20 et 50%). Du point de vue de la qualité olfactive/gustative, il est aussi difficile, de mettre en évidence des différences significatives entre les différents types de tanins œnologiques et le contrôle. Néanmoins, les tanins du raisin (pépins et pellicules) présentent un effet inhibiteur sur l'activité de la laccase, puisque l'activité résiduelle était plus faible avec leur présence dans le moût. En ce qui concerne les propriétés organoleptiques, les vins issus de raisins botrytisés (20% et 50%) se distinguent visuellement des témoins provenant de raisins sains (0%). En effet, ceux-ci présentent des teintes plus orangées et une couleur moins intense que le contrôle, caractéristique de l'action des laccases. Par ailleurs, afin d'étayer la discussion et la compréhension de la capacité antioxidasique des tanins œnologiques, leurs mécanismes d'action vis-à-vis des laccases ont été étudiés. Les résultats obtenus démontrent que les tanins œnologiques présentent une capacité différente à précipiter les laccases excrétées et/ou à inhiber leur activité. Tous les tanins œnologiques sont des inhibiteurs non compétitifs vis-à-vis des laccases de *Botrytis cinerea*, mais les gallotanins et les tanins de raisin sont les plus efficaces, que ce soit pour précipiter ou inhiber les laccases.

En conclusion, il sera recommandé à un vinificateur d'utiliser les ellagitanins lorsqu'il rencontre des problèmes d'oxydation dans les moûts et les vins, tandis que les gallotanins seront recommandés pour la stabilisation de la couleur. En cas de contamination par *B. cinerea*, les tanins de raisins seront conseillés en priorité, même si les gallotanins peuvent également être utilisés. Finalement, tous les résultats obtenus ont conduit à la modification des fiches 2.1.7 et 3.2.6 « Ajout de tanin » de la deuxième partie respectivement du chapitre 2 et du chapitre 3 du code international des pratiques œnologiques (**Annexe 1 et 2**).

À l'avenir, il sera également intéressant d'évaluer les différentes propriétés sur des mélanges des différents types de tanins pour voir l'effet combinatoire. En effet, une poudre commerciale constituée de tanins appartenant à différentes familles, peut éventuellement améliorer les propriétés associées spécifiquement à chacun des tanins. Par exemple, un mélange de gallotanins et d'ellagitanins peut présenter une bonne capacité de stabilisation de la couleur et de protection du vin contre l'oxydation, tandis qu'une simple poudre de gallotanins ou d'ellagitanins ne permet pas de combiner les deux fonctions.

Valorization of research work

1. Scientific publications

Gombau, J.; **Vignault, A.**; Pascual, O.; Canals, J.M.; Teissedre, P.-L.; Zamora, F. Influence of supplementation with different oenological tannins on malvidin-3-monoglucoside copigmentation. *BIO Web of Conferences* **2016**, *7*, 02033.

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Vignault, A.; González-Centeno, M.R.; Pascual, O.; Gombau, J.; Jourdes, M.; Moine, V.; Iturmendi, N.; Canals, J.M.; Zamora, F.; Teissedre, P.-L. Chemical characterization, antioxidant properties and oxygen consumption rate of 36 commercial oenological tannins in a model wine solution. *Food Chemistry* **2018**, *268*, 210–219.

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Abbreviations

a*	Red-greenness
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)
b*	Yellow-blueness
C^*_{ab}	Chroma
Cp/p	Copigment/pigment
CUPRAC	CUPric Reducing Antioxidant Capacity
DAD	Diode Array Detector
DPPH	2,2 DiPhenyl-1-PicrylHydrazyl
EC	European Commission
EIC	Extract Ion Chromatogram
ESI	ElectroSpray Ionization
ET	Ellagitannins
FRAP	Ferric Reducing Antioxidant Power
Glc	Glucose
GT	Gallotannins
h_{ab}	Hue
HPLC	High Pressure Liquide Chromatography
L*	Lightness
mDP	mean Degree of Polymerization
MS	Mass Spectrum
OCR	Oxygen Consumption Rate
OIV	Organization International of Vine and Wine
ORAC	Oxygen Radical Absorption Capacity
PC/PD	Procyanidins/Prodelphinidins
PF/PR	Profisetinidins/Prorobitenidins
QQQ	Triple quadrupole
Q-TOF	Quadrupole - Time of Flight
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SIM	Selective Ion Monitoring
SO_2	Sulfur dioxide
TPC	Total Phenolic Content
TPI	Total Polyphenol Index
UL	Units of Laccase
UPLC	Ultra High Pressure Liquide Chromatography
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
λ_{max}	Maximal absorbance wavelength
YPD	Yeast-Peptone-Dextrose
ΔE_{ab}	Colorimetric difference

General introduction and objectives

Phenolic compounds or polyphenols are some of the responsible molecules of the main organoleptic properties of wines. They are widely present in wine and are found in the flavonoid form through condensed tannins, anthocyanins or flavones [1]. They may nevertheless be present in a non-flavonoid form, such as phenol acids and stilbenes. These phenolic compounds are extracted from the solid parts of the grapes (skins and seeds), during fermentation and maceration. The phenolic acids consist of benzoic and hydroxycinnamic acids and hydroxycinnamate esters. These phenolic acids form part of the structure of hydrolysable tannins. The stilbenes consist of two aromatic rings connected by a double bond and the most important one is resveratrol. Anthocyanins are the red pigments responsible for the color of red wine, while flavones are the coloring matter of white wines [2].

The term "tannins" refers to a substance of organic origin that is found in most plants. We can then indifferently speak of vegetable tannins, polyphenols or even phenolic compounds. The wine's tannins come (mainly for red wines) from the skins (90%), seeds (5-8%) and grapes stems (3%). Tannins provide wines astringency, and due to their antioxidant property, they protect the wine during aging. Finally, their antiseptic action can contribute to the annihilation of certain bacteria considered harmful. Tannins can be defined as the “spinal column” of red wines, thanks to their relevance in the organoleptic properties of wine. The biosynthesis of tannins, their botanical origins, their concentrations and their compositions are highly important, to better understand the astringency, the mouthfeel and the color of red wines [3].

The oenological tannins are mainly used for finning white wines, but nowadays they are gaining interest in red winemaking. In fact, they are highly recommended by winemakers and are now an integral part of their toolbox. On the market, many exogenous tannins are available for the winemaker. Indeed, the various origins, types, structures, chemical compositions of oenological tannins offer a wide range of choices and properties. The final quality of the oenological tannins depends of the botanical origin, the obtaining process, the type of solvent extraction and finally, the extraction time. In addition, tannin quality will be conditioned by the richness in total polyphenols and more specifically, by the typology and concentration of active ingredient (tannins). Oenological tannins can present many interests, although some are still discussed, such as the stabilization of red wine color, the contribution to the structure of wine body, the anti-laccase activity and the protection of wines against oxidation [4]. The current method of the OIV (International Organization of Vine and Wine) to measure the tannin richness of commercial oenological tannins, presents incoherencies. Indeed, the results obtained measure all polyphenols and not specifically tannins. In addition, even they are used for many purposes in the wine industry, until 2019, oenological tannins, are only authorized to the clarification and finning of musts and wines. Therefore, it is necessary to develop a method that will then really determine the tannic richness of the oenological tannins diversity, in function of their origins and compositions.

Furthermore, it is indispensable to study oenological tannins potential properties and functionalities and to add them in the OIV International code of oenological practices with update resolutions to use them with legal and appropriate norms. Although oenological tannins are commonly employed to other purpose, there is lack of knowledge about their chemical composition, characterization and their real properties.

Actually, given the wide range of commercial tannins present in the market, the main goal of our work was to elucidate their composition according to the botanical origins as well as their potential properties and functionalities. The final objective being the modification of the International code of oenological practices as well as the International oenological codex in order to add and legalize new functionalities of the use of oenological tannins.

- In this way, **Chapter 3** will carry out an exhaustive study (36 oenological tannins) to characterize the oenological tannins and quantified the different compounds founds in each one. Additionally, to this, different rapid methods allowing to determine the richness of oenological tannins in "tannins" will be achieved and compared to the one proposed by the OIV.
- Concerning the different properties possibly conferred to the oenological tannins, three of them present higher interest for the manufacturers and winemakers. Indeed, oenological tannins are principally used in order to protect wines against oxidation. According to this, **Chapter 4**, will discussed the possible antioxidant capacity attributed to them as well as to evaluate their ability to directly consume oxygen.
- Another property of great interest for winemakers is the color stabilization of rosé and red wines. For these reason, **Chapter 5**, will explore and compare the effectiveness of different oenological tannins regarding color stabilization for different pH and ethanol content, as well as time function. The quantification of the malvidin-3-*O*-glucoside and its possible degradation products will be also achieved.
- In our days, infections of grapes by *B. cinerea* still cause huge economic damages each year. Thus, winemakers are looking for a tool healthier and eco-friendlier than sulfur dioxide to prevent the damages caused in wine. The aim of **Chapter 6**, will be to determine the ability of enological tannins to reduce the laccase activity produced by *B. cinerea* and, consequently, to protect the wine color against enzymatic browning and oxidasic haze. In addition, the understanding of oenological tannins action-mechanisms against laccases, will be also achieved.

Chapter 1: Bibliographical research

1. Introduction

Tannins (or tannoids) are a class of astringent, polyphenolic biomolecules that precipitate and bind to proteins and various other organic compounds including amino acids and alkaloids. The term tannin (from Anglo-Norman tanner, from Medieval Latin tannāre, from tannum, oak bark) refers to the use of oak and other bark in tanning animal hides into leather. By extension, the term tannin is widely applied to any large polyphenolic compound containing enough hydroxyls and other suitable groups (such as carboxyl) to form strong complexes with various macromolecules. The tannin compounds are widely distributed in many species of plants and have molecular weights ranging from 500 to over 3,000 (gallic acid esters) and up to 20,000 Da (proanthocyanidins) [5].

Tannin production starts at the beginning of the 19th century with the industrial revolution, to produce tanning material for leather. Before that time, processes used plant material and were long (up to six months). There was a collapse in the vegetable tannin market in the 1950s–1960s, due to the appearance of synthetic tannins, which were invented in response to a scarcity of vegetable tannins during World War II. Indeed, ellagic acid, gallic acid, and pyrogallic acid were first discovered by Henri Braconnot chemist, in 1831 [6]. After that, Julius Löwe was the first one to synthesize ellagic acid by heating gallic acid with arsenic acid or silver oxide [6]. Maximilian Nierenstein studied natural phenols and tannins from different plant species [7]. The discovery in 1943 by Martin and Synge of paper chromatography provided for the first time the means of surveying the phenolic constituents of plants and for their separation and identification. There was an explosion of activity in this field after 1945, including prominent work of Edgar Charles Bate-Smith and Tony Swain at Cambridge University [8]. In 1966, Edwin Haslam proposed a first comprehensive definition of plant polyphenols based on the earlier proposals of Bate-Smith, Swain and Theodore White, which includes specific structural characteristics common to all phenolic having a tanning property. It is referred to as the White–Bate-Smith–Swain–Haslam (WBSSH) definition [9].

This bibliographic study chapter presents the main phenolic compounds of grapevines and wines focusing on oenological tannins. Their origins, their structures, their properties, the manufacturing process and the current regulation applied to them will be discussed. The various properties associated to them as their interaction with proteins or their bacteriostatic effect will be presented as well as their impact on oxygen/metal. The implication of oenological tannins on color stabilization during the aging of red wine will also be discussed to better understand the evolution of the color. Finally, their properties both, mouthfeel and sensory will be also presented to better understand wines taste properties.

2. Phenolic compounds of grapevine and wine

Grapevine is a rich source of polyphenols, a class of molecules characterized by the presence of one or more aromatic rings. This family of secondary metabolites is the most common among plants with more than 8000 structures knowing today [10]. This diversity makes their classification difficult, explaining the different interpretation encountered in scientific publications. Some authors [11] classify polyphenols in 9 families based on the carbon skeleton of molecules (C6-C1, C6-C2, C6-C3, C6-C4, C6-C1-C6, C6-C2-C6, C6-C3-C6) (**Figure 1**) while others [12] use only 4 families (hydroxybenzoic acids, hydroxycinnamic acids, stilbenes and flavonoids).

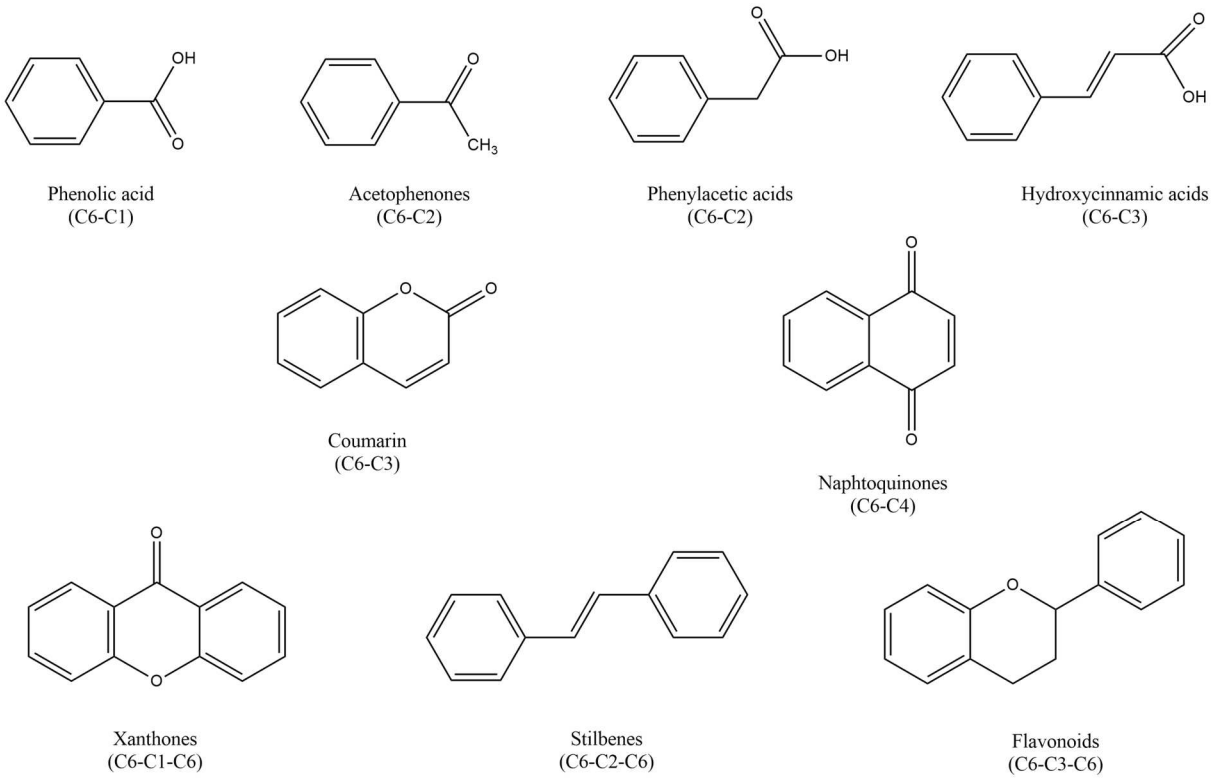


Figure 1: Classification of polyphenols according to their carbon skeleton

Polyphenols participate in red wines quality thanks to their contributions to wine color [13,14], sensory characteristics such as bitterness and astringency [15,16], oxidation reactions [17], interactions with proteins [18–20] and the qualitative evolution of wines during aging [21,22]. In addition to these properties, phenolic compounds also have beneficial effects on human health [23]. These compounds are extracted from the grapes during winemaking and they undergo an evolution during wines aging [24].

2.1. Classification and structures

In grapes and wines, polyphenols are generally classified only in two families which are flavonoids and non-flavonoids. For flavonoids, this family are further subdivided in two sub-families, tannins and anthocyanins that present a major importance in the wine and will be more detailed afterward.

2.1.1. Non-flavonoids compounds

Non-flavonoids compounds include, hydrolysable tannins, phenolic acids (mainly benzoic acids and hydroxycinnamic acid derivates) and stilbenes. The phenolic acids are present in red wines at a concentration of 100 to 200 mg/L meanwhile stilbenes are present at a concentration of 20 to 80 mg/L. In white wines phenolic acids are present at a concentration of 10 to 20 mg/L meanwhile stilbenes are present at a concentration of 0.5 to 1 mg/L [1].

2.1.1.1. Phenolic acids

Phenolic acids are represented by two major classes, benzoic acids of type C6-C1 and cinnamic acids of type C6-C3 presented in **Figure 2**.

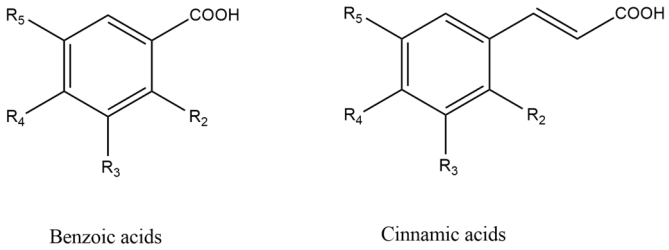
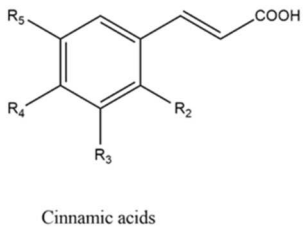


Figure 2: Structures of main phenolic acids

These come mainly from the grape pulp and come from the partial degradation of anthocyanins or the degradation of sugars. They can combine with esters, glycosides or form volatile phenols in case of microbial derivates [25]. Phenolic acids have biological and chemical properties and they contribute to color development and wines stabilization. At doses, where they are naturally present (100-200 mg/L), they have an impact on alcoholic fermentation and bacterial development, particularly during the malolactic fermentation.

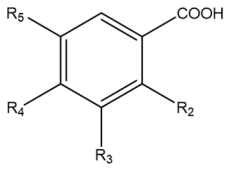
More specifically, hydroxycinnamic acids (**Figure 3**) are mainly found in the esterified form with tartaric acid but also with organic acids, sugars, polyamines and polysaccharides [26]. They participate in the formation of acylated anthocyanins by esterification of acetic, *p*-coumaric and caffeic acids on anthocyanins sugar. Among the cinnamic acids; some (*p*-coumaric acid and ferulic acid) can act as volatile precursors by leading ethyl phenol compounds (4-ethylphenol and 4-ethylgaïacol) with unpleasant odors (stable, animals, leather...) [27]. These also play a role in the browning of white must due to their oxidation [28], particularly involving caftaric acid [1].



	R_2	R_3	R_4	R_5
***p*-coumaric acid**	H	H	OH	H
Caffeic acid	H	OH	OH	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3

Figure 3: Structures of main cinnamic acids

Benzoic acids, are present mainly in grapes in the form of esterified glycosides **(Figure 4)**. In wine, they can be found in free form, which results from the hydrolysis of ester bonds or the degradation of anthocyanins [29].



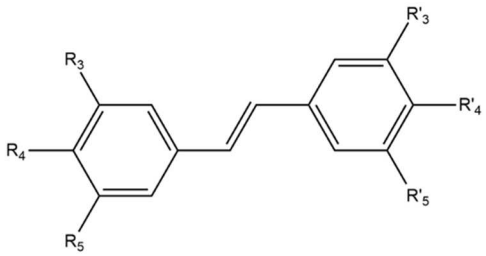
Benzoic acids

Name	R_2	R_3	R_4	R_5
p-hydroxybenzoic acid	H	H	OH	H
Protocatechic acid	H	OH	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Syringic acid	H	OCH_3	OH	OCH_3
Salicylic acid	OH	H	H	H
Gentic acid	OH	H	H	OH

Figure 4: Structures of main benzoic acids

2.1.1.2. Stilbenes

Stilbenes (**Figure 5**) are molecules produced by plants in response to bacterial, fungal, viral attacks and UV. This family groups many compounds according to the position and the number of hydroxyl groups, the steric conformation, the substitution by methoxy residues or sugars on a C6-C2-C6 skeleton. They consist of two aromatic rings connected by a double bond, which gives them a great reactivity.



Name	R_3	R_4	R_5	R_3'	R_4'	R_5'
Trans-resveratrol	OH	H	OH	H	OH	H
Piceid	OGlc	H	OH	H	OH	H
Piceatannol	OH	H	OH	OH	OH	H
Trans-pterostilbene	OCH_3	H	OCH_3	H	OH	H
Rhapontigenin	OH	H	OH	H	OCH_3	OH
Isorhapontigenin	OH	H	OH	OCH_3	OH	H
Trans-piceid	Glucoside	H	OH	H	OH	H
Astringin	Glucoside	H	OH	OH	OH	H

Figure 5: Structures of main stilbenes

In addition, there is also stilbenes in oligomeric forms (trimers to pentamers), with anti-oxidant properties regarding low density proteins (LDL). Stilbenes are present in grapes, wine and oak. In the grape, these molecules can polymerize to give pallidol, viniferins or hopeaphenol (resveratrol tetramer) [30]. Among the stilbenes, trans-resveratrol is a key compound whose healthy properties are widely studied [31–33].

2.1.2. Flavonoids compounds

Flavonoids, including flavones, flavanols, flavanonols, flavan-3-ols and anthocyanins are characterized by a C6-C3-C6 type structure, with two aromatic rings linked by three carbons (**Figure 6**).

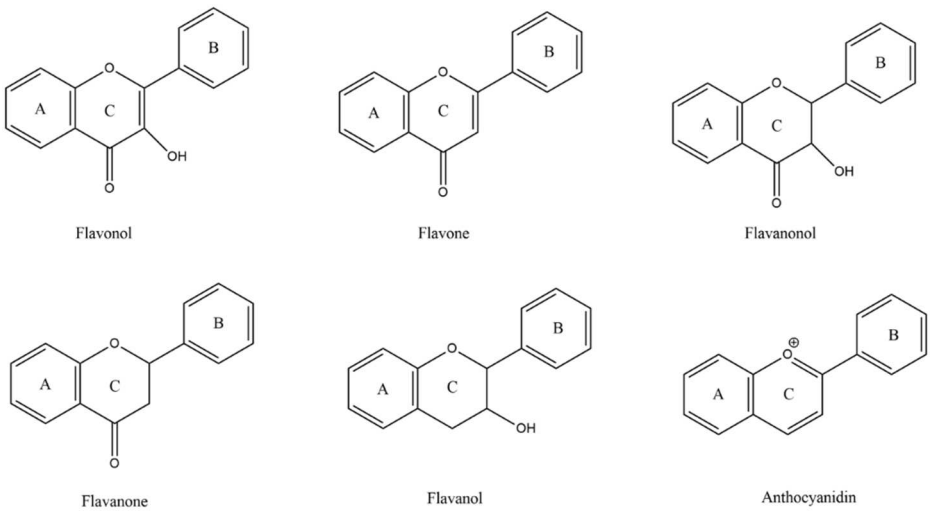


Figure 6: Structure of principal sub-family's compounds appertaining to flavonoids

The three cycles constituting this type of molecules are called phloroglucinol cycle for the ring A, catechol or pyrogalloyl ring, if respectively a hydrogen atom or a hydroxyl group is in position 5', for the ring B and pyranic cycle for the ring C. The classification of these families is organized around the degree of oxidation of the pyranic C cycle and the substitution of the C3 and C4 carbons of this heterocycle.

These secondary metabolites have a number of important roles in the plant kingdom that range from pigmentation to plant defenses against ultraviolet radiation (solar radiation with a wavelength between 10 and 400 nm) and pathogen attacks [34–36]. In this way, flavanols, flavones, flavanonols and flavanones are more or less intense yellow pigments in grapes, while anthocyanidin in glycoside form (anthocyanins) are the red/blue pigments of grapes. The flavan-3-ols family is constitutive of the condensed tannins of grapes.

The tannin and anthocyanin families are discussed in detail in Part 2.1.3 and 2.1.4 respectively of this chapter. In grapes and wine, they are mainly flavones, flavanols, flavan-3-ol and anthocyanins.

2.1.3. Tannins

The term "tannin" include a large number of molecules that by definition are capable of forming stable bonds with proteins [37], hence their ability to cause astringency [38], polysaccharides [39,40] and alkaloids.

Tannins are generally classifieds in two families, which are the hydrolysables and non-hydrolysables tannins since 1920 according to Freudenberg. More recently, authors defined the non-hydrolysables tannins as condensed tannins or proanthocyanidins [41].

Additionally, to this, some authors described separately tannins in four different groups instead of two, including condensed tannins, hydrolysable tannins, phlorotannins and complex tannins [42] (**Figure 7**).

Group	Monomer
Proanthocyanidins (1)	(+)-catechin R_1 = OH, R_2 = H
	(+)-afzelechin R_1 = H, R_2 = H
	(-)-gallocatechin R_1 = OH, R_2 = OH
Proanthocyanidins (2)	(-)-epicatechin R_1 = OH, R_2 = H
	(-)-epiafzelechin R_1 = H, R_2 = H
	(+)-epigallocatechin R_1 = OH, R_2 = OH
Hydrolysable tannins	Gallic acid
	Hexahydroxidiphenic acid
	Ellagic acid (formed after hydrolysis)
Phlorotannins	Phloroglucinol
Complex tannins	Acutissimin A and B

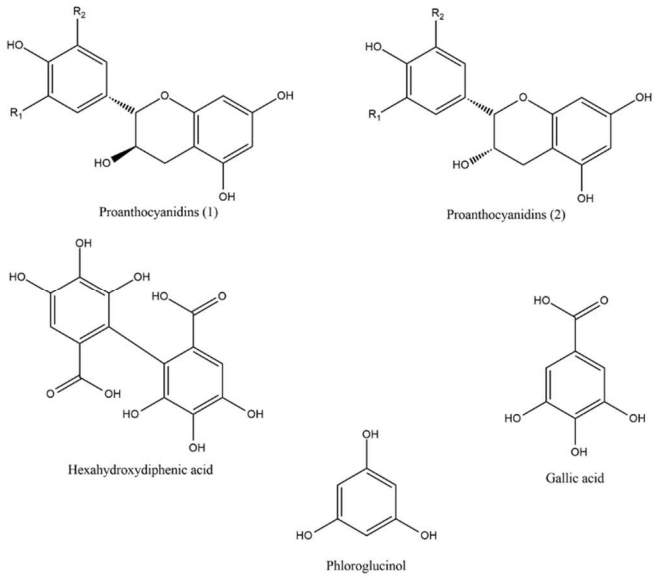


Figure 7: Tannins classification

Nonetheless, tannins can also be classified in three groups, according to the reactivity with the oral mucosa, which are hard, young and soft tannins [37].

2.1.3.1. Phlorotannins

Phlorotannins (**Figure 8**) are oligomers or polymers of units derived from phloroglucinol. They can be differentiated according on the basis of the nature of the intermonomeric and substitution profiles of the derived from phloroglucinol [43]. Phlorotannins are found exclusively in brown macroalgae.

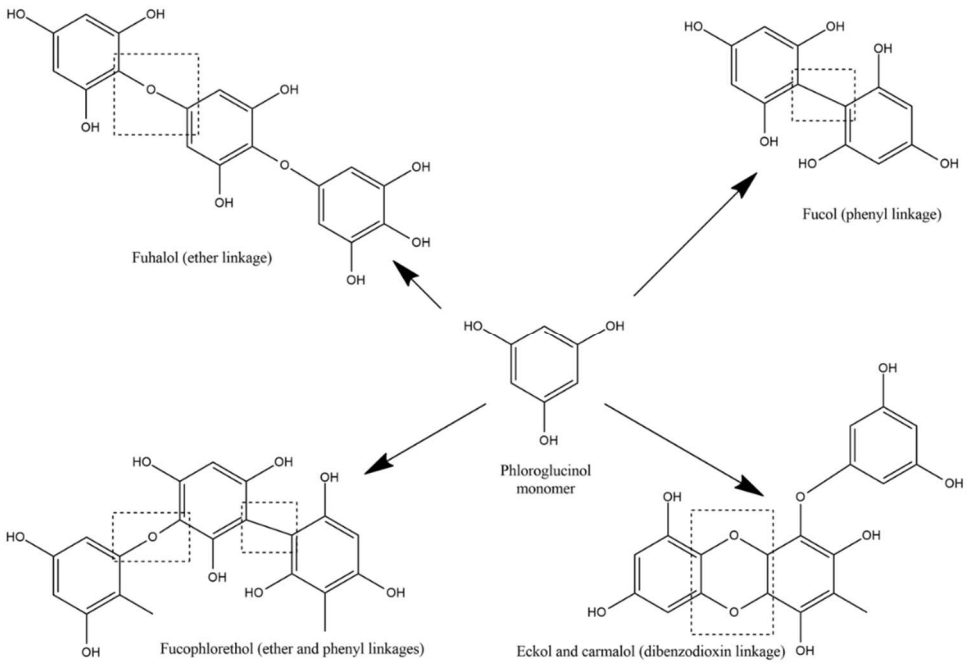


Figure 8: Structure of principal phlorotannins

The different units of phloroglucinol could only be linked by carbon-carbon, that is, phenyl linkage as in fucols, by a diaryl ether linkage as in fuhalols and phlorethols, by both phenyl and ether linkages as in fucophlorethols, and finally, a dibenzodioxin linkage can associate two phloroglucinol units as it is observed for eckols and carmalols [44].

2.1.3.2. Complex tannins

The complex tannins are defined as complex structures which contain structural elements of different tannin groups and other macromolecules. Indeed, they are hydrolysable tannins (gallotanins or ellagitannins) connected to a flavonoid unit through a C-glycosidic bond. Complex tannins can be formed during wine aging in barrels when the flavonoids come into contact with the released ellagitannins from the wood. The two best known complex tannin in wine, until now, are the acutissimin A and B (**Figure 9**) [45].

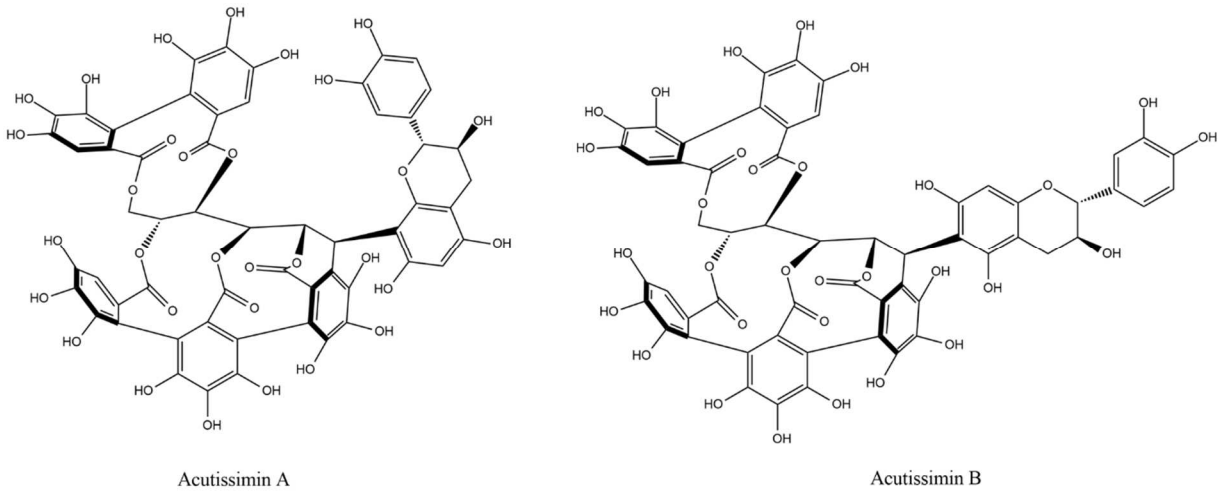


Figure 9: Structures of the two principal complexes tannins (Acutissimin A and B)

2.1.3.3. Condensed tannins

Condensed tannins also called proanthocyanidins, are oligomers or polymers of polyhydroxyflavan. The monomeric flavanols differ in their hydroxylation pattern in ring A and B and in the stereochemistry of C3. The most common monomers are the diastereomers (+)-catechin/(-)-epicatechin, (-) gallocatechin/(-)-epigallocatechin and (+)-afzelechin/(-)-epiafzelechin [42]. Their respective oligomers and polymers are called procyanidins, prodelphinidins, propelargonidins, profisetinidins, prorobitenidins, depending of the anthocyanidin that release after acidic hydrolysis [46].

The flavanol monomers are usually linked by two types of inter-flavonoid bonds: type A and B bonds. B-type proanthocyanidins linkages are between the C4 carbon of the higher unit and the C8 or C6 carbons of the lower unit (C4 $\rightarrow$ C6 or C4 $\rightarrow$ C8).

A-type proanthocyanidins have an ether linkage in addition to type B, which can be between the C2 carbon of the C ring of the upper unit and the hydroxyl function carried by the carbon C7 or C5 of the ring A of the lower unit (**Figure 10**) [42].

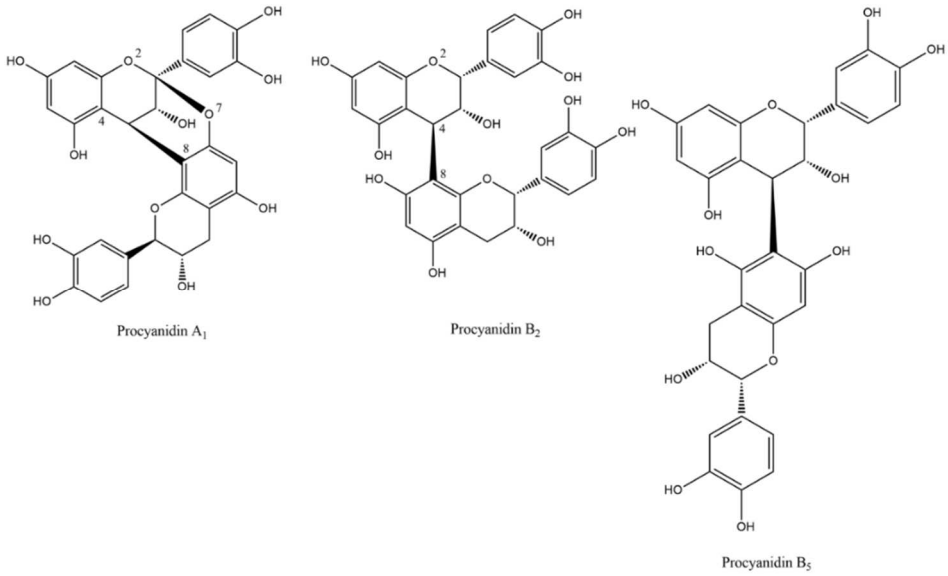
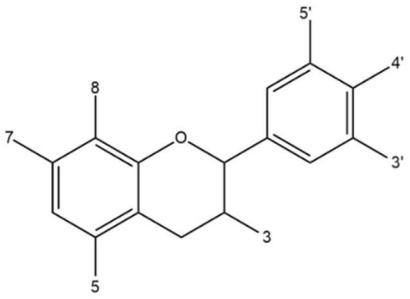


Figure 10: Structures of simple dimeric procyanidins with the different linkage type present in condensed tannins

The degree of polymerization varies over a broad range from dimers up to about 200 monomeric flavanol units [47]. B-type proanthocyanidins can be classified according to the hydroxylation pattern(s) of the chain-extender unit(s) as: procyanidins, prodelphinidins, propelargonidins, profisetinidins, prorobitenidins, proteracacinidins, promelacacinidins, procassinidins and probutinidins (**Figure 11**) [48]. In certain positions proanthocyanidins may sometimes be esterified with gallic acid or exceptionally with sugars.



Procyanidins (3,5,7,3',4'-pentahydroxylation)

Prodelphinidins (3,5,7,3',4',5'-hexahydroxylation)

Propelargonidins (3,5,7,4'-tetrahydroxylation)

Profisetinidins (3,7,3',4'-tetrahydroxylation)

Prorobinetinidins (3,7,3',4',5'-pentahydroxylation)

Proteracacinidins (3,7,8,4'-tetrahydroxylation)

Promelacacinidins (3,7,8,3',4'-pentahydroxylation)

Procassinidins (7,4'-dihydroxylation)

Probutinidins (7,3',4'-trihydroxylation)

Figure 11: Proanthocyanidins classification in relation to the hydroxylation pattern

Condensed tannins are non-hydrolysable compounds but when they suffered heat-treatment in acidic medium they can be depolymerized. This chemical depolymerization in an acid medium followed by an auto-oxidation generates anthocyanidins, hence the fact that they are also called "proanthocyanidins" [49]. They are usually named regarding the anthocyanidin that they released and the proanthocyanidin quantification method developed by Bate-Smith is based on this property [50].

In grapevines, condensed tannins are located mainly in the solid part as stems, seeds and skins although traces have sometimes been reported in the pulp [51]. In skins, tannins are mainly found in the hypodermic stratums of the inner cells [52,53]. They are in free form in the vacuoles which increase in size as one approaches the surface of the skin. Tannins can also be linked to polysaccharides of the cell walls or linked with the constitutive proteins of the internal surface of the vacuolar membranes [52,54]. In the seed, tannins are found in larger amounts at the outer and inner shells located under the epidermal cells and the cuticle. [54,55].

Skin-tannins differ significantly from seed-tannins regarding the mean degree of polymerization (mDP), the concentration in epigallocatechin subunits and in epicatechin-3-*O*-gallate. Firstly, the mDP of the skin-tannin is higher than the one of seed-tannin, with values ranging from 4.3 to 35.4 and from 2.0 to 8.8 units respectively [3]. In addition, these mDPs varied greatly during grape berry ripening and can even be divided by three between veraison and maturity [56,57]. Secondly, the tannins containing epigallocatechin subunits are only present in the skin [58]. Thirdly, seed-tannins generally have a greater proportion of epicatechin-3-*O*-gallate subunits, since this compound is generally in a very low proportion in skin-tannins [59].

More recently, apart from the B-type and A-type proanthocyanidins that have been widely shown in the literature, a new subclass of condensed tannins named “crowns proanthocyanidins” has been reported for the first time in the wine and in the vegetable kingdom [60,61]. These crown proanthocyanidins have the structural particularity of being cyclic and exclusively composed of B-type inter-flavonoid bonds. In addition, they have within their structures a relatively large cavity composed of four aromatic rings and several phenol functions. Crowned condensed tannins show specific properties since they are more polar and present higher stability during aging than B-type condensed tannins. In addition, they are partially resistant to phloroglucinolisis conditions, even if they contain only B-type inter-flavonoid bonds. A first test of crown tetramer biological activity shows an inhibitory activity of the aggregation of the β-amyloid peptide involved in Alzheimer's disease of interest [60]. Moreover, it has been shown that their concentrations are higher in Syrah wines than in Cabernet Sauvignon and Merlot wines. A strong correlation between the concentration of crown tannins and the intensity of astringency felt by the tasters has also been shown[62].

Other exogenous factors more or less responsible of the activation of tannin biosynthesis, such as the water status of the vine during flowering, the temperatures and the number of sunshine hours during maturation, will also play a major role [63,64].

Finally, condensed tannins are extracted during alcoholic fermentation and post-fermentation maceration to levels between 1 and 4 g/L in red wines and 100 to 300 mg/L in white wines [65]. This content varied according grape variety and the extraction methods [66], but also evolve during grape maturation, winemaking and aging process [67].

2.1.3.4. Hydrolysable tannins

Unlike grape tannins which are named condensed tannins, wood tannins are called hydrolysable tannins. This term refers to their ability to be hydrolyzed and to release either gallic acid or ellagic acid for gallotannins and ellagitannins respectively [68–70]. Today, more than 500 polyphenolic compounds derived from gallic acid or ellagic acid (**Figure 12**) have been isolated from various plants and well characterized [71,72].

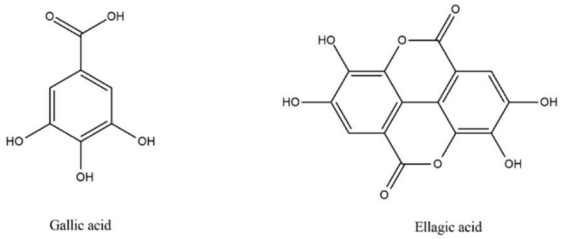


Figure 12: Monomers precursors of gallotannin (gallic acid) and ellagitannin (ellagic acid)

Hydrolysable tannins are synthesized in two major stages (**Figure 13**). First, a D-glucopyranose undergoes enzymatic galloylation with free gallic acid, which results in the formation of 1-*O*-galloyl-β-D-glucopyranose. Then, by successive enzymatic transgalloylation resulted in the successive formation of 1,6-*O*-digalloyl-β-D-glucopyranose, 1,2,6-*O*-trigalloyl-β-D-glucopyranose, 1,2,3,6-*O*-tetragalloyl-β-D-glucopyranose and finally 1,2,3,4,6-*O*-pentagalloyl-β-D-glucopyranose. This latter molecule is considered to be the precursor of gallotannins and ellagitannins [68,73]. Thanks to a galloyltransferase which esterifies one of the phenols of the galloyl groups by free gallic acid, the precursor of the gallotannins is obtained. On the other hand, by dehydrogenation (oxidative) and coupling between two adjacent galloyl units, 1,2,3,4,6-*O*-pentagalloyl-β-D-glucopyranose leads to the formation of tellimagrandin II, the precursor of ellagitannins.

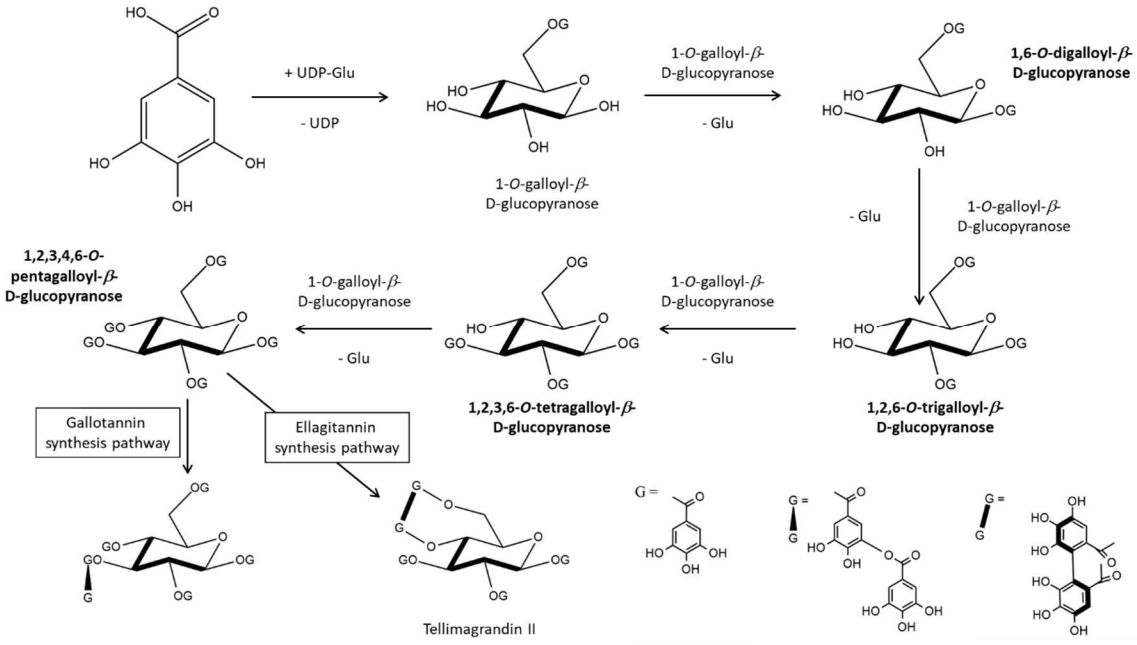


Figure 13: Synthesis pathway of gallotannin and ellagitannin precursors

Gallotannins are mainly composed of polygalloyl esters of quinic acid. Complete hydrolysis involving rupture of depside or ester bonds yields quinic and gallic acids [74]. More specifically, gallotannins consist of a central polyol, most often glucose, which is surrounded by several gallic acid units, and further gallic acid units can be attached through a depside bond. When the glucose core is esterified with five or fewer galloyl groups, the resulting compounds are defined as gallotannin precursors, which can also be considered as a part of gallotannins in a liberal sense [75,76] (**Figure 14**).

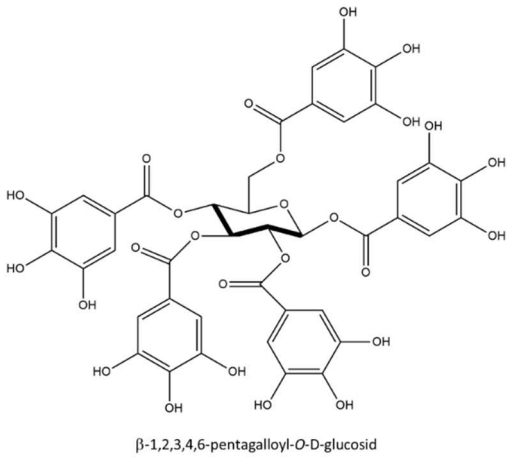


Figure 14: Structure of a gallotannin

Two different types of gallotannins are mostly found which are the tannins from nut gall and from tara tree. The nut gall is a nut-shaped gall caused by gall wasps on the oak and other trees. Galls from *Quercus infectoria* contain the highest naturally occurring level of tannin, with approximatively 50-70% of the total raw material [77]. *Quercus infectoria* (Cyprus oak) is a tree with a wide top that can grow to heights of 12 m and a diameter of 80 cm. It is found most predominantly in Turkey in the Marmara and the Black Sea regions [78]. *Quercus rubra* specie which is an important species in many forested ecosystems of eastern North America contain similar quantity of gallotannins than ellagitannins [79]. *Galla Chinensis* is also well known for having abundant gallotannins, which may represent up to 70% (w/w) of this plant material. *Galla Chinensis* is a traditional Chinese herb originating from the abnormal growth of the Rhus leaf tissue in response to secretion of parasitic aphids (family Pemphigidae) [80]. Additionally, to this, in Cæsalpinia spinosa (Molina) Kuntze, commonly known as tara, gallotannins are also present in major quantities. Tara is a small leguminous tree or thorny shrub from South America consisting of red or pale-yellow pods of 8-10 cm length. It is spread from the region of Venezuela, Colombia, Ecuador, Peru, Bolivia, until the north of Chile. Tara wildly grows in the Peruvian coast and Andean region at altitudes from 1000 to 2900 m above sea level [81].

Ellagitannins have a specific structure consisting of an open glucose chain esterified in position 4 and 6 by a hexahydroxydiphenoyl (HHDP) unit and a nonahydroxyterphenyl (NHTP) unit esterified in positions 2, 3, and 5 with a *C*-glycosidic bond between the carbon glucose and position 2 of the trihydroxyphenyl unit [82].

The vescalagin and its epimer, castalagin, are the first *C*-glycosidic ellagitannins to have been isolated and characterized 30 years ago, from *Castanea* (chestnut) and *Quercus* (oak) [83–85]. Six other ellagitannins were subsequently isolated, which are the grandinin and the roburin A, B, C, D and E. Grandinin and roburin E correspond to the monomers glycosylated by lyxose and xylose respectively. Roburin A and D correspond to the non-glycosylated dimers meanwhile roburin B and C correspond to the glycosylated dimers [86–88] (**Figure 15**).

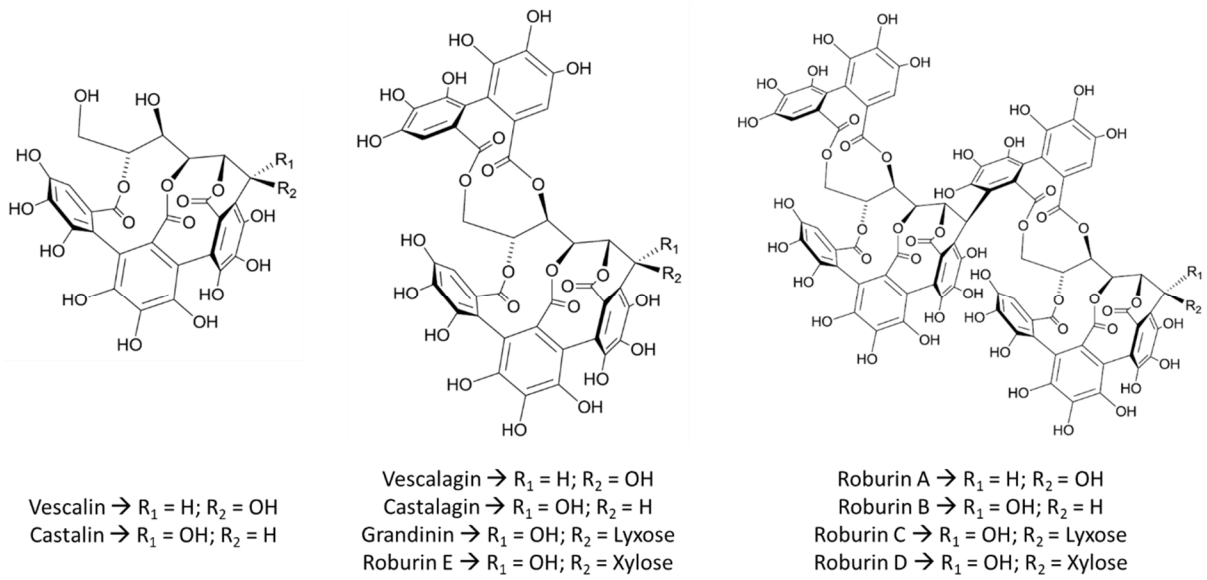


Figure 15: Structures of the eight principal ellagitannins of wood

Ellagitannins account for a large proportion of extractable compounds from the heart of wood, since they can be found at concentrations of between 4 or even more than 120 mg/g of dry wood [86]. However, the quantity and composition of ellagitannins depends on many factors such as the species, age or geographical origin. It should be take into account also the silvicultural practices, position of the piece of wood and the height in the trunk [89–91]. Additionally, the process of the barrel fabrication is also important (type, duration and power of heating) [89,92].

In oenology, three different species of oak are mostly used, which are *Quercus robur* (or pedunculate oak), *Quercus petraea* (or sessile oak) and *Quercus alba* (North American white oak) (**Figure 16**).

Figure 16: Different species of oak used mostly used in oenology (Quercus robur, petraea and alba)

Quercus robur area occupies all Europe since the Iberian Peninsula to the Urals. In France, it is found in the plains of the South-West and Center, on the edge of the Massif Central and in the plains and valleys of the North and East. This species is rich in phenolic compounds and ellagitannins but poor in *cis* and *trans*-whiskey lactones (coconut aroma marker). *Quercus petraea* implantation spreads from the extreme north of the Iberian Peninsula to the southern part of Scandinavia. In France it is present in the northern half of Massif Central and the Central Pyrenees. This oak species has a moderate content of ellagitannins, lactones and eugenol (with the aroma of "clove"). *Quercus alba* species is found in eastern USA, Southern Quebec, in the forests of Minnesota, Missouri and Appalachians. The presence of numerous thylles and its low content of ellagitannins slow down the transfer of oxygen leading oxygenation phenomena happening in cooperage. This essence is very rich in scopoletin and ferulic acid responsible for balsamic notes in wine, but also in whiskey-lactones. The use of American white oak is widespread in the United States. In Europe it is mainly used for the spirits breeding although is widely used in Spain for red wines. Ellagitannins are generally present in concentrations between 20 and 80 mg/g of wood but there is variability according to the species as mentioned above. In this way, pedunculate oak is generally richer in ellagitannins than sessile oak but a clear distinction between the two species cannot be established [86,89,92]. Indeed, the inter-individual variability is greater than the inter-species variability between *Quercus robur* and *Quercus petraea*. Indeed, for a single wine aged in the same batch of barrels, a great organoleptic heterogeneity due to the barrel is observed.

During wine aging in barrels, the contact between the wood and the wine is characterized by the dissolution of the main extractable constitutive of the wood and by the extraction of ellagitannins. Then, the concentration of ellagic acid increase during time and may be correlated with the hydrolysis of ellagitannins which, by spontaneous cleavage and reorganization of the 4,6-hexahydroxydiphenoyl group, releases an ellagic acid and vescalin or castalin in the case of vescalagin and castalagin respectively. One of the methods of determination of ellagitannins in fact, consists in causing this hydrolysis by placing them in acidic and hot medium [93].

2.1.4. Anthocyanins

Anthocyanins (from the Greek anthos = flower and kianos = blue) are the pigments that give to plants their color from pink to purple. They are responsible for the color of the grapevine and the wine.

2.1.4.1. Structures, location and content of anthocyanins

The anthocyanin molecule contains a flavylium nucleus with positively charged oxygen. Given the existence of conjugated double bonds, the charge is in fact delocalized over the entire cycle which is stabilized by resonance [2].

Anthocyanins are differentiated on the one hand by their degree of hydroxylation and methylation, and on the other hand by the nature of the oses bound to the molecule. Anthocyanidin is the chromophore moiety of the pigment. In heterosidic form (anthocyanins), these molecules are much more stable than in their aglycone form (anthocyanidins). The berries from *Vitis vinifera* contains the 15 anthocyanins which are found in the wine; cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, paeonidine-3-*O*-glucoside, petunidin-3-*O*-glucoside, and malvidin-3-*O*-glucoside, which is the predominant one [2,94] as well as their derivatives esterified on the glucoside unit at position 3 with acetic acid (cyanidin-3-*O*-acetylglucoside, delphinidin-3-*O*-acetylglucoside, paeonidine-3-*O*-acetylglucoside, petunidine-3-*O*-acetylglucoside, and malvidin-3-*O*-acetylglucoside) [94,95] or p-coumaric acid (cyanidin-3-*O*-(*p*-coumaroyl)glucoside, delphinidin-3-*O*-(*p*-coumaroyl)glucoside, paeonidine-3-*O*-(*p*-coumaroyl)glucoside, petunidin-3-*O*-(*p*-coumaroyl) glucoside, and malvidin-3-*O*-(*p*-coumaroyl glucoside) [94] (**Figure 17**). The presence of anthocyanins with glucoside units at the position 3 and 5 has been demonstrated for red wines obtained from hybrid grapes from different species of *Vitis vinifera* [96].

Groups	Compounds
Anthocyanidins	Cyanidin (R_1 = OH; R_2 = H)
	Delphinidin (R_1 = OH; R_2 = OH)
	Peonidin (R_1 = OCH_3; R_2 = H)
	Petunidin (R_1 = OCH_3; R_2 = OH)
	Malvidin (R_1 = OCH_3; R_2 = OCH_3)
Monoglucoside anthocyanins (R_3 = Glucose; R_4 = H)	Cyanidin-3-*O*-glucoside
	Delphinidin-3-*O*-glucoside
	Peonidin-3-*O*-glucoside
	Petunidin-3-*O*-glucoside
	Malvidin-3-*O*-glucoside
Acylated anthocyanins (R_3 = Glucose; R_4 = acetate)	Cyanidin-3-*O*-acetylglucoside
	Delphinidin-3-*O*-acetylglucoside
	Peonidin-3-*O*-acetylglucoside
	Petunidin-3-*O*-acetylglucoside
	Malvidin-3-*O*-acetylglucoside
Coumaroylated anthocyanins (R_3 = Glucose; R_4 = coumarate)	Cyanidin-3-*O*-(*p*-coumaroyl)glucoside
	Delphinidin-3-*O*-(*p*-coumaroyl)glucoside
	Peonidin-3-*O*-(*p*-coumaroyl)glucoside
	Petunidin-3-*O*-(*p*-coumaroyl)glucoside
	Malvidin-3-*O*-(*p*-coumaroyl)glucoside

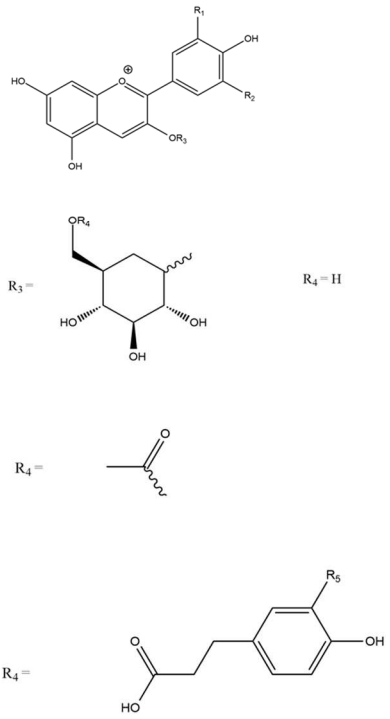


Figure 17: Structures of the monomeric anthocyanidins naturally occurring in Vitis vinifera wines and their corresponding anthocyanins

Anthocyanins are localized only in the vacuoles of the cells of the skins, except for the vines known as teinturier cultivars in which they are also found in the pulp [97,98]. The first anthocyanins of the grape berry start to appear about three weeks before the veraison (change of color of the berry), but it is really after this one that their concentrations increase significantly until maturity.

The anthocyanin concentration in grapes is strongly dependent on the level of maturity of the berry [99], but also on exogenous factors such as climatic conditions [100], water stress [101,102], temperature [103], intakes of nutrients and growth regulators [104,105], terroir and grape variety [106]. Anthocyanin concentrations in wines will depend on their contents in grape berries, on winemaking process (mainly temperature, % ethanol and maceration time), but also on pectin composition in celluloses and glucans of the cell walls of the film which will make their extraction more or less easy [107]. Malvidin-3-*O*-glucoside is the predominant anthocyanin in grapes (70-90%) in most of the existing cultivars. The concentrations of anthocyanins vary between 350 and 1100 mg/L in red wines made from grapes of the species *Vitis vinifera*.

2.1.4.2. Equilibrium of anthocyanins in function of pH

In their aglycone form (anthocyanidin), anthocyanins are unstable and their color in solution is pH dependent. Indeed, depending on the pH of the medium [108], anthocyanins will take different forms and therefore different color. In this way, they can be encountered in the flavylium cation A^+ form (red color), in the quinone bases AO form (blue-purple color), the carbinols base AOH form (colorless) or in the cis and trans chalcones C form (light yellow in color) (**Figure 18**).

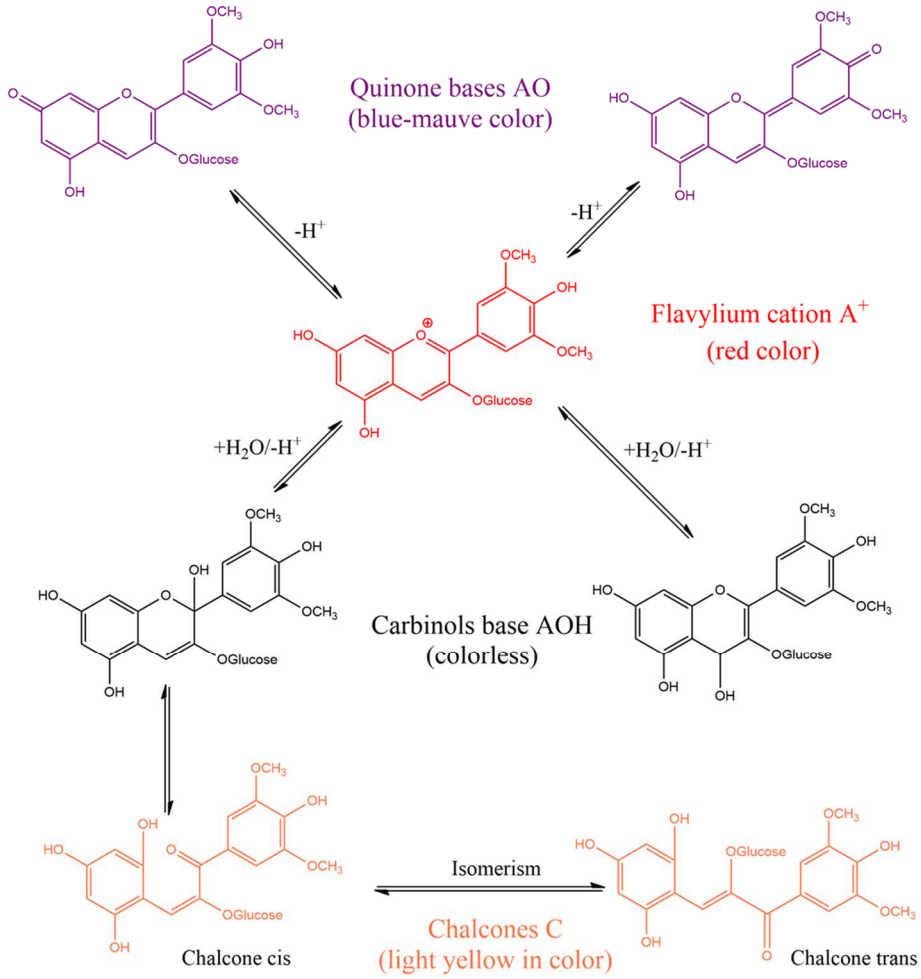


Figure 18: Equilibrium of anthocyanins in function of the pH

In the wine pH range, between 3.0 and 4.0, the four forms of anthocyanin co-exist (**Figure 19**), but the predominant form is the colorless AOH, which represents between 40 and 60% of all anthocyanins. The flavylium A^+ red form represents between 4 and 35% of anthocyanins over the same pH range. AO form also represent a small proportion between 8 and 15% of total anthocyanins. At pH 4, the chalcones forms have the majority chromophore, although they are weakly colored [94].

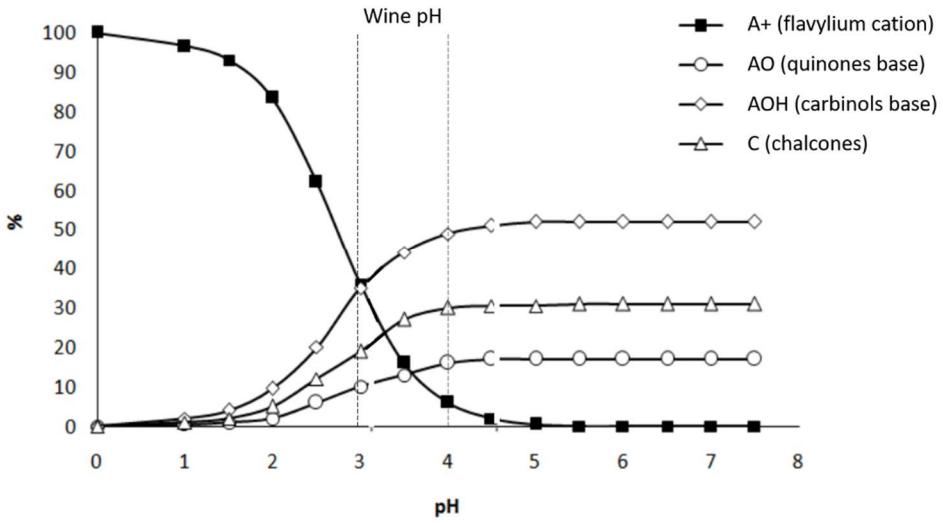


Figure 19: Evolution of the proportion of the different anthocyanins forms in function of pH

2.1.4.3. Equilibrium of anthocyanins in presence of SO_2

The amount of free sulfur dioxide is the most crucial factor that affects the color of young red wines. Indeed, in presence of sulfur dioxide (SO_2), free anthocyanin solutions are strongly bleached. In fact, in aqueous medium, sulfur dioxide combines with water to produce sulfurous acid (HSO_3^- and SO_3^{2-}) and this reaction is pH dependent [109]. According to this, bleaching phenomenon is due to the nucleophilic addition of HSO_3^- at the C4 or C2 position in the flavylium cation of the anthocyanin in red wines, though such transformation is also reversible (**Figure 20**).

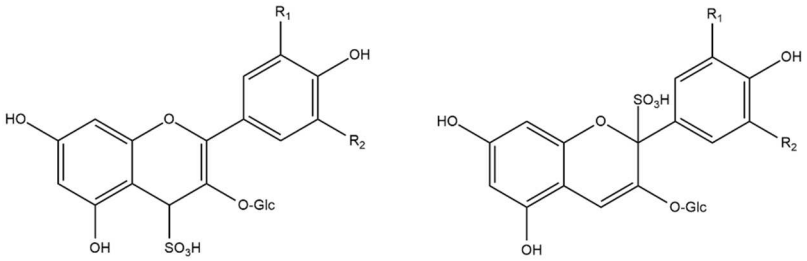


Figure 20: Structures of ASO_3H type colorless forms resulting from the reaction between anthocyanins and the HSO_3^- ion

At a pH around 3.5, about 96% of sulfuric acid is in the form of hydrogen sulfite anion (HSO_3^-), leading the bleaching of anthocyanins happening easily in wines. This substitution would, moreover, prevent the condensation of anthocyanins with other molecules. The bleaching effect of SO_2 on combined and polymerized anthocyanins is less or non-existent [2,110].

2.1.4.4. Anthocyanins derived pigments

Pyranoanthocyanins

Through the decrease of the anthocyanin content during the winemaking, the formation of another class of stable pigments over time is observed: the pyranoanthocyanins, responsible for the gradual evolution of the color of the red wines towards orange-red [111]. Indeed, this is the direct reaction between free anthocyanins and certain yeast by-products, such as acetaldehyde, pyruvic acid and vinyl phenols who lead to the formation of pyranoanthocyanins [112]. Generally, pyranoanthocyanins constitute one of the most important classes of anthocyanin-derived pigments occurring naturally in red wine [113–116]. Normally, they are cycloaddition products with an additional pyran ring. More specifically, the anthocyanins undergo a nucleophilic substitution of type 2 (SN_2 or bimolecular nucleophilic substitution) at the C4 position: the encounter between the anthocyanin in the flavylium cation form and the compound is done simultaneously. The transition state resulting from this reaction has an unstable carbocation, which leads to the formation of a fourth D ring involving the C5 hydroxyl group and the C4 of the anthocyanin heterocycle [117]. Thus, pyranoanthocyanins, compared to the free anthocyanins, present two heteroaromatic rings, with a dynamic equilibrium among different flavylium cation forms, as shown in **Figure 21**.

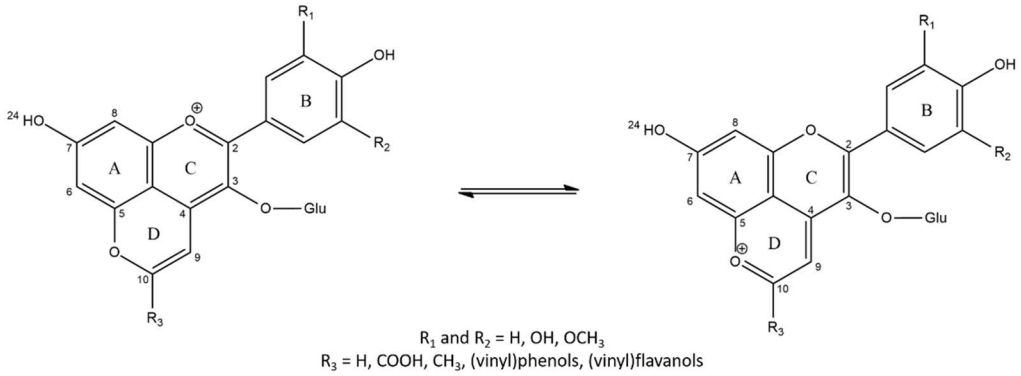


Figure 21: General structures of pyranoanthocyanins derived from anthocyanidin-3-O-glucoside

These new pigments are mainly formed from grape anthocyanins during the fermentation of must and later during the maturation and aging of red wines [117,118]. Additionally, some studies even reported that pyranoanthocyanins represented an important part of total pigment content in rosé wines, in blanc de noir base and sparkling wines [119].

- ♣ Structure and formation of Vitisins

Vitisins, the first group of pyranoanthocyanins to be identified in red wines are usually the most abundant pyranoanthocyanins [120–122]. During alcoholic fermentation, anthocyanins react with yeast metabolites such as pyruvic acid, acetaldehyde or acetoacetic acid. These low molecular weight metabolites are present in keto-enol equilibrium in hydroalcoholic solution but react preferentially with their enol tautomeric form.

After nucleophilic substitution, loss of water molecule and oxidation, Vitisins types are formed (**Figure 22**): carboxypyranoanthocyanins or Vitisin of type A [121,122], Vitisin of type B [123] and methylpyranoanthocyanins [118].

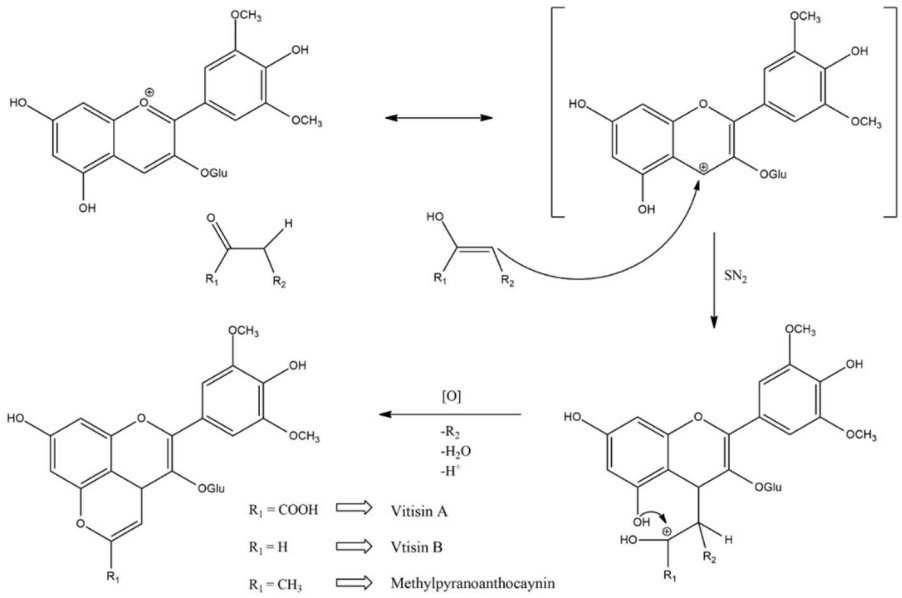


Figure 22: Mechanism of formation of the different vitisins: vitisin A, vitisin B and methylpyranoanthocyanins

Vitisin A is formed from malvidin-3-*O*-glucoside and pyruvic acid, meanwhile vitisin B is synthetized as the product of malvidin-3-*O*-glucoside and acetaldehyde. Methylpyranoanthocyanins are the products of the reaction between acetone or acetoacetic acid with an anthocyanin to form a product with a methyl moiety [118].

As well as malvidin-3-*O*-glucoside, other anthocyanin flavylium ions in red wines can also form the pyranoanthocyanins, even the acylated ones. Structurally, vitisins apparently have a greater conjugation system than their precursors as so should have a longer maximum absorption wavelength than free anthocyanins. However, results in real conditions, shown the opposite and surprisingly, vitisins have a lower maximum absorption wavelength and appear orange under the same pH conditions. An explanation is that the new formed pyran ring may balance with the B ring contribution resulting in a small decrease in the overall maximum absorption wavelength [124]. Several factors can affect the formation of the vitisins, including the pH and the temperature of the medium as well as the concentration in precursors (pyruvic acid, acetaldehyde, vinyl phenols…) [125,126]. Furthermore, some other factors which can influence the above-mentioned factors may also affect the production of vitisins. In this way, the yeast strain used in the alcohol fermentation, the lactic acid bacteria used in malolactic fermentation and the content of SO_2 may play a role [126,127].

- ♣ Structure and formation of Pinotins

Anthocyanins can react with hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic) or the products of their decarboxylation that form during fermentation (4-vinylphenols).

This results in the formation of hydroxyphenyl-pyranoanthocyanins also called pinotins [128,129]. They are named pinotins, since they were firstly isolated from Pinotage wines [129]. Indeed, in Pinotage, it is the form with a catechol that have been identified and named by the authors Pinotin A, hence the generalization of the "pinotin" for the other structures. At wine pH, the maximum wavelength of these pigments is between 505 and 508 nm, which gives them a red-orange color. Pinotins have different names depending on the nature of the radical present on the phenol nucleus. The basic structures of well-known hydroxyphenyl-pyranoanthocyanins are illustrated in **Figure 23**.

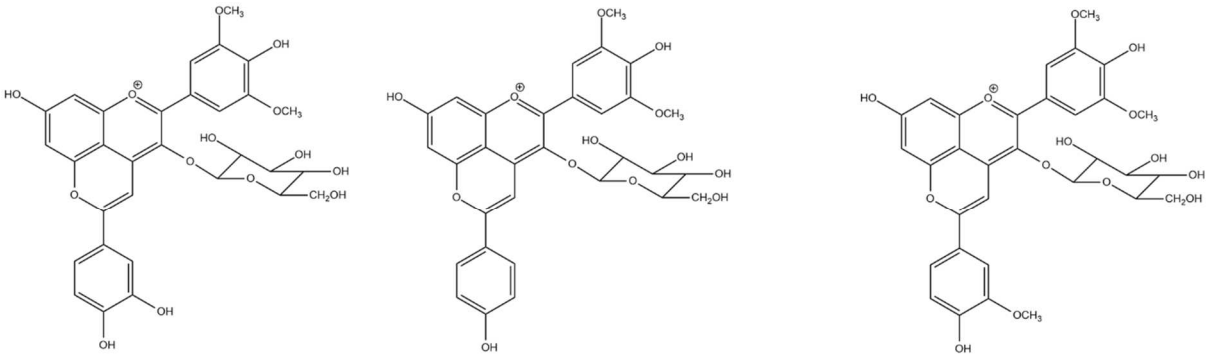


Figure 23: Structure of principal Pinotins

Pinotin A (pyranomalvidin-3-*O*-glucoside-catechol), the best-known pigment in this category, is formed from caffeic acid and malvidin-3-*O*-glucoside. The nucleophilic C2 position of the caffeic acid initially attacks the electrophilic C4 position of a malvidin-3-*O*-glucoside to form an electro deficient intermediate. Subsequently, the hydroxyl group at the C5 position of the anthocyanin moiety will intramolecularly react with this intermediate carbonium ion to form a pyran ring. After the further oxidation and decarboxylation, the final products are formed, as shown in **Figure 24** [128,130].

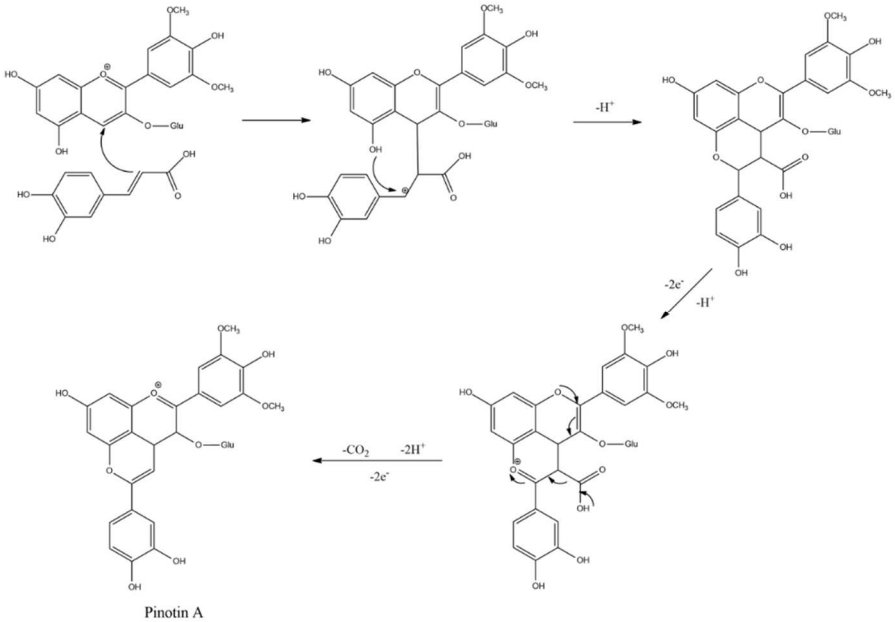


Figure 24: Mechanism of Pinotin A formation

Unlike vitisins, pinotins tend to be accumulated after the post-alcoholic fermentation. For example, the most rapid synthesis of pinotin A was observed in wines aged for 2 years at least where the malvidin-3-*O*-glucoside decreased to an extremely low level (5-10 mg/L at minimum are needed) while the concentration of caffeic acid remained very stable.

- ♣ Structure and formation of Flavanyl-pyranoanthocyanins

Another group of pyranoanthocyanins is the flavanyl-pyranoanthocyanins, also known as vinylflavanol-pyranoanthocyanins or pyranoanthocyanin-flavanols. This group, bearing a pyranoanthocyanin moiety directly linked to flavanols, naturally occurred in red wines. The cycloaddition is established between an anthocyanin (A) and an 8-vinylflavanol (F) either from the cleavage of flavanol oligomers linked by an ethylidene bridge [131], or from the cleavage of oligomers F-ethyl-F or F-ethyl-A. They also, can be synthetized in model wine solutions by cycloaddition between an anthocyanin and an 8-vinylflavanols (8-vinylcatechin or 8-vinylprocyanidin) mediated by acetaldehyde as shown in **Figure 25**.

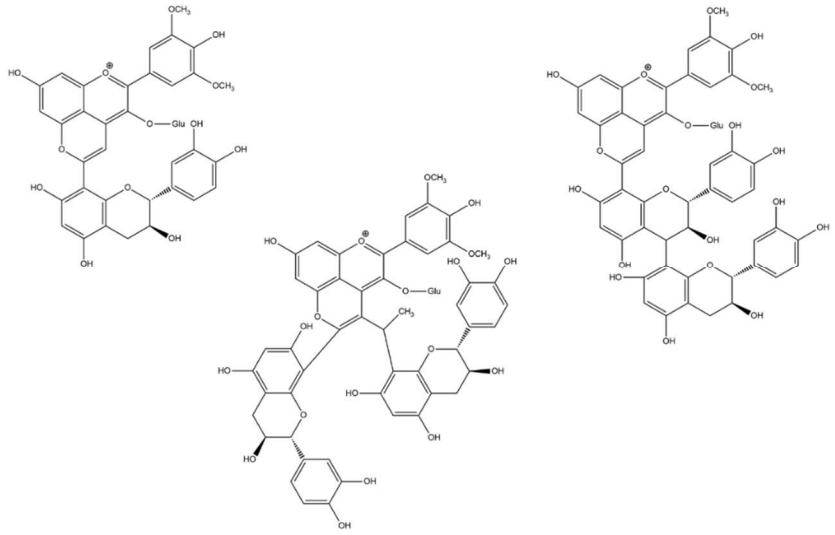


Figure 25:Structures of flavanyl-pyranoanthocyanins

Flavanyl-pyranoanthocyanins are formed by the cycloaddition reaction between vinylflavanols and anthocyanins, by a mechanism like that of hydroxyphenyl-pyranoanthocyanins with vinylphenols. The final oxidation step allows cyclization on flavylium and formation of the D cycle as presented in **Figure 26** [132].

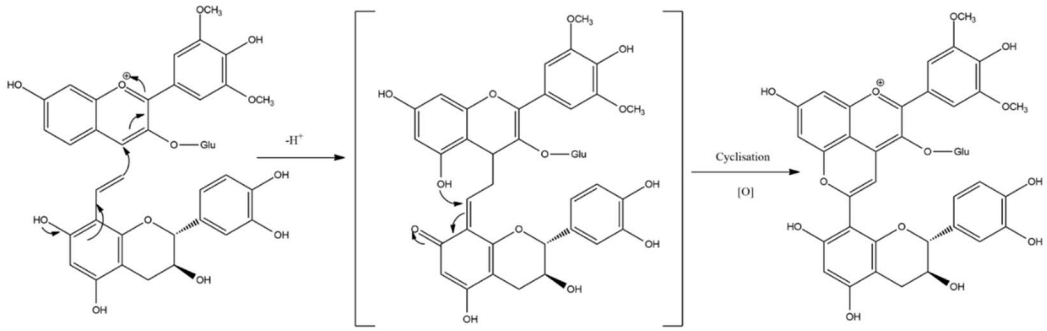


Figure 26: Formation mechanism of flavanyl-pyranoanthocyanins

The adducts of malvidin-3-*O*-glucoside and vinylcatechin, vinylepicatechin or vinylprocyanidin B2, were first detected and identified in model wine solutions [133]. Later, flavanyl-pyranoanthocyanins derived from malvidin-3-*O*-glucoside (as well as its acylated products) and the vinyl derivatives of (+)-catechin, (-)-epicatechin or oligomeric procyanidins up to tetramers were detected in red wines [133]. However, it should be highlighted, as important precursors, vinylflavanol adducts do not occur naturally in grapes. Like vitisins or pinotins, these pigments have a hypsochromic displacement of the maximum wavelength at values between 490 and 511 nm, which gives them an orange color. In addition, this hypsochromic shift of λ_{max} results in a more orange color, which is also more stable at varying pH values [112].

Adducts formation from pyranoanthocyanins

Pyranoanthocyanins, especially carboxy-pyranoanthocyanins, due to their electrophilic character, can also react with other compounds during wine aging to produce some other pyranoanthocyanin pigments. These new adducts present more complicated structures such as the oxovitisins, the portisins, or the pyranoanthocyanins dimers groups.

- ♣ Structure and formation of Oxovitisins

Oxovitisins are neutral structures of pyranone-anthocyanins, generally deriving directly from carboxy-pyranoanthocyanins and especially, from vitisin A. These new pigments present a yellowish color since they have a pronounced broad band around 370 nm in the UV-Vis spectrum. Pyranoanthocyanin precursors, undergoes the nucleophilic attack of water at the C10 position, leading to a hemiacetal form. Then, the resulting intermediate undertake a series of decarboxylation, oxidation and dehydration reactions to produce the final neutral pyranone-anthocyanins (**Figure 27**) [134].

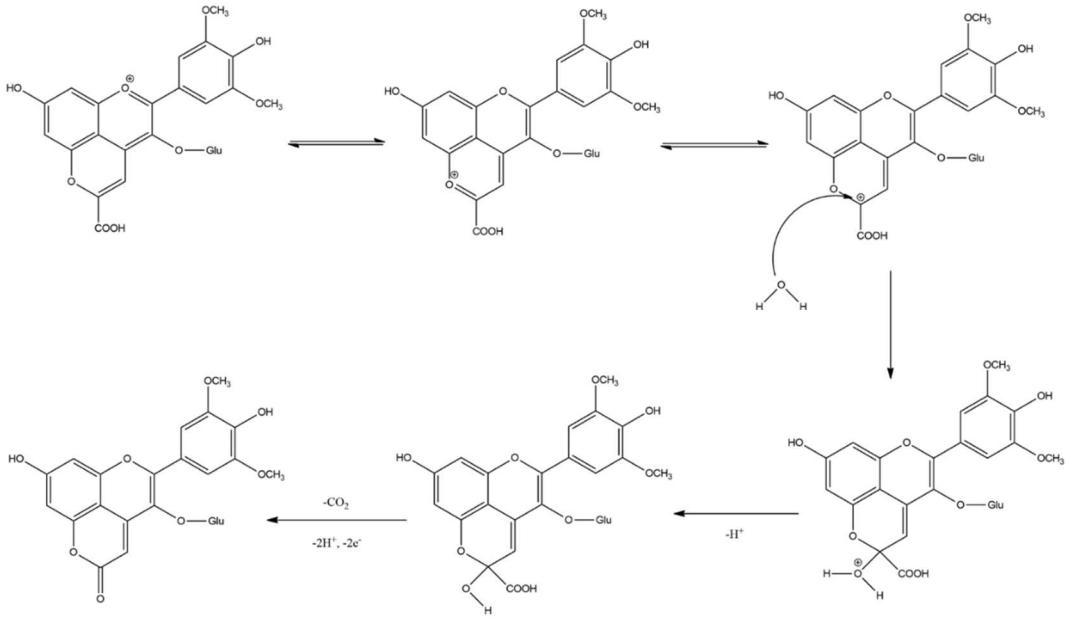


Figure 27: Formation mechanism of oxovitisins

Nevertheless, it has been recently reported that vitisin B types are not in equilibrium with the hemiacetal forms resulting from the nucleophilic attack by water. According to this, vitisins B may not easily take part in the formation of the oxovitisins pigments, contrary to the vitisins A [117,134].

- ♣ Structure and formation of Portisins

The portisins, also called flavanyl/phenyl-vinylpyranoanthocyanins, present a pyranoanthocyanin moiety linked through a vinyl bridge to a flavanol or phenol unit [135]. These new pigments have their maximum light absorption at a longer wavelength than the vitisins. In addition, portisins seems to have higher resistance regarding water attack of sulfur dioxide than their monomeric anthocyanin precursors. Portisins are derived through condensation of vitisin A and vinylphenolic compounds leading to create two types of portisins, A and B (**Figure 28**).

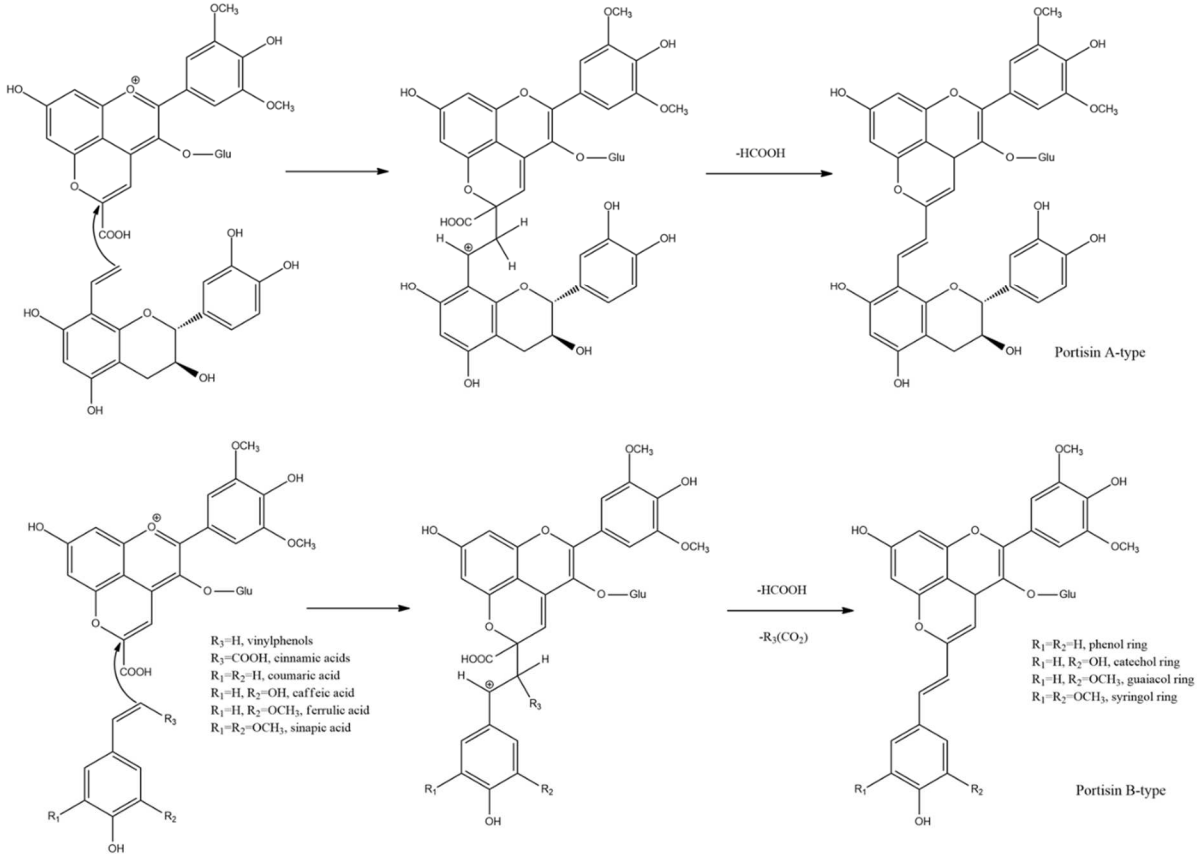


Figure 28: Formation mechanism of portisins A-type and B-type

Vitisin A reacts at the C10 position of the vinyl group of an 8-vinylflavanol adduct, leading to the formation of a portisins A (also called vinylpyranoanthocyanins) after losing formic acid group and being oxidized. Their λ_{max}, is close to 570 nm, corresponding to a rare intense blue-violet color [112]. This can be explained by the extensive conjugation of electron π which could confer a high stability at the pigment [136]. Although these compounds are in very low concentration in red wines, many A type portisins have been identified in Port wine fraction. Thus, their possible chromatic contribution should not be overlooked.

B type portisins, is the product of the reaction between a vitisin A and a vinylphenol or other phenolic groups such as catechol, syringol or guaiacol [137]. Their mechanism of formation is similar to the one of the portisins A, but it involves a decarboxylation. Indeed, the C10 position of vitisin A undergoes a nucleophilic attack of a hydroxycinnamic acid, followed by the loss of a formic acid group and then a decarboxylation [138]. It should be highlighted, that the color of B type portisins is quite different from the color of A type portisins, by showing a hypsochromic shift of the maximum absorption wavelength in the visible spectrum [138]. They present a purplish color (λ_{max} = 540 nm) and it is quite interesting to note that this pigment becomes blue when frozen in water, explained by a reversible physico-chemical modification due to electronic and vibratory effects.

- ♣ Structure and formation of Pyranoanthocyanins dimers

More recently, a new class of pyranoanthocyanin dimers has been characterized in a 9-years-old bottle of Port wine [139]. This new class, present an outstanding rare turquoise color with a maximum visible absorption wavelength at 680 and 730 nm [140]. Pyranoanthocyanins dimers result from the reaction between carboxy-pyranoanthocyanins and methyl-pyranoanthocyanins which are linked by methylene bridges as shown in **Figure 29**. More specifically, their formation involves a deprotonation of the methyl group on the methyl-pyranoanthocyanin and then, a nucleophilic attack of this group on the C10 position of the vitisin A. The dimer is finally obtained after the loss of a formic acid group [140].

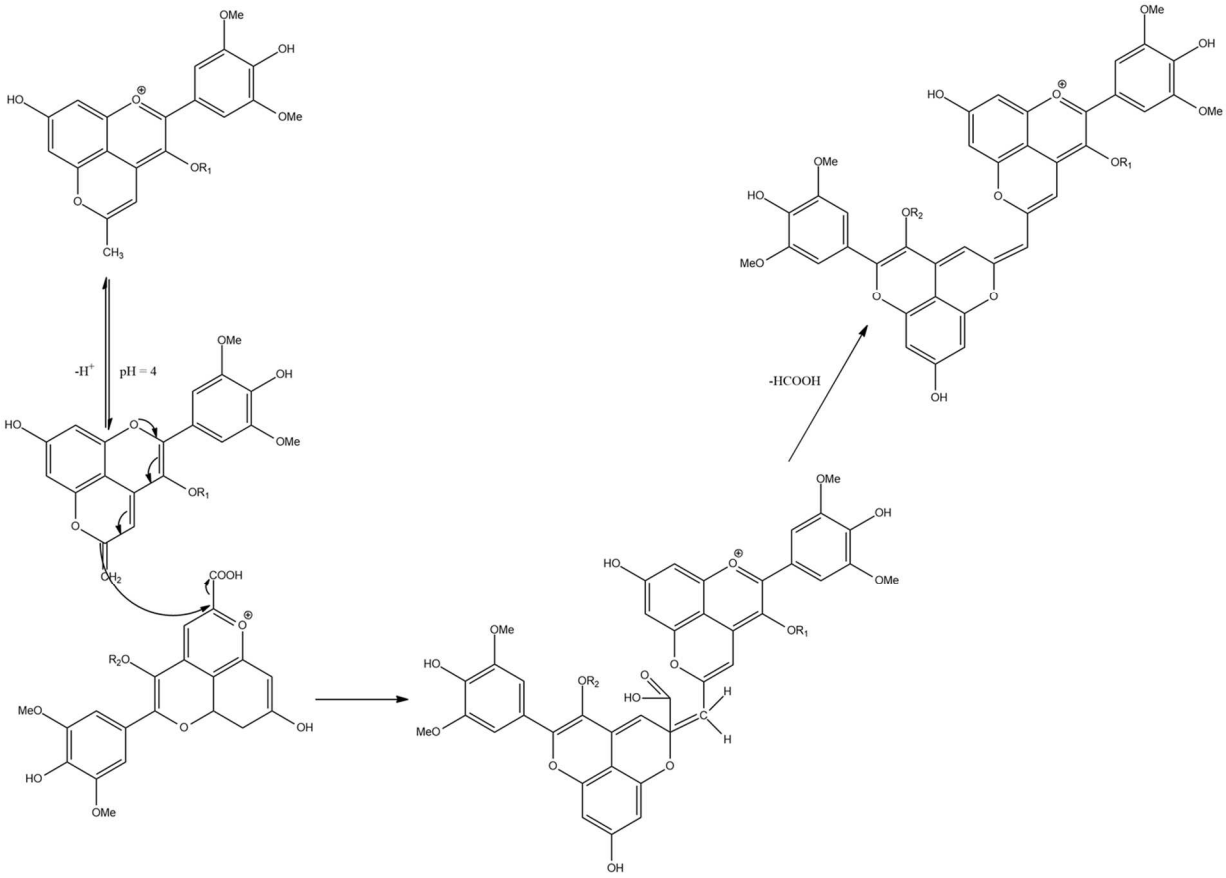


Figure 29: Formation mechanism of pyranoanthocyanin dimers

Other types of pyranoanthocyanins

In the same way as for wine, the manufacture of certain fruit or vegetable juices favors the formation of pyranoanthocyanins. Indeed, the process of manufacturing and storage of these beverages involves a decrease in anthocyanin composition [141], in favor of the formation of others pigments. Although there is a great diversity of anthocyanins, the various pyranoanthocyanins characterized in the juices converge to a common base: they are all hydroxyphenyl-pyranoanthocyanins. There is a great diversity of these pigments related to the form of anthocyanin.

2.1.4.5. Degradation of anthocyanins

In red wine, free anthocyanins evolve rapidly, since they are not particularly stable. After several years, although the wines remain red, there are almost no monomeric anthocyanins present. Indeed, their concentration decreases considerably during the first months of wine aging in barrels or bottles. This decrease is due, to combination reactions (polymerization) with various wine compounds and to degradation reactions [110]. The stability of monomeric anthocyanins in red wines depends on several factors: structure, pH of the wine, time and temperature of storage, oxidation status, light exposure and the composition of the solution [2]. Three types of reactions have been identified: thermal degradation, oxidative degradation and degradation in the presence of ketones.

Thermal degradation

Heating at 100 °C with an anthocyanin solution causes rapid and irreversible discoloration [142]. In addition, the degradation rate is correlated with the increase of temperature and anthocyanin concentration. The shift of equilibrium to chalcone form and the colorless forms of anthocyanins are the most likely explanation [2] even if glycoside hydrolysis may be another reasonable explanation [94]. Temperature is therefore a key point to consider for aging wines, which is why in general, the storage cellars are thermostated to keep an optimal and constant temperature all the year.

Oxidative degradation

In acidic hydroalcoholic solution exposed to light, as in red wines, anthocyanins in the hydrated forms can react with *o*-diquinones generated by enzymatic or non-enzymatic oxidation easily. Colorless and unstable compounds are produced, anthocyanins become discolored in a few days [94]. In such conditions, oxygen and light appear to act as catalysts, and a higher pH accelerates the reaction [143,144]. Anthocyanins with two adjacent hydroxyl groups on ring B (*o*-diphenols) can form *ortho*-quinones by oxidation. Malvidin-3-*O*-glucoside and paeonidine-3-*O*-glucoside, which are not *ortho*-diphenols, are more resistant to oxidation than cyanidin-3-*O*-glucoside during barrel aging of red wines [145,146].

Degradation in the presence of ketones

The presence of ketones is also a factor that can cause degradation of anthocyanins. In acidic and aqueous medium containing acetone, anthocyanins can react with them to give orange compounds [94]. Various mechanisms are proposed to explain this phenomenon, such as hydrolysis of anthocyanins and formation of dihydroflavonols; heterocycle breakdown of the anthocyanins with formation of benzoic acids; or else condensation with acetone via polarized double bond [2].

2.2. Physicochemical properties

Phenolic or polyphenolic compounds extracted from grapes (skins or seeds), or from wood barrel, can be directly involved and play an important role in the wine coloration by forming polymeric pigments with anthocyanins. Beside this, phenolic or polyphenolic compounds are also, involved as cofactors in copigmentation phenomenon.

2.2.1. Formation of covalent bonds

The formation of such pigments occurs by direct polymerization of anthocyanins and tannins (condensed or hydrolysable), as well as by the formation of the "bridge" with acetaldehyde or glyoxylic acid mediated polymeric pigments. The formation of this kind of bridge occurs only between anthocyanins and condensed tannins. These polymeric pigments are more stable than the monomeric anthocyanins and help stabilize wine color. Polymerization results in the chromophore of the anthocyanin being protected from water and nucleophilic attack, oxidation or other chemical modifications, such as the bleaching of sulfur dioxide. Additionally, to their condensation with anthocyanins, tannins can also polymerize directly between them or indirectly by condensation via acetaldehyde.

2.2.1.1. Directly condensed products of anthocyanins and hydrolysable tannins

It is value to point that anthocyanins may react with some non-flavonoid phenolic compounds to form complicated pigments. As mentioned in part 2.1.3.4, anthocyanins can react with C-glycosidic ellagitannins to generate anthocyanin-ellagitannin hybrid pigments such as pyranoanthocyanins. Additionally, to this, malvidin-3-*O*-glucoside can react with (-)-vescalagin, in acidic organic solution, to form desired condensation product. These new kind of pigment are called anthocyanoellagitannins and presents a visible absorption band, bathochromically shifted by more than 20 nm, inducing a deeper red-purple color [147]. Even if, such anthocyanin derivatives have not been observed in young red wines, it is not hard to imagine their detection and identification in red wines after wood aging in the future. Nevertheless, more recently, some authors have shown that a wine aged in low ellagitannin content barrels showed a higher percentage of loss than in medium with high ellagitannin content, and therefore contained significantly less monomeric anthocyanins at 12 months.

This results proved once again that ellagitannins in wine from barrel are capable to protect anthocyanins from their degradation by oxidation during aging, but not necessary interact with them to form new stable pigment [148].

2.2.1.2. Directly condensed products of anthocyanins and flavanols

The flavanols have three reaction sites: the phloroglucinol phenol A nucleus, the C heterocycle, and the *ortho*-di (or tri) hydroxyl phenolic B nucleus. Since the B-ring of the predominantly anthocyanin of the wine is dimethoxylated in 3' and 4', the reactivity of the anthocyanins will relate to the sites of the nucleus A and the heterocycle B. As a general rule, the kinetics of the various possible reactions are relatively slow, and their kinetics depend on the proportion of reactants present. On the ring A of anthocyanins as on that of flavanols, the hydroxyl groups in positions 5 and 7 have a mesomeric donor effect which induces negative charges at the top of carbons 6 and 8 and thus favors electrophilic substitutions. In this case, the flavonoid plays the role of nucleophile. In addition, the positive charge represented on the oxygen of flavylium A^+ can be relocated to C2 and C4, to give carbocation forms subject to nucleophilic additions. In particular, the nucleophilic attack of water and SO_2 leads to a colorless hemiacetal base and a colorless compound of bisulfite addition, respectively. Similarly, the breakdown of the proanthocyanidins C-C bonds, which occurs spontaneously during winemaking and maturation of wines, releases intermediate carbocations capable to react with nucleophiles. Anthocyanins (A) and tannins (T) can react to give rise to anthocyanin-tannin adducts according to two schemes which are A-type bonds and B-type bonds.

B-type bonds

- ♣ Adducts of type A^+-T

In the case of A^+-T adducts, the anthocyanin plays the role of electrophile while the tannin plays the role of nucleophile. Anthocyanin, in its flavylium form, reacts on an electronegative site such as carbon C6 or C8 of a flavanol to give an adduct. Initially, this adduct is generated as a colorless flavene complex named [(A(flavene)-T] [25]. This reaction would be followed by an oxidation which reintroduce a positive charge on the C ring of the anthocyanin and which would thus restore its red color (**Figure 30**).

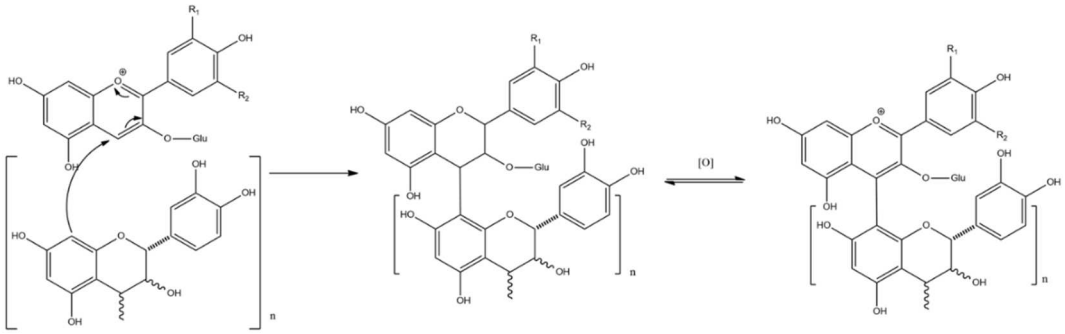


Figure 30: Mechanism of A-T adducts formation

As the reaction progresses, the balance between the flavylium form and the hemiacetal base shifts to the flavylium cation. In general, the substitution at C4 of the heterocycle confers a greater stability to the pigment obtained [112].

- ♣ Adducts of type T-A^+

In the case of T-A^+, anthocyanin in its hydrated form can react as a nucleophilic agent through its C8 or C6 carbons with the carbocation in C4 position resulting from acid cleavage of proanthocyanidins in wine. Such a nucleophilic attack results the formation of a B-type inter-unit bond leading to the formation of polymerized pigments with anthocyanin as a terminal unit in its flavylium (A^+) form (**Figure 31**).

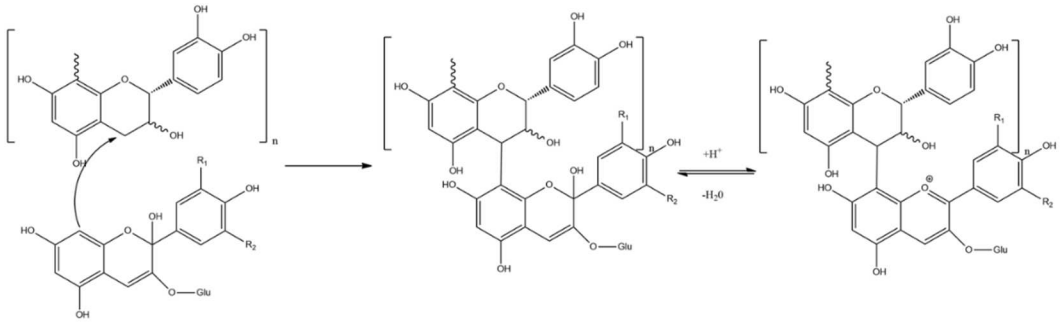


Figure 31: Mechanism of T-A adducts formation

Contrary to A^+-T adducts, in this case the polymerization cannot continue because the anthocyanin molecule is the terminal unit of the reaction chain and can only very easily be bound by its C8 or C6 carbons.

A-type bonds

This class has the C4-C8/C6 B-type bond and an additional ether linkage between the oxygen carried by the C5 or C7 carbons of the lower unit and the carbon C2 of the higher unit (**Figure 32**).

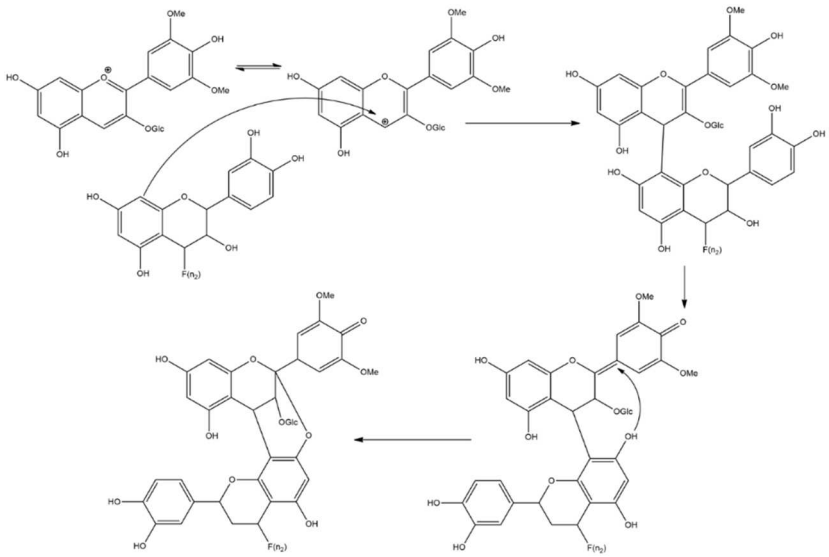


Figure 32: Mechanism of formation of A-type bonds

Nevertheless, several studies have shown the impossibility of distinguishing between adducts A(flavene)-F, FA adducts (flavene) and AF adducts (type A) in MS/MS and some attributions of structural fragments remain vague and controversial. The authors proposed using a chemical depolymerization strategy, since A(flavene)-F adducts depolymerize in acidic and hot condition while type A adducts resist to chemical depolymerization [62].

The formation of type A bond from type B bonding can also be achieved by oxidation. This oxidation can be done by free radicals or enzymes leading to the formation of a quinone on cycle B which will allow the nucleophilic attack of oxygen carried by the C7 or C5 carbons of the lower unit.

2.2.1.3. Direct interflavanoids polymerization (tannin-tannin adducts)

As explained for the direct condensed products of anthocyanin and flavanol two different types of bond can be involved in the direct interflavanoids polymerization of tannins which are the B-type and A-type bonds.

B-type bonds

The natural dimers belong to B-type procyanidins and consist of two flavanol units, referred to as the upper unit and lower unit with a carbon-carbon interflavan linkage between C4 of one unit and the C6 or C8 of another unit as shown in **Figure 33**. Four major isomers, which often occur in nature, are assigned to dimers B1, B2, B3 and B4 [149].

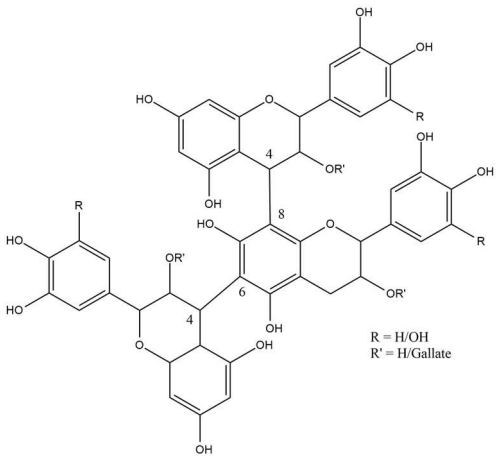


Figure 33: Interflavanoids B-type bonds (C4-C8/C6)

Polymerization and reversible depolymerization of proanthocyanidins are possible under wine conditions (pH 3.0-4.0). Indeed, in acidic condition, the B type bond C4-C8/C6 is cut off by releasing the unit lower in intact form and the upper unit with a carbocation located on the carbon C4. The carbocation thus formed can undergo the nucleophilic attack of the C8 or C6 carbons of another unit of flavanol or proanthocyanidin by forming proanthocyanidins again [62]. If the lower part released has a size smaller than the proanthocyanidin which comes to add to the carbocation, the degree of polymerization of the compound increases and decreases in the opposite case.

This polymerization is not without limit, since with the increase in polymerization, proanthocyanidins become more difficult to dissolve in alcoholic and aqueous media.

A-type bonds

This class has the C4-C8/C6 B-type bond and an additional ether linkage between the oxygen carried by the C5 or C7 carbons of the lower unit and the C2 carbon of the higher unit (**Figure 34**).

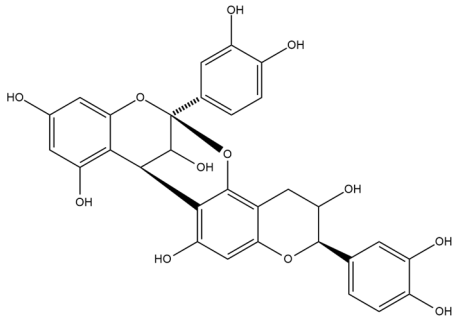


Figure 34: Interflavanoids A-type bonds (C4-C6; C2-O-C5)

Additionally, trimeric procyanidins, called procyanidins of type D, can be found with an inter-flavonoid binding of type A and another of type B.

2.2.1.4. Direct interanthocyanins polymerization (anthocyanin-anthocyanin adducts)

The presence of anthocyanin dimer and trimer was first identified in grape skins in 2004 and then in red wines. Five-years later, in 2009, 22 anthocyanins dimers were identified in red wines from Cabernet Sauvignon and Montepulciano varieties [150]. Only in 2013, a trimeric anthocyanin was identified for the first time in a young Port wine (**Figure 35**) [151].

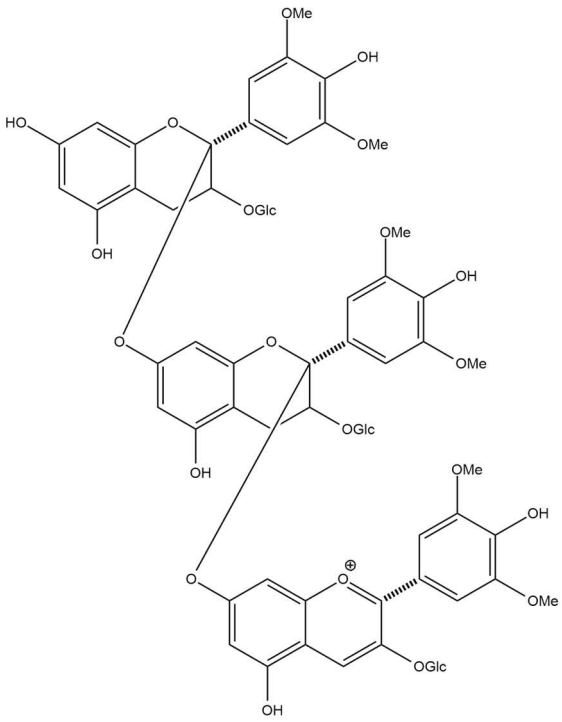


Figure 35: Structure of a trimeric anthocyanin with A-type bond

Since these structures were firstly detected in grape skins, it was postulated that they were initially present in grape skins and extracted during the winemaking process, make it their detection also possible later in wines [150].

2.2.1.5. Acetaldehyde or glyoxylic acid mediated polymeric products

In addition to direct condensations, indirect condensations via aldehydes between flavanol units and anthocyanin units have also been observed in red wine [131]. This condensation forms reddish or violet polymeric pigments in which the anthocyanin moiety is more protected against water attack and more stable regarding bleaching by sulfite dioxide. Nevertheless, this polymeric pigments are more sensitive to degradation in aqueous solution [112]. Of all the intermediates, acetaldehyde arising from yeast (metabolites generated during fermentation) or the oxidation of ethanol, is the most abundant aldehyde in wine [152]. This condensation, catalyzed by the acidity of the medium, involves the formation of a carbocation on acetaldehyde which undergoes a nucleophilic attack on the C8 or C6 carbons of a flavan-3-ol unit and leads to the formation of an intermediate having a benzyl alcohol. This alcohol can be dehydrated to generate a benzylic cation that can again undergo nucleophilic attack of an anthocyanin (in its hydrated form) lead to the formation of a bridge, so-called flavanol-bridge-ethylidene-anthocyanin (**Figure 36**) [153].

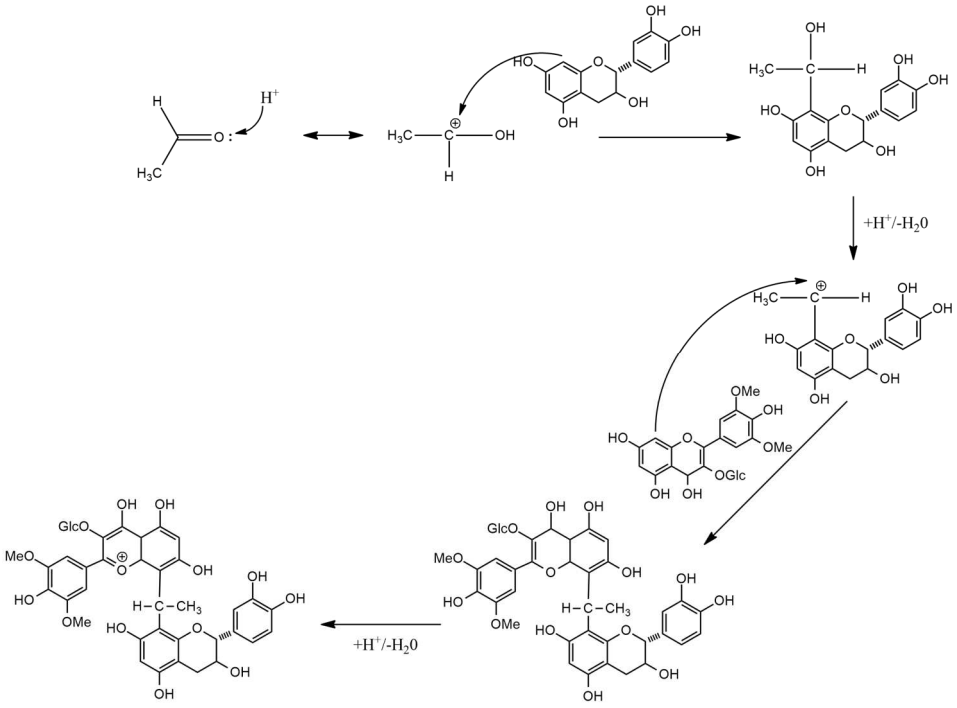


Figure 36: Acetaldehyde mediated polymeric products (anthocyanin-flavanol)

In addition to acetaldehyde, since glyoxylic acid bear an aldehyde moiety, it can also be involved in indirect condensation of anthocyanins and flavan-3-ols [112]. Iron catalyzed oxidation of tartaric acid produce glyoxylic acid which can attacks the nucleophilic C6 or C8 position in the ring A of anthocyanins and flavanols leading to the production of a carboxy methane bridged oligomers.

According to the relative concentration of wine in tartaric acid and ferrous ions, this reaction may be quite important during wine aging and probably competes with the acetaldehyde-mediated polymerization [112].

The ethylidene bridge can also bind two anthocyanin units based on the same mechanism. Three different forms depending on the pH were detected for this kind of compound: the bicharged ion m/z = 506.1419 contains two anthocyanins in the flavylium form, the monocharged ion m/z = 1011.2765 contains an anthocyanin in the flavylium form and another in quinone form and the monocharged ion m/z = 1029.2870 contains one anthocyanin in hydrated form and another in flavylium form.

The ethylidene bridge and the carboxy methane bridged (glyoxylic acid) can also bind two flavanol units according to the same mechanism with that for one unit of flavanol and one unit of anthocyanin.

2.2.1.6. Xanthylium pigments

Xanthylium pigments are one of the important groups of polyphenolic pigments and have various sources in red wines. In general way, they are derived by direct or indirect condensation of anthocyanins and/or flavanols and present different color going from yellow to red (**Figure 37**).

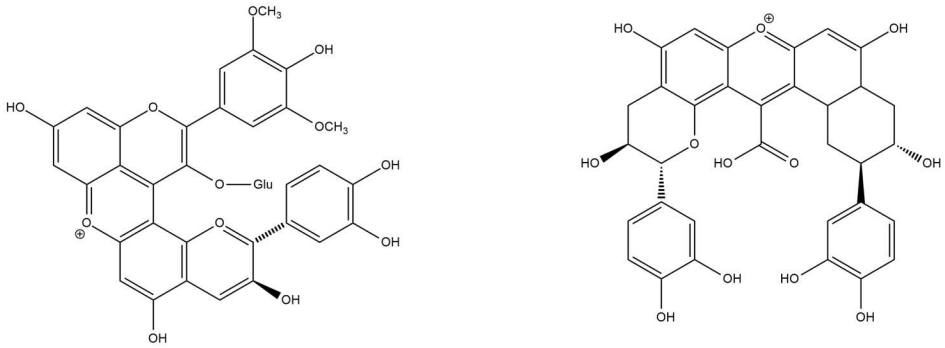


Figure 37: Examples of xanthylium salts

Yellow-orange xanthylium pigments can be generated by the A-T adducts after further structural rearrangements, such as the formation of a new heterocyclic pyran ring between the two parent rings (A ring of the anthocyanin and flavanol). In the same way, xanthylium can also be formed directly from two flavanol units instead of one anthocyanin and one flavanol unit.

In addition, xanthylium pigments can also derived from indirect condensation such as from the glyoxylic acid mediated dimer of flavanols and anthocyanins.

2.2.2. Copigmentation

Copigmentation is an important phenomenon to consider when assessing the color of a wine, although its only seems to occur in young wines. Indeed, the color of the anthocyanin pigments is depending on the pH, but also on the interaction with other organic non-colored compounds present in the medium, called copigments [154].

The copigments form with the anthocyanins (under flavylium cation or quinone base form) complexes structures, called "sandwich" by non-covalent bonds (Hydrophobic and Van der Waals interactions) allowing the establishment of a stacking π-π (**Figure 38**).

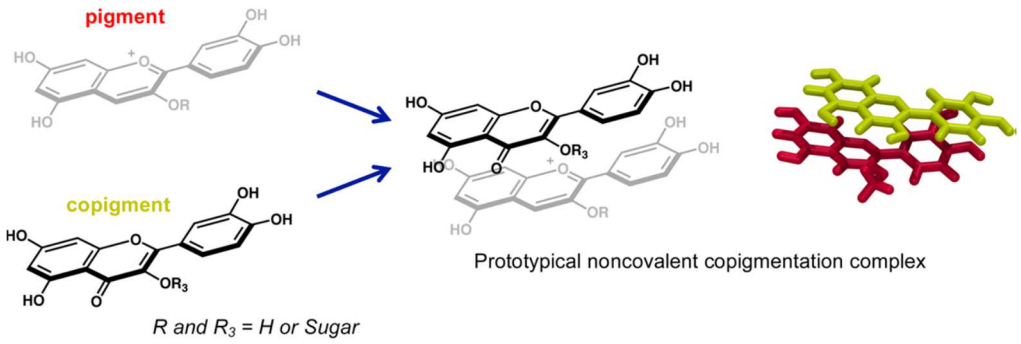


Figure 38: Copigmentation phenomenon by Trouillas et al., (2016)

The formation of stacking complexes leads to a bathochromic effect (displacement of the maximum absorption wavelengths at higher wavelengths) by shifting the balance between the different forms of anthocyanins (A^+ and AO) and a hyperchromic effect (increased color intensity). This kind of reaction protects the flavylium cation from nucleophilic attack of water, thus preventing its discoloration [155]. The copigmentation phenomenon is mainly influenced by pigment and copigment concentration, copigment patterns and structures, pigment/copigment ratio, and environmental factors such as pH, temperature, ethanol content and ionic strength [156].

Finally, the copigmentation can be intermolecular as well as intramolecular. In the case of intermolecular copigmentation, non-covalent Van der Waals and hydrophobic interactions allow the establishment of π-π stacking. In the case of intramolecular copigmentation, when the anthocyanin is coumaroylated, copigmentation occurs between the anthocyanin and the coumaric acids of the same molecule. At high concentrations, anthocyanins can also associate themselves to form dimers by hydrophobic packing resulting in an intensification of color [25].

2.3. Organoleptic and sensory properties

The contents and the qualitative profile of the tannins influence wine organoleptic qualities. Indeed, tannins contributes to structure, body and mouthfeel of wines and can be defined as the spinal column. Nevertheless, astringency and bitterness perception must be balanced to have a great wine. This balance will depend mainly on the quantity and quality of tannins solubilized in the wine. By their properties to polymerize with anthocyanins as described previously, they will also play a role in wine color. The terms "soft" and "hard" tannin are often used in wine tasting to try to approximate their quality.

2.3.1. Astringency

Although the exact mechanisms of astringency are not well understood, astringency is generally considered to be from an organoleptic point of view, a tactile sensation perceived in the mouth as a dryness and roughness of the buccal wall [157]. It is a sensation that you do not perceive instantly but whose persistence is long once appeared [158]. In addition, the astringency with repeated injection tend to augment until saturation phenomenon.

From a chemical point of view, astringency is not defined as a sensation but as a capacity of tannins, to precipitate proteins. Oligomers and polymers of condensed tannins are the principle responsible of the astringency of red wines. Furthermore, the sensation of astringency coming from these tannins, is directly a function of their mDP and the percentage of galloylation [159]. Moreover, the astringency perception diminishes with the augmentation of the concentration in ethanol and/or augmentation of wine pH. Indeed, the ethanol limit the precipitation of the proteins when they encounter tannins, allowing the conservation of the lubricating characteristics of saliva. Finally, it has been found that the tannins polymers are just as astringent as their monomeric form, unlike tannin-anthocyanin polymers which are less astringent than the original tannins [159].

2.3.2. Bitterness

Bitterness, unlike astringency, is not a sensation but a taste perceived by tongue receptors. It is perceived by the taste buds present in taste receptors of the tongue. Each buds of taste consists of approximately 50-100 taste receptors and is innervated by multiple taste fibers that transmit nerve signals to the brain [138]. Bitter taste receptor cells are mainly located at the back of the tongue near the throat. In humans, each bitter taste receptor cell contains about 25 bitter (type G) receptors coupled to proteins encoded by a TAS2Rs gene family [62]. Moreover, bitterness can come from different origins (clover, wood and glycerol) and therefore be considered either a defect or a quality. The compounds responsible for the bitterness are mainly flavan-3-ols whether in the monomeric or polymeric form, as well as the phenolic acids. That said, it has been found that flavanol monomers are bitterer than their polymers, thus explaining the loss of bitterness during wine aging [158]. Additionally, as for the astringency, bitterness increases with the concentration in ethanol.

2.3.3. Color

The color component among others, is crucial from a consumer point of view for the choice of a wine. It is therefore important to understand its evolution and stabilization over time. It has therefore been found that the combination of anthocyanins and tannins gives rise to more intense orange-red compounds than free anthocyanin. In contrast, reactions combining anthocyanins with tannins through the ethanal, give rise to compounds of red-violet color.

Moreover, products from cyclo-addition via vinylphenol, pyruvate or ethanal, for their part are yellow-orange in color. Finally, the copigmentation phenomenon results in a bathochromic effect (spectrum shift towards the highest wavelengths) and intensification of color as explained previously.

3. Oenological tannins

There is in fact a wide range of oenological tannins available in the market which differ in chemical structure (condensed and hydrolysable tannins), botanical origin (grape seed or grape skin, oak wood, exotic wood, nut gall…) and/or preparation process. The term of oenological tannin includes all the products resulting from the solid/liquid extraction of fresh materials or dry plants, naturally rich in phenolic compounds. In this fraction, the tannins usually represent the most important part. According to this they present different properties and possible use in winemaking.

3.1. Origins

Oenological tannins come from a lot of source (botanical origin), since a multitude of trees and shrubs contain tannins. Indeed, for both hydrolysable and condensed tannins, they are many species rich in tannins. The main sources used until today in winemaking are, mimosa, acacia, oak bark, quebracho wood, chestnut wood and tara. Nut gall is also a non-negligible source of tannin deriving from the formation of a tumor on different types of wood. In addition of them, the by-products of grapevine are also used with an important presence on the market of grape-skin, grape-seed and grape tannins. Idem the by-products of green tea, are very rich in tannins [160].

As regards the tannin origin by location, the areas of strong industrial production worldwide today are Brazil, South Africa, India, Zimbabwe, Tanzania and Argentina. Concerning the production in Europe, France, Spain and Italy are huge producer of commercial tannins with many factories working on this subject. There are many other small to very small producers a bit everywhere, for example, oak tannin factory in Poland or chestnut factory in Slovenia [160].

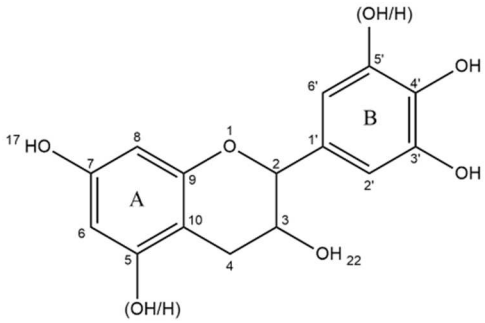
Tannin from different botanical origin, have very different commercial values and shows varied chemical and technological properties. In this way, it is fundamental in order to optimize and help the consumer choice to have the exact knowledge concerning their sources [161]. However, few studies have highlighted the lack between the information provided by the supplier on the label or data sheet and the real content of the commercial products [162]. The botanical origin of the different oenological tannins can be controlled on the one hand, thanks to molecular markers such as the quercitol (oak wood), pinitol (gall nut) or even arabitol (quebracho). On the other hand, it can be controlled by the composition of monosaccharides (xyloses, fructose, glucose, arabinose) [163]. The traceability of tannins can also be achieved regarding their mineral element profile or their carbon isotopic ratio.

3.2. Structures

Oenological tannins are divided into two classes, which are the condensed tannins and hydrolysable tannins, as well as the “natural” tannins.

Hydrolysable tannins are classified into two subfamilies, gallotannins and ellagitannins. Gallotannins are polymers formed by esterification between D-glucose and gallic acid. Tannic acid is the commercial name for gallotannin extract comprising mixtures of polygalloyl quinic acid ester or polygalloyl glucoses [164]. The main sources of commercial gallotannins are nut galls and tara. Ellagitannins are polymers of ellagic, gallic and/or hexahydroxidiphenic acids [163]. To be more precise, a nonahydroxyterphenoyl unit (NHTP) is esterified in positions 2, 3 and 5 with a C-glycosidic bond, while an open-chain glucose is esterified in positions 4 and 6 with a hexahydroxydiphenoyl unit (HHDP) forming the chemical structure of ellagitannins [165]. The main sources of commercial ellagitannins are oak and chestnut.

Condensed tannins, also known as proanthocyanidins, come from different botanical origins, such as grapes, quebracho, mimosa and acacia. They differ mainly regarding the monomer released after acidic cleavage (**Figure 39**), the degree of polymerization (mDP), and their levels of galloylation and ramification [163].



Resorcinol ring	Ring A with only one hydroxyl group on C7
Phloroglucinol ring	Ring A with two hydroxyl groups on C5 and C7
Catechol ring	Ring B with two hydroxyl group on C3’ and C4’
Pyrogallol ring	Ring B with three hydroxyl group on C3’, C4’ and C5’

Figure 39: Structure of oenological flavonoids

The prodelphinidins, contain a phloroglucinol A ring and a pyrogallol B ring meanwhile procyanidins, contain a phloroglucinol A ring and a catechol B ring. Prorobitenidins, contain a resorcinol A ring and a pyrogallol B ring meanwhile profisetinidin contain a resorcinol A ring and a catechol B ring.

Grape-skin tannins are composed of procyanidins and prodelphinidins because their acidic cleavage gives cyanidin and delphinidin, whereas grape-seed tannins are composed only of procyanidins. Grape-skin tannins have a high mDP and a low level of galloylation, while grape-seed tannins have a lower mDP and a high level of galloylation [58]. Quebracho tannins are profisetinidins, because their acidic cleavage gives fisetinidin, and they have a high level of ramification, while mimosa tannins are prorobitenidins because they release robinetinidin [46]. Less is known about acacia tannins, but it seems they are composed of a mixture of profisetinidins, prorobitenidins and prodelphinidins [166].

3.3. Manufacturing process

Imagine the preparation of an infusion, as it could be tea. The leaves are left to be infused, in hot water, and the tea is released slowly. The same thing happens for tannin. For example, in the production of chestnut or quebracho tannin, the wood is shredded and left to macerate in hot water, releasing an intense aroma. The tannin gets transferred to this surrounding water in a completely spontaneous way. There is no need to force this process, since it happens naturally, and it does not need any chemical additives. When all the tannin is released in the water, we are already in the presence of a natural extract ready to be used in liquid form. Then the liquid can be dehydrated in order to keep the active ingredients in their initial state. The time between extraction and obtaining powders should be as short as possible. Indeed, their high reactivity gives them a great ability to hydrolyze and oxidize in solution under ambient conditions.

As an example, the extraction of tannin from wood will be explained step by step. In this case, six stage are necessary from the beginning to the packing.

The first step consists in resting the lumber; the trunks which derive from sustainable forest management and in compliance with local forestry legislation, are piled up and left to rest in the open air. This period will allow the wood to be processed more easily. The second step consist in debarked and shredded the trunk into small pieces called chips, to increase the possible surface then in contact with the water, to facilitate the release of tannin. In the third step came the extraction; the wood chips are loaded in big autoclaves with hot water over 100 °C. The parameters can change depending on the type of wood used and the industry. The tannin extraction process requires only hot water, without the addition of any chemical additive. Then in the fourth step, the aqueous solution of tannin is then cooled down to room temperature to precipitate the non-completely soluble substances and impurities contained in the raw material. The tannin is then purified and processed according to the destination of use. The liquid concentrated tannin is now ready to be used and could be commercialized as it is. Nevertheless, as explained before to facilitate its transportation, storage and usability, the liquid is turned into powder in a fifth step through a spray-drying process. Finally, the tannin powder is ready to be packed and shipped on the final stage.

3.4. Current regulations

Oenological tannins are regulated in the same way as the other inputs of the sector by the International Oenological Codex. The Codex Alimentarius classifies oenological tannins in the category of “food additive n°. 181” (Codex Alimentarius Commission 2009), but also in the category of processing aids (Codex Alimentarius Commission 2010), which is confusing. The main distinction between food additives and processing aids is that “additives” have a technological function in the final food, whereas “processing aids” do not.

The addition of tannins is permitted in Europe, South Africa, USA, Australia and New Zealand [163]. However, some differences in the regulation appears among these countries. More particularly, USA allows tannin addition for both clarification and adjustment of tannin content, with the restriction of no adding color. South Africa only authorized the addition of tannin if it's not "foreign to wine" meaning if it's a by-product of the grape who have been used in the winemaking. Australia and New Zealand authorized the use of tannin only as an additive meanwhile European Union authorized them as processing aids and additive. Oenological tannins are also allowed in organic farming for the European Union and Australia, if they are come from biological vintage (EC, 2012).

Codex also provides well-established criteria for controlling impact, botanical origin, extraction solvent and metal content (**Table 1**) [167].

Table 1: Current Codex Alimentarius regulation of oenological tannins

<table>
<tr><th>Botanical origin</th><th>Regulation</th><th>Extraction solvents</th><th>Regulation</th><th>Colorant power</th><th>Regulation</th><th>Metal content</th><th>Regulation</th></tr>
<tr><td>Grapes</td><td>> 50 mg/g eq. catechin</td><td rowspan="5">Only water</td><td rowspan="3">Solubility index < 5</td><td rowspan="4">Yellow</td><td rowspan="4">< + 1.5 on the initial value</td><td rowspan="2">Arsenic</td><td rowspan="2">< 3 mg/Kg</td></tr>
<tr><td>Nut galls</td><td>4 to 8 mg/g eq. digallic acid</td></tr>
<tr><td>Exotic woods</td><td>Presence of an absorption peak between 270 and 280 nm</td><td>Iron</td><td>< 50 mg/Kg</td></tr>
<tr><td>Oak</td><td>> 4 µg/g of scopoletin</td><td rowspan="2">Extractability index > 0.05</td><td rowspan="2">Mercury</td><td rowspan="2">< 1 mg/Kg</td></tr>
<tr><td>Chestnut</td><td>< 4 µg/g of scopoletin</td><td>Red</td><td>< + 0.05 on the initial value</td></tr>
</table>

3.5. Properties and utilizations

The main goal of the addition of oenological tannins, is to help the winemaker to overcome the difference in time, which is increasingly accentuated between technological maturity and phenolic maturity [168]. On the other hand, it must be considered that the effects of oenological tannins are first and foremost dependent on the quality of the harvest. Their role will always be the same, but with an impact and a force, more or less important according to the vintages. Nevertheless, it should be highlighted that each kind of tannins due to its structure present different ability regarding the diverse properties.

3.5.1. Proteins interaction

The affinity of tannins for the proteins, is well known since many years, conferring to them their astringent character. Tannins-proteins interactions are principally non-covalent hydrophobic interactions which may then be stabilized by hydrogen bonds. Likewise, the precipitation of tannins-proteins complex is due to formation of appropriate hydrophobic surface on the complex and affected by proteins types (size, conformation and charge of the protein), tannins types (size, length and flexibility), and the amount and stereo specificity of binding sites on both proteins and tannins molecules [169]. Nevertheless, tannins and proteins can be linked by three different types of bounds, which are hydrophobic bounds, ionic bounds or hydrogen bounds. The nature of the interaction depends greatly on the structure of the tannins and the proteins involved. Indeed, it has been reported that gallotannin, which is very nonpolar, precipitates by forming a hydrophobic coat around the protein, whereas the much more polar condensed tannins forms hydrogen-bonded cross-links between protein molecules [170].

In addition, tannins can be used in prevention of protein haze caused by over-fining when white wines are treated with gelatin [171,172]. Indeed, protein haze remains one of the key potential instabilities in white wine production and requires costly treatment with bentonite [173]. In this way, oenological tannins can present a great alternative [174].

They also can be used when proteins are in excess in white or rosé wines of quality. In this case, the most efficient ones are the grape-seed and grape-skin tannins, since condensed tannins are the only ones able to flocculate proteins.

3.5.2. Bacteriostatic effects

Malolactic-fermentation is an important stage during winemaking. Indeed, this second fermentation is essential for red wines. It is generally not sought for white wines and rosé wines, in which it is desired to preserve a certain vivacity, brought by malic acid. However, in Champagne, it is usually sought to prevent it from taking place during the foam or aging which would cause difficulties during riddling. It is therefore important to determine in what extent the use of oenological tannins could act on the engagement and unfolding of the malolactic fermentation (MLF).

Some preliminary results conducted in 2000 [175], shown the effect of the gallic acid and the (+)-catechin which are two constitutive units of the hydrolysable and condensed tannins respectively on the growth of *Oenoccocus oeni* strain. In this study, both gallic acid and (+)-catechin seems to present a stimulating effect by increasing the growth of the lactic bacteria.

Two years later another study [176] have shown that, only nut gall tannin have an effect and can completely blocks malolactic fermentation which is in total contradiction.

This effect is, however, noticeable only in two types of situation: either in use at a very high dose (200 g/hl) or in use on white or rosé wine at lower doses (from 50 g/hl) and associated with 3 g/hl of SO_2. The other tannins (except for skin tannin, which still have no effect) only serve to delay the start of the malolactic fermentation from a use dose of 200 g/hl on red and 50 g/hl on white wines. According to this, the use of oenological tannins, at the end of alcoholic fermentation, at current doses of use (5 to 30-40 g/hL), on red wine, does not therefore seems to run the risk of blocking the MLF. On white and rosé wines, it remains to be verified whether it is possible to associate some of these tannins used with doses of 50 g/hl with SO_2 in order to avoid a departure in uncontrolled malolactic fermentation. However, it is important to take into account the possible organoleptic effects of this technique on the final products.

A more recent study [177], conducted in 2015 present some contradictory results with the previous one but in accordance with the preliminary study of 2000. The authors demonstrate the positive impact of oenological tannins on bacterial growth, survival and malolactic activity in Carr culture medium in presence of two different strains of *Oenoccocus oeni*. This effect was then confirmed when bacteria were inoculated into the white wine. In this study, one grape-seed and one grape-skin tannin have been used, and no significant difference between them have been noticed. This means that the effect of the different grape tannins on the bacterial growth was not a function of their composition (mDP and percentage of galloylation).

To conclude, really few studies have been conducted regarding the possible bacteriostatic effect of oenological tannins and present contradictory results. Further studies are needed to prove the positive or negative effect of oenological tannins on the malolactic fermentation. For this purpose, it would be interesting to realize different experimentation in presence of different lactic bacteria and the different commercial tannins available on the market.

3.5.3. Impact on oxygen/metals

The impact of oenological tannins on oxygen/metals, involved different properties, which are the antioxidant capacity, the antioxidasic activity, the ability to scavenge superoxide radical, the prevention of oxidative damage mediated by Fenton-based reactions, the ability to chelate metals and the direct consumption of dissolved oxygen.

Exhausted woods represent an interesting byproduct of tannin industrial production processes, with high efficiency regarding superoxide scavenging assays [178], leading the possibility to use commercial tannins from wood for this purpose. Furthermore, two by-products (pomace and lees) from *Vitis vinifera* L. cv Pinot noir presented also a great ability to scavenge superoxide radicals [179]. In pomace, flavanols were present in majority meanwhile in lees flavanols were the most represented mostly with catechin.

These results mean that the use of oenological tannins coming from grape by-products should also be able to scavenge superoxide radicals since they will also be rich in flavanols and flavonols.

It has been widely reported that loosely bound iron, can react with hydrogen peroxide to produce the short-lived and highly reactive hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction [180]. These hydroxyl radicals can in turn oxidize wine compounds with critical damages. Subsequently polyphenols containing a catechol group or a galloyl group are oxidized to semiquinone radicals and benzoquinones whereas oxygen is reduced to hydrogen peroxide, as the entire process is dependent on the redox cycle of Fe^{3+}/Fe^{2+} and Cu^{2+}/Cu^{+}. The quinones which are formed from polyphenolic oxidation are unstable and possess an electrophilic character, thus they continue reacting and can spontaneously combine with nucleophilic compounds [181]. Few years ago, a scientific paper have evidence the ability of oenological tannins to prevent oxidative damage mediated by Fenton-based reactions [182]. More specifically, commercial tannins composed mainly of ellagic acid provided the greatest results regarding this property.

Concerning antioxidant capacity, various studies have been conducted on different plants containing tannins [183]. A first one [184], compared the possible antioxidant capacity of thirty plant extract and results have shown that oak, pine and cinnamon aqueous extracts possessed the highest antioxidant capacity. A second one [185], which was more centered on the different woods used in cooperage and the effect of the botanical species of wood, shown that *Quercus robur* and *Castanea sativa* were the greatest species. In addition, the authors have shown that phenolic acids, including gallic acid, protocatechuic, *p*-coumaric and ellagic acid and all the ellagitannins were the major woods contributors to antioxidant capacity. A third one [186], focused on the antioxidant capacity of stem by-products and the influence of the grapevine variety. The obtained results shown that stems from Callet, Syrah, Premsal Blanc, Parellada, and Manto Negro varieties yielded the greatest antioxidant capacities, whereas Chardonnay and Merlot stems presented the lowest values. According to these different studies it can be expected, that commercial tannins extracted from grapes or woods will present antioxidant capacity and will differ regarding the botanical origin of the wood and the grapevine. All measurements of antioxidant capacity were consistent with the content and composition of the phenolic compounds detected in samples.

In addition, antioxidant activity of oenological tannins can result from their capacity to chelate bivalent transition metal ions as iron, copper and zinc. These ions can engender highly reactive $OH^{\bullet}$ radicals by Fenton reactions in the same way as mentioned above. Chelating agents, which stabilize pro oxidative transition metal ions by complexing them, are regarded as secondary antioxidants. Some studies have reported the ability of pure commercial products to form stable complexes with Fe(II) [187,188]. More recent study [189], has demonstrated the ability of buckwheat and buckwheat groats tannins fractions to chelate iron, copper and zinc.

The same author has also, demonstrated the ability of tannins from hazelnuts, walnuts and almonds to complexed metals [190]. Nevertheless, it is also shown that these tannins complexed copper ions more effectively than iron and zinc. Almond tannins were able to chelate around 90% of zinc meanwhile hazelnut tannins cannot be able to chelate zinc. More recently, a research has proved, the ability of ellagitannins to chelate metals [191]. All these research lead to expect from the oenological tannins, whatever they are (condensed or hydrolysable), a possible ability to chelate metals.

In addition to present antioxidant capacity and ability to chelate metals, oenological tannins could present an ability to directly consume the oxygen. Indeed, a first study conducted in our laboratory have demonstrated that ellagitannins released by the barrels during wine aging, were able to consume directly the oxygen [192]. Moreover, the rate of oxygen consumption was higher when French oak was used instead of American oak, demonstrating the importance of the botanical origin of the used wood. A second study [164], also conducted in our research group, have proved that oenological tannins were able to consume directly the oxygen, ellagitannins being the most efficient ones. Nevertheless, the study, as a preliminary one was conducted with only one commercial tannin of each botanical origin.

Regarding the antioxidasic activity (anti-laccase activity), a recent study [193] have highlighted that some natural compounds can inhibit *Botrytis cinerea* at different stage of its development. Indeed, 3-phenyl-1-propanol presented the most potent inhibition of in vitro germination, germ tube development, and sporulation. Nevertheless, it had lower protection of leaves and postharvest fruit in plant infection. Isoeugenol and 1-phenylethanol displayed lower inhibition of in vitro germination and sporulation, but at the highest concentrations, they inhibited germ tube elongation. In addition, a recent overview reported the antifungal activity of various compounds deriving from the plant such as stilbenes, resveratrol, pinosylin or pterostilbene [194].

These properties in general have been widely described even if they have not been conducted using many samples and from different botanical origins. However, the antioxidant capacity [182], the direct oxygen consumption and the antioxidasic activity have been described regarding “natural” tannins but few studies present the effect of oenological tannins regarding these properties.

3.5.4. Impact on color/pigments

It is widely accepted that tannins, contribute in general to color stability, even more so to its temporary increase due to copigmentation. Grape tannins do not increase the brown shade of the wines unlike to the ellagitannins which accentuate the dark shade. Gallotannins, seems to present an intermediate effect, between grape tannins and ellagitannins. Independently of the exogenous tannin, the tanned wines develop a better resistance to “tiling”, since the orange or yellow shades are very slow to appear. This statement is right even for wines produce from grape varieties considered as little tannic and easily oxidizable as Grenache or Gamay for example [195].

Preliminary studies, have shown that the red color of wine increased after tannin addition because of the copigmentation effect [176,196]. Then complementary study [197] revealed that after five days of maceration with tannin addition, the total anthocyanins concentration was higher as well as the color intensity. The authors also suggest that the copigmentation processes was responsible of this increment. Nevertheless, they highlight the fact that, the better initial color of wines treated with oenological tannins can also be due to a temporary inhibition of oxidation rather than to copigmentation. In addition, in this experimentation, after 8-months in bottle, wines supplemented by oenological tannins presented lower color intensity but higher percentage of yellow color [197]. They attribute this increment of yellow color to an increase of polymeric tannins. Indeed, the addition of condensed tannins may have desequilibrated the anthocyanin/tannin ratio of the wine and favored the polymerization of tannins inducing an increase in yellow color.

Few years later (2012), another scientific publication [198], highlight the importance of the timing of tannin addition on color stability. Their results shown that an addition during pre-fermentation had a more significant influence on color stabilization than an addition during post-fermentation.

Finally, in a study of 2013, condensed tannin exhibited better effect than hydrolysable tannin regarding improvement and stabilization of wine color [199]. Condensed tannin probably slows down the degradation of anthocyanins (more specifically pyranoanthocyanins), leading perhaps to more copigmented anthocyanins and polymer pigment. Nonetheless, in this study, authors did not analyze the contribution of free anthocyanins, polymeric pigments, and copigmented anthocyanins to the color of wine. So there is still more work to do in order to confirm these findings, since they represent an important part of the color.

3.5.5. Sensory-mouthfeel properties

Wine aromas are made up of several hundred volatile compounds, who give to each wine, is specify and make him distinguishable of the other ones. In this way, oenological tannins can be added, to improve the aromatic composition of the wines. In addition, they can be added to complete the body structure and mouthfeel of the wines.

An interesting preliminary studied [195], have evaluate the impact of the diverse type of oenological tannins regarding their possible sensory and mouthfeel properties. This work has shown that, all the tannins increase the impression of acidity of the wines at the tasting, and more especially the gallotannins which are described as more aggressive than the other ones. In addition, nut gall and chestnut tannins naturally contain bitter substances that the taster perceives very quickly. This bitter character greatly limits the usable doses as well as the types of wine that can be treated with these tannins. On the contrary, oak tannins and grape tannins (skin and seed) are much less bitter and available to be used by the winemaker in an easier way.

Concerning astringency, the sensation will be greater with condensed tannins than with hydrolysable tannins. It should be highlighted that over time the combination between tannins and polysaccharides, will change the sensation of astringency tower a feeling of “maturity” of wine. The authors have also noted that an addition of well-chosen tannins (able to blend with the original material) makes it possible to completely improve the perception of the structure by the taster [195]. Nevertheless, if the tannin added choose cannot blend with the “natural tannins” present in the wine, tasters will first perceive the structure of the product and then, after a phase of hollow, they will perceive the exogenous tannins.

Few years later, another studied [197] has shown somewhat contradictory results in which wines treated with exogenous tannins, presented less harmony, equilibrium and persistence, accompanied by an increase of herbaceous aromas and astringency. In this case, only one gallotannin and a condensed tannin were tested in several wines, and it seems that they always found an increase of the astringency, dryness, and bitterness parameters. The results obtained means, that an addition of exogenous tannins does not improve wines body, nevertheless, only two tannins were tried.

A more recent study has proved that an addition of grape-seed tannin can contribute positively to the wine aroma and mouth feeling. Indeed, the authors have shown an improvement by this tannin addition of the fruity, wine body, astringency, color intensity and aroma quality of the wine. In addition, they have also observed a slight decrease of vegetable aroma. Moreover, a supplementation of wine with oak tannin, induced an increase of the astringency, which can affect the balance of young red wine [200].

These results proved that an addition of oenological tannin in winemaking can improve the sensory and mouthfeel properties of a wine, but they must be used in reasonable quantity to not induce organoleptic deviation. For example, doubling tannin concentration (e.g. 100 mg/L tannin addition to a wine containing 100 mg/L tannin) would likely provide a substantial improvement. This would probably not be the case, however, when 100 mg/L is added to a wine containing 1000 mg/L of tannins. In fact, although tannins contribute to the structure of wine, they are not always perfectly integrated and wines may lose their equilibrium, resulting in a hardening of wine and an increase in bitter sensations. Finally, it is important to choose the right tannin adapted to the grapevine cultivar and to the intrinsically properties of the wine.

Chapter 2: General experimental section

This chapter will present the common methods used in all the following chapters, and the specific methods corresponding to only one chapter will be described in the corresponding one.

1. Solvents and reagents

The 2,2'azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (≥ 98%, HPLC), (±)-6-hydroxy-2,5,7,8 tetramethylchromane-2-carboxylic acid (Trolox) (≥ 97%, ACS reagent), 2,2-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (≥ 97%, ACS reagent), gallic acid (≥ 99%, HPLC), copper (II) sulfate pentahydrate (≥ 98%, ACS reagent), iron (III) chloride hexahydrate (≥ 97%, ACS reagent), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (≥ 98%, ACS reagent), copper (II) chloride dihydrate (≥ 99%, ACS reagent), neocuproine (≥ 98%, ACS reagent), Folin-Ciocalteu reagent (≥ 98%, ACS reagent), L(+)-tartaric acid (≥ 99.5%, ACS reagent), sodium hydroxide (≥ 97%, ACS reagent), sodium carbonate (≥ 99.5%, ACS reagent), potassium persulfate (≥ 99%, ACS reagent), sodium phosphate monobasic (≥ 98%, ACS reagent), phosphate buffer solution (≥ 98%, ACS reagent), polyvinylpolypyrrolidone (PVVP), methyl-cellulose (viscosity 15 cP), ascorbic acid (≥ 99%, ACS reagent), phloroglucinol (≥ 99%, HPLC), ammonium sulfate (≥ 98%, ACS reagent), sodium acetate (≥ 99%, Molecular biology), Tween 80, syringaldazine (≥ 99%, Molecular biology), glycerol (≥ 99%, Molecular biology), L-histidine (≥ 99%, ACS reagent), copper sulfate (≥ 99.9%, Trace metal basis), sodium nitrate (≥ 99%, ACS reagent), sodium chlorate (≥ 98%, ACS reagent), potassium chlorate (≥ 99%, ACS reagent), calcium chloride dihydrate (≥ 99%, ACS reagent), iron (II) sulfate heptahydrate (≥ 99%, ACS reagent), potassium dihydrogen phosphate (≥ 99.5%, ACS reagent), magnesium sulfate heptahydrate (≥ 98%, ACS reagent), Bradford reagent, bovine serum albumin (BSA) (≥ 96%, ACS reagent) and ammonium acetate (≥ 98%, Molecular biology) were provided by Sigma Aldrich (St. Quentin, Fallavier, France). Malvidin-3-*O*-glucoside (≥ 95%, HPLC) and (-)-epicatechin (≥ 99%, HPLC) were purchased from Extrasynthese (Genay, France). Fluorescein (≥ 98%, ACS reagent), sodium acetate (≥ 99%, ACS reagent) and 2,4,6-triazine-s-tripyridyl (TPTZ) (≥ 99%, ACS reagent) were from Fluka Analytical (Munich, Germany). Sodium dihydrogen phosphate (≥ 98%, ACS reagent) and disodium hydrogen phosphate (≥ 99%, ACS reagent) were supplied by VWR Prolabo Chemicals (Fontenay-sur-Bois, France). D-(+)-glucose (≥ 99%, ACS reagent), peptone (≥ 99%, ACS reagent), agar (≥ 99%, ACS reagent) and yeast extract (≥ 99%, ACS reagent) was provided by Panreac (Barcelona, Spain). Potassium metabisulfite (≥ 98%, ACS reagent) was obtained from Acros Organics (Madrid, Spain).

Deionized water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Methanol (HPLC grade), ethanol (HPLC grade), formic acid, acetic acid, hydrochloric acid and acetaldehyde were purchased from Prolabo-VWR (Fontenay-sous-Bois, France). Acetic acid (HPLC grade), absolute ethanol and hydrochloric acid (HPLC grade) were obtained from Fisher Scientific (Illkirch, France).

Water (Optimal® LC / MS), methanol (Optimal® LC / MS), acetonitrile (Optimal® LC / MS) and formic acid (Optimal® LC / MS) were purchased from Fisher Scientific (Geel, Belgium) and used for UPLC-UV analyses coupled to high-resolution mass spectrometer.

Yeast (Zymaflore® Spark), bentonite (MICROCOL® ALPHA) and nutrients (Nutristart®) were provided by Laffort (Floirac, France).

2. Oenological tannins

Thirty-six commercial tannins are considered in the whole manuscript. Specifically, the following are analyzed: 17 proanthocyanidins comprising nine procyanidins/prodelphinidins (three from grapes, four from grape-seeds and two from grape-skin) and eight profisetinidins/prorobitenidins (two from acacia and six from quebracho), and 19 hydrolysable tannins comprising eight gallotannins (four from nut galls and four from tara) and 11 ellagitannins (eight from oak and three from chestnut).

These oenological tannins were provided by eight different international oenological companies: Laffort (Floirac, France), Agrovin (Ciudad Real, Spain), Sofralab (Magenta, France), Institut Oenologique de Champagne (IOC) (Epernay, France), Esseco (Trecate Novara, Italy), AEB (Brescia, Italy), Erblsöh (Geisenheim, Germany) and Vason (Verona, Italy).

3. U/HPLC systems coupled to different detectors (DAD, fluorescence or MS)

All the systems were HPLC or UPLC coupled to DAD detector. The ionization source was always and ESI source. Regarding the analyzer, two different types were used, which were the time-of-flight (TOF) or the triple-quadrupole (QQQ). Time-of-flight (TOF) mass detectors offer the advantage of high resolution and good sensitivity, since all generated ions are retained for detection, unlike quadrupole filters which retain only one at each moment.

3.1. System 1: HPLC-DAD-fluorescence-ESI-Quadrupole (mDP / anthocyanins)

The Thermo Scientific Accela system UPLC-DAD-fluorescence-ESI-MS (Quadrupole) consisted of a quaternary pump (Accela 600 Pump, 920175), a column compartment with sample injector (Accela Autosampler, 750466) and a diode array detector (PDA Detector, 930079) that was coupled to either an ESI-Quadrupole mass spectrometer (Accela MSQ Plus, MSQ 20808) or a Thermo Scientific high-sensitivity fluorescence detector (Dionex UltiMate 3000 FLD-3000). These different modules were controlled by Xcalibur software and Chromeleon software.

3.2. System 2: HPLC-DAD-ESI-QQQ (characterization of hydrolysable tannins)

The HPLC-DAD-ESI-QQQ system used was an Agilent 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6460-triple quadrupole (QQQ) mass spectrometer detector (Agilent, Waldbronn, Germany).

The QQQ used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI). These different modules were controlled by MassHunter software.

3.3. System 3: HPLC-DAD-ESI-QTOF (identification of degradation product of malvidin-3-*O*-glucoside)

The HPLC-DAD-ESI-QTOF used was an Agilent 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6545 quadrupole-time of flight (Q-TOF) mass spectrometer detector (Agilent, Waldbronn, Germany). The Q-TOF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source. These different modules were controlled by MassHunter software.

3.4. System 4: UPLC-DAD-ESI-QTOF (characterization of condensed tannins)

The UPLC-DAD-ESI-Q-TOF system used was an Agilent 1290 Infinity liquid chromatography system consisting of a 1290 Infinity binary pump, a 1290 Infinity Thermostated Column Compartment, a 1290 Infinity Auto sampler and a 1290 Infinity Diode Array Detector which was coupled to an ESI-Q-TOF mass spectrometer (Agilent 6530 Accurate Mass). These different modules were controlled by MassHunter software.

4. Colorimetry using the CIELAB method

The CIELAB colorimetry method is used for different experimental sections of the manuscript. Also, it is important to describe the system and the calculation method, since the CIELAB space is a complex one.

The CIELAB model, adopted by the CIE in 1976, is an alternative to the XYZ model. It represents the same color space but uses a different representation model. Like CIE XYZ, it models human vision and is independent of any material. The CIELAB model builds on the theory of opponents by drawing inspiration from Richard Hunter's former Model Lab (1942). In order to not confuse it with its predecessor, it is noted with asterisks L*a*b*. But today it is simply called CIELAB or CIE LAB, the official name being "1976 CIE L*a *b *".

4.1. CIELAB system

The CIELAB coordinates are defined here according to three axes (**Figure 40**). The X axis corresponds to the evolution of the color passing from green (- a*) to red (+ a*). This means that the more you move positively on the X axis, the closer you get to the red color. The Y axis for its part ranges from L* = 0 to L* = 100%. This axis corresponds to the transition from black to white and therefore to clarity. This means that the closer you get to zero on this axis, the more intense and marked the color will be. Conversely, the closer we get to 100%, the closer we get to the white, so the color will be much softer.

Finally, the Z axis corresponds to the transition from blue (- b*) to yellow (+ b*). This means that the more we move negatively on the Z axis and the closer we get to a blue color.

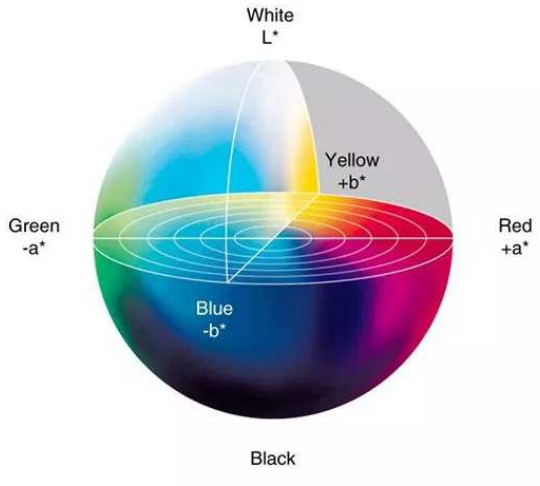


Figure 40: CIELAB color space

In addition, colors are generally classified according to three criteria: hue (color), lightness (clarity) and chroma. Lightness (L*) defines, the property according to which each color can be considered as equivalent to a member of the gray scale, between black and white. Color clarity increases or decreases along a vertical axis.

The hue and the chroma are analogous to polar coordinates and can be defined in the CIELAB space (**Figure 41**). The hue (h_{ab}) is the attribute according to which colors have been traditionally defined as red, green, blue or yellow for example. Indeed, h = 0 ° for the red hue and the direction + a*; h = 90 ° for the yellow hue and the direction + b*; h = 180 ° for the green tint and the direction - a*; h = 270 ° for the blue hue and the - b* direction. The chroma (C^*_{ab}), as color intensity is an attribute that allows each hue to be determined by its degree of difference in comparison to a gray color with the same lightness. The Chroma can increase or decrease with respect to a vertical point.

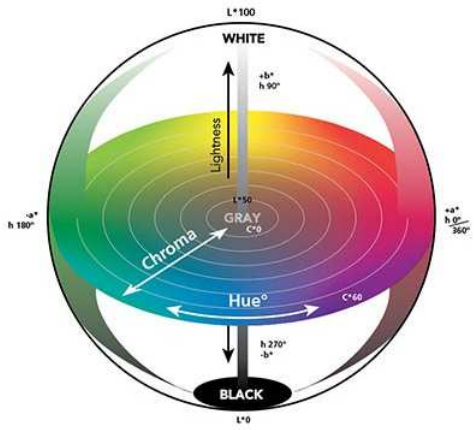


Figure 41: Representation of C^*_{ab} (chroma) and h_{ab} (hue) coordinates

Finally, Lightness (L*) and Chroma (C^*_{ab}) describe quantitative attributes of the color, whereas the red-greenness component (a*), the yellow-blueness (b*) and hue (h_{ab}) describe qualitative attributes.

4.2. Calculation methods

The calculation methods have been done according to the method proposed by the OIV (OIV-MA-AS2-11). In this way the different calculation are presented in **Table 2** and were realized using the free available MSCV software (https://www.unirioja.es/color/descargas.shtml). X, Y and Z are the trichromatic components defined by the International Commission of Lightning (CIE, 1976).

Table 2: Calculation of CIELAB parameters

Parameters	Symbol	Calculation
Lightness	L*	$L^* = 116\,(Y/Yn)^{1/3} - 16$
Red-greenness	a*	$a^* = 500\,[(X/Xn) - (Y/Yn)]$
Yellow-blueness	b*	$b^* = 200 - [(Y/Yn)^{1/3} - (Z/Zn)^{1/3}]$
Chroma	C^*_{ab}	$C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$
Hue	h_{ab}	$h^* = tg^{-1}\,(b^*/a^*)$
Colorimetric difference	ΔE_{ab}	$\Delta E_{ab} = (L^{*2} + a^{*2} + b^{*2})^{1/2}$

5. Winemaking trials of botrytized wines supplemented by oenological tannins

In this part, we explain how the white, "rosé" and red wines were obtained for all the experimentations conducted in the Chapter 6.

5.1. Selection of the *Botrytis cinerea* strain

The *B. cinerea* single-spore isolate 213, originally isolated from grapevine leaves in 1998, was selected from the collection of UMR (Research Mixed Unit) SAVE INRA, Bordeaux [201]. It was selected because of its virulence on grapevine leaves and berries and because it is a II-*transposa* type strain [201–203]. Indeed, there are three main genetic type of *B. cinerea* strains which are Group I, Group II-*vacuma* and Group II-*transposa*. The II-*transposa* group is the most virulent one and seems well-adapted to the grapes because it is dominant at every phenological stage and isolated from over wintering canes more than other types.

5.2. Small-scale white and "rosé" winemaking trials

In this section, it is explaining how the grapes were sampling and inoculated in order to process the healthy and botrytized grape-juice. Then the elaboration process of the white and "rosé" wines botrytized or not and supplemented or not by oenological tannins is explain.

5.2.1. Grape sampling

During the 2017 vintage, healthy grapes (*Vitis vinifera* cv. Muscat d'Alexandrie) were collected on September 18th (around 50 kg) from the experimental vineyard, planted in 1992, at the Enology Faculty of the University Rovira i Virgili in Constantí (AOC Tarragona; 41°8'54.17" N and 1°11'53.89" E).

The vineyard is at 87 m above sea level, and groundwater is located at a depth of around 4 m. The vines were trained on a vertical trellis system and arranged in rows 2.80 m apart, with 1.20 m spacing between vines. They were pruned using a double "Cordon de Royat" system, with 16 buds, 8 on each cane. Half of the grapes were kept at 4 °C to obtain healthy grape juice, whereas the other half was inoculated with *B. cinerea* strain 213.

5.2.2. Inoculation of white grapevine with *Botrytis cinerea*

The pathogen (*B. cinerea* strain 213) was inoculated on Yeast Peptone Dextrose (YPD) Petri plates (20 g/L of peptone and glucose, 10 g/L of yeast extract and 17 g/L of agar in distillated water) and grown about 1 week at 20 °C in an incubator. Half of the grapes harvested (around 25 kg) were placed in five plastic boxes (600x400x200 mm) and inoculated by spraying a spore suspension (1.10^6 conidia/mL, 1 drop of Tween 80 and 50 g/L of glucose in sterilized water) until the complete fruit surface was covered by the spore suspension. The plastic boxes containing the grapes were then incubated for around 3 weeks, at 20 °C, surrounded by two plastic boxes containing sterile water to maintain the humidity (90-100 %).

5.2.3. Recovery of healthy and botrytized grape juice

The healthy grapes were crushed and pressed to obtain a healthy grape juice using a small pneumatic press (Venmhidrprei-040, Invia, Vilafranca del Penedès, Spain). The juice was also recovered under dry ice to keep the grape juice protected from oxidation and was slightly sulfited (60 mg of $K_2S_5O_7$/L). This healthy grape juice was settled with previous addition of 20 mg/L of pectolytic enzymes at 4 °C for 18 hours.

The botrytized grapes were previously sorted visually to remove undesirable rotten berries due to other fungal development, notably by *Penicillium* spp. (blue-green color) or other fungal species (*Alternaria* spp. or *Clostridium* spp.), or acetic bacteria (red-pink color). Then, these selected botrytized grapes were crushed and pressed to obtain the botrytized grape juice using a small pneumatic press. The juice was also recovered under dry ice in order to keep it protected from oxidation, but without any addition of sulfur dioxide in order not to inhibit laccase activity. This botrytized grape juice was centrifuged at 8,500 rpm for 5 minutes.

Both grape juices, healthy and botrytized, were then immediately stocked in glass bottles at - 4 °C.

Two types of winemaking trials were performed using the grape juices obtained from the healthy and botrytized grapes. The first trial was carried out directly with the white grape juices (white winemaking), whereas the second trial was performed with the white grape juices supplemented with malvidin-3-*O*-glucoside (pseudo- "rosé" winemaking) in order to approach what happens in "rosé" winemaking.

This strategy was selected instead of using real "rosé" winemaking because our goal was to determine the protective effect of the different enological tannins on the color of anthocyanins without the presence of the natural proanthocyanidins from seeds and skins that can also exert an inhibitory effect on laccase activity.

5.2.4. Elaboration of white wines

All winemaking trials were carried out in plastic tubes of 30 mL which were used as fermentation vessels. Working solutions of the five enological tannins were prepared at 10 g/L in a model wine solution (12 % vol. of ethanol, 4 g/L of tartaric acid, pH adjusted to 3.5) for supplementing the different winemaking trials. The healthy grape juice was used without any treatment. However, the botrytized juice was drastically treated with PVPP (160 g/L) and centrifuged for 10 minutes at 8,500 rpm to eliminate the interferences that its dark brown color could cause in the final wines.

In the case of fermentations without laccase addition, 22 mL of healthy grape juice were added to each vessel. In the case of fermentations with laccase addition, 17 mL of healthy grape juice were supplemented with 5 mL of botrytized grape juice (corresponding to a 1.5 UL/mL of final volume). Immediately, to both fermentations, 1 mL of deionized water was added for the controls or 0.5 mL of each tannin solution and 0.5 mL of deionized water (corresponding to a dose of 20 g/hL), or 1 mL of each tannin solution (corresponding to a dose of 40 g/hL). All the vessels were inoculated with 1 mL of a solution containing the yeast (10 g/L of *Saccharomyces cerevisiae*, Zymaflore Spark®, Laffort, Floirac, France) and 1 mL of nutrient solution (10 g/L of Nutrient complex combining diammonium phosphate, inactivated yeasts and thiamine, Nutristart®, Laffort, Floirac, France).

The fermentations were carried out at room temperature (20 ± 2 °C) and were monitored by weighing the vessels. Residual sugars were determined to ensure that alcoholic fermentations were finished. All these fermentations were performed in triplicate.

5.2.5. Elaboration of "rosé" wines

All winemaking trials were carried out in plastic tubes of 30 mL which were used as fermentation vessels. Working solutions of the five enological tannins were prepared at 10 g/L in a model wine solution (12 % vol. of ethanol, 4 g/L of tartaric acid, pH adjusted to 3.5) for supplementing the different winemaking trials. The healthy grape juice was used without any treatment. However, the botrytized juice was drastically treated with PVPP (160 g/L) and centrifuged for 10 minutes at 8,500 rpm to eliminate the interferences that its dark brown color could cause in the final wines.

In the case of fermentations without laccase addition, 21 mL of healthy grape juice were added to each vessel. In the case of fermentations with laccase addition, 16 mL of healthy grape juice were supplemented with 5 mL of botrytized grape juice (corresponding to a 1.5 UL/mL of final volume).

Immediately, to both fermentations, 1 mL of deionized water was added for the controls or 0.5 mL of each tannin solution and 0.5 mL of deionized water (corresponding to a dose of 20 g/hL), or 1 mL of each tannin solution (corresponding to a dose of 40 g/hL). Additionally, to approach the "rosé" winemaking, 1ml of malvidin-3-*O*-glucoside was added to reach a final concentration of 50 mg/L. All the vessels were then inoculated with 1 mL of a solution containing the yeast (10 g/L of *Saccharomyces cerevisiae*, Zymaflore Spark®, Laffort, Floirac, France) and 1 mL of nutrient solution (10 g/L of Nutrient complex combining diammonium phosphate, inactivated yeasts and thiamine, Nutristart®, Laffort, Floirac, France).

The fermentations were carried out at room temperature (20 ± 2 °C) and were monitored by weighing the vessels. Residual sugars were determined to ensure that alcoholic fermentations were finished. All these fermentations were performed in triplicate.

5.3. Microvinification of red winemaking trials

The red winemaking was carried out at higher scale than for the white and "rosé" winemaking. According to this, the grape sampling, the inoculation and the elaboration of the red wines was some kind different from the two previous ones. This microvinifications were performed in duplicates.

5.3.1. Grape sampling

Mature grapes (*Vitis vinifera*, cv. Merlot) were collected from vineyard from Saint-Émilion (France) in 2017. The vineyard was planted on sandy-gravely or chalky-clay soils and the planting density presented a minimum density at planting of 5,500 vines/ha.

5.3.2. Inoculation of red grapevine with *Botrytis cinerea*

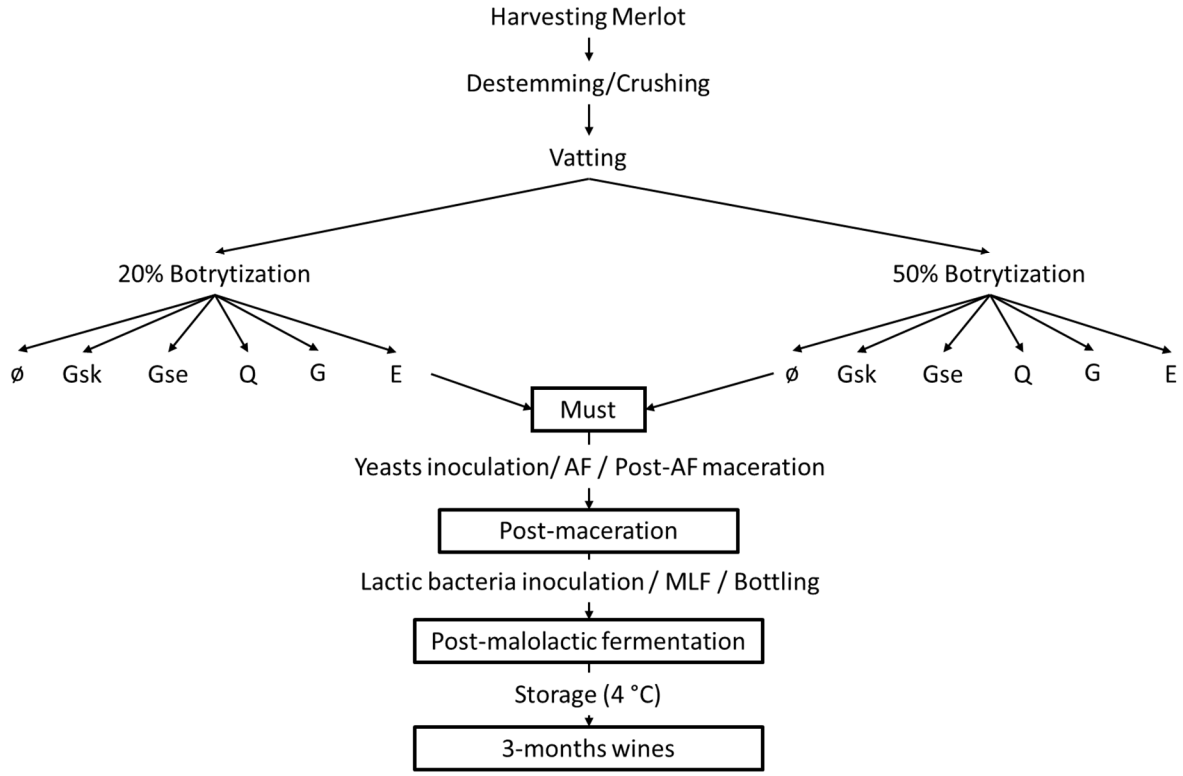
A part of healthy grape bunches was then inoculated, by spraying a spore and mycelium suspension from *Botrytis cinerea* strain 213 (1.4 x 10^5 spores/mL) until the total recovery of the fruit surface. Grape bunches were incubated in obscurity at 20 °C in a moist chamber (90% moisture) to improve mold development. A visual sorting of berries was then performed to remove undesirable rotten berries following a fungal development (blue-green color) due to *Penicillium* spp., *Alternaria* spp. or *Clostridium* spp. as well as development of acetic bacteria (red-pink color). Botrytized grapes were used for small-scale winemaking.

5.3.3. Elaboration of red wines

5.3.3.1. Vatting

In 2017, fruits totally recovered by mold were added to healthy fruits to make 3 different types of box (0, 20 or 50% of botrytized grapes) containing 9 kg of grapes in total. Each '9-kg grapes' batch was mechanically crushed, destemmed and collected in a 10-L aluminum tank.

A 3 g/hL dose of aqueous bisulfite solution 18% (Laffort, Bordeaux, France) was added only in control tanks (botrytization rate 0%). Five different oenological tannins were added in other tanks at a concentration of 100 g/hL. These commercial tannins were chosen to be representative of the different botanical origin mainly used, which are, grape-seed, grape-skin, quebracho, ellagitannin and gallotannin. The different modalities prepared are presented in **Figure 42**.



Ø: no addition of tannins. Gsk: grape-skin tannin; Gse: grape-seed tannin. Q: quebracho tannin; G: gallotannin; E: ellagitannin; AF: alcoholic fermentation. MLF: malolactic fermentation.

Figure 42: Modalities of small-scale winemaking from 20% and 50% botrytized grapes with or without tannins addition

5.3.3.2. Alcoholic fermentation

Saccharomyces cerevisiae yeasts (Zymaflore®FX10, Laffort) were prepared by rehydration in a warm water (37 °C) for 15 min. Rehydrated yeasts were applied to each fermentation tank in order to reach concentrations of 20 g/hL. An alcoholic fermentation activator (Thiazote®, Laffort) was added with the leaven when nitrogen was lower than 140 mg/L and was added during fermentation (when 1,06 kg/L density reached) to complete nutriments at 200 mg/L. Fermentations were conducted at 25 °C and temperature and density were monitored daily in each tank. The cap was punched down daily and an intake of 2 to 4 mg/L O_2 was brought when density reached 1.02. When densities were stable (0.993-0.997) for each batch and concentrations of reducing sugars were lower than 2 g/L, a 2 days' post-fermentation maceration was conducted at 30 °C under CO_2.

5.3.3.3. Malolactic fermentation

Wines were separated from the pomaces by moderate manual pressing and poured into 5 and 10 L glass bottles. Malolactic fermentations were conducted by inoculating a commercial *Oenococcus oeni* (Lactoenos® SB3 Direct, Laffort) at 1 g/hL. The decrease in malic acid concentrations was followed by enzymatic kit (R-Biopharm, Saint Didier au Mont d'Or, France). When they were lower than 0.2 g/L, finished wines were racked in 0.75 L bottles and 2 g/hL of aqueous bisulfite solution 18% was added. Bottles were stored at 4 °C. There were thirteen tanks in duplicate (1 x 2 with botrytization rate of 0%, 6 x 2 with botrytization rate of 20% and 6 x 2 with botrytization rate of 50%). Sampling was realized at different steps of winemaking: vatting (VAT), after post-alcoholic fermentation maceration (MAC), after malolactic fermentation (MALO), and 3 months after bottling.

5.3.3.4. Wines

All samples, musts and wines, were centrifuged 10 min at 4,500 rpm before analyses except for HPLC separation analyses and filtered on syringe with 0.45 mm pore size diameter filters.

5.3.3.5. Classical oenological analyses

In musts, total acidity, pH, organic acids (tartaric, malic, lactic and gluconic acids) concentrations, reducing and total sugars and consecutive probable alcohol were determined by Foss WineScanTM 79000 (Foss, Nanterre, France). For wines, reducing sugars, total acidity, pH, organic acids (tartaric, malic, lactic, gluconic) concentrations, residuals sugars, alcohol (% vol.) were measured by IRTF Foss WineScanTM 79000 (Foss, Nanterre, France). The results of this classical analysis are presented in **Table 3**.

Table 3: Classical oenological analysis of control musts and 3-month wines botrytized at 0, 20 and 50%

	Musts			3-month wines		
% *Botrytis*	**0%**	**20%**	**50%**	**0%**	**20%**	**50%**
Oenological parameters						
pH	4.8 ± 0.0	4.8 ± 0.0	4.7 ± 0.0	3.7 ± 0.0	3.8 ± 0.0	3.8 ± 0.0
Total acidity (g/L H_2SO_4 eq)	3.4 ± 0.0	3.7 ± 0.2	3.7 ± 0.3	3.5 ± 0.1	3.2 ± 0.1	3.6 ± 0.2
Density (g/dm³)	1104.7 ± 0.0	1103.5 ± 0.0	1106.3 ± 0.2	991 ± 0	993 ± 0	995 ± 1
Alcohol (% vol.)	-	-	-	12.8 ± 0.1	12.2 ± 0.0	12.3 ± 0.0
Volatile acidity (g/L H_2SO_4 eq.)	0.45 ± 0.00	0.53 ± 0.00	0.72 ± 0.04	0.34 ± 0.01	0.52 ± 0.02	0.90 ± 0.20
Sugars						
Reducing sugars (g/L)	167.4 ± 0.0	162.8 ± 0.4	164.6 ± 1.3	1.6 ± 0.1	1.4 ± 0.1	1.6 ± 0.3
Total sugars (g/L)	194.4 ± 0.0	192.2 ± 0.1	197.8 ± 0.0	6.2 ± 0.3	7.7 ± 0.2	10.2 ± 1.1
Organic acids						
Tartaric acid (g/L)	3.8 ± 0.0	3.4 ± 0.0	2.6 ± 0.2	2.0 ± 0.1	2.2 ± 0.0	2.4 ± 0.3
Malic acid (g/L)	2.0 ± 0.0	2.8 ± 0.1	2.6 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Lactic acid (g/L)	2.1 ± 0.0	1.8 ± 0.0	1.9 ± 0.1	1.9 ± 0.0	1.7 ± 0.0	1.5 ± 0.0

6. Statistical analysis

All the chemical and physical data are expressed as mean values ± standard deviation. The statistical analyses were carried out using the XLSTAT 2017 statistical package or RStudio software. The normality and homoscedasticity of the data were tested for all parameters by using the Shapiro-Wilk test and Levene's test respectively. When populations were distributed normally and presented homogeneity in variance, parametric tests (ANOVA and Tukey) were used to evaluate the existence and degree of significant differences. In contrast, when populations were not distributed normally and/or presented heterogeneity in variance, non-parametric tests (Kruskal-Wallis and Pairwise-Wilcox) were used. For the analysis of the "Botrytis", "tannin" and "Botrytis/tannin interaction" effects, we performed 2-factors ANOVAs despite the non-homoscedasticity of the variables (only 2 tanks per modality), normality being verified or low standard deviation (p-value > 0.001 for the Shapiro-Wilks test). Principal component analyses (PCA) were also performed using the XLSTAT 2017 statistical package or RStudio software, as well as Friedman test for the tasting.

Differences were statistically significant at p-value < 0.05 or p-value < 0.01 or p-value < 0.001 depending on the selectivity searched.

Chapter 3: Chemical characterization of oenological tannins

1. Introduction

The term 'tannins' includes different phenolic compounds that bind to and precipitate proteins from different vegetal origins such as nut galls, tara, oak, chestnut, as well as grape seeds and skins or other plant sources such as quebracho, mimosa and acacia [163]. Regarding this wide range of plant sources, tannins are usually classified into two families: hydrolysable and condensed tannins.

Hydrolysable tannins are classified into two subfamilies, gallotannins and ellagitannins. Gallotannins are polymers formed by esterification between D-glucose and gallic acid. Tannic acid is the commercial name for gallotannin extract comprising mixtures of polygalloyl quinic acid ester or polygalloyl glucoses [164]. The main sources of commercial gallotannins are nut galls and tara. Ellagitannins are polymers of ellagic, gallic and/or hexahydroxidiphenic acids [163]. To be more precise, a nonahydroxyterphenoyl unit (NHTP) is esterified in positions 2, 3 and 5 with a C-glycosidic bond, while an open-chain glucose is esterified in positions 4 and 6 with a hexahydroxydiphenoyl unit (HHDP) forming the chemical structure of ellagitannins [165]. They constitute one of the most important families of tannins with many biological features, such as antioxidant capacity [204]. The main sources of commercial ellagitannins are oak and chestnut woods.

Condensed tannins, come from different botanical origins, such as grapes, quebracho, mimosa and acacia. They are polymers of flavan-3-ol units, differing mainly by the monomer released after acidic cleavage, the degree of polymerization (mDP), and their levels of galloylation and ramification [163]. Condensed tannins are also known as proanthocyanidins, because they release anthocyanins by acidic cleavage in accordance with the Bate-Smith reaction. Grape-skin tannins are composed of procyanidins and prodelphinidins because their acidic cleavage gives cyanidin and delphinidin, whereas grape-seed tannins are composed only of procyanidins. Grape-skin tannins have a higher mDP and a low level of galloylation, while grape-seed tannins have a lower mDP and a high level of galloylation [58]. Quebracho tannins are profisetinidins, because their acidic cleavage gives fisetinidin, and they have a high level of ramification, while mimosa tannins are prorobitenidins because they release robitenidin [46]. Less is known about acacia tannins, but it seems they are composed of a mixture of profisetinidins, prorobitenidins and prodelphinidins [166].

According to the wide range of commercial tannins present in the market and their great chemical diversity, the main goal of this chapter is to carry out an exhaustive study to determine the richness in tannins and phenolic compounds of the thirty-six tannins used in the next chapters. Additionally, it is necessary and important to characterize also them by determining their composition in ellagitannins, gallotannins, procyanidins, prodelphinidins, prorobitenidins and profisetinidins.

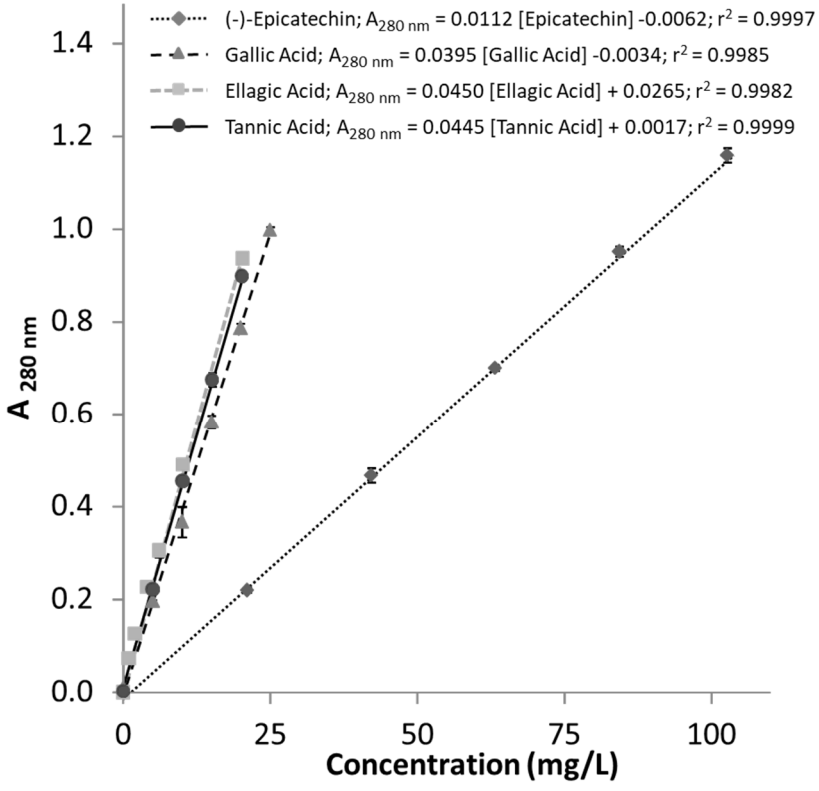
2. Materials and methods

2.1. Determination of polyphenol and tannin contents

All the oenological tannins were characterized using the analytical methods described below to determine their richness. In this part various methods were used to compare them and determine the most suitable one. In this way the richness in tannins and/or polyphenols of all the oenological tannins were determined. For this purpose, solutions of 2 g/L of each tannin were prepared in a synthetic model wine solution (12% vol. of ethanol, 4 g/L tartaric acid adjusted to pH 3.5 with sodium hydroxide). All the analyses were carried out at least in triplicate.

2.1.1. Total Polyphenol Index (TPI)

The total polyphenol index (TPI) was analyzed by measuring the 280 nm absorbance of a 1:100 dilution of tannin solutions with a spectrophotometer, using a 10 mm quartz cuvette and multiplying the absorbance value by 100 [171]. The tannin richness (g of tannin/100 g of commercial product) of the different oenological tannins was estimated by interpolating the TPI in two different calibration curves according to tannin type and regarding the original weight of the sample. **Figure 43** shows the A_{280} nm calibration curves for (-)-epicatechin, gallic acid, ellagic acid and tannic acid.



All data are the mean ± SD of three replicates.

Figure 43: A_{280nm} calibration curves of (-)-epicatechin, gallic acid, ellagic acid and tannic acid

These clearly indicate that gallic acid, ellagic acid and tannic acid have similar absorptivity coefficients (expressed in L/mg.cm), whereas that of (-)-epicatechin is around 4-fold times lower.

Proanthocyanidins were therefore interpolated in a (-)-epicatechin calibration curve because this is the main subunit of condensed tannins. In contrast, gallotannins are composed mainly of glucose and gallic acid and should therefore be interpolated in a gallic acid calibration curve or, even better, a tannic acid calibration curve, since this tannic acid is available at a high purity level. Ellagitannins, on the other hand, are composed mainly of glucose and ellagic acid and consequently should be interpolated in an ellagic acid calibration curve. The impossibility of obtaining commercial vescalagin or another pure ellagitannin in enough quantity and the poor solubility of ellagic acid led us use tannic acid as standard for ellagitannins too.

2.1.2. Folin-Ciocalteu assay

The total phenolic content (TPC) was measured using the Folin-Ciocalteu reagent in accordance with the proposed method using 96-well microplates [205]. Stock solutions were diluted around 10 times to reach the interval of sensitivity for the method. For the measurement, 20 µL of gallic acid standard solution (0.00625–0.2 mg/L) or oenological tannin solution, 100 µL of diluted Folin-Ciocalteu reagent (1:10, v/v with distillated water) and 80 µl of 7.5% (w/v) Na_2CO_3 buffer were placed in each well. The reduction of phosphotungstate-phosphomolybdate heteroply acid salts at alkaline pH was measured at 760 nm and 25 °C after 30 min of reaction. The reagent blank was obtained by the addition of 20 µL of distillated water instead of sample. The results are expressed as richness (g of gallic acid equivalents/100 g of commercial product).

2.1.3. Bate-Smith assay

All the oenological tannins were analyzed using an adaptation of the Bate-Smith method [206]. Briefly, a fixed volume of oenological tannin solutions (20 µL), water (1.48 mL) and hydrochloric acid (1.5 mL, 37%) were placed in two tubes. Tube A was then placed in an ice bath (0 °C) while tube B was placed in warm bath (100 °C). After standing in their respective bath for 30 min, 600 µL of ethanol was added to the samples to stop the reaction. The proanthocyanidins concentration (g/L) was obtained by multiplying the difference in absorbance at 550 nm (ΔA_{550}) between tube B and tube A by 19.33, which is the absorptivity coefficient of cyanidin after the acidic cleavage of the condensed tannins (Bate-Smith reaction). The results are expressed as richness (g of tannin/100 g of commercial product).

2.1.4. Tannin analysis by the methyl-cellulose precipitation method

All the oenological tannins were analyzed using the methyl-cellulose precipitation method [207]. Briefly, a fixed volume of oenological tannin solutions (125 µL for condensed tannins and 75 µL for ellagitannin and gallotannin) were placed in two centrifuge tubes. Tube A received an addition of 1 mL of methylcellulose solution (0.4%, w/v) and 1 mL of saturated ammonium sulfate solution. The final volume was made up to 5 mL with distillated water.

Tube B was prepared similarly, but the added methyl cellulose solution was omitted and replaced with distillated water. After standing at room temperature for 20 min, the samples were centrifuged, and absorbance measured at 280 nm. The difference in absorbance (ΔA_{280}) between tube B and tube A corresponds to the tannin content of the sample. The tannin richness (g of tannin/100 g of commercial product) of the condensed tannins was estimated by interpolating ΔA_{280} in a (-)-epicatechin calibration curve, while the hydrolysable tannin richness was estimated using tannic acid as calibration standard, as described for the TPI analysis.

2.1.5. Analysis of proanthocyanidins following acid catalysis with phloroglucinol

Acid-catalyzed depolymerization of proanthocyanidins in the presence of an excess of phloroglucinol (phloroglucinolisis method) was used to analyze the proanthocyanidin content, monomeric composition and mDP of all the oenological condensed tannins [208]. A 0.1 N solution of hydrochloric acid in methanol containing 10 g/L of ascorbic acid and 50 g/L of phloroglucinol was prepared. 200 µL of commercial tannin solution (10 g/L) and 200 µL of the previous solution were incubated at 50 °C for 20 min, then combined with 5 volumes of 40 mM aqueous sodium acetate to stop the reaction. Phloroglucinol adducts were analyzed by reversed-phase HPLC using an Xterra RP18 (100×4.6 mm, 3.5 µm) column protected by a guard column with the same characteristic. A binary gradient with mobile phases containing 1% v/v aqueous acetic acid (mobile phase A) and methanol (mobile phase B) was used. The elution conditions were 1.0 mL/min; 5% B for 25 min; a linear gradient from 5 to 20% B in 20 min; a linear gradient from 20 to 32% B in 15 min; and a linear gradient from 32 to 100% B in 2 min. The column was then washed with 100% B for 5 min and reequilibrated with 5% B for 5 min before the next injection. The volume injection was 20 µL and the eluting peaks were monitored at 280 nm. To calculate the apparent mean degree of polymerization (mDP), the sum of all subunits (flavan-3-ol monomer and phloroglucinol adduct, expressed in moles) was divided by the sum of all flavan-3-ol monomers (expressed in moles). The richness (g of proanthocyanidin/100 g of commercial product) was calculated considering the sum of all subunits (excluding the phloroglucinol portion in the phloroglucinol adducts) as regards the original weight of the commercial product.

2.1.6. Tannin analysis by the OIV method

All the oenological tannins were analyzed by official OIV method [209]. First, a stock solution of tannins (solution A) was prepared by adding 6 g of tannins to 950 mL of warm distillated water (60-70 °C). After 30 min at room temperature, the solution was cooled in a bath thermostated at around 20 °C and the volume completed to 1 L. Total solids (TS) measurement was done by determining the dry weight of 25 mL of solution A after complete evaporation in an aluminium dish.

Soluble solids (SS) measurement was done similarly with the supernatant of 25 mL of solution A after centrifuging (4000 rpm; 5 min) and filtering (0.22 µm GSWP pore size diameter, Millipore, Tullagreen, Ireland) to eliminate possible insoluble substances. Finally, the non-phenolic solids (NPS) were determined using an SPE column with polyvinylpolypyrrolidone (PVPP). 7.0 g of PVPP was rehydrated in the column with a 20% hydroalcoholic solution for 15 min, then placed on the vacuum manifold. The column was activated by carrying out three washes (50 mL of ethanol (20% v/v), 50 mL of distillated water and 50 mL of solution A). 30 mL of solution A was then put at the top of the column and the eluate was collected in a flask. This was stopped when the liquid reached the level of the upper frit and 25 mL of the eluate was taken and transferred to an aluminum dish to determine the dry weight. A blank measurement (BK) was also taken by doing the same as for the NPS but adding 30 mL of distillated water instead of solution A. The tannin richness (% tannins) was estimated using the following equation:

$$\%Tannins = (SS - NPS - BK) / TS \times 100$$

2.2. Molecular characterization

In addition to the previous results obtained in the first part, all the oenological tannins were characterized using the analytical methods described below to determine their composition. In this part various methods were used in order to determine for each tannin in function of its family its composition in the main compounds characteristics of an ellagitannin, gallotannin, procyanidin/prodelphinidin or profisetinidin/prorobitenidin. All the analyses were carried out in triplicate.

2.2.1. Hydrolysable tannins

Concerning hydrolysable tannins, the main compounds characteristics of an ellagitannin and a gallotannin were quantified. Vescalagin, castalagin, grandinin and roburin A, B, C, D and E were quantified for each ellagitannin (chestnut and oak tannins) analyzed.

Concerning gallotannins (nut gall and tara tannins), gallic acid and digallic acid were quantified as the principal compounds present in both nut gall and tara tannins. Then, quinic acid, 3-galloylquinic acid, 4-galloylquinic acid and 5-galloylquinic acid were quantified since they are known to be representative of a tara tannin. Trigalloyl-glucose, tetragalloyl-glucose, pentagalloyl-glucose, hexagalloyl-glucose, heptagalloyl-glucose, octagalloyl-glucose, nonagalloyl-glucose and decagalloyl-glucose have been quantified as they are known to be representative of a nut gall tannin. Nevertheless, all these compounds were quantified in both tannins, nut galls and tara because they are present in both type of tannins.

2.2.1.1. Ellagitannins

An aqueous solution of 5 g/L of each ellagitannin (chestnut and oak tannins) was prepared and filtered on 0.45 µm (pore size diameter) whatman filters. The HPLC was a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6460-triple quadrupole (QQQ) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was MassHunter Workstation (version B.08.00). The QQQ used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the negative ionization mode and the following parameters were set: capillary voltage, 4500 V; fragmentor, 135; gas temperature, 350 °C; drying gas, 5 L/min; nebulizer, 50 psi; sheath gas temperature, 250 °C; sheath gas flow, 10 L/min; acquisition range, 100-2000 m/z; and fixed collision energy of 8 V. Samples were analyzed by injection (10 µL) on a Phenomenex Kinetex column (150 × 3.0 mm, 2.6 µm particle size; Phenomenex, Torrance, CA, USA) thermostated at 30 °C. The solvent system, at a flow rate of 0.4 mL/min, was water acidified with 0.1% of formic acid (solvent A) and methanol acidified with 0.1% of formic acid (solvent B). The elution gradient was (time, % of solvent A): 0 min, 99.0%; 2 min, 98.0%; 5 min, 97.0%; 6 min, 96.5%; 7 min, 96.0%; 8 min, 95.5%; 10 min, 95.0%; 14 min, 90.0%; 17 min, 85.0%; 23 min, 80.0%; 35 min, 1.0% and then, 10 min equilibrium time was left between analysis. Compounds were identified using the "Scan Segments" that allowed to enter the exact mass of the compounds searched. Quantification was performed using the ESI-SIM of each corresponding mass, which are presented in **Table 4**.

Table 4: Main compounds present in ellagitannins

Compounds	Chemical formula	m/z	2 (m/z)
Castalagin	$C_{41}H_{26}O_{26}$	934.1	
Vescalagin	$C_{41}H_{26}O_{26}$	934.1	
Roburin A	$C_{82}H_{50}O_{51}$	-	925.2
Roburin B	$C_{87}H_{58}O_{55}$	-	991.2
Roburin C	$C_{87}H_{58}O_{55}$	-	991.2
Roburin D	$C_{82}H_{50}O_{51}$	-	925.2
Roburin E	$C_{46}H_{34}O_{30}$	1066.1	
Grandinin	$C_{46}H_{34}O_{30}$	1066.1	

Figure 44 and **Figure 45** shows the obtained chromatogram for a chestnut and oak tannin as an example. Vescalagin calibration curve was prepared at 200, 100, 50, 40, 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 mg/L and was used as external standard. All the samples were analyzed in triplicates. Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000.

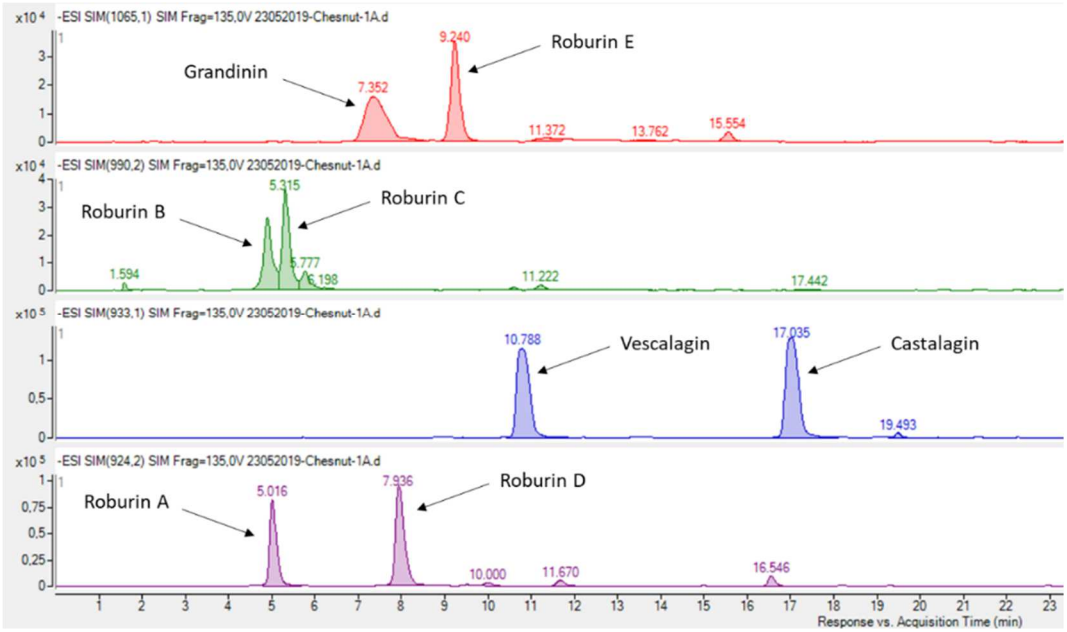


Figure 44: Example of an ESI-SIM spectra of a chestnut tannin

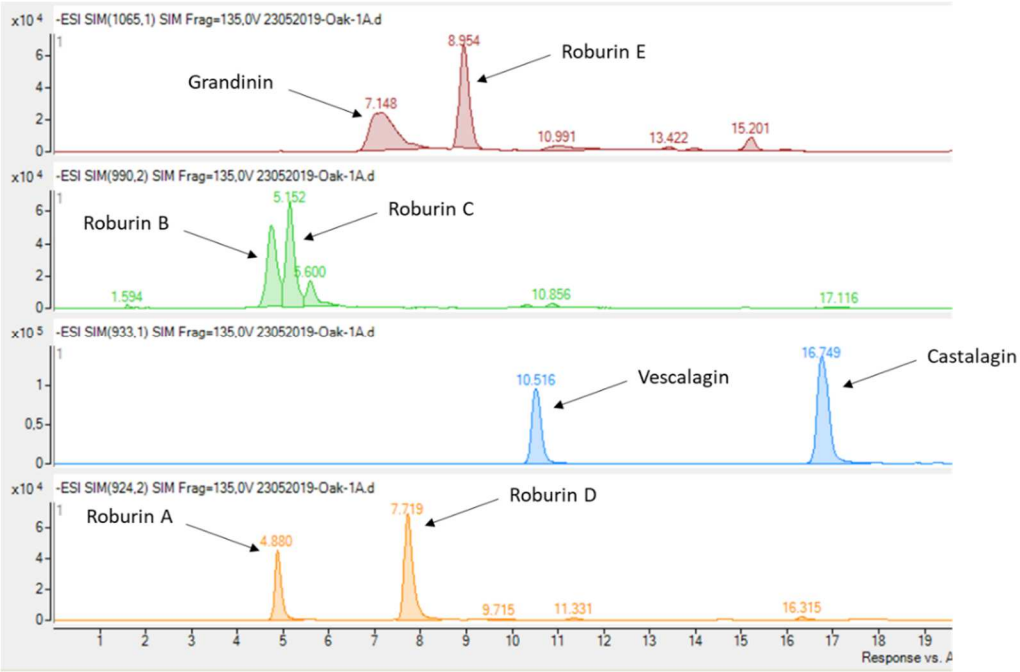


Figure 45: Example of an ESI-SIM spectra of an oak tannin

2.2.1.2. Gallotannins

An aqueous solution of 5 g/L of each gallotannin (nut gall and tara tannins) was prepared and filtered on 0.45 µm (pore size diameter) whatman filters. The HPLC was a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6460-triple quadrupole (QQQ) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was MassHunter Workstation (version B.08.00).

The QQQ used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the negative ionization mode and the following parameters were set: capillary voltage, 3500 V; fragmentor, 135; gas temperature, 300 °C; drying gas, 5 L/min; nebulizer, 45 psi; sheath gas temperature, 250 °C; sheath gas flow, 10 L/min; acquisition range, 100-2000 m/z; and fixed collision energy of 8 V. Samples were analyzed by injection (10 µL) on a Phenomenex Kinetex column (150 × 3.0 mm, 2.6 µm particle size; Phenomenex, Torrance, CA, USA) thermostated at 30 °C. The solvent system, at a flow rate of 0.4 mL/min, was water acidified with 0.1% of formic acid (solvent A) and methanol acidified with 0.1% of formic acid (solvent B). The elution gradient was (time, % of solvent A): 0 min, 99.0%; 2 min, 98.0%; 5 min, 97.0%; 6 min, 96.5%; 7 min, 96.0%; 8 min, 95.5%; 10 min, 95.0%; 14 min, 90.0%; 17 min, 85.0%; 23 min, 10.0%; 25 min, 8.0%; 29 min, 5.0%; 34 min, 1.0%; 45 min, 99.0% and then, 10 min equilibrium time was left between analysis. Compounds were identified using the "Scan Segments" that allowed to enter the exact mass of the compounds searched. Quantification was performed using the ESI-SIM of each corresponding mass, which are presented in **Table 5**.

Table 5: Main compounds present in gallotannins

Compounds	Chemical formula	m/z
Gallic acid	$C_7H_6O_5$	170.014
Digallic acid	$C_{14}H_{10}O_9$	322.225
Quinic acid	$C_7H_{12}O_6$	192.167
3-galloylquinic acid	$C_{28}H_{24}O_{18}$	648.089
4-galloylquinic acid	$C_{35}H_{28}O_{22}$	800.099
5-galloylquinic acid	$C_{42}H_{32}O_{26}$	952.692
Trigalloyl-glucose	$C_{27}H_{24}O_{18}$	636.471
Tetragalloyl-glucose	$C_{34}H_{28}O_{22}$	788.576
Pentagalloyl-glucose	$C_{41}H_{32}O_{26}$	940.681
Hexagalloyl-glucose	$C_{48}H_{36}O_{30}$	1092.786
Heptagalloyl-glucose	$C_{55}H_{40}O_{34}$	1244.891
Octagalloyl-glucose	$C_{62}H_{44}O_{38}$	1396.996
Nonagalloyl-glucose	$C_{69}H_{48}O_{42}$	1548.101
Decagalloyl-glucose	$C_{76}H_{52}O_{46}$	1701.206

Gallic acid calibration curve was prepared at 100, 75, 50, 25, 10, 5 and 1 mg/L and was used as external standard.

Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000. **Figure 46** and **Figure 47** shows the obtained chromatogram for a nutgall and a tara tannin as an example. All the samples were analyzed by triplicate.

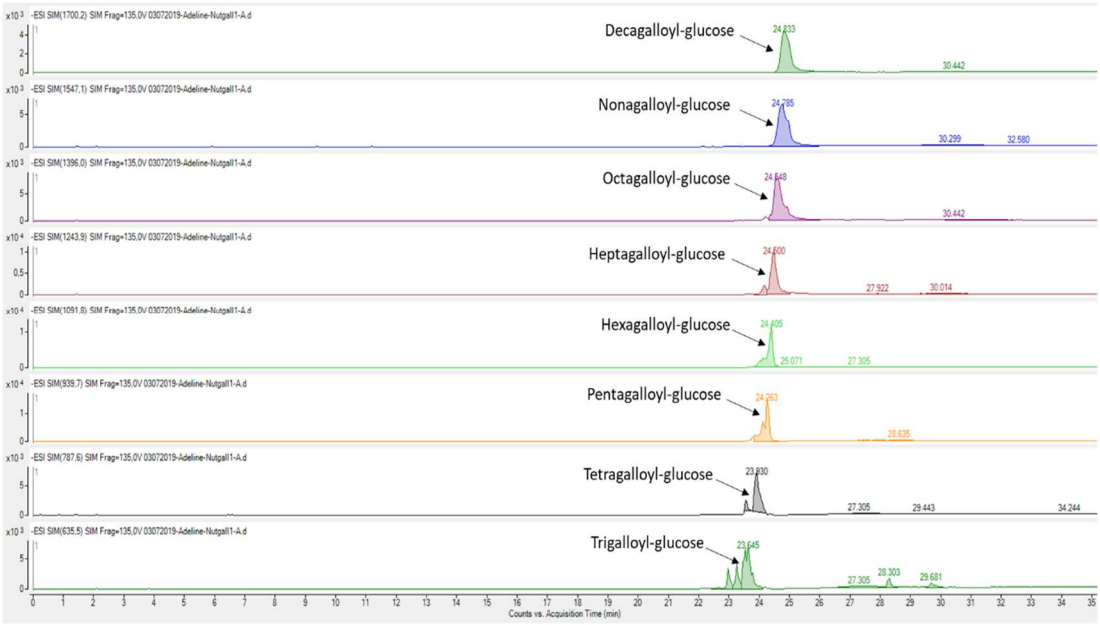


Figure 46: Example of an ESI-SIM spectra of nut gall tannin

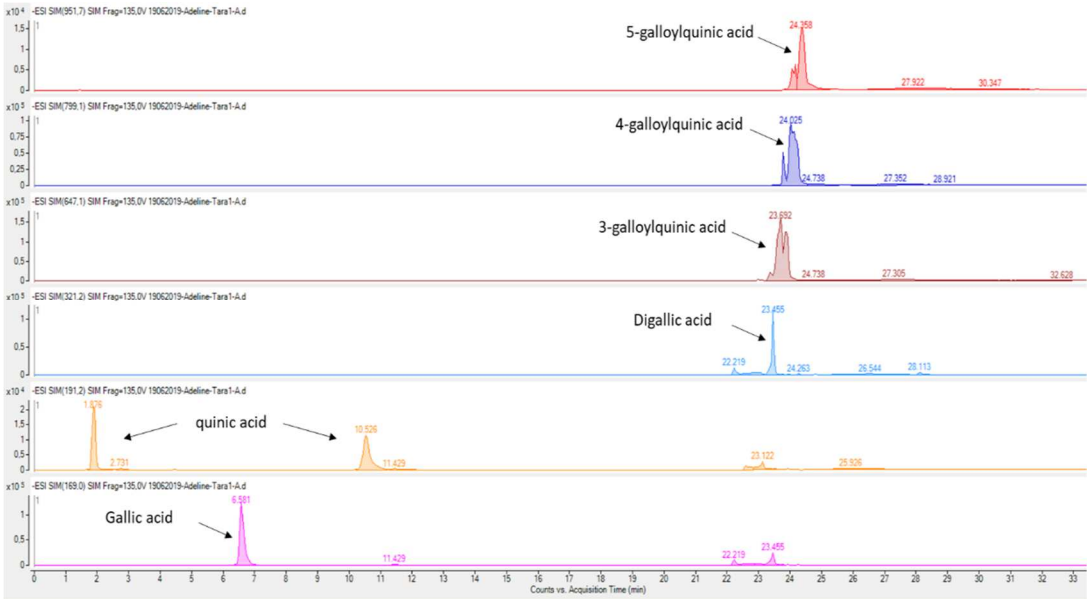


Figure 47: Example of an ESI-SIM spectra of tara tannin

2.2.2. Condensed tannins

Concerning condensed tannin, the main compounds characteristics of a procyanidins/prodelphinidins tannins and a profisetinidins/prorobitenidins tannins were quantified.

In this way, for a procyanidins/prodelphinidins tannin (grape, grape-skin and grape-seed tannins), (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, dimers B1, B2, B3 and B4, B2-3-*O*-gallate and trimers were quantified.

Concerning profisetinidins/prorobitenidins (quebracho and acacia tannins) various compounds were quantified. Indeed, (+)-catechin and (-)-epicatechin were quantified as monomers constitutive of both types of tannins (quebracho and acacia).

Then the dimers, trimers and tetramers of (+)-catechin and fisetinidinol were quantified since they are known to be representative of a quebracho tannin. Concerning acacia tannins, (-)-epicatechin-3-*O*-gallate, present in grape tannins were also found and quantified. Additionally, dimers of fisetinidol-gallocatechin and dimers of robinetinidol-catechin were quantified as well as chalcan-flavan dimers also called Gambiriin. Finally, trimers of catechin-fisetinidol-robinetinidol or gallocatechin-fisetinidol-fisetinidol and trimers of robinetinidol-robinetinidol-catechin or gallocatechin-fisetinidol-robinetinidol were found and quantified.

2.2.2.1. Procyanidins/Prodelphinidins

An aqueous solution of 0.5 g/L of each procyanidins/prodelphinidins tannins (grape, grape-seed and grape-skin tannins) was prepared and filtered on 0.45 µm (pore size diameter) whatman filters. The UPLC was a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6530-quadrupole-time of flight (Q-TOF) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was MassHunter Workstation (version B.08.00). The Q-TOF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the negative ionization mode and the following parameters were set: capillary voltage, 3500 V; fragmentor, 200; gas temperature, 300 °C; drying gas, 9 L/min; nebulizer, 25 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; acquisition range, 100-3000 m/z. Samples were analyzed by injection (10 µL) on an Eclipse Plus C-18 column (2.1 × 100 mm, 1.8 µm particle size; Agilent, France). The solvent system, at a flow rate of 0.3 mL/min, was water acidified with 0.1% of formic acid (solvent A) and methanol acidified with 0.1% of formic acid (solvent B). The elution gradient was (time, % of solvent A): 0 min, 99.0%; 0.5 min, 94.0%; 20 min, 50.0%; 25 min, 0.0%; 32 min, 94.0% and then, 10 min equilibrium time was left between analysis. Compounds were identified using the "Scan Segments" that allowed to enter the exact mass of the compounds searched. Quantification was performed using the ESI-EIC of each corresponding mass, which are presented in **Table 6**.

Table 6: Main compounds present in procyanidins/prodelphinidins (grape, grape-skin and grape-seed tannins)

Compounds	Chemical formula	m/z
(+)-catechin	$C_{15}H_{14}O_6$	290.072
(-)-epicatechin	$C_{15}H_{14}O_6$	290.072
(-)-epicatechin-3-*O*-gallate	$C_{22}H_{18}O_{10}$	442.084
Dimer B1	$C_{30}H_{26}O_{12}$	578.137
Dimer B2	$C_{30}H_{26}O_{12}$	578.137
Dimer B3	$C_{30}H_{26}O_{12}$	578.137
Dimer B4	$C_{30}H_{26}O_{12}$	578.137
B2-3-*O*-gallate	$C_{37}H_{30}O_{16}$	730.148
Trimers	$C_{45}H_{38}O_{18}$	866.198

(+)-catechin calibration curve was prepared at 100, 80, 60, 40, 20, 10 and 5 mg/L and was used as external standard.

Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000. **Figure 48**, **Figure 49** and **Figure 50** shows the obtained chromatogram for a grape, grape-skin and grape-seed tannin as an example. All the samples were analyzed by triplicate.

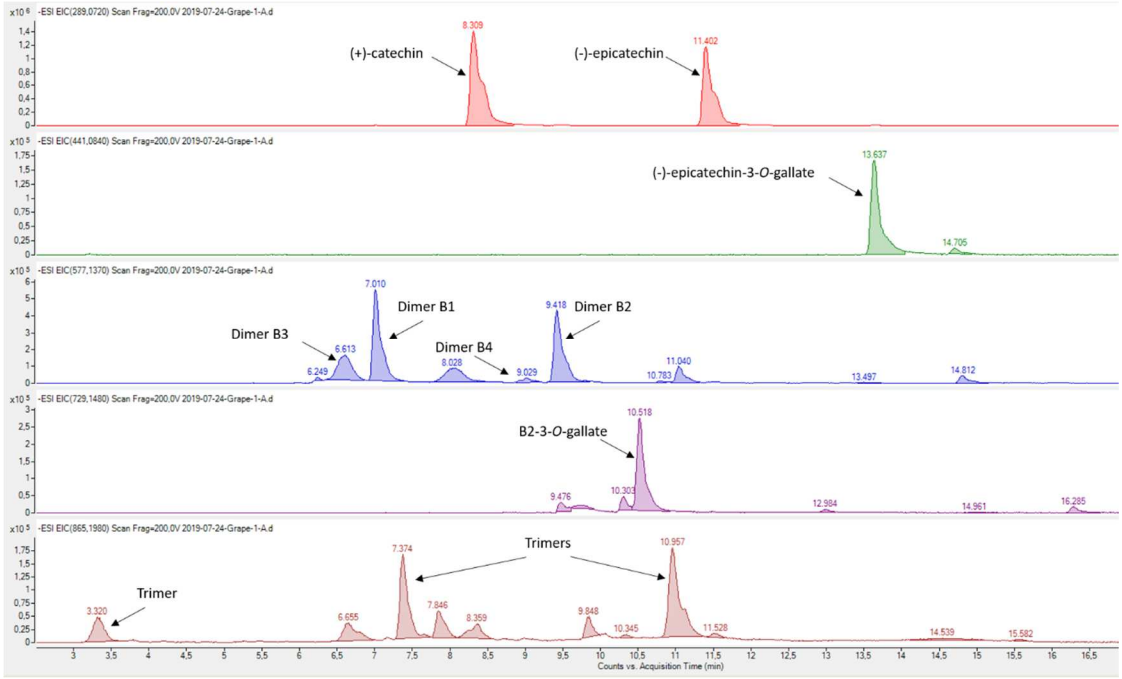


Figure 48: Example of an ESI-EIC spectra of a grape tannin

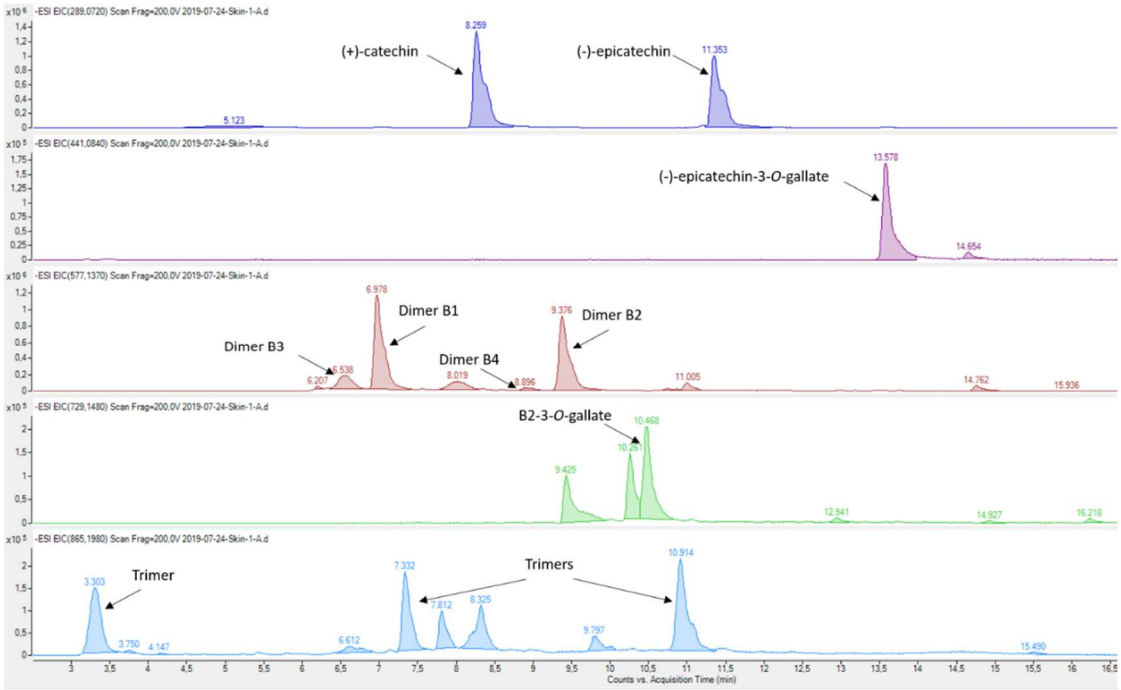


Figure 49: Example of an ESI-EIC spectra of a grape-skin tannin

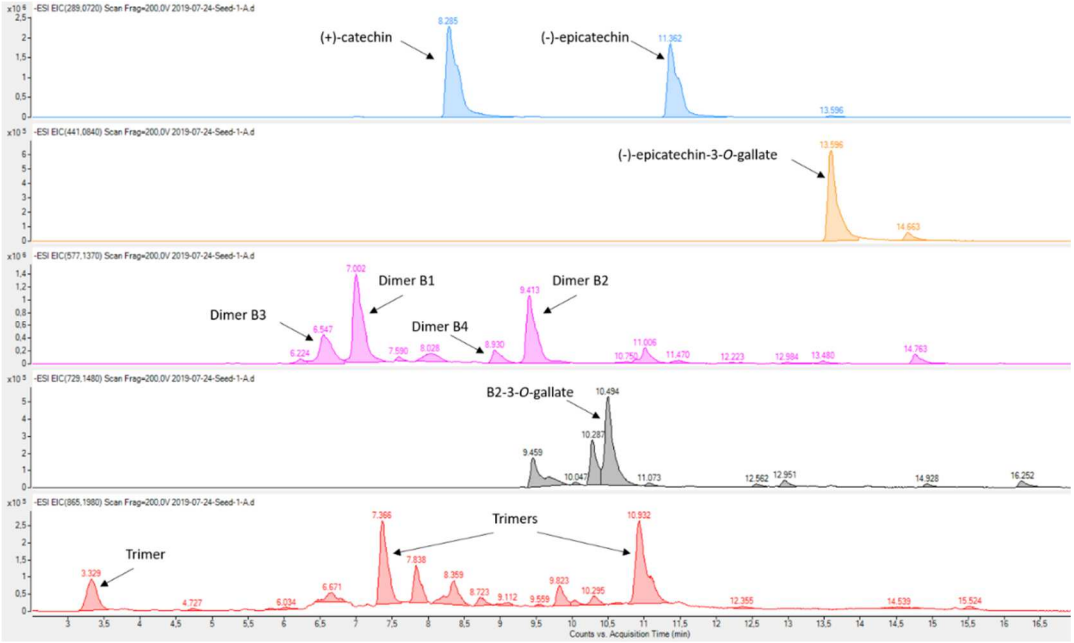


Figure 50: Example of an ESI-EIC spectra of a grape-seed tannin

2.2.2.2. Profisetinidins/Prorobitenidins

An aqueous solution of 0.5 g/L of each profisetinidins/prorobitenidins tannins (acacia and quebracho tannins) was prepared and filtered on 0.45 µm (pore size diameter) whatman filters. The UPLC was a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6530-quadrupole-time of flight (Q-TOF) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was MassHunter Workstation (version B.08.00). The Q-TOF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the negative ionization mode and the following parameters were set: capillary voltage, 3500 V; fragmentor, 200; gas temperature, 300 °C; drying gas, 9 L/min; nebulizer, 25 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; acquisition range, 100-3000 m/z. Samples were analyzed by injection (10 µL) on an Eclipse Plus C-18 column (2.1 × 100 mm, 1.8 µm particle size; Agilent, France). The solvent system, at a flow rate of 0.3 mL/min, was water acidified with 0.1% of formic acid (solvent A) and methanol acidified with 0.1% of formic acid (solvent B). The elution gradient was (time, % of solvent A): 0 min, 99.0%; 0.5 min, 94.0%; 20 min, 50.0%; 25 min, 0.0%; 32 min, 94.0% and then, 10 min equilibrium time was left between analysis. Compounds were identified using the “Scan Segments” that allowed to enter the exact mass of the compounds searched. Quantification was performed using the ESI-EIC of each corresponding mass, which are presented in **Table 7** and **Table 8**.

Table 7: Main compounds present in quebracho tannins

Compounds	Chemical formula	m/z
(+)-catechin	$C_{15}H_{14}O_6$	290.072
(-)-epicatechin	$C_{15}H_{14}O_6$	290.072
Dimers catechin-fisetinidol	$C_{30}H_{26}O_{11}$	562.142
Trimers catechin-fisetinidol	$C_{45}H_{38}O_{16}$	834.209
Tetramers catechin-fisetinidol	$C_{60}H_{50}O_{21}$	1106.270

Table 8: Main compounds present in acacia tannins

Compounds	Chemical formula	m/z
(+)-catechin	$C_{15}H_{14}O_6$	290.072
(-)-epicatechin	$C_{15}H_{14}O_6$	290.072
(-)-epicatechin-3-*O*-gallate	$C_{22}H_{18}O_{10}$	442.372
Dimers fisetinidol–gallocatechin	$C_{30}H_{26}O_{12}$	578.142
Dimers robinetinidol–catechin	$C_{30}H_{26}O_{12}$	578.142
Chalcan-flavan dimers (Gambiriin)	$C_{30}H_{28}O_{12}$	580.150
Trimers catechin-fisetinidol-robinetinidol or gallocatechin-fisetinidol-fisetinidol	$C_{45}H_{38}O_{17}$	850.200
Trimers robinetinidol-robinetinidol-catechin or gallocatechin-fisetinidol-robinetinidol	$C_{45}H_{38}O_{18}$	866.198

(+)-catechin calibration curve was prepared at 100, 80, 60, 40, 20, 10 and 5 mg/L and was used as external standard.

Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000. **Figure 51** and **Figure 52** shows the obtained chromatogram for a quebracho and an acacia tannin as an example. All the samples were analyzed by triplicate.

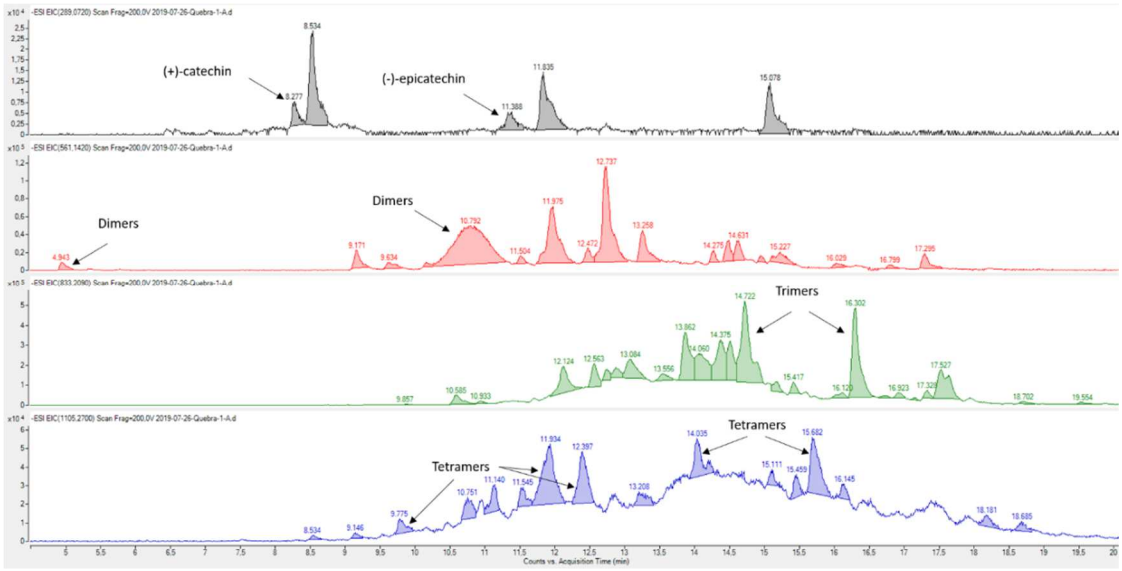


Figure 51: Example of an ESI-EIC spectra of a quebracho tannin

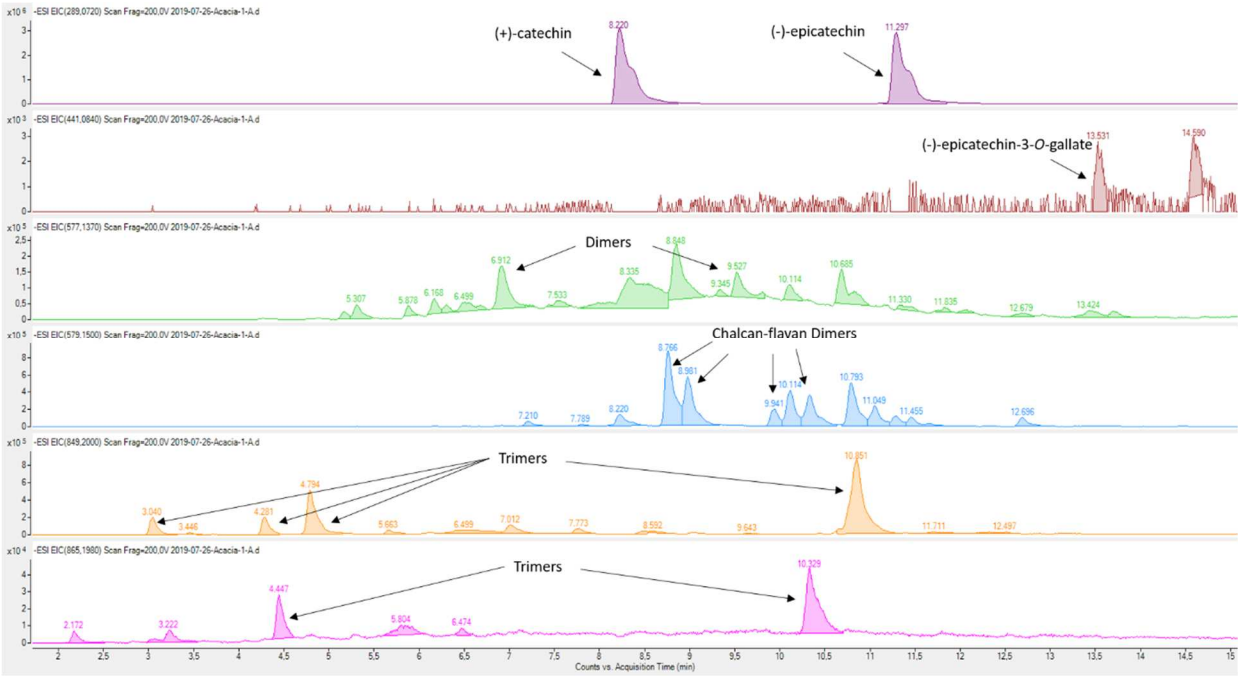


Figure 52: Example of an ESI-EIC spectra of an acacia tannin

3. Results and discussion

3.1. Determination of polyphenol and tannin contents

Table 9 shows the polyphenol and tannin contents of the 36 oenological tannins. The different tannins were grouped in 2 families of condensed tannins: procyanidins/prodelphinidins (PC/PD) and profisetinidins/prorobitenidins (PF/PR); and 2 families of hydrolysable tannins: gallotannins (GT) and ellagitannins (ET). Each family was in turn classified according to different botanical origin.

Indeed, procyanidins/prodelphinidins include tannins from grapes, grape-seeds and grape-skins meanwhile profisetinidins/prorobitenidins include tannins from acacia and quebracho.

Concerning hydrolysables tannins, ellagitannins include tannins from chestnut and oak meanwhile gallotannins include tannins from nut galls or tara. Richness was expressed in all cases as a percentage (%w/w; g of tannin per 100 g of commercial product).

Table 9: Chemical characterization of the different oenological tannins

	Tannins	Folin-Ciocalteu		TPI				Methyl-cellulose				Bate-Smith		Phloroglucinolisis						OIV Method	
		Richness (%)		Richness (%) as EC		Richness (%) as TA		Richness (%) as EC		Richness (%) as TA		Richness (%)		Richness (%)		mDP		%Gal		%Pro	Richness (%)	
Procyanidins/ Prodelphinidins (PC/PD)	grape 1	47.9 ± 0.8		50.9 ± 6.4				44.5 ± 3.1				68.0 ± 0.2		31.3 ± 0.9		3.9 ± 0.1		36.2 ± 0.8		1.5 ± 0.3	59.6 ± 2.4	
	grape 2	65.0 ± 1.3		66.9 ± 4.0				98.3 ± 6.4				102.0 ± 0.1		29.6 ± 0.3		5.9 ± 0.1		47.8 ± 0.3		nd	72.6 ± 3.3	
	grape 3	58.2 ±0.7		55.7 ± 2.4				74.5 ± 3.6				84.3 ± 0.3		30.1 ± 0.5		3.2 ± 0.1		38.0 ± 0.8		0.0 ± 0.0	73.9 ± 3.8	
	AV Grapes	**57.0 ± 8.6**	**A**	**57.8 ± 8.2**	**A**			**72.5 ± 27.0**	**A**			**84.8 ± 17.0**	**A**	**30.3 ± 0.9**	**B**	**4.3 ± 1.4**	**A**	**40.7 ± 6.2**	**A**	**0.8 ± 1.1**	**68.7 ± 7.9**	**A**
	seed 1	39.7 ± 1.7		39.9 ± 2.1				47.1 ± 1.7				53.9 ± 0.2		30.6 ± 0.4		2.4 ± 0.1		28.1 ± 0.7		nd	57.4 ± 3.9	
	seed 2	63.2 ± 3.1		54.0 ± 3.7				76.2 ± 9.4				38.9 ± 3.0		30.4 ± 0.1		4.3 ± 0.4		44.3 ± 0.4		0.3 ± 0.1	69.2 ± 2.4	
	seed 3	78.9 ± 4.4		60.8 ± 0.6				74.5 ± 4.5				95.4 ± 1.6		31.3 ± 0.9		4.1 ± 0.2		19.4 ± 0.3		1.7 ± 0.8	74.8 ± 5.5	
	seed 4	55.2 ± 1.0		51.2 ± 1.9				69.2 ± 3.9				94.7 ± 1.5		31.6 ± 1.5		4.8 ± 0.1		29.3 ± 0.4		N.D.	73.1 ± 0.7	
	AV Seeds	**59.2 ± 16.3**	**A**	**51.5 ± 8.7**	**A**			**66.8 ± 13.4**	**A**			**70.7 ± 28.7**	**A**	**31.0 ± 0.6**	**B**	**3.9 ± 1.0**	**AB**	**30.3 ± 10.3**	**A**	**1.0 ± 1.0**	**68.7 ± 7.8**	**A**
	skin 1	53.2 ± 0.8		41.8 ± 1.6				48.9 ± 3.6				65.5 ± 0.8		31.7 ± 0.3		3.4 ± 0.0		34.2 ± 0.5		nd	61.1 ± 2.4	
	skin 2	53.8 ± 0.6		49.4 ± 2.2				64.7 ± 3.8				88.4 ± 0.2		31.0 ± 0.2		4.7 ± 0.0		40.3 ± 0.6		nd	74.0 ± 1.9	
	AV Skins	**53.5 ± 0.4**	**A**	**45.6 ± 5.3**	**A**			**56.8 ± 11.2**	**A**			**76.9 ± 16.2**	**A**	**31.4 ± 0.5**	**B**	**4.1 ± 0.9**	**A**	**37.3 ± 4.3**	**A**	**nd**	**67.6 ± 9.2**	**A**
	AV PC/PD	**56.6 ± 2.9**	**α**	**51.6 ± 6.1**	**β**			**65.4 ± 7.9**	**β**			**77.5 ± 7.0**	**χ**	**30.9 ± 0.5**		**4.1 ± 0.2**		**36.1 ± 5.3**		**0.9 ± 0.2**	**68.3 ± 0.7**	**αβ**
Profisetinidins/ Prorobitenidins (PF/PR)	acacia 1	44.7 ± 1.7		29.9 ± 0.8				21.2 ± 0.9				80.7 ± 0.2		nd		nd		nd		nd	40.9 ± 1.4	
	acacia 2	40.5 ± 1.8		27.1 ± 5.0				30.8 ± 3.0				37.5 ± 0.3		nd		nd		nd		nd	37.1 ± 0.3	
	AV Acacia	**42.6 ± 2.9**	**A**	**28.5 ± 2.0**	**A**			**26.0 ± 6.8**	**A**			**59.1 ± 30.6**	**A**	**nd**		**nd**		**nd**		**nd**	**39.0 ± 2.7**	**A**
	quebracho 1	44.5 ± 3.2		51.5 ± 0.6				78.8 ± 3.9				57.3 ± 0.6		nd		nd		nd		nd	72.5 ± 1.9	
	quebracho 2	56.8 ± 3.1		55.5 ± 1.1				87.0 ± 3.1				48.6 ± 3.0		nd		nd		nd		nd	71.6 ± 4.0	
	quebracho 3	47.0 ± 3.1		50.3 ± 1.0				71.9 ± 3.4				51.8 ± 2.9		nd		nd		nd		nd	65.9 ± 7.2	
	quebracho 4	53.9 ± 2.0		63.4 ± 4.5				82.1 ± 3.1				63.1 ± 0.3		nd		nd		nd		nd	78.6 ± 0.3	
	quebracho 5	50.1 ± 1.2		62.5 ± 1.1				73.1 ± 6.5				52.5 ± 0.3		nd		nd		nd		nd	79.5 ± 6.9	
	quebracho 6	65.8 ± 2.8		98.7 ± 4.6				110.1 ± 4.0				29.4 ± 0.1		nd		nd		nd		nd	82.2 ± 1.1	
	AV Quebracho	**53.0 ± 7.7**	**A**	**63.7 ± 18.0**	**B**			**83.8 ± 14.0**	**B**			**50.4 ± 11.5**	**A**	**nd**		**nd**		**nd**		**nd**	**75.0 ± 6.1**	**B**
	AV PF/PR	**47.8 ± 7.3**	**α**	**46.1 ± 24.9**	**β**			**54.9 ± 40.9**	**β**			**54.8 ± 6.1**	**β**	**nd**		**nd**		**nd**		**nd**	**57.0 ± 25.5**	**α**
Gallotannins (GT)	nut galls 1	53.8 ± 1.4				48.0 ± 3.2				82.9 ± 4.1		4.8 ± 0.5									72.6 ± 3.3	
	nut galls 2	54.3 ± 2.4				51.5 ± 2.7				61.9 ± 4.2		0.0 ± 0.7									84.6 ± 5.3	
	nut galls 3	56.0 ± 1.0				47.5 ± 0.5				66.6 ± 13.3		0.0 ± 0.6									72.9 ± 3.4	
	nut galls 4	76.6 ± 2.1				45.8 ± 1.7				87.9 ± 11.6		2.8 ± 0.7									84.2 ± 2.4	
	AV Nut galls	**60.2 ± 11.0**	**A**			**48.2 ± 2.4**	**A**			**74.8 ± 12.5**	**A**	**1.9 ± 2.3**	**A**								**78.6 ± 6.7**	**A**
	tara 1	62.7 ± 1.5				41.7 ± 0.3				49.5 ± 1.5		0.0 ± 0.1									87.5 ± 7.7	
	tara 2	69.2 ± 2.6				43.9 ± 3.1				50.6 ± 4.0		2.3 ± 0.3									85.6 ± 7.2	
	tara 3	66.6 ± 0.9				45.1 ± 1.9				50.4 ± 3.7		2.2 ± 0.3									83.2 ± 1.1	
	tara 4	81.2 ± 3.9				51.6 ± 1.4				100.3 ± 1.8		0.1 ± 0.4									83.3 ± 3.4	
	AV Tara	**69.9 ± 8.0**	**A**			**45.6 ± 4.3**	**A**			**62.7 ± 25.1**	**A**	**1.1 ± 1.3**	**A**								**84.9 ± 2.1**	**A**
	AV NG/T (GT)	**65.0 ± 6.9**	**α**			**46.9 ± 1.9**	**β**			**68.8 ± 8.6**	**β**	**1.5 ± 0.5**	**α**								**81.8 ± 4.5**	**β**
Ellagitannins (ET)	chestnut 1	65.9 ± 0.8				21.3 ± 1.1				14.9 ± 1.0		11.5 ± 0.6									69.3 ± 3.4	
	chestnut 2	44.0 ± 2.2				18.6 ± 1.0				12.5 ± 1.1		8.7 ± 0.9									67.5 ± 2.2	
	chestnut 3	64.5 ± 2.5				21.7 ± 0.4				16.2 ± 1.0		0.1 ± 0.0									69.8 ± 3.0	
	AV Chestnut (C)	**58.1 ± 12.3**	**A**			**20.5 ± 1.7**	**A**			**14.5 ± 1.9**	**A**	**6.8 ± 6.0**	**A**								**68.9 ± 1.2**	**A**
	oak 1	41.5 ± 0.8				16.1 ± 1.7				12.0 ± 1.4		17.0 ± 0.2									55.7 ± 2.2	
	oak 2	30.2 ± 0.6				19.4 ± 0.5				18.0 ± 3.5		1.8 ± 0.3									66.1 ± 1.0	
	oak 3	72.3 ± 2.7				17.3 ± 0.4				12.4 ± 0.8		0.1 ± 2.9									72.0 ± 4.5	
	oak 4	48.1 ± 0.8				19.8 ± 0.4				16.4 ± 1.9		16.2 ± 0.5									66.9 ± 2.1	
	oak 5	54.4 ± 7.0				17.2 ± 0.6				10.6 ± 2.0		6.9 ± 0.2									65.5 ± 4.1	
	oak 6	54.2 ± 1.3				32.3 ± 2.9				12.1 ± 0.7		0.0 ± 0.7									66.7 ±4.3	
	oak 7	26.4 ± 0.3				18.1 ± 0.2				11.5 ± 0.6		6.9 ± 0.3									73.5 ± 4.4	
	oak 8	53.7 ± 1.4				17.1 ± 0.9				10.6 ± 1.2		10.2 ± 9.9									61.9 ± 4.6	
	AV Oak (O)	**47.6 ± 14.8**	**A**			**19.7 ± 5.3**	**A**			**13.0 ± 2.7**	**A**	**7.4 ± 6.7**	**A**								**66.0 ± 5.5**	**A**
	AV C/O (ET)	**52.9 ± 7.4**	**α**			**20.1 ± 0.6**	**α**			**13.7 ± 1.1**	**α**	**7.1 ± 0.4**	**α**								**67.5 ± 2.0**	**αβ**

All data are the mean ± SD of three replicates. TPI: Total Phenolic Index, EC: (-)-epicatechin, TA: Tannic Acid, mDP: medium Degree of Polymerization, %Gal: % of galloylation, %Pro: % of proanthocyanidin, nd: not detectable. Different capital letters indicate the existence of significant differences between tannins of the same family ($p < 0.05$). Different Greek letters indicate the existence of significant differences between tannin ($p < 0.05$).

When tannin richness was calculated by the Folin-Ciocalteu method using gallic acid as the calibration standard, the average richness of all the oenological tannins studied was 55.6 ± 2.2% with a minimal value of 30.2 ± 0.6% and a maximal value of 81.2 ± 3.9%. However, no significant differences were found between the richness of the different tannin families, which ranged from 47.8 ± 7.3% in the case of PF/PR tannins to 65.0 ± 6.9% for GT. Neither was any significant difference detected between the different botanical origins of the tannins in each family. The lack of significance in the richness of any of the different tannin types is probably due to the non-specificity of the Folin-Ciocalteu method, since this analytical procedure determines not only tannins but other phenolic compounds as well [210].

The second method used for estimating tannin richness was TPI. According to the data, the average richness of the tannins was somewhat lower than that obtained by the Folin-Ciocalteu method (41.2 ± 14.3%). The richness of the different tannins ranges from 16.1 ± 1.7 to 98.7 ± 4.6%, explains the high standard deviation and indicates the existence of a wide variability among oenological tannins. No significant differences were found between the richness of the two families of condensed tannins (PC/PD: 51.6 ± 6.1%; PF/PR: 46.1 ± 24.9%) and gallotannins (GT: 46.9 ± 1.9%). However, the richness of the ellagitannins was significantly lower (ET: 20.1 ± 0.6%). Within the PF/PR family, the richness of the quebracho tannins (63.7 ± 18.0%) was significantly higher than that of the acacia tannins (28.5 ± 2.0%). In contrast, no significant differences were found between the different botanical origins of the other tannin types. It would therefore appear that the TPI method, using the different calibration curves according to tannin type, is more discriminating than the Folin-Ciocalteu method even though it is not specifically for tannins but detects all types of phenolic compounds.

The methyl-cellulose precipitation method was also used for determining the richness of the different oenological tannins. In theory this method is more specific for tannin estimation than the previous two [207] since it only analyzes the phenolic compounds that precipitate with methylcellulose rather than all those present in the commercial product. The average richness obtained for all the tannins by this method was 50.7 ± 17.8%, an intermediate value between those obtained by the Folin-Ciocalteu and TPI methods, with values ranging between 10.6 ± 1.2% and 110.1 ± 4.0%. This again confirms the wide heterogeneity of the oenological tannins. As with the TPI method, no significant differences were found between the richness of the family of condensed tannins (PC/PD: 65.4 ± 7.9%; PF/PR: 54.9 ± 40.9%) and gallotannins (GT: 68.8 ± 8.6%), while the richness of ellagitannins was significantly lower (ET: 13.7 ± 1.1%). Within each family of tannins, no significant differences were observed with regards to botanical origin, except for the PF/PR tannins. The high standard deviation detected in this family was due to the great differences observed between quebracho (83.8 ± 14.0%) and acacia tannins (26.0 ± 7.0%).

The Bate-Smith reaction was then applied, nevertheless, is specific reaction for condensed tannins [210] and consequently it makes no sense applying this method to hydrolysable tannins.

However, due to the great heterogeneity of the commercial tannins, it was interesting to apply the reaction to check all the commercial tannins. Indeed, it is a simple way to discriminate the two tannin families (hydrolysable and condensed tannins). As expected, this method provided significantly higher richness for condensed tannins (61.2 ± 23.1%) than for hydrolysable tannins (4.3 ± 4.0%). The very low richness detected in hydrolysable tannins may be because these commercial tannins contain a very low proportion of proanthocyanidins. However, it is more likely to be the result of other unspecific reactions that can cause browning under strong reaction conditions (100 °C for 30 min) like the Maillard reaction. Since the Bate-Smith reaction is followed by an increase in absorbance at 550 nm, the appearance of brown pigments can lead to an overestimation. According to this method the richness of PC/PD tannins was significantly higher than that of PF/PR (77.5 ± 7.0% and 44.9 ± 7.8% respectively). The lower richness of PF/PR tannins may be due to a lower real tannin content, but it must be highlighted that the other methods detected no significant differences between PF/PR and PC/PD tannins either. Another possible explanation could be that PF/PR are more resistant to acidic cleavage [211], thereby reducing the proportion of anthocyanins generated by the Bate-Smith reaction.

The analysis of proanthocyanidins by acidic cleavage in the presence of an excess of phloroglucinol and the subsequent HPLC analysis of the subunits obtained (phloroglucinolisis method) was applied only to the condensed tannins. In fact, the method is specifically for these kinds of tannins and because the Bate-Smith reaction gave very low richness for all hydrolysable tannins. All the PC/PD family of tannins gave rise to similar richness regardless of whether they came from grape-seeds, grape-skins or the whole berry, with an average of 30.9 ± 0.5% for a range of values between 29.6 ± 0.3% and 31.7 ± 0.3%. In contrast, when this method was applied to the PF/PR tannin family the results were below the detection limit. Other authors have also reported that quebracho tannins could not be analyzed by acidic cleavage in the presence of another nucleophile like toluene-α-thiol [209,212]. This together with the lower yield for these tannins obtained by the Bate-Smith reactions indicates that profisetinidins and prorobitenidins are more resistant to acidic cleavage regardless of the nucleophile. Finally, the current OIV method was also applied to determine the richness of the different oenological tannins. This method determines all phenolic compounds and not just specifically tannins since it is based on the gravimetric measurement of all the phenolic compounds retained by a column of PVPP. The average richness obtained by this method was 68.7 ± 10.2% with a minimal value of 37.1 ± 0.3% and a maximal value of 87.5 ± 7.7%. No significant differences were found between condensed tannins (62.7 ± 8.0%) and hydrolysable tannins (74.7 ± 10.1%). Overall, the richness of the PC/PD family of tannins was like that of the PF/PR tannins. Moreover, no significant differences were found between the different sources (grape, grape-seeds or grape-skins) in the case of PC/PD tannins.

However, quebracho tannins have significantly higher richness than acacia tannins (75.0 ± 6.1% and 39.0 ± 2.7% respectively) within the PF/PR family of tannins. Of the hydrolysable tannins, gallotannins have significantly higher richness than ellagitannins (81.8 ± 4.5% and 67.5 ± 2.0% respectively). No statistical differences were found among the different botanical origins in either type of hydrolysable tannin.

Overall, these results are very complex because each analytical method provides different results, which is only to be expected given that each method has a different chemical basis. The Folin-Ciocalteu, TPI and OIV methods are in fact, not specifically for tannins because they analyze all types of phenolic compounds, whereas the Bate-Smith reactions and phloroglucinolisis are specifically for condensed tannins. The methyl-cellulose precipitation method, on the other hand, determines all types of tannins because it measures the difference in absorbance at 280 nm before and after precipitating them with methyl-cellulose.

For these reasons, and to better understand the information provided by each of these analytical methods for the different types of oenological tannins, a principal component analysis (PCA) was performed using those parameters.

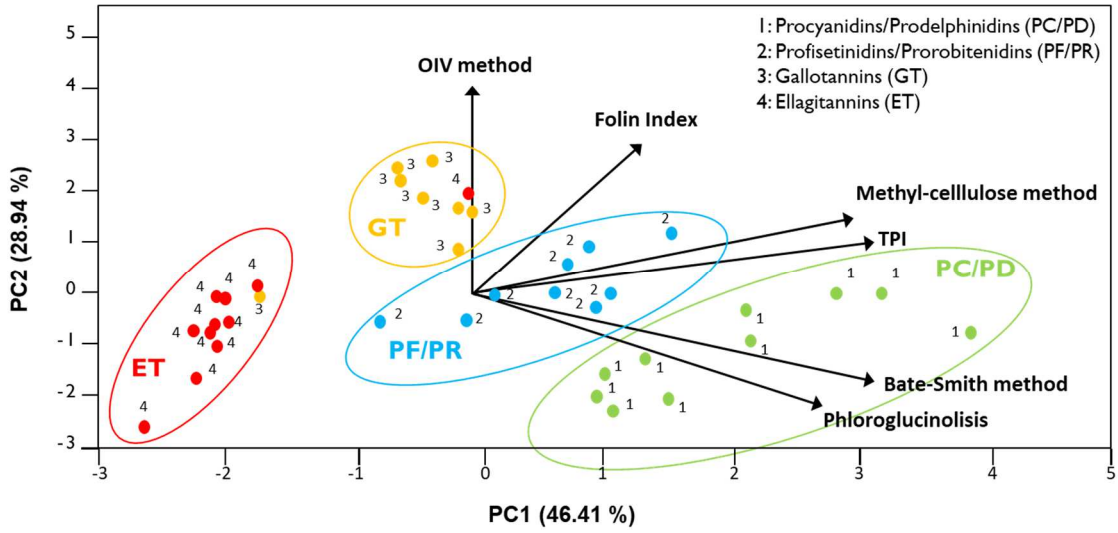


Figure 53: Plot of varimax-rotated principal component analysis of tannins richness in function of the used method

Figure 53 shows the plot of varimax rotated PCA for the different oenological tannins. The first component explains 46.41% of the variance and the second 28.94%, which means that the aggregate variance explained by these two components was 75.35%. PCA enabled the different oenological tannins to be separated with only two incorrect classifications (one gallotannin was classified as ellagitannin and one ellagitannin as gallotannin). PC1 placed ellagitannins on the left meanwhile procyanidins/prodelphinidins tannins were placed on the right. Gallotanins and profisetinidins/prorobitenidins were place in the center.

PC2 enabled gallotannins and profisetinidins/prorobitenidins to be separated, locating gallotannins above profisetinidins/prorobitenidins. The loadings are presented as arrows, the length and direction of which indicate the contribution made by both components. Hence the methyl-cellulose, TPI, Bate-Smith and phloroglucinolisis methods contribute mainly to PC1, the OIV method to PC2 and the Folin-Ciocalteu to both components. These data simply indicate that the samples placed more to the right have higher richness with the methyl-cellulose, TPI, Bate-Smith and phloroglucinolisis methods and that samples located further up have higher richness with the OIV method. This meaning that with all these methods, in general ellagitannins presented the lowest richness meanwhile procyanidins/prodelphinidins presented the highest richness.

3.2. Molecular characterization

The molecular characterization was achieved for the thirty-six oenological tannins according to their family. As explained in the material and methods, four different methods were used, one for each family (ellagitannins, gallotannins, procyanidins/prodelphinidins and profisetinidins/prorobitenidins). According to this, it was possible to determine the molecular composition of each tannin according to the major compounds known to be present in this kind of structure.

3.2.1. Hydrolysable tannins

Concerning hydrolysable tannins, molecular characterization from one hand was done for the ellagitannins (oak and chestnut tannins) and from the other hand done for the gallotannins (tara and nut gall tannins).

3.2.1.1. Ellagitannins

Regarding ellagitannins, the eight-principal molecular ellagitannins (castalagin, vescalagin, roburin A, B, C, D, E and grandinin) were quantified for the three chestnut tannins and the eight oak tannins, using vescalagin as standard.

Table 10 show the concentration (mg equivalent vescalagin/g of tannins) of the main compounds present in ellagitannins and the total concentration in molecular ellagitannins.

In general, castalagin and vescalagin were the two compounds mainly present in ellagitannins independently if they are extract from chestnut or oak wood. Indeed, the concentrations were ranking from 57.4 ± 1.8 to 94.5 ± 1.4 mg eq. vescalagin/g of tannins and from 35.2 ± 1.0 to 70.6 ± 0.9 mg eq. vescalagin/g of tannins for castalagin and vescalagin respectively. The only exceptions were tannins oak 2 and oak 4 which presented poor richness for these two compounds with values ranking from 7.6 ± 0.1 to 7.7 ± 0.2 and from 7.9 ± 0.1 to 8.6 ± 0.2 mg eq. vescalagin/g of tannins for castalagin and vescalagin respectively.

Additionally, it should be highlighted that in the case of chestnut tannins, roburin B and C were the two molecular ellagitannins with the lowest concentration in the extracts with values ranking from 9.2 ± 0.4 to 11.7 ± 0.5 and from 7.5 ± 0.2 to 9.6 ± 0.3 mg eq. vescalagin/g of tannins respectively. On the contrary, in the case of oak tannins roburin B and C were present in low concentrations but roburin A was the lowest present in the extracts with values ranking from 2.2 ± 0.1 to 32.1 ± 0.4 mg eq. vescalagin/g of tannins. Once again oak 2 and oak 4 tannins presents differences from the others oak tannins with very low concentration in roburin D additionally to the very low concentration in roburin A as the other oak tannins. Regarding the total concentration in molecular ellagitannins, oak tannins present in general higher concentration than chestnut tannins with values ranking from 227.0 ± 8.9 to 409.1 ± 7.2 and from 184.8 ± 5.0 to 247.4 ± 3.1 mg eq. vescalagin/g of tannins respectively. Nevertheless, oak 2 and oak 4 present lower total richness (74.0 ± 2.2 and 67.4 ± 2.2 mg eq. vescalagin/g of tannins respectively) in molecular ellagitannins than all the other tannins independently if they are chestnut or oak tannins. In order to compare easily oak and chestnut tannins and to determine if the botanical origin (chestnut or oak) of the ellagitannins have a major importance, two groups were made with from one hand the three chestnut tannins and from the other hand the eight oak tannins.

In this way, **Table 11** present the mean of the concentration of the main compounds present in oak and chestnut tannins. Additionally, as commented previously, oak 2 and oak 4 tannins present significant difference with the rest of the oak tannins, leading us to calculate also the mean for oak tannins without them.

Results shows that chestnut tannins were significantly poorest in roburin B, C, E and grandinin than oak tannins considering oak 2 and oak 4 tannins or not. Concerning castalagin, vescalagin, roburin A and roburin D no significant differences were noted between oak and chestnut tannins, once again with or without oak 2 and oak 4 tannins in the mean. Considering or not, oak 2 and oak 4, in the mean of oak tannins, results remain the same between oak and chestnut tannins for each compound. Indeed, concentration in roburin B, C, E and grandinin are significantly different between mean of oak (with or without oak 2 and 4) and chestnut tannins meanwhile concentration in castalagin, vescalagin, roburin A and D were not significantly different. Nevertheless, the comparison of the total concentration in molecular ellagitannin present significant differences between oak and chestnut tannins without oak 2 and oak 4 tannins. On the contrary, when oak 2 and oak 4 tannins are considering in the calculation, no significant differences were observed between oak and chestnut tannins.

The differences between oak 2 and oak 4 with the rest of oak tannins can be easily explained by the fact that the different commercial tannins studied came from different companies meaning different raw material, process of extraction and preparation of the powder [163].

Additionally, as shown previously, ellagitannins concentrations in wood depend of the species of oak/chestnut, the origin of the tree, the rain washing during the staves seasoning, their degradation by micro-organisms, and their chemical oxidation [213]. Concentration in ellagitannins depends also of the treatment during the processing of wood in cooperage [92]. Indeed, an older wood extract presents a lower quantity of extractible ellagitannins meaning a lower richness in the final powder (commercial tannin). Additionally, higher is the drying time of wood lower is the quantity of extractible ellagitannin, meaning again a lower richness at the end. More especially, castalagin and vescalagin are the two more impacted compounds regarding their diminution with the drying time of wood. The rest of the compounds are also impacted but in lower proportion [214].

To conclude, it is necessary to consider all the tannins from the same botanical origin. Indeed, significant differences noted between oak tannins could be explained by their differential process of fabrication. Moreover, ellagitannins can be grouped even if they came from 2 different botanical origins (chestnut or oak wood) since no significant differences are observed between their total concentration in the eight mains molecular ellagitannins. Nevertheless, it should be highlighted and not omitted that chestnut tannins present lower quantity of roburin B, C, E and grandinin.

Table 10: Concentration (mg equivalent vescalagin/g of tannins) of the main compounds present in ellagitannins and total concentration in molecular ellagitannins

Compounds (mg eq vescalagin / g of tannins)	Chestnut 1		Chestnut 2		Chestnut 3		Oak 1		Oak 2		Oak 3		Oak 4		Oak 5		Oak 6		Oak 7		Oak 8	
Castalagin	72.3 ± 0.9	C	56.1 ± 1.9	E	57.4 ± 1.8	E	61.9 ± 4.0	DE	7.7 ± 0.2	F	84.7 ± 1.4	B	7.6 ± 0.1	F	74.7 ± 1.3	C	94.5 ± 1.4	A	77.6 ± 5.3	C	64.1 ± 0.6	D
Vescalagin	63.7 ± 0.7	AB	47.4 ± 1.3	CD	46.8 ± 0.8	CD	35.2 ± 1.0	E	7.9 ± 0.1	F	57.5 ± 1.0	BC	8.6 ± 0.2	F	52.7 ± 0.8	BCD	70.6 ± 0.9	A	55.1 ± 1.4	BCD	44.3 ± 0.4	D
Roburin A	25.2 ± 0.4	B	15.8 ± 0.3	F	15.2 ± 0.2	F	11.8 ± 0.5	G	2.2 ± 0.1	H	23.7 ± 0.3	C	2.3 ± 0.1	H	18.8 ± 0.4	E	32.1 ± 0.4	A	21.6 ± 1.2	D	16.2 ± 0.1	F
Roburin B	11.7 ± 0.5	G	9.2 ± 0.4	H	9.9 ± 0.2	H	22.1 ± 0.7	D	17.0 ± 0.6	E	37.6 ± 0.4	A	14.7 ± 0.1	F	29.8 ± 0.5	C	39.2 ± 0.6	A	35.3 ± 1.0	B	30.9 ± 0.5	C
Roburin C	9.6 ± 0.3	F	7.5 ± 0.2	G	8.4 ± 0.1	FG	20.1 ± 0.5	E	8.7 ± 0.2	FG	33.8 ± 0.5	A	7.1 ± 0.1	G	26.0 ± 0.5	D	34.1 ± 0.3	A	32.0 ± 1.3	B	29.4 ± 0.5	C
Roburin D	36.4 ± 0.1	BC	26.5 ± 0.7	DE	26.9 ± 0.5	DE	24.4 ± 0.8	E	2.1 ± 0.1	F	38.8 ± 0.5	B	2.1 ± 0.1	F	35.1 ± 0.5	C	48.8 ± 1.6	A	35.2 ± 1.9	C	27.0 ± 0.1	D
Roburin E	13.5 ± 0.2	G	11.2 ± 0.1	H	11.6 ± 0.0	H	25.5 ± 0.4	D	19.8 ± 0.4	E	43.2 ± 0.5	A	17.5 ± 0.4	F	34.3 ± 1.2	C	40.0 ± 1.4	B	40.0 ± 0.7	B	32.8 ± 0.5	C
Grandinin	14.9 ± 0.1	E	11.1 ± 0.0	EF	11.7 ± 0.1	EF	25.9 ± 1.1	D	8.7 ± 0.7	F	60.1 ± 1.9	A	7.5 ± 1.2	F	32.5 ± 2.0	C	49.9 ± 0.7	B	56.4 ± 3.4	A	47.7 ± 0.3	B
Total	**247.4 ± 3.1**	**E**	**184.8 ± 5.0**	**F**	**187.9 ± 3.7**	**F**	**227.0 ± 8.9**	**E**	**74.0 ± 2.2**	**G**	**379.4 ± 6.4**	**B**	**67.4 ± 2.2**	**G**	**303.8 ± 7.2**	**D**	**409.1 ± 7.2**	**A**	**353.2 ± 16.2**	**C**	**292.4 ± 3.0**	**D**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

Table 11: Mean of concentration (mg equivalent vescalagin/g of tannins) of the main compounds present in ellagitannins and total concentration in molecular ellagitannins

Mean Compounds (mg eq vescalagin / g of tannins)	Mean Chestnut Tannins			Mean Oak Tannins		Mean Oak Tannins WITHOUT Oak 2 and 4	
Castalagin	61.9 ± 9.0	A	α	59.1 ± 33.4	A	76.3 ± 12.3	α
Vescalagin	52.6 ± 9.6	A	α	41.5 ± 22.9	A	52.6 ± 12.0	α
Roburin A	18.7 ± 5.6	A	α	16.1 ± 10.4	A	20.7 ± 7.0	α
Roburin B	10.3 ± 1.3	B	β	28.3 ± 9.4	A	32.5 ± 6.3	α
Roburin C	8.5 ± 1.1	B	β	23.9 ± 10.9	A	29.2 ± 5.4	α
Roburin D	29.9 ± 5.6	A	α	26.7 ± 16.9	A	34.9 ± 8.7	α
Roburin E	12.1 ± 1.2	B	β	31.6 ± 9.7	A	36.0 ± 6.4	α
Grandinin	12.6 ± 2.0	B	β	36.1 ± 20.7	A	45.4 ± 13.5	α
Total	**206.7 ± 35.4**	**A**	**β**	**263.3 ± 131.4**	**A**	**327.5 ± 66.2**	**α**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between mean of chestnut and mean of oak tannins for each compound dosed ($p < 0.05$).
Different Greek letters indicate the existence of significant differences between mean of chestnut and mean of oak tannins without oak 2 and 4 for each compound dosed ($p < 0.05$).

3.2.1.2. Gallotannins

Regarding gallotannins, the fourteen principals molecular gallotannins (gallic, digallic and quinic acid; 3,4 and 5-galloylquinic acid; tri, tetra, penta, hexa, hepta, octa, nona and decagalloyl-glucose) were quantified for the four tara tannins and the four nut gall tannins, using gallic acid as standard.

Table 12 shows the concentration (mg equivalent gallic acid/g of tannins) of the main compounds present in gallotannins and the total concentration in molecular gallotannins.

The different compounds quantified were grouped in three sum (sum 1, 2 and 3), since the compounds present in sum 2 have been described as typical of a tara tannin [215] and the compounds present in sum 3 have been described as typical of a nut gall tannin [216,217]. Concerning gallic acid and digallic acid grouped in sum 1, they are known to be present in both type of tannins (tara and nut gall).

The main compounds present in gallotannins differ according to the botanical origin. Indeed, in the case of the tara tannins, sum 2 corresponding to quinic acid, 3-galloylquinic acid, 4-galloylquinic acid and 5-galloylquinic acid was the most abundant. The concentrations were ranking from 131.0 ± 1.5 to 163.2 ± 3.9 mg eq. gallic acid/g of tannins. An interesting point in this sum 2, is that 3-galloylquinic acid and 4-galloylquinic acid have more weight than quinic acid and 5-galloylquinic acid.

On the contrary, in the case of nut gall tannins, sum 1 corresponding to gallic and digallic acid was the most abundant. The concentrations were ranking from 69.6 ± 1.4 to 149.9 ± 3.8 mg eq. gallic acid/g of tannins. The only exception was nut gall 2 which presented also great richness in sum 1, but higher richness in sum 2 with values of 102.3 ± 2.8 and 121.5 ± 5.1 mg eq. gallic acid/g of tannins respectively.

Additionally, it should be highlighted that independently of the botanical origin (tara or nut gall), sum 3 corresponding to tri, tetra, penta, hexa, hepta, octa, nona and decagalloyl-glucose was the lowest abundant. The concentrations were ranking from 0.9 ± 0.2 to 1.6 ± 0.2 mg eq. gallic acid/g of tannins for tara and from 15.6 ± 0.3 to 47.6 ± 1.0 mg eq. gallic acid/g of tannins for nut gall. Once again, nut gall 2 tannin was quite different regarding the rest of nut gall tannins with low value of sum 3.

Regarding the total concentration in molecular gallotannins, tara tannins present in general higher concentration than nut gall tannins with values ranking from 181.1 ± 3.4 to 233.6 ± 8.2 and from 122.0 ± 3.7 to 239.4 ± 8.2 mg eq. gallic acid/g of tannins respectively. Nevertheless, nut gall 2 tannin present higher richness than the rest of nut gall tannin and even more so than the tara tannins with a total concentration in gallotannins of 239.4 ± 8.2 mg eq. gallic acid/g of tannins.

As done previously for ellagitannins, in order to compare easily tara and nut gall tannins and to determine if the botanical origin (tara or nut gall) of the gallotannins have a major importance, two groups were made with from one hand the four tara tannins and from the other hand the four nut gall tannins.

In this way, **Table 13** presents the mean of the concentration of the main compounds present in tara and nut gall tannins. Additionally, as commented previously, nut gall 2 tannin present significant difference with the rest of the nut gall tannins, leading us to calculate also the mean for nut gall tannins without him.

Results shows that tara tannins were significantly poorest in sum 1 and 3 than nut gall tannins considering nut gall 2 or not in the mean. To the opposite, results shows that tara tannins were significantly richer in sum 2 than nut gall tannins considering nut gall 2 or not in the mean. Nevertheless, the comparison of the total concentration in molecular gallotannin present significant differences between tara and nut gall tannins without nut gall 2 in the calculation of the mean. On the contrary, when nut gall 2 is considering in the calculation, no significant differences were observed between tara and nut gall tannins.

As explain previously in the case of ellagitannins, the differences between nut gall 2 and the rest of nut gall tannins can be explained by the different process used by the companies to product commercial tannins.

Nut galls are pathological excrescences formed on young branches or twigs of plants, induced by insect attacks, as a result of deposition of the eggs. They are a lot of different nut galls but specifically, two different nut galls as a source of tannin which are Turkish gallnuts (*Gallae Turcicae*) and Chinese gallnuts (*Gallae Chinensis*) [218]. According to this, the chemical composition of nut galls varies depending on the gall-forming agent and the type of plant.

Concerning Tara (*Caesalpinia spinosa*) is a small leguminous tree native of Peru and widely spread in Latin America, from Venezuela to northern Chile [219]. According to this, the chemical composition varies depending the region of the plantation. Nevertheless, this variation between two tara tannins are not so different as in the case of two nut galls tannins.

To conclude, it is necessary as for ellagitannins, to consider all the tannins from the same botanical origin. Indeed, significant differences noted between nut gall tannins could be explained by their differential process of fabrication. Moreover, gallotannins can be grouped even if they came from 2 different botanical origins (tara or nut gall) since no significant differences are observed between their total concentration in gallotannins. Nevertheless, it should be highlighted and not omitted that tara tannins present lower quantity of sum 1 and 3 and higher quantity in sum 2 than nut gall tannins.

Table 12: Concentration (mg equivalent gallic acid/g of tannins) of the main compounds present in gallotannins and total concentration in molecular gallotannins

Compounds (mg eq gallic ac/g of tannins)	Tara 1		Tara 2		Tara 3		Tara 4		Nut gall 1		Nut gall 2		Nut gall 3		Nut gall 4	
Gallic acid	35.5 ± 1.8		58.7 ± 1.9		24.2 ± 1.0		23.0 ± 0.7		87.8 ± 2.4		72.4 ± 2.3		67.1 ± 3.7		37.3 ± 0.8	
Digallic acid	17.1 ± 0.8		16.5 ± 0.6		24.6 ± 0.5		15.8 ± 4.1		62.2 ± 1.4		29.9 ± 0.5		44.7 ± 1.0		32.2 ± 0.6	
Sum 1	**52.6 ± 2.6**	**E**	**75.1 ± 2.5**	**D**	**48.8 ± 1.5**	**E**	**38.8 ± 4.7**	**F**	**149.9 ± 3.8**	**A**	**102.3 ± 2.8**	**C**	**111.8 ± 4.6**	**B**	**69.6 ± 1.4**	**D**
Quinic acid	10.0 ± 0.5		8.3 ± 0.3		2.4 ± 0.1		3.2 ± 0.1		0.1 ± 0.0		6.5 ± 0.2		0.1 ± 0.0		0.5 ± 0.0	
3-galloylquinic acid	95.2 ± 4.8		95.4 ± 2.1		64.3 ± 0.0		103.3 ± 0.8		0.4 ± 0.1		56.6 ± 3.1		3.0 ± 3.4		3.2 ± 1.3	
4-galloylquinic acid	51.1 ± 4.1		47.9 ± 2.9		57.9 ± 1.3		50.9 ± 2.7		0.5 ± 0.1		54.4 ± 1.8		1.7 ± 1.9		1.1 ± 0.1	
5-galloylquinic acid	5.7 ± 0.5		5.4 ± 0.2		6.5 ± 0.1		5.9 ± 0.3		0.1 ± 0.0		3.9 ± 0.1		0.2 ± 0.1		0.1 ± 0.0	
Sum 2	**162.0 ± 9.9**	**A**	**157.0 ± 5.5**	**A**	**131.0 ± 1.5**	**B**	**163.2 ± 3.9**	**A**	**1.1 ± 0.2**	**C**	**121.5 ± 5.1**	**B**	**5.0 ± 5.4**	**C**	**4.8 ± 1.4**	**C**
Trigalloyl-glucose	1.2 ± 0.1		1.3 ± 0.1		1.1 ± 0.4		0.8 ± 0.2		3.4 ± 0.1		1.5 ± 0.0		2.5 ± 0.2		5.9 ± 0.1	
Tetragalloyl-glucose	0.1 ± 0.0		0.0 ± 0.0		0.1 ± 0.0		0.0 ± 0.0		2.5 ± 0.2		0.3 ± 0.0		1.7 ± 0.1		6.9 ± 0.2	
Pentagalloyl-glucose	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		5.2 ± 0.2		0.6 ± 0.0		3.5 ± 0.0		11.8 ± 0.2	
Hexagalloyl-glucose	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		4.2 ± 0.1		1.2 ± 0.0		2.7 ± 0.1		10.1 ± 0.4	
Heptagalloyl-glucose	0.1 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		4.1 ± 0.2		2.7 ± 0.1		4.1 ± 0.1		7.7 ± 0.1	
Octagalloyl-glucose	0.1 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		5.0 ± 0.2		4.0 ± 0.1		4.9 ± 0.1		3.9 ± 0.1	
Nonagalloyl-glucose	0.1 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0		4.6 ± 0.1		3.6 ± 0.0		4.5 ± 0.1		1.2 ± 0.0	
Decagalloyl-glucose	0.0 ± 0.0		0.0 ± 0.0		0.1 ± 0.0		0.0 ± 0.0		2.8 ± 0.1		1.7 ± 0.0		2.8 ± 0.0		0.2 ± 0.0	
Sum 3	**1.6 ± 0.2**	**E**	**1.4 ± 0.1**	**E**	**1.3 ± 0.5**	**E**	**0.9 ± 0.2**	**E**	**31.9 ± 1.1**	**B**	**15.6 ± 0.3**	**D**	**26.7 ± 0.7**	**C**	**47.6 ± 1.0**	**A**
Total Sum	**216.2 ± 12.8**	**AB**	**233.6 ± 8.2**	**A**	**181.1 ± 3.4**	**C**	**202.9 ± 8.9**	**BC**	**183.0 ± 5.1**	**C**	**239.4 ± 8.2**	**A**	**143.5 ± 10.7**	**D**	**122.0 ± 3.7**	**D**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

Table 13: Mean of concentration (mg equivalent gallic acid/g of tannins) of the main compounds present in gallotannins and total concentration in molecular gallotannins

Mean Compounds (mg eq gallic ac/g of tannins)	Mean Tara Tannins			Mean Nut gall Tannins		Mean Nut gall Tannins WITHOUT Nut gall 2	
Sum 1	53.8 ± 15.3	B	β	108.4 ± 33.0	A	110.4 ± 40.2	α
Sum 2	153.3 ± 15.1	A	α	33.1 ± 59.0	B	3.6 ± 2.2	β
Sum 3	1.3 ± 0.3	B	β	30.5 ± 13.3	A	35.4 ± 10.9	α
Total Sum	**208.5 ± 22.1**	**A**	**α**	**172.0 ± 51.6**	**A**	**149.5 ± 30.9**	**β**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between mean of tara and mean of nut gall tannins for each compound dosed ($p < 0.05$).
Different Greek letters indicate the existence of significant differences between mean of tara and mean of nut gall tannins without nut gall 2 for each compound dosed ($p < 0.05$).

3.2.2. Condensed tannins

Concerning condensed tannins, molecular characterization from one hand was done for the procyanidins/prodelphinidins (grape, grape-seed and grape-skin tannins) and from the other hand done for the profisetinidins/prorobitenidins (quebracho and acacia tannins).

3.2.2.1. Procyanidins/Prodelphinidins

Regarding procyanidins/prodelphinidins, (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, dimers B1, B2, B3 and B4, B2-3-*O*-gallate and trimers were quantified, for the three grape tannins, the four grape-seed tannins and the two grape-skin tannins, using (+)-catechin as standard.

Table 14 show the concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in procyanidins/prodelphinidins tannins and the total concentration in molecular tannins.

In general, the monomers ((+)-catechin and (-)-epicatechin) were the compounds mainly present in procyanidins/prodelphinidins tannins independently if they are extract from the whole grape, grape-skin or grape-seed. The concentrations in monomers were ranking from 22.3 ± 0.2 to 209.6 ± 1.5 mg eq. (+)-catechin/g of tannins. It should be highlighted that tannins from grapes seems to present in general higher richness than grape-seed tannins, themselves richer than grape-skin tannins. In addition, after monomers, dimers and trimers were the compounds founds in the highest concentration in the different type of tannins. Indeed, the concentrations were ranking from 23.7 ± 4.9 to 74.3 ± 0.5 and from 6.8 ± 0.1 to 28.5 ± 0.3 mg eq. (+)-catechin/g of tannins for dimers and trimers respectively. Concerning dimers and trimers, contrary to monomers, the different tannins seems to present similar concentrations independently of their botanical origin. The (-)-epicatechin-3-*O*-gallate and the B2-3-*O*-gallate were present in low concentrations in all the tannins. Indeed, the concentrations were ranking from 0.1 ± 0.0 to 15.4 ± 0.3 and from 0.3 ± 0.0 to 24.5 ± 0.4 mg eq. (+)-catechin/g of tannins respectively. The different type of tannins seems to present similar concentrations of (-)-epicatechin-3-*O*-gallate meanwhile grape tannins seems to present higher concentration of B2-3-*O*-gallate than grape-skin and grape-seed tannins.

Moreover, making the comparison of the total concentration of the different compounds, grape tannins seems to be richer than grape-seed tannins, themselves richer than grape-skin tannins. The values were ranking from 111.5 ± 3.0 to 347.1 ± 0.6 mg eq. (+)-catechin/g of tannins, from 59.3 ± 2.0 to 210.7 ± 13.1 mg eq. (+)-catechin/g of tannins and from 63.6 ± 0.3 to 135.4 ± 2.5 mg eq. (+)-catechin/g of tannins for grape, grape-seed and grape-skin tannins respectively. Finally, it should be highlighted that grape 3 presented higher richness in all compounds than grape 1 and grape 2 tannins. In addition, seed 3 and seed 4 presented lower richness in all compounds than seed 1 and seed 2 as well as skin 1 which was richer than skin 2.

In this way, in order to compare easily grape, grape-seed and grape-skin tannins and to determine if the botanical origin of the procyanidins/prodelphinidins have a major importance, three groups were made with the three grape tannins, the four grape-seed tannins and the two grape-skin tannins.
According to this, **Table 15** present the mean of the concentration of the main compounds present in grape, grape-skin and grape-seed tannins.
Results shows that for all the compounds and the total concentrations, no significant differences were observed between the three different botanical origin. Indeed, even if grape tannins seem to present higher richness, the variability inside each botanical origin involved high differences between each commercial tannin. In this way, not all the grape tannins were richer than all the grape-seed or grape-skin tannins. This can be easily explained by the fact that the different commercial tannins studied came from different companies meaning different raw material, process of extraction and preparation of the powder. In fact, commercial tannins prepared from grape, grape-skin and grape-seed, can find their origin from various grapevine, meaning raw material more or less rich in the different compounds. For example, the red varieties are known to be richer than the rosé varieties and even more so than the white grapevines [220]. In addition, including for the same grapevine variety, difference in richness and mDP have been highlighted in a study conducted on different Cabernet sauvignon grapes meaning that two commercial tannins extracted from the same variety can also present difference [51].
To conclude, commercial tannins extracted from grape, grape-skin or grape-seed cannot be separated and should be considered as one family since no significant differences were observed between them. At the end, the variability between two grape tannins is similar to the variability between one grape tannin and those from grape-skin or grape-seed tannin. An important think to noted, is that the quantification of the monomers, dimers and trimers of the grape, grape-skin and grape-seed tannins, allowed us only to quantified around 20% of the total extract.

Table 14: Concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in procyanidins/prodelphinidins and total concentration in molecular procyanidins/prodelphinidins

Compounds (mg eq catechin / g of tannins)	grape 1		grape 2		grape 3		seed 1		seed 2		seed 3		seed 4		skin 1		skin 2	
Monomers	69.8 ± 2.3	C	109.0 ± 1.4	B	209.6 ± 1.5	A	113.2 ± 2.1	B	110.7 ± 1.1	B	24.6 ± 0.7	F	31.4 ± 1.9	E	61.7 ± 1.1	D	22.3 ± 0.2	F
(-)-epicatechin-3-*O*-gallate	3.9 ± 0.1	E	6.2 ± 0.2	C	10.3 ± 0.2	B	15.4 ± 0.3	A	10.4 ± 0.3	B	0.6 ± 0.0	F	0.1 ± 0.0	F	4.0 ± 0.1	E	5.3 ± 0.0	D
Dimers	23.7 ± 4.9	D	36.4 ± 1.0	C	74.3 ± 0.5	A	61.3 ± 11.9	B	53.6 ± 1.1	B	26.2 ± 0.9	CD	30.0 ± 2.1	CD	53.9 ± 1.1	B	27.2 ± 0.0	CD
B2-3-*O*-gallate	5.0 ± 0.0	E	14.3 ± 0.2	B	24.5 ± 0.4	A	7.6 ± 0.1	D	10.5 ± 0.3	C	0.4 ± 0.1	H	0.3 ± 0.0	H	2.8 ± 0.0	F	2.0 ± 0.0	G
Trimers	9.1 ± 0.1	D	15.6 ± 0.5	B	28.5 ± 0.3	A	13.3 ± 0.1	C	15.3 ± 0.4	B	7.6 ± 0.4	E	9.5 ± 0.7	D	12.9 ± 0.2	C	6.8 ± 0.1	E
Total	**111.5 ± 3.0**	**E**	**181.5 ± 2.9**	**C**	**347.1 ± 0.6**	**A**	**210.7 ± 13.1**	**B**	**200.6 ± 2.2**	**B**	**59.3 ± 2.0**	**F**	**71.2 ± 4.7**	**F**	**135.4 ± 2.5**	**F**	**63.6 ± 0.3**	**D**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

Table 15: Mean of concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in procyanidins/prodelphinidins and total concentration in molecular procyanidins/prodelphinidins

Means Compounds (mg eq catechin / g of tannins)	Mean Grape Tannins		Mean Seed Tannins		Mean Skin Tannins	
Monomers	129.5 ± 72.1	A	70.0 ± 48.6	A	42.0 ± 27.9	A
(-)-epicatechin-3-*O*-gallate	6.8 ± 3.2	A	6.6 ± 7.5	A	4.7 ± 0.9	A
Dimers	44.8 ± 26.3	A	42.8 ± 17.3	A	40.5 ± 18.9	A
B2-3-*O*-gallate	14.6 ± 9.8	A	4.7 ± 5.2	A	2.4 ± 0.6	A
Trimers	17.7 ± 9.8	A	11.4 ± 3.5	A	9.8 ± 4.3	A
Total	**213.4 ± 121.0**	**A**	**135.5 ± 81.3**	**A**	**99.5 ± 50.7**	**A**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

3.2.2.2. Profisetinidins/Prorobitenidins

Concerning profisetinidins/prorobitenidins, various compounds were quantified. (+)-catechin and (-)-epicatechin were quantified as monomers constitutive of both types of tannins (quebracho and acacia). Then for quebracho tannins, the dimers, trimers and tetramers of (+)-catechin and fisetinidinol were quantified. For acacia tannins, (-)-epicatechin-3-*O*-gallate, dimers of fisetinidol-gallocatechin and dimers of robinetinidol-catechin, chalcan-flavan dimers and trimers of catechin-fisetinidol-robinetinidol or gallocatechin-fisetinidol-fisetinidol and trimers of robinetinidol-robinetinidol-catechin or gallocatechin-fisetinidol-robinetinidol were quantified, using (+)-catechin as standard.

Quebracho and acacia tannins, are considered to be profisetinidins/prorobitenidins tannins since they present similar structure and they contain both (+)-catechin, (-)-epicatechin and fisetinidin as monomers. Nevertheless, acacia tannins contain also monomers of robinetinidin differentiating them from quebracho tannins. In this way, the different dimers, trimers or tetramers found in acacia and quebracho tannins did not have the same structure based on the different monomers. Indeed, some authors [212] have reported the presence of robinetinidol in quebracho extracts but more recent study [221] present contradictory results indicating the presence of fisetinidol instead of robinetinidol.

Table 16 show the concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in quebracho tannins and the total concentration in molecular tannins. First of all, it is important to note that the total concentration in tannins of the quebracho extracts were very low with values ranging from 18.2 ± 0.6 to 38.8 ± 0.6 mg equivalent (+)-catechin/g of tannins. This results are quite curious but in agreement with previous ones [162], and can be explained by the fact that quebracho tannins are made of large polymers which are difficult to break. It has been demonstrated that quebracho tannins cannot be break, also with the phloroglucinolisis or thioacidolysis methods [212]. In addition, the presence of monomers was quasi inexistent with values around 0 mg equivalent (+)-catechin/g of tannins for the six different commercial quebracho tannins. Furthermore, concentration in dimers and tetramers were also quite low with values ranking from 1.8 ± 0.1 to 7.1 ± 0.2 mg equivalent (+)-catechin/g of tannins and from 1.3 ± 0.2 to 3.6 ± 0.3 mg equivalent (+)-catechin/g of tannins respectively. It is not surprising to obtained few quantity of dimers since it has been also reported, meanwhile, the low values of tetramers were more curious since they should be close to the ones obtained for trimers [222]. Trimers, were the compounds which presented the highest concentration in the total extract with values ranking from 13.5 ± 0.3 to 27.4 ± 0.2 mg equivalent (+)-catechin/g of tannins. In fact, the concentration in trimers correspond to 70-80% of the total concentration of the extracts. Finally, it is important to remind that the total concentrations obtained for the quebracho tannins were not representative of the real concentration of the total extract. As mentioned above with this method, it is not possible to detect all the tannins present in the extract and more specifically, the polymers of higher degrees such as pentamers or hexamers for example.

Indeed, when the results are compared with the previous ones obtained by the different methods used to characterized the commercial tannins (**Table 9**), in the case of the Bate-Smith reaction based on acidic hydrolysis, low richness was obtained for the quebracho tannins. Nevertheless, with the methyl-cellulose which is a more specific method, great richness were obtained confirming that the quantification obtained in **Table 9** is not representative enough of the total concentration of the extract.

Table 17 show the concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in acacia tannins and the total concentration in molecular tannins. Contrary to quebracho tannins, the main compounds present in acacia tannins were the monomers. Indeed, there concentrations were ranking from 131.2 ± 5.5 to 172.9 ± 2.2 mg equivalent (+)-catechin/g of tannins representing more than 70% of the total concentration of the extract. This results are in accordance with previous ones described in the literature in which they have demonstrated that *Acacia Catechu* heartwood, was composed predominantly by catechin and epicatechin [223]. In addition to the monomers, chalcan-flavan dimers and trimers were also found in great quantity with values ranking from 10.1 ± 3.8 to 23.6 ± 3.1 mg equivalent (+)-catechin/g of tannins and from 20.6 ± 0.4 to 42.8 ± 8.0 mg equivalent (+)-catechin/g of tannins respectively. Concerning the dimers, very low levels were found in both acacia tannins with no significant differences, which agree with previous study reporting their presence but in few quantities in acacia wood [223]. Furthermore, (-)-epicatechin-3-*O*-gallate, have been detected in the extracts but the quantification of the peaks gives values around 0 mg equivalent (+)-catechin/g of tannins. This can be explained by the fact that this compound is present in acacia tree but in majority in the leaves and not in the wood parts [223], which is the part commonly used to produce commercial acacia tannins.

Finally, significant differences were obtained between the two different commercial acacia tannins regarding the main compounds (monomers, chalcan-flavan dimers and trimers) and the total concentration. Acacia tannin number 1 reaching always similar or higher quantity of the different compounds than acacia tannin number 2. In fact, these differences can be explained since there is a wide variety of acacia tannin according to the different species of acacia tree. Indeed, only in Australia there is more than 600 different species of acacia conducted to a great variety in composition and richness in the different compounds [224]. For example, *Acacia catechu* and *Acacia confuse* contain mainly procyanidins meanwhile *Acacia auriculiformis*, *Acacia mangium* and *Acacia mearnsii* contain mainly prorobitenidins, as major flavanol units [225]. Since the specific origin of the commercial extract have been not provided it is not possible to affirm that they came from two different species, even if it can be widely supposed. To conclude, even if the richness of quebracho and acacia tannins were highly different, due to the impossibility of the method to quantify all the tannins in the quebracho extracts, these two different types of tannins present similarity in their composition.

Table 16: Concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in quebracho tannins and total concentration in molecular quebracho tannins

Compounds (mg eq catechin / g of tannins)	Quebracho 1		Quebracho 2		Quebracho 3		Quebracho 4		Quebracho 5		Quebracho 6	
Monomers	0.2 ± 0.0	C	0.1 ± 0.0	C	0.1 ± 0.0	C	0.7 ± 0.1	A	0.8 ± 0.0	A	0.5 ± 0.0	B
Dimers	3.3 ± 0.2	C	1.9 ± 0.2	D	1.8 ± 0.1	D	7.1 ± 0.2	A	5.3 ± 0.2	B	3.0 ± 0.2	C
Trimers	16.1 ± 0.3	C	15.9 ± 0.5	C	16.3 ± 0.1	C	27.4 ± 0.2	A	20.6 ± 0.9	B	13.5 ± 0.3	D
Tetramers	3.0 ± 0.4	AB	2.7 ± 0.1	BC	2.6 ± 0.0	BC	3.6 ± 0.3	A	2.1 ± 0.3	C	1.3 ± 0.2	D
Total	**22.5 ± 0.7**	**C**	**20.7 ± 0.5**	**C**	**20.9 ± 0.0**	**C**	**38.8 ± 0.6**	**A**	**28.8 ± 0.6**	**B**	**18.2 ± 0.6**	**D**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

Table 17: Concentration (mg equivalent (+)-catechin/g of tannins) of the main compounds present in acacia tannins and total concentration in molecular acacia tannins

Compounds (mg eq catechin / g of tannins)	Acacia 1		Acacia 2	
Monomers	172.9 ± 2.2	A	131.2 ± 5.5	B
(-)-epicatechin-3-*O*-gallate	0.0 ± 0.0	A	0.2 ± 0.1	A
Dimers	3.2 ± 0.4	A	4.7 ± 1.3	A
Chalcan-flavan dimers	23.6 ± 3.1	A	10.1 ± 3.8	B
Trimers	42.8 ± 8.0	A	20.6 ± 0.4	B
Total	**242.4 ± 4.8**	**A**	**166.7 ± 10.2**	**B**

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between tannin for each compound dosed ($p < 0.05$).

4. Conclusion

The first part of this chapter allowed to separate the four families of oenological tannins (ellagitannins, gallotannins, procyanidins/prodelphinidins and profisetinidins/prorobitenidins) according to the different methods used to determine their richness in tannins. Nevertheless, the results obtained are very complex since each analytical method supplies different results and different information concerning their richness in tannins. Indeed, Folin Ciocalteu, TPI and OIV methods provide richness in phenolic compounds and not specifically richness in tannins as wanted. The Bate-Smith reactions and phloroglucinolisis were only specifically for condensed tannins. The only method probably more specifically of tannins and for all type of tannins (condensed and hydrolysable) was the methyl-cellulose precipitation. In fact, this method measures the difference in absorbance at 280 nm before and after precipitating tannins with methyl-cellulose. According to these first results it was necessary to determine in a second part, the molecular composition of each tannin in function of its family.

In this way, it was possible to determine more specifically the difference between two tannins appertaining to the same family regarding the composition in principal monomers, dimers or polymer characteristic of this family. The obtained results for hydrolysable tannins shows for both ellagitannins and gallotannins that all the tannins from the same botanical origin have to be considered as a one family. Indeed, no significant differences were noted between oak and chestnut as well as between tara and nut galls. The only few significant differences highlighted between two tannins of the same botanical origin are due to the fabrication process which differs according to the suppliers. Concerning condensed tannins, for the procyanidins/prodelphinidins, the same conclusions were obtained than for hydrolysables tannins, since no significant differences were observed between grape, grape-skin and grape-seed tannins. Regarding profisetinidins/prorobitenidins, results are quite different as it is really difficult to quantify all the quebracho extract with this method, nevertheless, quebracho and acacia tannins present similarity in their composition more than with the rest of the commercial tannins, leading us to join them in the same family.

To conclude, according to the various methods used to characterize the thirty-six oenological tannins, in the next chapters, they will be grouped in four families regarding their botanical origin which are ellagitannins (oak and chestnut), gallotannins (tara and nut gall), procyanidins/prodelphinidins (grape, grape-seed and grape-skin) and profisetinidins/prodelphinidins (quebracho and acacia). Their possible properties will be elucidated according to this groups even though special attention will be given to the differences that may be due to their specific botanical origin (oak vs chestnut for example).

Chapter 4: Antioxidant properties and oxygen consumption rate of oenological tannins

1. Introduction

Polyphenols are known for their antioxidant properties and more specifically, among them a particular class called tannins. Tannins are constituted by one or several rather hydrophobic aromatic nuclei bearing polar hydroxyl groups conferring to them the antioxidant properties. Studies have demonstrated that the tannins antioxidant properties come from their catechol moieties (i.e. –OH groups at *ortho* positions in aromatic rings) [226]. Antioxidant capacity of tannins coming from various plants or source have been widely described [227,228] as well as in red wines [204,229] and white wines [230].

In this way, one of the main reasons that oenological tannins are widely used in wineries it's because they possess antioxidant capacity. It is generally accepted that they are very useful in protecting grape juice and wine against oxidation, avoiding browning [163,231]. Nevertheless, there are a quite few references about the antioxidant properties of commercial tannins [182,232,233]**.** Moreover, some researchers have shown that different antioxidant assays (FRAP, ABTS, DPPH, CUPRAC, ORAC…) produce different, and sometimes, contradictory results [182,185]. In this way, they insist on the importance of using several methods to asses this parameter, in order to obtain robust results. Recently, a new method based on high-pressure flow injection with an electrochemical detector (ECD) has been also proposed [234]. The phenolic compounds which contribute to the antioxidant activity are oxidized by the potential put on the ECD. This is a new simple tool to determine the antioxidant activity of oenological tannins and a faster method than the spectrophotometric one, since only 3 minutes are required for the analysis.

Recently new methods have been proposed in order to determine complementary properties to the antioxidant ones. A method based on cyclic voltammetry has been proposed to determine the oxidation potential of flavonoids [235]. The results obtained were in great agreement with the ones obtained by the FRAP assay.

Nevertheless, all these methods are indirect methods and none of them really measure the direct oxygen consumption. More recently, a new method proposed to measure the oxygen consumption rate (OCR) of two hydrolysable tannins and three condensed tannins [164].

Given the wide range of commercial tannins present in the market, the main goal of this chapter is to carry out an exhaustive study using many samples (36 commercial tannins). For this purpose, the antioxidant capacity was achieved by five different methods (ABTS, CUPRAC, DPPH, FRAP and ORAC) and the kinetics of oxygen consumption was measured in a model wine solution using non-invasive method based on luminescence.

2. Materials and methods

2.1. Determination of antioxidant capacity

Antioxidant capacity was measured using 96-well microplates and five different methods: ABTS, CUPRAC, DPPH, FRAP and ORAC. Each assay was performed as following the methods described by González-Centeno et al [186]. Stock solutions (1.5 mg/10 mL) of oenological tannins were prepared in model wine solution. In the case of the ORAC assay, a dilution of the stock solution of between 1/30 and 1/100 in deionized water was necessary. Trolox, used as a standard, was also prepared in model wine solution (0.25-1.25 mM for the ABTS, CUPRAC and FRAP assays; 0.1-1 mM for the DPPH assay and 1.5-40 µM for the ORAC assay). Except for ORAC assay, a reagent blank was taken by adding 190 µL of working solution in each well. Then 10 µL of deionized water, Trolox solution or oenological tannin solution was added to each well and spectrophotometric measurements were performed at 25 °C after 30 min of reaction. Fluorometric ORAC assay was performed at 37 °C and lasted 1.5 h.
All the results, expressed as mg of Trolox equivalents/g tannin (dry matter), are the average of six replicates.

2.1.1. ABTS assay

ABTS reagent was prepared by mixing in equal volumes a solution of $ABTS^{\bullet+}$ (7 mM) and an aqueous solution of persulfate potassium (2.45 mM) and then allowing it to react at room temperature in darkness for 12-16 h. After that, the working solution was diluted with deionized water to obtain an absorbance of 0.9 ± 0.01 units at 734 nm. The measurement was monitored at this wavelength.

2.1.2. CUPRAC assay

The CUPRAC reagent was prepared by mixing equal volumes (1:1:1) of a Cu (II) chloride dihydrate aqueous solution (10 mM), a neocuproine solution in ethanol (7.5 mM) and ammonium acetate buffer (1 M, pH 7). The measurement was monitored at 450 nm.

2.1.3. DPPH assay

The DPPH reagent (6.10^{-5} M) was freshly prepared in methanol. The measurement was monitored at 515 nm.

2.1.4. FRAP assay

FRAP reagent was prepared by mixing acetate sodium buffer solution (300 mM, pH 3.6), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) (10 mM) and iron (III) chloride hexahydrate aqueous solution (20 mM) at a ratio of 10:1:1. The measurement was monitored at 593 nm.

2.1.5. ORAC assay

The ORAC assay was carried out as it described [184]. All reagents were prepared in phosphate buffer (75 mM, pH 7.4). First, 30 µL of deionized water, Trolox standard solution or oenological tannin solution was placed in each well. 180 µL of fluorescein (117 nM, final concentration) and 90 µL of AAPH (40 mM) were then added to each well. Fluorescence was monitored at 485 (excitation) and 530 nm (emission). The area under the curve (AUC) was calculated for each sample by integrating their relative fluorescence curves. The net AUC of the oenological tannins was calculated by subtracting the AUC from the blank and then correlating with the Trolox calibration curve.

2.2. Determination of the oxygen consumption rate (OCR)

The OCR was performed following the non-invasive luminescence technique [164]. Clear glass bottles (0.75 L) provided with a pill (PreSens PrecisionSensing GmbH, order code: SPPSt3-NAU-D5-CAF; batch number: 1203-01_PSt3-0828-01, Regensburg, Germany) to measure dissolved oxygen were used. The pills were all placed at the middle of the bottle. The experiments were conducted in a model wine solution composed of 12% vol. of ethanol, 4 g/L of tartaric acid and pH adjusted to 3.5. This was supplemented with 3 mg Fe (III)/L in the form of iron (III) chloride hexahydrate, and 0.3 mg Cu (II)/L in the form of copper (II) sulfate pentahydrate to mimic as closely as possible real wine conditions. The model wine solution was saturated with oxygen by bubbling with air for 10 min. The bottles with the inserted pill were first filled with 20 mL of the model wine solution (control) or with the different oenological tannins dissolved in 20 mL of model wine solution. Immediately afterwards, the bottles were filled with the oxygen-saturated solution to almost overflowing, to minimize the volume of headspace. The bottles were hermetically sealed and carefully shaken to fully homogenize. The oxygen measurements started 1 h later and were performed every two days. Bottle temperature was maintained at 20 °C the whole time. Two different experimental setups were performed: the first one to evaluate the influence of the oenological tannin doses and the second one to evaluate the influence of the type of oenological tannin. All the experiments were made and analyzed in triplicate.

2.2.1. Influence of the oenological tannin doses

Four different doses of oenological tannins (25, 50, 75 and 100 g/hL) from the two usual botanical origins (grape skin and oak) were used to evaluate the influence of the oenological tannin and to identify the best dose for performing the next experiment. The concentrations used are somewhat higher than those usually applied to must and wines under real winemaking conditions to shorten the experimentation time.

2.2.2. Influence of the botanical origin of the oenological tannins

Once the best oenological tannin dose was estimated, the influence of botanical origin on oxygen consumption was studied. To this end the oxygen and tannin concentrations were fixed at 8 mg/L and 100 g/hL, respectively. All the commercial tannins of various botanical origins (quebracho, tara, nut gall, oak, chestnut, acacia, grape-seed and grape-skin) were considered for this experiment.

3. Results and discussion

3.1. Antioxidant capacity

Table 18 shows the antioxidant capacity of the different oenological tannins assessed by different analytical methods (ABTS, ORAC, FRAP, CUPRAC and DPPH). The aim of these analyses was to provide a way to classify the different oenological tannins according to their antioxidant properties.

First of all, it should be highlighted that no significant differences were observed for the different tannins appertaining to the same family leading us the possibility to group them into family. Indeed, for each method studied no significant differences were observed for the procyanidins/prodelphinidins, between tannins from grape, grape-seed or grape-skin as well as for profisetinidins/prorobitenidins, between tannins from acacia or quebracho. Concerning hydrolysable tannins, the same effect was observed inside the ellagitannins with no significant differences between chestnut and oak tannins as well as for gallotannins with no significant differences between nut gall and tara tannins. Nevertheless, in general terms, the different antioxidant capacity tests produced different and sometimes contradictory results for the different tannin families. These results are in accordance with previous ones obtained, which reported the existence of variation between the different antioxidant test used [182,185]. Additionally, in all cases DPPH assay give always the lowest values of antioxidant capacity meanwhile ABTS and ORAC give the highest values in the case of hydrolysable tannins and condensed tannins respectively.

Briefly, the ABTS and FRAP tests gave the highest values to both hydrolysable tannins (gallotannins and ellagitannins), and these were followed in decreasing order by the procyanidins/prodelphinidins and profisetinidins/prorobitenidins tannins. The CUPRAC test also gave the highest value to ellagitannins but placed gallotannins in second position followed by procyanidins/prodelphinidins and profisetinidins/prorobitenidins tannins. No significant differences were found among the different types of tannins when using the DPPH test, although the antioxidant capacity of hydrolysable tannins tended to be higher than that of condensed tannins. Overall, the ABTS, FRAP, CUPRAC and DPPH tests indicate that hydrolysable tannins have a higher antioxidant capacity than condensed tannins, although these differences are not always significant.

These results are in accordance with previous one obtained were the results collected for DPPH, ABTS and FRAP were comparable with high correlation between the methods [184]. In contrast, the ORAC test supplied contradictory results since it gave the highest antioxidant capacity to profisetinidins/prorobitenidins tannins followed by, in decreasing order, procyanidins/prodelphinidins tannins, gallotannins and ellagitannins. Other authors [164,182] have obtained similar results, concluding that each antioxidant assay yields different information of a complementary nature. Indeed, these results can be explained since the different methods measure different chemical reaction/activity leading us to classify them such as HAT (hydrogen atom transfer) or ET (electron transfer) based assays [236]. ET-based assays include FRAP, CUPRAC, ABTS and DPPH methods, even if the latter two are considered as borderline between ET and HAT based assays. FRAP and CUPRAC results are based on an absorbance increase meanwhile, ABTS and DPPH measure the discoloration of radical reagents as a reduction reaction with antioxidants. Nevertheless, in general terms these four methods measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentration. In contrary, ORAC assay requires expensive equipment and is longer to perform, but is to date the only method that takes free radical action to completion and uses the AUC (area under the curve) technique for quantitation [237,238].

Table 18: Antioxidant capacity of oenological tannins from different botanical origins

	Tannins	ABTS		ORAC		FRAP		CUPRAC		DPPH	
		as mgTrolox/gTannins									
Procyanidins/ Prodelphinidins (PC/PD)	grapes 1	1464 ± 79		2065 ± 190		960 ± 95		1128 ± 17		521 ± 65	
	grapes 2	1930 ± 56		1715 ± 159		1620 ± 128		1678 ± 118		646 ± 53	
	grapes 3	1741 ± 55		2593 ± 220		1642 ± 115		1537 ± 49		611 ± 25	
	AV Grapes	**1712 ± 234**	**A**	**2124 ± 442**	**A**	**1407 ± 388**	**A**	**1448 ± 285**	**A**	**592 ± 65**	**A**
	seeds 1	1652 ± 53		2261 ± 245		1314 ± 40		1401 ± 125		608 ± 49	
	seeds 2	2514 ± 154		5993 ± 247		1358 ± 54		1675 ± 64		1376 ± 65	
	seeds 3	1425 ± 18		2589 ± 258		1056 ± 36		1072 ± 42		804 ± 36	
	seeds 4	1684 ± 103		1761 ± 154		1354 ± 93		1416 ± 50		595 ± 54	
	AV Seeds	**1819 ± 477**	**A**	**3151 ± 1925**	**A**	**1270 ± 144**	**A**	**1391 ± 247**	**A**	**846 ± 366**	**A**
	skins 1	1768 ± 46		2004 ± 230		1409 ± 69		1354 ± 142		624 ± 60	
	skins 2	1852 ± 46		2308 ± 258		1382 ± 45		1396 ± 94		534 ± 34	
	AV Skins	**1810 ± 60**	**A**	**2156 ± 215**	**A**	**1396 ± 19**	**A**	**1375 ± 30**	**A**	**579 ± 63**	**A**
	AV PC/PD	**1780 ± 60**	**αβ**	**2477 ± 584**	**αβ**	**1358 ± 76**	**αβ**	**1404 ± 38**	**β**	**672 ± 150**	**α**
Profisetinidins/ Prorobitenidins (PF/PR)	acacia 1	1381 ± 43		5856 ± 559		1054 ± 26		1100 ± 38		718 ± 31	
	acacia 2	1441 ± 41		1835 ± 141		1261 ± 81		1218 ± 64		539 ± 48	
	AV Acacia	**1411 ± 42**	**A**	**3845 ± 2843**	**A**	**1158 ± 147**	**A**	**1159 ± 84**	**A**	**628 ± 126**	**A**
	quebracho 1	1262 ± 57		1313 ± 193		1145 ± 44		1054 ± 23		453 ± 31	
	quebracho 2	1388 ± 47		5859 ± 141		831 ± 20		1022 ± 39		817 ± 13	
	quebracho 3	1392 ± 71		1810 ± 197		1117 ± 71		1086 ± 78		483 ± 36	
	quebracho 4	1490 ± 129		2515 ± 247		1489 ± 51		1319 ± 28		538 ± 29	
	quebracho 5	1758 ± 67		1725 ± 104		1510 ± 55		1307 ± 43		672 ± 47	
	quebracho 6	1887 ± 86		970 ± 165		1729 ± 137		1580 ± 38		1028 ± 120	
	AV Quebracho	**1529 ± 242**	**A**	**2365 ± 1789**	**A**	**1303 ± 329**	**A**	**1228 ± 215**	**A**	**665 ± 223**	**A**
	AV PF/PR	**1470 ± 84**	**α**	**3105 ± 1046**	**β**	**1230 ± 103**	**α**	**1193 ± 49**	**α**	**647 ± 26**	**α**
Gallotannins (GT)	nut galls 1	1681 ± 59		1355 ± 8		1430 ± 51		1285 ± 58		706 ± 32	
	nut galls 2	2075 ± 155		1672 ± 248		1548 ± 43		1386 ± 89		770 ± 78	
	nut galls 3	2182 ± 48		1758 ± 79		3019 ± 105		2069 ± 72		767 ± 24	
	nut galls 4	2559 ± 111		2394 ± 89		1039 ± 23		1466 ± 38		1461 ± 52	
	AV Nut galls (NG)	**2124 ± 361**	**A**	**1795 ± 435**	**A**	**1759 ± 868**	**A**	**1552 ± 353**	**A**	**926 ± 358**	**A**
	tara 1	2252 ± 87		1725 ± 122		2787 ± 113		1706 ± 27		1144 ± 101	
	tara 2	2380 ± 138		977 ± 81		1934 ± 78		1618 ± 97		1121 ± 74	
	tara 3	2616 ± 54		1240 ± 151		1660 ± 106		1381 ± 134		1135 ± 38	
	tara 4	2972 ± 55		2855 ± 146		1752 ± 30		1268 ± 119		2088 ± 68	
	AV Tara (T)	**2555 ± 316**	**A**	**1699 ± 830**	**A**	**2033 ± 515**	**A**	**1493 ± 203**	**A**	**1372 ± 477**	**A**
	AV NG/T (GT)	**2339 ± 305**	**β**	**1747 ± 68**	**α**	**1896 ± 194**	**β**	**1523 ± 41**	**χ**	**1149 ± 316**	**α**
Ellagitannins (ET)	chestnut 1	1957 ± 32		857 ± 50		1489 ± 102		1529 ± 63		880 ± 63	
	chestnut 2	2268 ± 128		2083 ± 281		1790 ± 117		1634 ± 77		1359 ± 44	
	chestnut 3	2439 ± 84		2536 ± 284		2194 ± 84		1770 ± 54		1602 ± 61	
	AV Chestnut (C)	**2221 ± 244**	**A**	**1825 ± 868**	**A**	**1824 ± 354**	**A**	**1644 ± 121**	**A**	**1280 ± 367**	**A**
	oak 1	1862 ± 151		865 ± 108		2038 ± 64		1578 ± 57		703 ± 54	
	oak 2	1482 ± 80		4008 ± 285		1662 ± 46		1176 ± 37		574 ± 52	
	oak 3	2250 ± 71		2238 ± 217		1408 ± 91		1650 ± 90		1493 ± 44	
	oak 4	2252 ± 101		804 ± 92		2311 ± 76		1654 ± 180		914 ± 85	
	oak 5	2389 ± 100		392 ± 48		3292 ± 110		2123 ± 49		811 ± 39	
	oak 6	2593 ± 54		384 ± 58		2816 ± 140		2023 ± 174		882 ± 25	
	oak 7	1167 ± 60		2928 ± 130		861 ± 33		1438 ± 19		880 ± 19	
	oak 8	2242 ± 91		814 ± 97		2321 ± 81		1664 ± 185		924 ± 90	
	AV Oak (O)	**2030 ± 487**	**A**	**1554 ± 1346**	**A**	**2089 ± 778**	**A**	**1663 ± 301**	**A**	**898 ± 269**	**A**
	AV C/O (ET)	**2125 ± 136**	**β**	**1690 ± 192**	**α**	**1956 ± 187**	**β**	**1654 ± 13**	**δ**	**1089 ± 271**	**α**

All data are the mean ± SD of six replicates. AV: Average. Different capital letters indicate the existence of significant differences between tannins of the same family ($p < 0.05$). Different Greek letters indicate the existence of significant differences between tannins of different families ($p < 0.05$).

In order to better understand the information provided by each of these analytical methods for the different types of oenological tannins, a principal component analysis (PCA) was performed using those parameters.

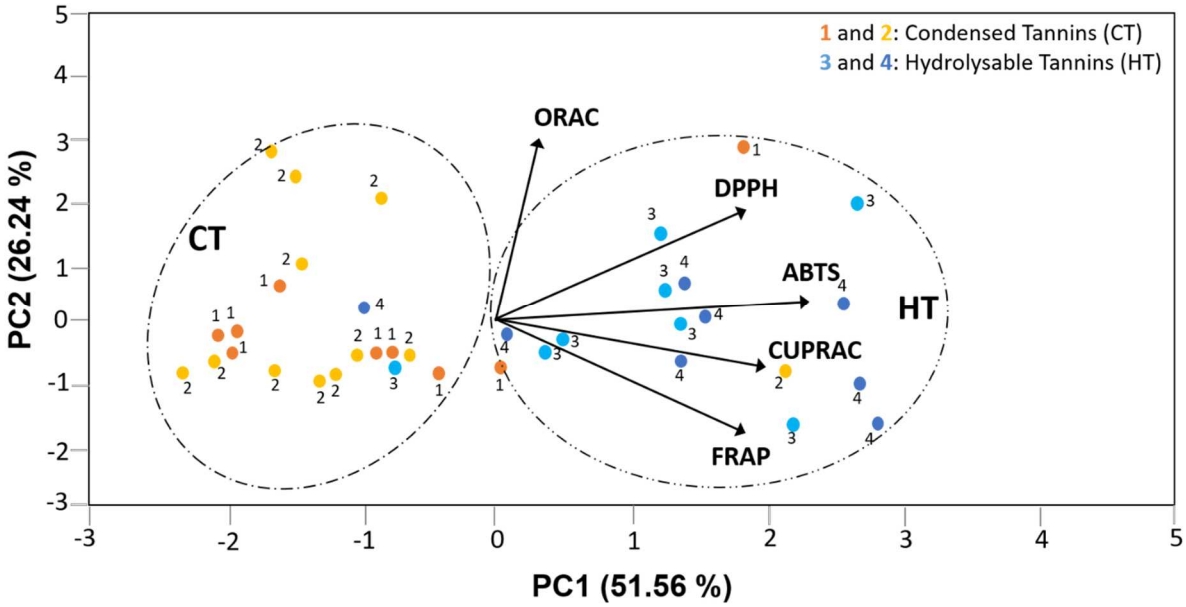


Figure 54: Plot of principal components analysis of antioxidant capacity in function of the used method

In this way, **Figure 54** shows the PCA applied to all the oenological tannins using the different antioxidant capacities. The first component explains 51.56% of the variance and the second 26.24%, so the aggregate variance explained by these two components totals 77.80%. The PC1 enables separation between hydrolysable tannins (on the right) and condensed tannins (on the left) with only five incorrect classifications (three condensed tannins were classified as hydrolysable tannins and two hydrolysables tannins as condensed tannins). The PC1 simply indicates that hydrolysable tannins have a higher antioxidant capacity than condensed tannins according to the ABTS, CUPRAC, FRAP, and DPPH assays. In contrast the PC2, which is explained mainly by the ORAC test, did not improve the separation between tannins obtained with PC1. Consequently, it is possible to distinguish between hydrolysable and condensed tannins according to their antioxidant capacities, but not possible to differentiate between procyanidins/prodelphinidins and profisetinidins/prorobitenidins tannins or between gallotannins and ellagitannins.

3.2. Oxygen consumption rate (OCR)

Our research team recently developed a method for measuring the oxygen consumption rate (OCR) of different oenological tannins [164] and suggested that oenological tannins should be classified in terms of their effectiveness to consume oxygen and thus protect wine against oxidation. A preliminary study was carried out with some representative samples to select the most suitable concentration for all tannins given their wide variety.

Figure 55 shows as an example, the oxygen consumption kinetics of a condensed tannin (procyanidins/prodelphinidins: skin 2) and a hydrolysable tannin (ellagitannins: oak 4) at different concentrations in an oxygen-saturated model wine solution at 20 °C.

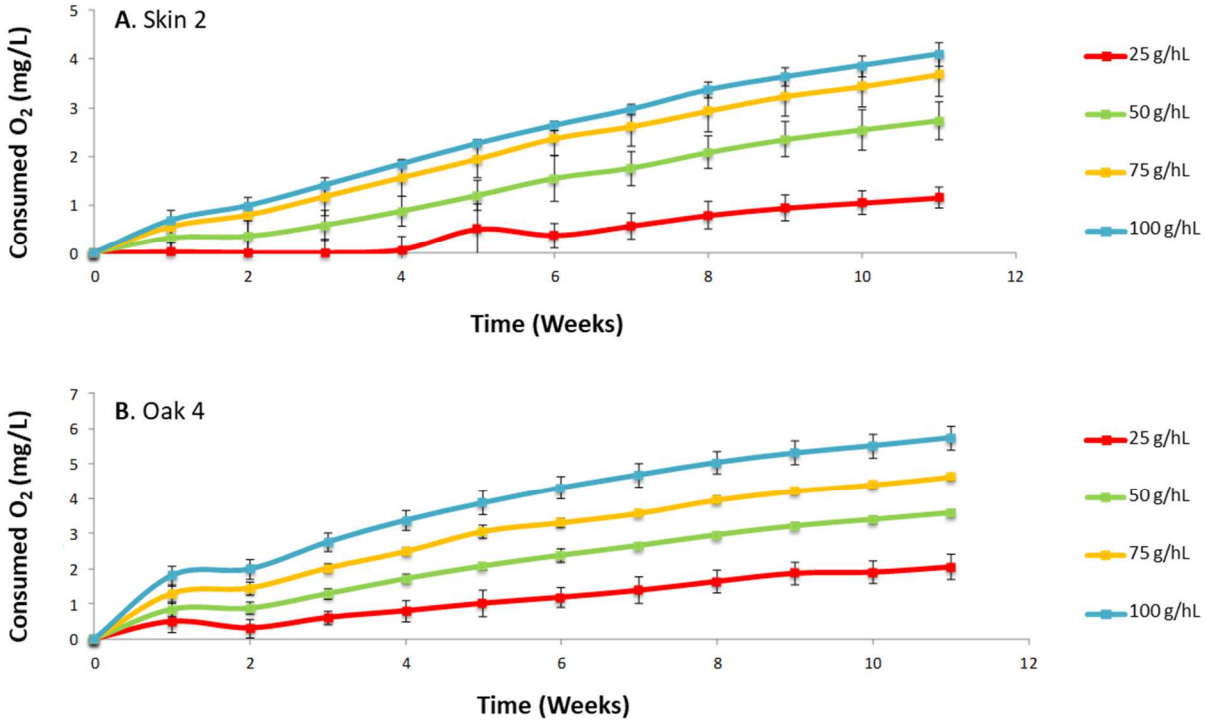


Figure 55: Influence of oenological tannin concentrations on oxygen consumption in an oxygen-saturated model wine solution at 20 °C

The oxygen consumption of the control model wine solution (without addition of any antioxidant) was very low and can be considered negligible (data not shown). In contrast, the supplementation with oenological tannins resulted in an oxygen consumption that was clearly influenced by the nature and dosage of the added antioxidant. However, the quantitative comparison of the oxygen consumption kinetics of these compounds is not evident from looking at these graphics. A kinetic modelization is therefore necessary in order to better quantify the antioxidant effectiveness of the various oenological tannins. Different mathematical regression approaches were tried (lineal, logarithmic, exponential...), but none of them provided satisfactory results (data not shown). After considering other possibilities, an acceptable modelization was obtained when the inverse of consumed oxygen was plotted versus the inverse of time.

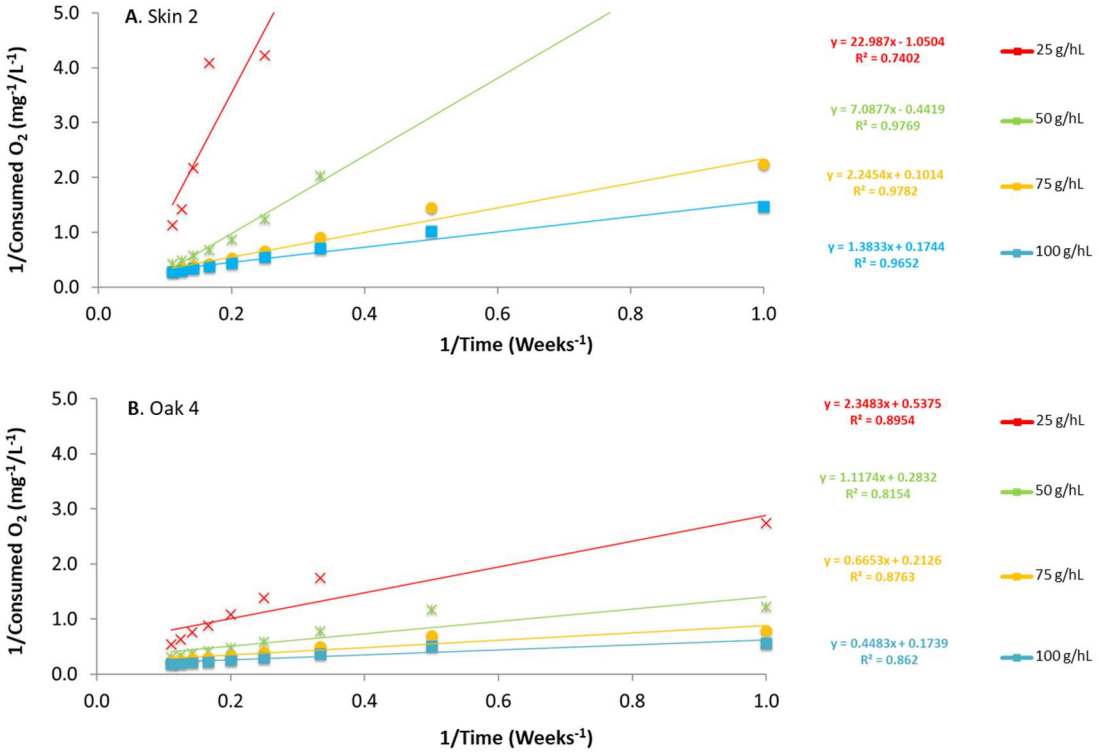


Figure 56: Influence oenological tannin concentrations on the oxygen consumption rate (OCR) in an oxygen-saturated model wine solution at 20 °C

Figure 56 shows the results obtained for skin 2 (**A**) and oak 4 (**B**). Satisfactory lineal regression coefficients were attained, thus confirming that this mathematical model works quite well. Similar results were obtained for the other oenological tannins tested but not shown in this figure so as not to overcomplicate the graphic. Their OCRs are shown below **(3.2.2)**. According to this modelization, the following equation can be established: $1/[O_2] = A/t + B$. This equation describes the relationship between the consumed oxygen versus time (**Figure 57**), which also shows how the consumed oxygen can be cleared up, how the first derivative is found, and finally how the OCR at time zero ($OCRt_0$) can be determined. These correspond to the inverse of the slope of the initial equation.

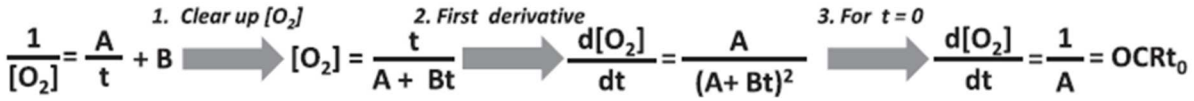


Figure 57: Calculation of the Oxygen Consumption Rate at time zero (OCRt0)

Using this procedure, it is possible to determine the relationship between the $OCRt_0$ and the antioxidant concentration for all the antioxidants studied.

Figure 58 show the representation of the $OCRt_0$ versus the concentration of skin 2 (**A**) and oak 4 (**B**) respectively. These graphics clearly indicate that the $OCRt_0$ is lineally dependent on the antioxidant concentration, considering that the linear regression coefficients are reasonably good (0.9621 and 0.9909 respectively for skin 2 and oak 4).

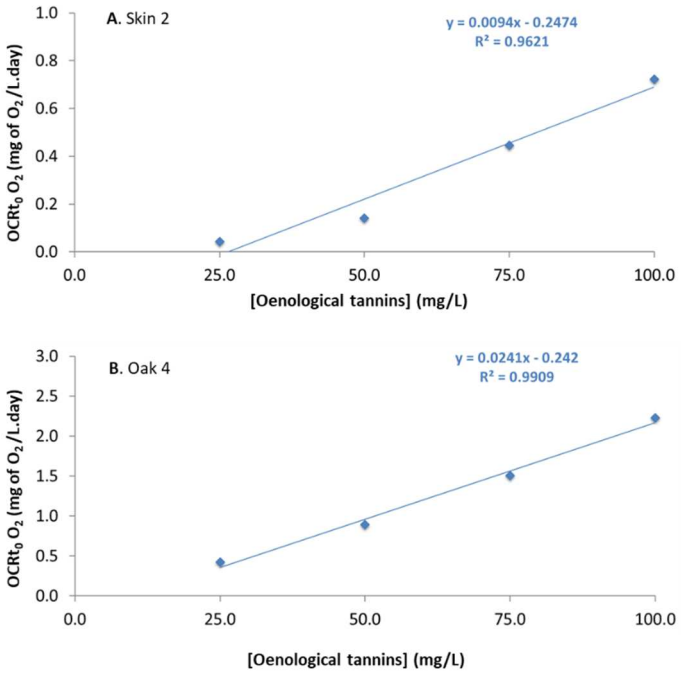


Figure 58: Influence of oenological tannins concentration on the OCRt0 in an oxygen-saturated model wine solution at 20 °C

Moreover, the slope of the straight lines provides the real OCR in function of the antioxidant concentration. Similar behaviors were obtained for all the other oenological tannins, enabling calculation of the OCR of each antioxidant expressed as milligram of oxygen per day and per gram of antioxidant.

3.2.1. Influence of the oenological tannin doses

Figure 59 shows the $OCRt_0$ obtained for a condensed tannin (procyanidins/prodelphinidins: skin 2) and a hydrolysable tannin (ellagitannins: oak 4). The results indicate that in both tannins types the $OCRt_0$ was higher when the concentration was greater. In line with these results, the highest concentration (100 g/hL) was selected for the following analysis to have higher precision and shorten the measurement times.

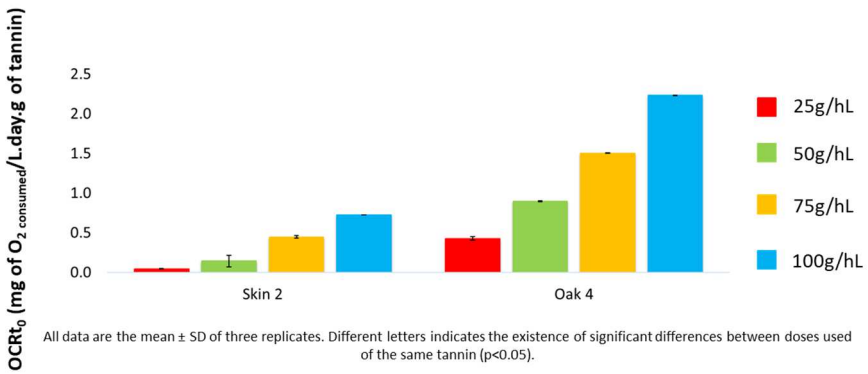


Figure 59: Influence of the tannin concentration on the oxygen consumption rate (OCR) in an oxygen-saturated model wine solution

3.2.2. Influence of the botanical origin of the oenological tannins

Table 19 shows the $OCRt_0$ obtained for each one of the thirty-six oenological tannins. The results present no significant differences between tannins of the same botanical origin. Indeed, the results obtained for grape tannins were no significantly different from the ones obtained for grape-seed and grape-skin tannins. The same observation is true for acacia and quebracho tannins, for nut gall and tara tannins and for chestnut and oak tannins. The fact that no significant differences were obtained between tannins from different botanical origin but appertaining to the same family lead us to classify them according their family in order to better understand the results and made the comparison between the different families of oenological tannins easier. Once the oenological tannins grouped by family, significant differences concerning the $OCRt_0$ were observed. Indeed, ellagitannins and gallotannins were significantly different between them and between the procyanidins/prodelphinidins (PC/PD), profisetinidins/prorobitenidins (PF/PR) tannins. Only the two families corresponding to procyanidins/prodelphinidins (PC/PD), profisetinidins/prorobitenidins (PF/PR) were no significantly different between them.

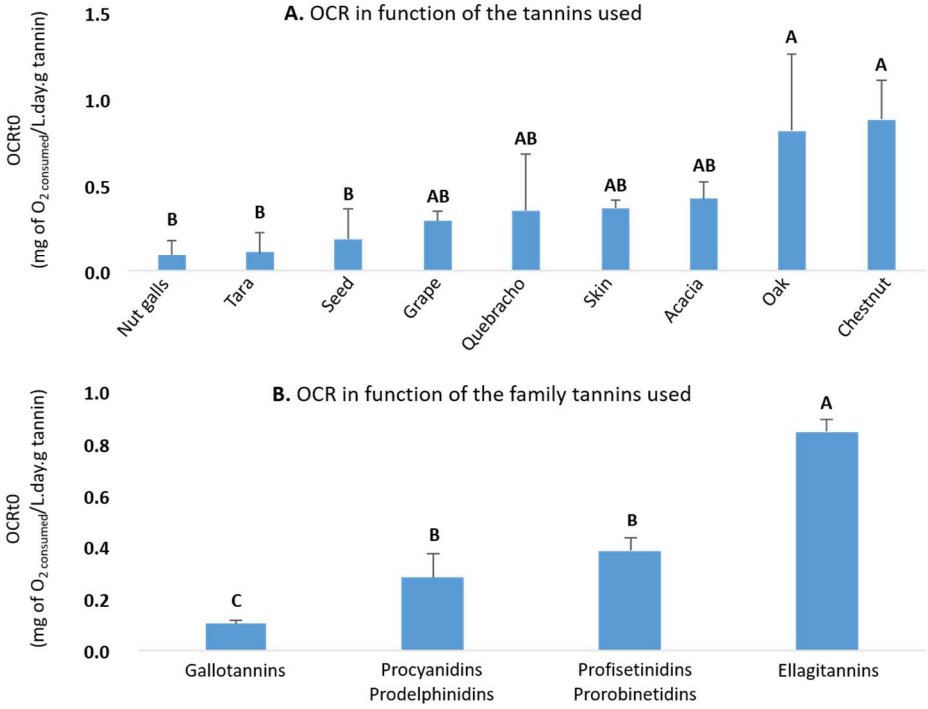
Table 19: Oxygen consumption rate (OCRt0) of the different oenological tannins in function of their botanical origin and family

Tannins		$OCRt_0$ (mg of O_2/L.day.$g_{tannins}$)			
Procyanidins/ Prodelphinidins (PC/PD)	grapes 1	0.27	±	0.15	
	grapes 2	0.25	±	0.00	
	grapes 3	0.35	±	0.09	
	AV Grapes	**0.29**	**±**	**0.06**	**A**
	seeds 1	0.34	±	0.04	
	seeds 2	0.33	±	0.08	
	seeds 3	0.03	±	0.02	
	seeds 4	0.02	±	0.00	
	AV Seeds	**0.18**	**±**	**0.18**	**A**
	skins 1	0.40	±	0.05	
	skins 2	0.33	±	0.05	
	AV Skins	**0.36**	**±**	**0.05**	**A**
	AV PC/PD	**0.28**	**±**	**0.09**	**β**
Profisetinidins/ Prorobitenidins (PF/PR)	acacia 1	0.49	±	0.02	
	acacia 2	0.35	±	0.07	
	AV Acacia	**0.42**	**±**	**0.10**	**A**
	quebracho 1	0.35	±	0.01	
	quebracho 2	0.53	±	0.14	
	quebracho 3	0.04	±	0.00	
	quebracho 4	0.05	±	0.01	
	quebracho 5	0.90	±	0.24	
	quebracho 6	0.23	±	0.01	
	AV Quebracho	**0.35**	**±**	**0.33**	**A**
	AV PF/PR	**0.38**	**±**	**0.05**	**β**
Gallotannins (GT)	nut galls 1	0.00	±	0.00	
	nut galls 2	0.17	±	0.06	
	nut galls 3	0.17	±	0.02	
	nut galls 4	0.02	±	0.01	
	AV Nut galls (NG)	**0.09**	**±**	**0.09**	**A**
	tara 1	0.27	±	0.05	
	tara 2	0.04	±	0.01	
	tara 3	0.11	±	0.04	
	tara 4	0.01	±	0.00	
	AV Tara (T)	**0.11**	**±**	**0.11**	**A**
	AV NG/T (GT)	**0.10**	**±**	**0.01**	**χ**
Ellagitannins (ET)	chestnut 1	0.96	±	0.23	
	chestnut 2	1.05	±	0.06	
	chestnut 3	0.62	±	0.07	
	AV Chestnut (C)	**0.88**	**±**	**0.23**	**A**
	oak 1	0.35	±	0.05	
	oak 2	0.52	±	0.08	
	oak 3	1.01	±	0.18	
	oak 4	1.53	±	0.01	
	oak 5	0.58	±	0.00	
	oak 6	0.68	±	0.14	
	oak 7	0.46	±	0.07	
	oak 8	1.36	±	0.01	
	AV Oak (O)	**0.81**	**±**	**0.44**	**A**
	AV C/O (ET)	**0.84**	**±**	**0.05**	**α**

All data are the mean ± SD of three replicates. AV: average. Different capital letters indicate the existence of significant differences between tannins of the same family ($p < 0.05$). Different Greek letters indicate the existence of significant differences between tannins of different families ($p < 0.05$).

In order to better visualize the results, tannins were grouped first of all by botanical origin **Figure 60 (A)** and then by family **Figure 60 (B)**.

Figure 60 (A) compares the $OCRt_0$ of the different oenological tannins according to their botanical origin. Overall the data shows that chestnut and oak tannins have the highest $OCRt_0$, followed in decreasing order by acacia, grape-skin, quebracho and grape. Grape-seed, tara and nut gall tannins presented the lowest $OCRt_0$ compared to the rest of the oenological tannins used. The differences were only significant between chestnut/oak and grape-seed/tara/nut gall tannins, probably because of the wide variability observed in the richness of the different types of commercial tannins (see chapter 3).



All data are the mean ± SD of three replicates. Different letters indicates the existence of significant differences between tannins of different family **(A)** (p<0.05). Different letters indicates the existence of significant between different families of tannins **(B)** (p<0.05).

Figure 60: Oxygen consumption rate (OCR) of the different oenological tannins in function of their nature and botanical origin

Figure 60 (B) shows the results obtained when these oenological tannins are grouped by family according to chemical structure independently of botanical origin: procyanidins/prodelphinidins (PC/PD), profisetinidins/prorobitenidins (PF/PR), gallotannins (GT) and ellagitannins (ET). These data clearly show that ellagitannins are the most efficient at consuming oxygen (0.84 ± 0.05 mg of O_2/L by day and by g of product), followed in decreasing order by PF/PR tannins (0.38 ± 0.05 mg of O_2/L.day.$g_{tannins}$) and PC/PD tannins (0.28 ± 0.09 mg of O_2/L.day.$g_{tannins}$). Finally, gallotannins (GT) are the least effective type for consuming oxygen (0.10 ± 0.01 mg of O_2/L.day.$g_{tannins}$). The differences regarding direct oxygen consumption ($OCRt_0$) were only not significant between procyanidins/prodelphinidins (PC/PD) and profisetinidins/prorobitenidins (PF/PR).

This could be explaining by the fact that these two families present similar composition and structure. Nevertheless, the differences were significant between the ellagitannins, gallotannins and PF/PR; PC/PD tannins. Other researchers, reported similar results using just one tannin from each family type [164]. This study confirms that ellagitannins are the most effective of the various oenological tannins, followed in decreasing order by condensed tannins (PC/PD and PF/PR) and finally gallotannins in terms of protecting the wine against chemical oxidation using a large sample of commercial products.

4. Conclusion

It can be concluded that it is necessary to combine different antioxidant assay in order to be closer as possible of the reality of the different mechanism/reaction that can happen in must and wine. Indeed, we have observed and demonstrated the importance and diversity of each method, leading us to use a combination of them more than only one as a reference. Moreover, the new method proposed, corresponding to the direct consumption of oxygen (OCR) is necessary in order to measure also the O_2 consumed directly by a non-invasive approach. At the end, the combination of antioxidant capacity and oxygen consumption data makes it possible to classify the various families of oenological tannins and provides useful information regarding their protective effect against wine oxidation. More precisely, the different oenological tannins can be classified using principal component analysis based on their antioxidant capacities. Indeed, condensed tannins and hydrolysable tannins can be separate regarding their antioxidant capacities. In general terms, hydrolysable tannins have a higher antioxidant capacity than condensed tannins according to all the analytical methods except for ORAC. Nevertheless, only with the antioxidant capacity it was not possible to separate inside of the hydrolysable tannins, gallotannins from ellagitannins and inside the condensed tannins, procyanidins/prodelphinidins from profisetinidins/prorobitenidins. In contrast, ellagitannins and gallotannins behave completely differently in terms of their ability to consume oxygen, with ellagitannins being the most effective and gallotannins the least. This method allowed us to separate gallotannins from ellagitannins. Nevertheless, condensed tannins were being always at an intermediate level with no significant difference between procyanidins/prodelphinidins and profisetinidins/prorobitenidins. This study therefore confirms that ellagitannins are the most effective of the various oenological tannins, followed in decreasing order by condensed tannins (procyanidins/prodelphinidins and profisetinidins/prorobitenidins) and finally gallotannins in terms of protecting the wine against chemical oxidation. Complementary studies are needed to determine the effect of the various oenological tannins in inhibiting polyphenol oxidases (tyrosinase and laccase) to prevent the enzymatic browning of musts and wines. The study of oenological tannins as antioxidasic compounds is presented in the **chapter 6**.

Chapter 5: Color stabilization properties of oenological tannins

1. Introduction

Anthocyanins are the main compounds responsible for the color of red wines. They are the major natural pigments in red wines, reaching typically 500 mg/L in young red wines [96], but their concentration depends on the grape variety and the growing conditions [239]. In red wines from *Vitis vinifera* grapes, the main monomeric anthocyanins are the malvidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, pelargodinin-3-*O*-glucoside, peonidin-3-*O*-glucoside and petunidin-3-*O*-glucoside [155,240]. These anthocyanins are also present in acylated forms with acetic, coumaric and caffeic acids [112]. More derivatives of anthocyanins can be extracted or formed during the fermentation process [241]. The extraction and formation of new pigments or copigment complexes permit the color stabilization of red wine during the aging process since the monomeric anthocyanin content decreases constantly. Different phenomena can occur naturally to stabilize the color in red wines. Anthocyanins can form association structures with other colorless compounds (intermolecular copigmentation), with other anthocyanins (self-association), or in the case of coumaroylated and cafeoylated anthocyanins, the acylated esterified group can be associate with the pyrylium ring of the same anthocyanidin molecule (intramolecular copigmentation) [154,242]. The copigmentation phenomenon is characterized by the formation of a "sandwich complex," meaning an hydrophobic interaction (π-π stacking) between the B ring of the anthocyanin and the copigment (phenolic compounds, for example) [154]. These complexes adopt a sandwich-like structure that protects the flavylium cation against nucleophilic attack by water, avoiding the formation of the colorless hemiketal form [243]. Consequently, copigmentation increases wine color intensity (hyperchromic effect), but it can also change the color hue through a bathochromic shift [244]. Finally, the formation of new pigments can occur, providing a large variety of pigments such as pyranoanthocyanins and polymeric anthocyanins [112]. The mechanism of pigment formation or copigmentation differs with respect to the botanical origins of oenological tannins since they present different chemical structure. In this way, condensed tannins can combine directly or indirectly with anthocyanins while hydrolysable tannins cannot participate in condensation reactions with anthocyanins. However, hydrolysable tannins, can participate in copigmentation reactions, as well as protect wine anthocyanins from oxidation since they may regulate oxidation-reduction phenomena happening in wines [4]. Furthermore, the color stabilization of red wine depends not only on the anthocyanin content and the presence of copigment or yeast by-products, but also on the wine conditions such as pH level and ethanol content [245].

Among the functions attributed to oenological tannins, their enhancing effect on red wine color and stability is probably one of the main reasons for their potential use in winemaking. Red wine color plays a very important role for consumers in their acceptance and perception of the product [246].

For this reason, winemakers are interested in gaining a better understanding of the role played by polyphenols, and more particularly, the interactions between tannins/anthocyanins to produce deeply colored wines with great color stability during aging [246]. To achieve this goal, winemakers apply different oenological practices, for example, "reduced volume of juice" or thermovinification [247], addition of pectinolytic enzymes [248] and oak aging. Despite the various tools available to winemakers, none of these treatments have provided clear results without secondary problems or effects. The addition of oenological tannins has been proposed in the past, but due to the abundant diversity, their effects are not so well studied and clear.

For these reasons, the aim of this chapter was to give model standards in terms of copigmentation proportion and level for the major anthocyanin of wine, malvidin-3-*O*-glucoside. Additionally, this chapter aimed to verify and confirm the effectiveness of different botanical origin of oenological tannins on wine color stability to be applied as a new tool by winemakers. For this purpose, the experimentations will be conducted at different pH, different ethanol contents and at different times.

2. Materials and methods

The full absorbance spectrum in the visible range (400-800 nm) of all samples was measured with a spectrophotometer, using a quartz cell (10 mm of path length). In all cases, the spectrum of the solution containing only the copigment (oenological tannins or (-)-epicatechin) was subtracted from the spectrum of the corresponding mix copigment/pigment (oenological tannins/malvidin or (-)-epicatechin/malvidin). This subtraction was made to avoid the interferences due to the natural color of each copigment. Then, the absorbance at 520 nm (A_{520nm}) and the CIELAB color space were calculated with an MSCV software [249]. The comparison of the A_{520nm} and CIELAB color space between copigment/pigment and pigment allowed us to determine the hypochromic/hyperchromic (variation of λ_{max}) and hypsochromic/bathochromic (variation of ε_{max}) effects of each oenological tannin.

2.1. Copigmentation effects of oenological tannins in model wine solution supplemented by malvidin-3-*O*-monoglucoside

In this part, different parameters which can influence the copigmentation phenomenon are studied. For this purpose, three parameters have been studied, which are the botanical origin of the tannins, the pH and ethanol content of the matrix.

2.1.1. Influence of the botanical origin of oenological tannins

The thirty-six oenological tannins were used to determine the influence of the botanical origin on the copigmentation effect. A model wine solution (12% vol. of ethanol, 4 g/L of tartaric acid and at pH 3.5) was prepared and supplemented with 0.1, 0.2 and 0.4 g/L of each of the thirty-six commercial tannins and (-)-epicatechin (copigments). The (-)-epicatechin was used as reference standard.

Simultaneously, solutions containing 50 mg/L of malvidin-3-*O*-monoglucoside (pigment) and 0.1, 0.2 and 0.4 g/L of commercial tannins or (-)-epicatechin were prepared to reach copigment/pigment ratios of 2, 4 and 8 respectively. Finally, a solution containing only malvidin-3-*O*-monoglucoside was prepared as positive control. Then, 1.5 mL of each solution was placed in Eppendorf tubes and maintained under airtight conditions. A week later, the full absorbance spectrum in the visible range (400-800 nm) was measured to determine the CIELAB color space and the effectiveness of copigmentation (Cp).

2.1.2. Influence of copigment/pigment ratio and pH

Five out of the thirty-six previously tested oenological tannins (1 grape-seed, 1 grape-skin, 1 quebracho, 1 ellagitannin and 1 gallotannin) representing the most common botanical origins were used to facilitate these experiments. For each oenological tannin considered, three different model wine solutions (4 g/L of tartaric acid and 12% vol. of ethanol) were prepared with different pH levels (3.1, 3.5 and 3.9) to observe the effect of pH on color stabilization. All samples were prepared as in the previous assay (2.1.1). As already stated, the samples were maintained under airtight conditions and the full absorbance spectrum was measured a week later.

2.1.3. Influence of copigment/pigment ratio and ethanol content

The experimentation was conducted as described (2.1.2) but in this case, three different model wine solutions (4 g/L of tartaric acid and pH adjusted at 3.5) were prepared with different ethanol contents (10, 12 and 14% vol. of ethanol) to observe the effect of ethanol on color stabilization. All the samples were prepared as in the previous assay (2.1.1). As stated previously, the samples were maintained in airtight conditions and the full absorbance spectrum was measured a week later.

2.2. Influence of time contact between oenological tannins and malvidin-3-*O*-glucoside on color stabilization

In this part, subsequently to the first part in which were tested the effect of the botanical origin, ethanol content and pH level, the time contact between oenological tannins and malvidin-3-*O*-glucoside was also studied. According to this, time contact influence was first estimated regarding the color stabilization with the analysis of color component and the determination of the copigmentation index. Then, the quantification of malvidin-3-*O*-glucoside and its degradation products were achieved.

2.2.1. Analysis of color component

Five out of the thirty-six oenological tannins (same tannins as in paragraph 2.1.2 and 2.1.3) were used to determine the influence of the time contact on the copigmentation effect. A model wine solution (12% vol. of ethanol, 4 g/L of tartaric acid and adjusted at pH 3.5) was prepared and supplemented with 0.1, 0.2 and 0.4 g/L of each of the thirty-six commercial tannins and (-)-epicatechin (copigments).

The (-)-epicatechin was used as reference standard. Simultaneously, solutions containing 50 mg/L of malvidin-3-*O*-monoglucoside (pigment) and 0.1, 0.2 and 0.4 g/L of commercial tannins or (-)-epicatechin were prepared to reach copigment/pigment ratios of 2, 4 and 8 respectively. Finally, a solution containing only the malvidin-3-*O*-monoglucoside was prepared as positive control. Then, 1.5 mL of each solution was placed in Eppendorf tubes and maintained under airtight conditions. After 1, 7, 14 and 21 days, the full absorbance spectrum in the visible range (400-800 nm) was measured to determine the CIELAB color space and A_{520nm}.

2.2.2. Copigmentation index

For the copigmentation effectiveness, the previous samples were also used and CIELAB coordinates were determined as well as previously (2.2.1). The following equation (**Figure 61)** was proposed as an index to measure comparatively the effectiveness as copigments of the different commercial tannins [250].

$$\text{Copigmentation index} = 100 \text{ x } \frac{\Delta E_{ab} \text{ x TS} - \Delta E_{ab} \text{ x CS}}{\Delta E_{ab} \text{ x CS}}$$

$$\Delta E_{ab} = \sqrt{(L_2^* - L_1^*) + (a_2^* - a_1^*) + (b_2^* - b_1^*)}$$

Figure 61: Equation of the copigmentation index and the difference of color between two samples (ΔE_{ab})

ΔE_{ab} x CS is the total color difference between the control solution malvidin-3-*O*-monoglucoside (CS) and a pure white color solution.

ΔE_{ab} x TS is the total color difference between the solution of malvidin-3-*O*-monoglucoside containing 0.4 g/L of commercial tannin (TS) and a pure white color solution.

The CIELAB coordinates of a pure white color solution are L* = 100.00, a* = 0.00 and b* = 0.00.

2.2.3. Quantification of malvidin-3-*O*-glucoside by HPLC-DAD

The quantification was purchased in order to determine and estimate the possible losses of malvidin-3-*O*-glucoside due to oxidation or the possible formation of polymeric pigments between malvidin-3-*O*-glucoside and oenological tannins.

The quantification of malvidin-3-*O*-glucoside was achieved according to the method described on the literature [251]. This quantification was carried out after 1, 7, 14 and 21 days after the sample preparation by reverse-phase HPLC analyses with an Agilent 1200 series liquid chromatograph (HPLC-DAD) using an Agilent Zorbax Eclipse XDB-C18 (4.6 x 250 mm, 5 µm particle size) column (Agilent Technologies, Santa Clara, USA). The injection volume was 40 µL. The solvent system, at a flow rate of 1.5 mL/min, was water acidified with 10 % of formic acid (solvent A) and water with methanol acidified with 10 % of formic acid (45/45/10, v/v/v) (solvent B). The elution gradient was (time, % of solvent A): 0 min, 80.0%; 15 min, 60.0%; 25 min, 20.0%; 33 min, 80.0% and then, 10 min equilibrium time was left between analysis.

For this purpose, a calibration curve of malvidin-3-*O*-glucoside was prepared at 0, 0.25, 0.5, 0.75 and 1 g/L. All samples were analyzed in triplicates. Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000.

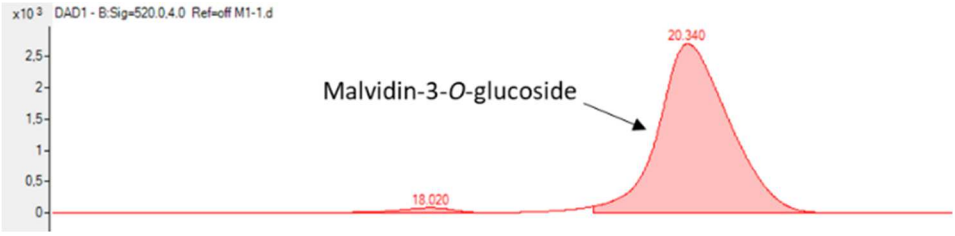


Figure 62: Example of HPLC chromatogram (DAD-520nm) of malvdin-3-O-glucoside

2.2.4. Characterization of malvidin-3-*O*-glucoside degradation products

Samples were prepared as previously explained and filtered on 0.45 µm whatman filters. The HPLC was a 1260 Infinity high performance liquid chromatography system coupled to a diode array detector (DAD) and a 6545 quadrupole-time of flight (Q-TOF) mass spectrometer detector (Agilent, Waldbronn, Germany). The control software was MassHunter Workstation (version B.08.00). The Q-TOF used a Dual Jet Stream Electrospray Ionization (Dual AJS-ESI) source operated in the positive ionization mode and the following parameters were set: capillary voltage, 3500 V; fragmentor, 100; gas temperature, 300 °C; drying gas, 9 L/min; nebulizer, 40 psi; sheath gas temperature, 400 °C; sheath gas flow, 10 L/min; acquisition range, 100-1700 m/z; and fixed collision. Samples were analyzed by injection (20 µL) on a ZORBAX Eclipse XDB-C18 (2.1 × 150 mm; 3.5 µm particle, Agilent USA, Santa Clara-California), at 40 °C. The solvent system, at a flow rate of 0.3 mL/min, was water acidified with 1% of formic acid (solvent A) and methanol with acetonitrile acidified with 1% of formic acid (30/70/1%; v/v) (solvent B). The elution gradient was (time, % of solvent A): 0 min, 99.0%; 2 min, 98.5%; 25 min, 97.0%; 45 min, 65.0%; 55 min, 20.0%; 60 min, 95.0% and then, 10 min equilibrium time was left between analysis. Compounds were identified using the algorithm "Find by Formula" that evaluated the mass accuracy together with the isotopic relative abundance and isotopic separation. Quantification was performed using the ESI-EIC of each corresponding mass, which are presented in **Table 20**.

Table 20: Degradation products of malvidin-3-O-glucoside

Compounds	Chemical formula	m/z
Formylphloroglucinol	$C_7H_6O_4$	155.120
Syringic acid	$C_9H_{10}O_5$	199.174

Quantification of degradation products was realized using a calibration curve of syringic acid at 1, 2.5, 5, 10, 20 and 30 mg/L. Malvidin-3-*O*-glucoside at 84 mg/L was used as external standard.

Detection and quantification thresholds of the methods have been evaluated according to the recommendation of the resolution OIV OENO 7/2000. **Figure 63** shows the obtained chromatogram for the malvidin-3-*O*-glucoside and an oak tannin as an example. All the samples were analyzed by triplicate.

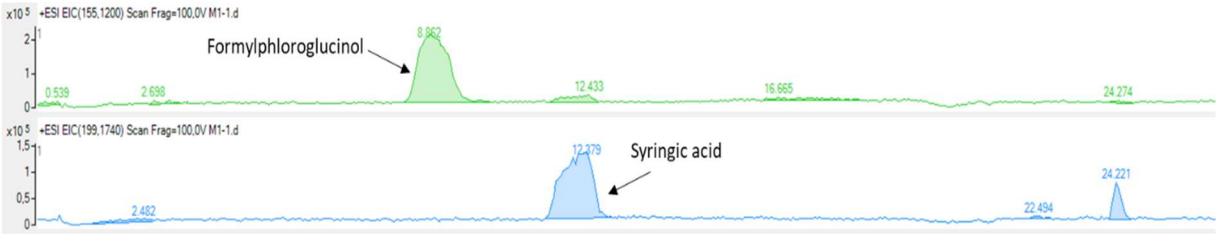


Figure 63: Example of an ESI-EIC spectra of malvidin-3-O-glucoside degradation products

3. Results and discussion

The copigmentation phenomenon represents a very important parameter regarding the aging of wines, since it permits to protect and stabilize their color. Nevertheless, it cannot be forgotten the importance also of the formation of new pigments conducting in some cases to an improvement of the color and in other cases to a decrease of the color. For these reasons, in this chapter the two phenomena have been studied and will be discussed in order to elucidate the role of the oenological tannins as copigments as well as in formation of new pigments.

3.1. Copigmentation effects of oenological tannins in model wine solution supplemented by malvidin-3-*O*-monoglucoside

In this first part, the effect of the oenological tannins as copigments is discussed, considering the influence of their botanical origin as well as the pH and ethanol content of the matrix used. Indeed, various authors have commented on the influence of the intrinsic conditions of red wine, specifically pH level [252,253] and ethanol content [143,245,254], on color stabilization.

3.1.1. Influence of the botanical origin of oenological tannins

This first experiment allowed us to determine the most efficient oenological tannins family with respect to color improvement according to their botanical origin under typical oenological conditions. **Table 21** displays the results obtained for each one of the 36 oenological tannins and (-)-epicatechin. In the case of the parameter b*, we observed a significant difference between grape-skin and grape-seed tannins and between acacia and quebracho tannins. Significant differences for the parameter L* were also observed between tannins within the same family in the case of the nut gall and tara tannins. Some variations can be noted between tannins from the same botanical origin, but in most of the cases, no contrasts have been found between tannins of the same family. In all cases, significant differences were noted between the diverse families of tannins.

In fact, some compelling disparities were even noted between two tannins from the same botanical origin, but the inequalities between families were more revealing. Indeed, we can expect a difference regarding the properties according to the richness in tannins of the commercial extract (Chapter III), but once again (as seen in Chapter IV), this property is similar for tannins of the same chemical origin. Tannins from different chemical family's present characteristics richness as their molecular compositions change. In fact, all the results obtained lead us to conclude that it is more appropriate to classify the oenological tannins according to their botanical family than by the plant source from which they originate. It is the chemical structure of the commercial tannins rather than their plant source which dictates their properties. In addition, their chemical structure better explains the ability of some commercial tannins to consume oxygen or protect other compounds from the oxidation, and the ability of others to stabilize the color of the wines. Actually, in the case of winemaking, a winemaker chooses the tannins according to their family, since in most cases; the equipment to test the purity of each tannin is not available.

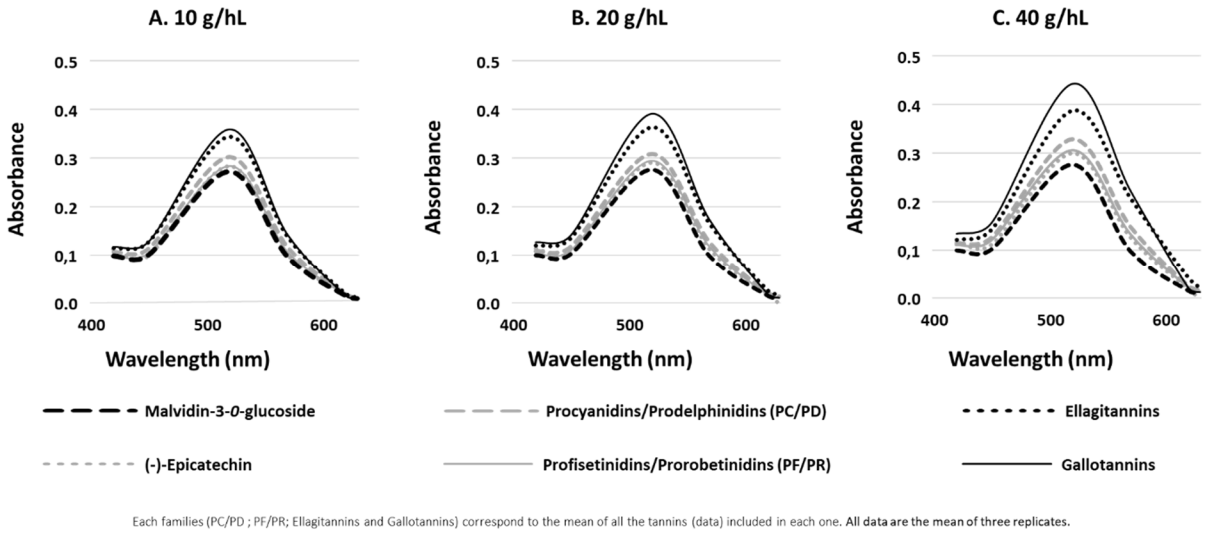
Table 21: Copigmentation effectiveness (Cp) of (-)-epicatechin or oenological tannins regarding CIELAB parameters

	Tannins	A_{520nm}		L*		a*		b*		h_{ab}		C^*_{ab}	
	(−)-Epicatechin	**0.061 ± 0.008**	**B**	**−1.39 ± 0.06**	**A**	**2.29 ± 0.02**	**A**	**−1.15 ± 0.09**	**B**	**−8.9 ± 0.2**	**A**	**2.51 ± 0.03**	**A**
Procyanidins/ Prodelphinidins (PC/PD)	Grape 1	0.141 ± 0.004		-2.09 ± 0.60		2.26 ± 0.50		-1.00 ± 0.04		-7.2 ± 0.3		2.45 ± 0.05	
	Grape 2	0.145 ± 0.002		−1.47 ± 0.60		1.56 ± 0.10		−0.86 ± 0.06		−7.2 ± 0.5		1.71 ± 0.10	
	Grape 3	0.167 ± 0.002		−1.84 ± 0.00		1.93 ± 0.20		−0.88 ± 0.05		−7.5 ± 0.4		2.01 ± 0.06	
	AV Grapes	**0.151 ± 0.014**	**A**	**−1.80 ± 0.31**	**A**	**1.92 ± 0.35**	**A**	**−0.91 ± 0.07**	**B**	**−7.3 ± 0.2**	**A**	**2.06 ± 0.37**	**A**
	Seed 1	0.128 ± 0.001		−1.04 ± 0.00		1.06 ± 0.60		−0.47 ± 0.05		−5.2 ± 0.5		1.26 ± 0.06	
	Seed 2	0.174 ± 0.007		−2.70 ± 0.00		2.43 ± 0.50		−1.02 ± 0.04		−7.9 ± 0.5		2.49 ± 0.04	
	Seed 3	0.073 ± 0.002		−0.90 ± 0.00		1.40 ± 0.60		−0.63 ± 0.04		−6.0 ± 0.3		1.62 ± 0.04	
	Seed 4	0.054 ± 0.002		−0.63 ± 0.00		1.25 ± 0.70		−0.52 ± 0.08		−5.3 ± 0.8		1.45 ± 0.03	
	AV Seeds	**0.107 ± 0.055**	**A**	**−1.32 ± 0.94**	**A**	**1.54 ± 0.61**	**A**	**−0.66 ± 0.25**	**A**	**−6.1 ± 1.2**	**A**	**1.70 ± 0.54**	**A**
	Skin 1	0.195 ± 0.022		−1.96 ± 0.07		1.81 ± 0.04		−0.76 ± 0.02		−6.7 ± 0.9		2.04 ± 0.13	
	Skin 2	0.163 ± 0.002		−2.49 ± 0.07		2.37 ± 0.08		−1.40 ± 0.09		−11.3 ± 0.8		2.65 ± 0.05	
	AV Skins	**0.179 ± 0.022**	**A**	**−2.22 ± 0.37**	**A**	**2.09 ± 0.40**	**A**	**−1.08 ± 0.45**	**B**	**−9.0 ± 3.3**	**A**	**2.35 ± 0.43**	**A**
	AV PC/PD	**0.146 ± 0.036**	**β**	**−1.78 ± 0.45**	**α**	**1.85 ± 0.28**	**β**	**−0.88 ± 0.21**	**α**	**−7.5 ± 1.5**	**α**	**2.04 ± 0.32**	**χ**
Profisetinidins/ Prorobitenidins (PF/PR)	Acacia 1	0.125 ± 0.018		−1.41 ± 0.06		0.91 ± 0.08		−0.53 ± 0.14		−4.6 ± 0.5		1.07 ± 0.06	
	Acacia 2	0.084 ± 0.006		−1.18 ± 0.14		1.18 ± 0.07		−0.18 ± 0.09		−1.4 ± 0.8		1.20 ± 0.08	
	AV Acacia	**0.105 ± 0.029**	**A**	**−1.30 ± 0.16**	**A**	**1.05 ± 0.20**	**A**	**−0.36 ± 0.25**	**A**	**−3.0 ± 2.3**	**A**	**1.14 ± 0.09**	**A**
	Quebracho 1	0.078 ± 0.079		−0.97 ± 0.07		1.14 ± 0.04		−0.83 ± 0.06		−7.9 ± 0.7		1.33 ± 0.04	
	Quebracho 2	0.088 ± 0.047		−1.23 ± 0.07		1.80 ± 0.04		−2.27 ± 0.05		−8.1 ± 0.6		1.28 ± 0.11	
	Quebracho 3	0.062 ± 0.007		−1.09 ± 0.06		0.97 ± 0.13		−0.93 ± 0.05		−9.2 ± 1.6		1.39 ± 0.08	
	Quebracho 4	0.076 ± 0.004		−1.13 ± 0.06		1.13 ± 0.15		−0.99 ± 0.19		−9.6 ± 0.8		1.32 ± 0.16	
	Quebracho 5	0.118 ± 0.010		−1.49 ± 0.10		1.55 ± 0.11		−1.63 ± 0.11		−14.4 ± 0.5		1.91 ± 0.12	
	Quebracho 6	0.173 ± 0.002		−2.65 ± 0.06		2.72 ± 0.03		−2.14 ± 0.07		−16.0 ± 0.3		3.17 ± 0.03	
	AV Quebracho	**0.099 ± 0.041**	**A**	**−1.43 ± 0.63**	**A**	**1.55 ± 0.65**	**A**	**−1.46 ± 0.64**	**B**	**−10.9 ± 3.5**	**B**	**1.74 ± 0.74**	**A**
	AV PF/PR	**0.102 ± 0.004**	**β**	**−1.36 ± 0.09**	**α**	**1.30 ± 0.36**	**β**	**−0.91 ± 0.78**	**α**	**−6.96 ± 5.5**	**α**	**1.44 ± 0.42**	**χ**
Gallotannins (GT)	Nut gall 1	0.302 ± 0.014		−2.76 ± 0.00		4.84 ± 0.09		−2.48 ± 0.10		−15.6 ± 0.1		5.33 ± 0.10	
	Nut gall 2	0.517 ± 0.029		−4.86 ± 0.21		7.01 ± 0.05		−3.78 ± 0.11		−20.0 ± 0.1		7.72 ± 0.06	
	Nut gall 3	0.234 ± 0.007		−2.78 ± 0.06		5.11 ± 0.04		−2.41 ± 0.04		−15.2 ± 0.2		5.59 ± 0.15	
	Nut gall 4	0.467 ± 0.025		-4.36 ± 0.07		6.69 ± 0.12		−4.01 ± 0.05		−23.5 ± 0.1		7.55 ± 0.13	
	AV Nut galls	**0.380 ± 0.134**	**A**	**−3.69 ± 1.08**	**A**	**5.91 ± 1.10**	**A**	**−3.17 ± 0.84**	**A**	**−18.6 ± 3.9**	**A**	**6.55 ± 1.26**	**A**
	Tara 1	0.520 ± 0.047		−4.94 ± 0.07		7.38 ± 0.03		−4.21 ± 0.07		−14.9 ± 0.1		5.61 ± 0.03	
	Tara 2	0.409 ± 0.009		−5.18 ± 0.06		7.42 ± 0.07		−4.29 ± 0.06		−12.9 ± 0.1		4.61 ± 0.06	
	Tara 3	0.588 ± 0.008		−5.45 ± 0.06		8.44 ± 0.07		−5.65 ± 0.04		−16.4 ± 0.1		5.82 ± 0.09	
	Tara 4	0.512 ± 0.004		−4.90 ± 0.06		7.41 ± 0.06		−4.22 ± 0.06		−14.8 ± 0.2		8.59 ± 0.06	
	AV Tara	**0.507 ± 0.074**	**A**	**−5.12 ± 0.25**	**B**	**7.66 ± 0.52**	**A**	**−4.59 ± 0.71**	**A**	**−14.7 ± 1.4**	**A**	**6.16 ± 1.71**	**A**
	AV GT	**0.443 ± 0.121**	**α**	**−4.40 ± 1.06**	**β**	**6.79 ± 1.23**	**α**	**−3.88 ± 1.05**	**β**	**−16.7 ± 3.4**	**β**	**6.35 ± 1.41**	**α**
Ellagitannins (ET)	Chestnut 1	0.400 ± 0.012		−4.03 ± 0.07		4.06 ± 0.09		−3.48 ± 0.06		−22.8 ± 0.1		4.79 ± 0.09	
	Chestnut 2	0.219 ± 0.005		−3.48 ± 0.12		3.96 ± 0.04		−3.46 ± 0.02		−23.5 ± 0.1		4.72 ± 0.13	
	Chestnut 3	0.219 ± 0.009		−3.54 ± 0.10		4.13 ± 0.08		−3.48 ± 0.06		−23.3 ± 0.2		4.88 ± 0.08	
	AV Chestnut	**0.279 ± 0.105**	**A**	**−3.68 ± 0.30**	**A**	**4.05 ± 0.08**	**A**	**−3.47 ± 0.01**	**A**	**−23.2 ± 0.4**	**A**	**4.80 ± 0.08**	**A**
	Oak 1	0.222 ± 0.007		−3.06 ± 0.07		5.98 ± 0.10		−2.19 ± 0.08		−15.3 ± 0.1		3.83 ± 0.11	
	Oak 2	0.307 ± 0.089		−3.66 ± 0.07		6.35 ± 0.09		−5.32 ± 0.09		−36.8 ± 0.0		5.28 ± 0.08	
	Oak 3	0.212 ± 0.014		−2.79 ± 0.00		5.28 ± 0.10		−2.18 ± 0.03		−22.4 ± 0.1		3.03 ± 0.10	
	Oak 4	0.290 ± 0.036		−3.91 ± 0.21		3.89 ± 0.13		−6.16 ± 0.07		−27.9 ± 0.1		4.27 ± 0.08	
	Oak 5	0.173 ± 0.018		−2.44 ± 0.12		5.08 ± 0.08		−2.30 ± 0.07		−17.9 ± 0.1		2.73 ± 0.13	
	Oak 6	0.262 ± 0.008		−2.76 ± 0.00		3.09 ± 0.05		−2.32 ± 0.08		−11.2 ± 0.1		2.57 ± 0.13	
	Oak 7	0.272 ± 0.006		−2.91 ± 0.07		4.00 ± 0.11		−3.18 ± 0.04		−10.0 ± 0.1		2.78 ± 0.07	
	Oak 8	0.353 ± 0.090		−2.96 ± 0.07		5.38 ± 0.08		−2.41 ± 0.04		−10.2 ± 0.1		2.86 ± 0.12	
	AV Oak	**0.261 ± 0.058**	**A**	**−3.06 ± 0.49**	**A**	**4.88 ± 1.12**	**A**	**−3.26 ± 1.58**	**A**	**−19.0 ± 9.6**	**A**	**3.42 ± 0.96**	**A**
	AV ET	**0.270 ± 0.068**	**αβ**	**−3.37 ± 0.52**	**β**	**4.46 ± 1.02**	**α**	**−3.37 ± 1.33**	**β**	**−21.1 ± 8.2**	**β**	**4.11 ± 1.03**	**β**

All data are the mean ± SD of three replicates. AV: average; L*: Lightness; C^*_{ab}: Chroma; h_{ab}: hue; a*: red-greenness; b*: yellow-blueness; A_{520nm}: absorbance at 520 nm. Different capital letters indicate the existence of significant differences between tannins of the same family ($p < 0.05$). Different Greek letters indicate the existence of significant differences between tannins of different families ($p < 0.05$).

Thus, to better visualize and facilitate the understanding of the following figures, tannins were grouped according to their family. Procyanidins/Prodelphinidins (PC/PD) include tannins from grapes, grape-seeds and grape-skins, while profisetinidins/prorobitenidins (PF/PR) include tannins from acacia and quebracho. Gallotannins (GT) are comprised of tannins from nut gall and tara, while ellagitannins (ET) are comprised of tannins from oak and chestnut.

The full spectra within the visible range (400-800 nm) of the control sample (malvidin-3-*O*-glucoside) and solutions containing the different doses of the 36 oenological tannins and (-)-epicatechin after one week of experimentation are presented in **Figure 64**. In all cases, the presence of copigments brought about an increase in the absorbance at visible range, an effect which was dose-dependent. The higher the dose, the higher is the absorbance of each compound. The increase of the Cp/p ratio induced an increment of the magnitude of the absorbance and even more so a raise of the hyperchromic and bathochromic shifts. In our experimentation, the concentration in pigment (malvidin-3-*O*-monoglucoside) was always the same (50 mg/L) meaning that the copigmentation effect depends only on the copigment concentration. Moreover, the higher the concentration of copigments (commercial tannins), the higher are the changes regarding hyperchromic and bathochromic shifts. Previous studies have reported the same effects [144,156]. This increment and dose dependence are more evident in the case of hydrolysable tannins (gallotannins and ellagitannins). Hydrolysable tannins reached higher absorbance values (between A_{520nm} = 0.35 to 0.45) than condensed tannins (between A_{520nm} = 0.27 to 0.32) in all the cases. Regarding the highest dose (40 g/hL), gallotannins had an A_{520nm} one and a half times higher than condensed tannins.



Each families (PC/PD ; PF/PR; Ellagitannins and Gallotannins) correspond to the mean of all the tannins (data) included in each one. All data are the mean of three replicates.

Figure 64: Visible spectra of model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented with 10, 20 or 40 g/hL of (-)-epicatechin and oenological tannins after one week of experimentation

Since the spectrum view only gives an idea of the color, the CIELAB color space was measured to better understand this phenomenon. Lightness (L*) and Chroma (C^*_{ab}) describe quantitative attributes of the color, whereas hue (h_{ab}) describe qualitative attributes [255].

The evaluation of these parameters makes it possible to determine the hyperchromic and bathochromic effects produced by the presence of the various copigments.

The red-greenness (a*), yellow-blueness (b*) and lightness (L*) components of the solutions of malvidin-3-*O*-glucoside enriched with increasing concentrations of (-)-epicatechin and oenological tannins are displayed in **Figure 65**. Positive values of a* are in the direction of redness and negative values in the direction of the greenness. Positive values of b* are in direction of "yellowness", and negative for "blueness". Finally, low values in L* point towards black while high values point towards white [246]. In general, all tannins yielded a positive effect as copigments and their effect on the color was dose-dependent since the higher the dose, the higher the resulting displacement. Clear differences in effects were observed between oenological tannins. Gallotannins were found to have the highest effect followed in decreasing order by ellagitannins, procyanidins/prodelphinidins (PC/PD) and profisetinidins/prorobitenidins (PF/PR). Condensed tannins (PC/PD and PF/PR) presented weak displacement, even though a higher shift towards red was observed. Moreover, gallotannins and ellagitannins presented similar movement towards blue (b*) and black (L*) while gallotannins presented higher displacement towards red (a*). The incrementation of the a* value was around 1.5 points in the case of ellagitannins versus 3 points for gallotannins. Finally, in the cases of gallotannins and ellagitannins, the dose-dependence seemed to be linear with a constant increment of the CIELAB color space according to the increment of the dose employed. These results confirm those obtained from absorbance measurements in **Figure 64**, indicating that hydrolysable tannins, especially gallotannins, are most effective as copigments because their hyperchromic (decrease of L*) and bathochromic (decrease of b*) effects are considerably higher.

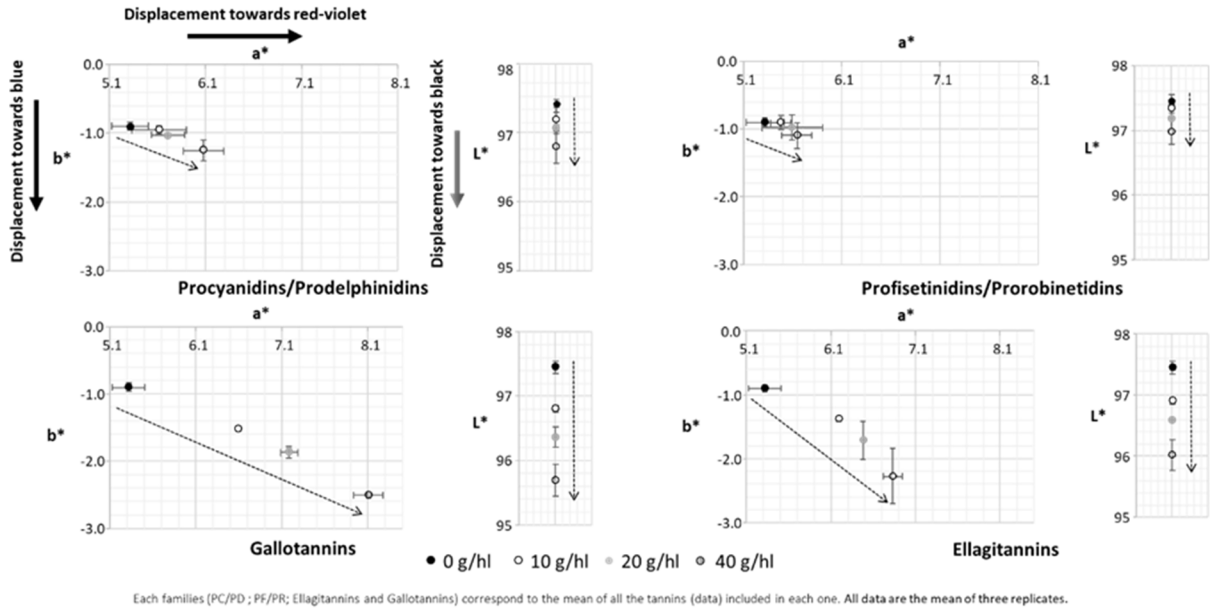


Figure 65: CIELAB color space of model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented by increasing concentration of (-)-epicatechin and oenological tannins after one week of experimentation

To better visualize the results obtained in **Figure 64** and **Figure 65**, a plot of principal components analysis (PCA) was constructed by regrouping all the color parameters evaluated (A_{520nm}, a*, b*, h_{ab}, C^*_{ab} and L*). PCA was performed on the variables and the observations of model wine solutions of malvidin-3-*O*-glucoside supplemented with increasing concentrations of (-)-epicatechin and oenological tannins, the results of which are displayed in **Figure 66**. Axis 1 and 2 explained in total 93.62% of the PCA, meaning a great separation between the different samples and a great explanation of this separation by the variables. Nevertheless, it should be highlighted that the first axis explains by himself almost the total of the PCA with a score of 84.20%. Only the variable h_{ab} was well explained by the second axis.

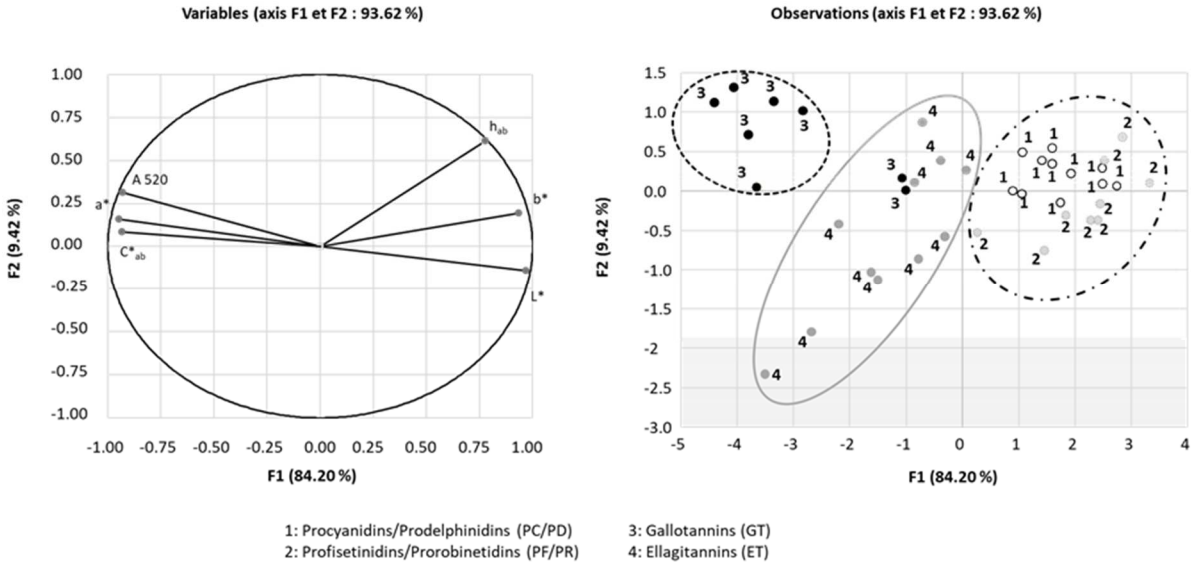


Figure 66: Plot of principal components analysis of model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented by increasing concentrations of (-)-epicatechin and oenological tannins after one week of storage

Three different clusters are clearly visible: the first containing samples 1 and 2 corresponding to condensed tannins (PC/PD and PF/PR), the second comprised mainly of sample 4 (ellagitannins), and the third containing sample 3 (gallotannins). The first cluster is placed to the right as the variables b*, L* and h_{ab}, indicating that condensed tannins induce a displacement toward yellow, weaker color and provide a low impact on color stabilization. Conversely, because the third cluster is placed to the top left as the variable A_{520nm}, a*, C^*_{ab} and at the opposite of L* and b*, it can be concluded that gallotannins induce a displacement toward red, blue and a darkness color meaning high color stabilization. The second cluster is placed to the left, but closer to the center of the PCA, meaning displacement towards red and blue, but to a lesser extent than gallotannins. These results confirm that the botanical origin of oenological tannins influence their effectiveness regarding color stabilization and that gallotannins are the most influential, followed by ellagitannins and condensed tannins.

3.1.2. Influence of copigment/pigment ratio and pH

The first experiment (3.1.1) allowed us to determine the most efficient oenological tannins family with respect to color improvement according to their botanical origin. Additionally, these effects were also confirmed under others oenological conditions. Different pH levels (3.1, 3.5, 3.9) were tested with a constant content of ethanol (12% vol.). In fact, many authors have previously studied the impact of the pH level but not so well in the presence of commercial tannins [256–258].

Figure 67 shows an example (pH 3.5 with ellagitannins) of the plots of A_{520nm} and CIELAB color space versus copigment/pigment ratio for all oenological tannins and (-)-epicatechin. These plots allowed us to determine the copigmentation effectiveness (Cp), since the slope of the straight line indicates the extent to which copigmentation occurs according to the copigment/pigment ratio. Values of the slopes indicate the effectiveness of (-)-epicatechin and oenological tannins in improving the color. For A_{520nm}, C^*_{ab} and a*, a higher Cp indicates a greater impact while for b*, L* and h_{ab}, a lower Cp indicates a greater impact.

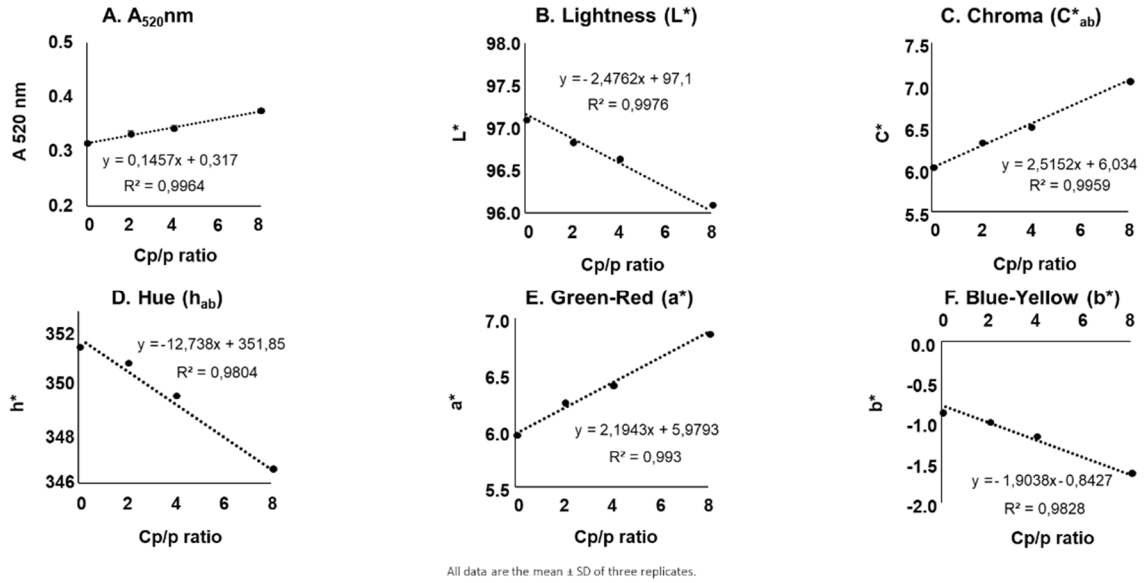


Figure 67: Example (case of ellagitannins at pH 3.5 and 12% of ethanol) of the determination of the Cp by the representation of A_{520nm} (A) and CIELAB color space (B, C, D, E, F) versus copigment/pigment ratio of model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented by (-)-epicatechin or oenological tannins

The determination of the Cp and r^2 were made by A_{520nm} and CIELAB color space for each pH level. In this way, **Table 22** presents Cp and r^2 values determined for model wine solutions of malvidin-3-*O*-glucoside (50 mg/L) at different pH levels supplemented with an increasing concentration of (-)-epicatechin and oenological tannins.

In 72% of the cases (13/18 possibilities), supplementation with gallotannin yielded the highest hyperchromic (increasing values of A_{520nm} and a* and decreasing values in L*) and bathochromic effects (decrease in b* and increase in C^*_{ab}).

Significant differences were noted between the different pH levels, pH 3.1 yielding the greatest color stabilization effect for (-)-epicatechin, grape-seed and grape-skin, whereas this effect was producing at pH 3.5 for quebracho, gallotannin and ellagitannin. In all cases, pH 3.9 was found to be the least suitable pH for the color stabilization between oenological tannins and malvidin-3-*O*-glucoside solution, although h_{ab} values were greater. In general, an increase in pH resulted in a decrease in Cp as reported for other phenolic compounds [245]. These results are also in accordance with the fact that anthocyanins are more stable at lower pH levels and thus, lower pH is favorable for color stabilization [144,259]. At pH 3.5, all tested oenological tannins, except for grape-seed, were found to induce a better color stabilization effect than (-)-epicatechin (the reference). At pH 3.1, grape-seed and quebracho tannins were weaker than (-)-epicatechin while at pH 3.9 only gallotannin and ellagitannin were more effective than (-)-epicatechin. This denotes that in general, oenological tannins present a higher capacity to stabilize the color of wines than (-)-epicatechin, which is naturally present in wine and known to be a great copigment.

Table 22: Copigmentation effectiveness (Cp) of model wine solutions of malvidin-3-O-glucoside (50 mg/L) at different pH levels, supplemented with increasing concentration of (−)-epicatechin and oenological tannins after one week of experimentation

		A_{520nm}			L*			C^*_{ab}			h_{ab}			a*			b*		
	Tannins	**Cp**	**r^2**		**Cp**	**r^2**		**Cp**	**r^2**		**Cp**	**r^2**		**Cp**	**r^2**		**Cp**	**r^2**	
pH 3.1	**(-)-Epicatechin**	0.12 ± 0.00	0.9997	**C α**	−1.68 ± 0.00	0.9881	**C β**	2.40 ± 0.02	0.9978	**D α**	−5.50 ± 0.00	0.9985	**AB β**	2.19 ± 0.02	0.9976	**AB α**	−1.53 ± 0.02	0.9828	**A β**
	Grape−seed	0.11 ± 0.00	0.9908	**D α**	−1.55 ± 0.01	0.9993	**B β**	1.95 ± 0.01	0.9925	**E α**	−6.95 ± 0.00	0.9987	**AB αβ**	1.70 ± 0.01	0.9925	**AB α**	−1.70 ± 0.04	0.9842	**AB β**
	Grape−skin	0.25 ± 0.01	0.9768	**A α**	−3.07 ± 0.00	0.9856	**F β**	3.97 ± 0.00	0.9807	**B α**	−4.96 ± 0.00	0.9750	**B α**	3.71 ± 0.00	0.9779	**AB α**	−2.07 ± 0.02	0.9989	**AB β**
	Quebracho	0.02 ± 0.00	0.8904	**E χ**	−0.92 ± 0.00	0.9758	**A α**	0.13 ± 0.04	0.7953	**F β**	−7.87 ± 0.00	0.9946	**AB α**	−0.16 ± 0.03	0.8585	**B β**	−1.61 ± 0.04	0.9933	**AB αβ**
	Gallotannin	0.22 ± 0.00	0.8446	**B β**	−3.02 ± 0.00	0.8318	**E αβ**	4.49 ± 0.03	0.8377	**A αβ**	−5.36 ± 0.00	0.9173	**AB α**	3.98 ± 0.02	0.8384	**A αβ**	−3.18 ± 0.04	0.9953	**B αβ**
	Ellagitannin	0.13 ± 0.00	0.8334	**C β**	−2.71 ± 0.00	0.8824	**D β**	2.52 ± 0.01	0.8371	**C α**	−13.05 ± 0.00	0.9955	**A αβ**	2.00 ± 0.01	0.8233	**AB αβ**	−3.09 ± 0.02	0.9952	**AB β**
pH 3.5	**(−)−Epicatechin**	0.06 ± 0.00	0.8940	**E χ**	−0.92 ± 0.00	0.9231	**A α**	1.20 ± 0.03	0.8937	**D β**	−4.06 ± 0.00	0.9763	**B αβ**	1.12 ± 0.03	0.8744	**A β**	−0.62 ± 0.03	1.0000	**AB αβ**
	Grape−seed	0.07 ± 0.00	0.9681	**E β**	−1.00 ± 0.00	1.0000	**B αβ**	1.28 ± 0.02	0.9619	**D αβ**	−3.01 ± 0.01	0.9776	**AB α**	1.23 ± 0.02	0.9702	**AB αβ**	−0.52 ± 0.04	0.8378	**A α**
	Grape−skin	0.12 ± 0.00	0.9581	**D β**	−1.91 ± 0.00	0.9791	**C αβ**	2.01 ± 0.06	0.9660	**C αβ**	−7.74 ± 0.01	0.9617	**C αβ**	1.83 ± 0.05	0.9610	**AB αβ**	−1.22 ± 0.06	0.8009	**AB αβ**
	Quebracho	0.17 ± 0.00	0.9251	**B α**	−2.33 ± 0.00	0.9018	**D β**	2.50 ± 0.06	0.9525	**B α**	−8.74 ± 0.01	0.9995	**D αβ**	2.38 ± 0.06	0.9562	**AB α**	−1.15 ± 0.08	0.7987	**AB α**
	Gallotannin	0.31 ± 0.00	0.9885	**A α**	−4.14 ± 0.00	0.9832	**F β**	6.23 ± 0.03	0.9897	**A α**	−16.75 ± 0.01	0.9516	**F β**	5.62 ± 0.02	0.0885	**B α**	−3.41 ± 0.05	0.9986	**B β**
	Ellagitannin	0.15 ± 0.00	0.9964	**C α**	−2.48 ± 0.00	0.9976	**E αβ**	2.52 ± 0.02	0.9959	**B α**	−12.74 ± 0.00	0.9828	**E α**	2.19 ± 0.01	0.9930	**AB α**	−1.90 ± 0.01	0.9998	**AB αβ**
pH 3.9	**(−)-Epicatechin**	0.08 ± 0.00	1.0000	**C β**	−1.08 ± 0.00	0.9826	**C αβ**	1.26 ± 0.01	0.9922	**C αβ**	−3.79 ± 0.00	0.9932	**A α**	1.20 ± 0.01	0.9908	**AB αβ**	−0.45 ± 0.02	0.9967	**AB α**
	Grape-seed	0.05 ± 0.00	0.9873	**D χ**	−0.79 ± 0.00	0.9973	**A α**	0.71 ± 0.01	0.9304	**F β**	−7.63 ± 0.07	1.0000	**B β**	0.61 ± 0.02	0.9131	**B β**	−0.62 ± 0.07	0.8536	**AB αβ**
	Grape-skin	0.06 ± 0.01	0.9873	**D χ**	−0.92 ± 0.00	0.8811	**B α**	0.85 ± 0.02	0.9996	**E β**	−11.63 ± 0.01	0.9001	**C β**	0.70 ± 0.01	0.7340	**AB β**	−0.15 ± 0.13	0.9251	**A α**
	Quebracho	0.05 ± 0.00	0.9434	**D β**	−1.75 ± 0.00	0.9643	**E αβ**	0.98 ± 0.03	0.8275	**D αβ**	−11.92 ± 0.01	0.9826	**D β**	0.80 ± 0.03	0.9943	**AB αβ**	−3.07 ± 0.05	0.9588	**B β**
	Gallotannin	0.15 ± 0.00	0.9920	**A χ**	−2.08 ± 0.00	0.9952	**F α**	3.04 ± 0.03	0.9954	**A β**	−16.14 ± 0.02	0.9924	**E αβ**	2.74 ± 0.02	0.9922	**A β**	−1.75 ± 0.05	0.9969	**AB α**
	Ellagitannin	0.10 ± 0.00	0.9510	**B χ**	−1.65 ± 0.00	0.9443	**D α**	1.62 ± 0.01	0.9615	**B α**	−18.34 ± 0.02	0.9968	**F β**	1.33 ± 0.01	0.9692	**AB β**	−1.51 ± 0.07	0.9898	**AB α**

All data are the mean ± SD of three replicates. L*: Lightness; C^*_{ab}: Chroma; h_{ab}: hue; a*: red-greenness; b*: yellow-blueness; A_{520nm}: absorbance at 520 nm. Different capital caster letters indicate the existence of significant differences between tannins for the same pH level ($p < 0.05$). Different Greek letters indicates the existence of significant differences between pH level for the same tannin ($p < 0.05$).

3.1.3. Influence of copigment/pigment ratio and ethanol content

The first experiment (3.1.1) allowed us to determine the most efficient oenological tannins family with respect to color improvement according to their botanical origin. Additionally, these effects were also confirmed under others oenological conditions. Different ethanol content (10% vol., 12% vol., 14% vol.) were tested with a constant pH level (3.5). In fact, the impact of the ethanol content has not been well studied in literature, and even more so in presence of commercial tannins as copigments.

Figure 68 shows an example (14% vol. of ethanol with ellagitannin) of the plots of A_{520nm} and CIELAB color space versus copigment/pigment ratio for all oenological tannins and (-)-epicatechin. These plots allowed us to determine the copigmentation effectiveness (Cp), since the slope of the straight line indicates the extent to which copigmentation occurs according to the copigment/pigment ratio. Values of the slopes indicate the effectiveness of (-)-epicatechin and oenological tannins in improving the color. For A_{520nm}, C^*_{ab} and a*, a higher Cp indicates a greater impact while for b*, L* and h_{ab}, a lower Cp indicates a greater impact.

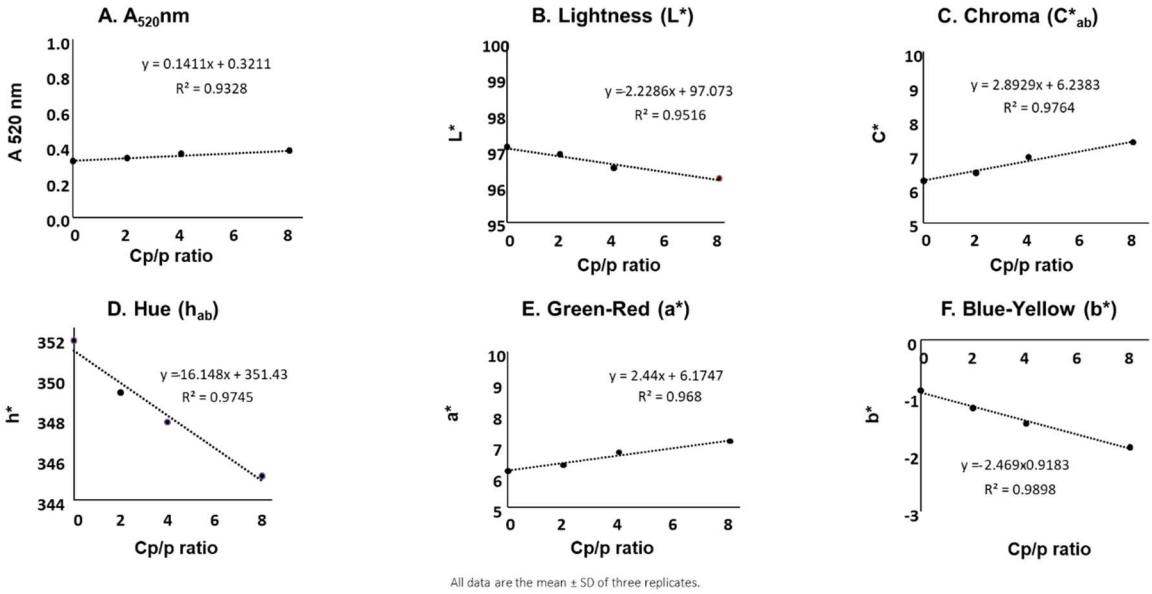


Figure 68: Example (case of ellagitannins at 14% of ethanol and pH 3.5) of the determination of the Cp by the representation of A_{520nm} (A) and CIELAB color space (B, C, D, E, F) versus copigment/pigment ratio of model wine solution of malvidin-3-O-glucoside (50 mg/L) supplemented by (-)-epicatechin or oenological tannins

The determination of the Cp and r^2 were made for A_{520nm} and CIELAB color space for each ethanol content (10, 12 and 14%) in the enriched model wine solutions of malvidin-3-*O*-glucoside. In this way, **Table 23** presents the Cp and r^2 values determined for the model wine solutions of malvidin-3-*O*-glucoside (50 mg/L) at different ethanol content supplemented with an increasing concentration of (-)-epicatechin and oenological tannins.

Table 23: Copigmentation effectiveness (Cp) of model wine solutions of malvidin-3-O-glucoside (50 mg/L) at different ethanol (EtOH) contents, supplemented with increasing concentrations of (−)-epicatechin and oenological tannins after one week of experimentation

		A_{520nm}				L^*				C^*_{ab}				h_{ab}				a^*				b^*			
	Tannins	**Cp**	**r^2**			**Cp**	**r^2**			**Cp**	**r^2**			**Cp**	**r^2**			**Cp**	**r^2**			**Cp**	**r^2**		
EtOH 10%	**(-)-Epicatechin**	0.15 ± 0.00	0.9999	**C**	**α**	−1.75 ± 0.00	0.9993	**B**	**β**	2.77 ± 0.02	0.9989	**C**	**α**	−7.02 ± 0.01	0.9717	**B**	**β**	2.62 ± 0.02	0.9980	**C**	**α**	−1.19 ± 0.04	0.9828	**C**	**β**
	Grape−seed	0.12 ± 0.00	0.9991	**D**	**α**	−1.46 ± 0.00	0.9909	**A**	**β**	1.83 ± 0.03	0.9983	**E**	**α**	−6.27 ± 0.01	0.9777	**A**	**αβ**	1.71 ± 0.02	0.9984	**E**	**α**	−0.94 ± 0.04	0.9842	**A**	**αβ**
	Grape−skin	0.14 ± 0.00	0.9849	**C**	**α**	−2.11 ± 0.00	0.9861	**D**	**β**	1.97 ± 0.01	0.9827	**D**	**α**	−8.50 ± 0.01	0.9967	**C**	**β**	2.15 ± 0.01	0.9857	**D**	**α**	−1.27 ± 0.04	0.9989	**B**	**β**
	Quebracho	0.12 ± 0.01	0.9775	**D**	**αβ**	−2.03 ± 0.00	0.9798	**C**	**αβ**	1.09 ± 0.05	0.8761	**F**	**α**	−14.97 ± 0.02	0.9875	**D**	**β**	1.53 ± 0.05	0.9521	**F**	**α**	−1.94 ± 0.13	0.9933	**C**	**β**
	Gallotannin	0.47 ± 0.00	0.9983	**A**	**α**	−6.30 ± 0.00	0.9979	**F**	**β**	8.33 ± 0.02	0.9979	**A**	**α**	−25.68 ± 0.01	0.9424	**F**	**β**	9.39 ± 0.02	0.9983	**A**	**α**	−5.49 ± 0.07	0.9953	**E**	**β**
	Ellagitannin	0.23 ± 0.00	0.9983	**B**	**α**	−3.48 ± 0.00	0.9996	**E**	**β**	4.43 ± 0.02	0.9858	**B**	**α**	−20.82 ± 0.00	0.9993	**E**	**β**	3.82 ± 0.01	0.9871	**B**	**α**	−3.31 ± 0.02	0.9952	**D**	**α**
EtOH 12%	**(−)−Epicatechin**	0.10 ± 0.00	0.9845	**D**	**αβ**	−1.42 ± 0.00	0.9935	**B**	**αβ**	1.78 ± 0.16	0.9267	**C**	**α**	−3.55 ± 0.01	0.9705	**A**	**α**	1.93 ± 0.11	0.9778	**B**	**αβ**	−0.73 ± 0.12	1.0000	**C**	**α**
	Grape−seed	0.07 ± 0.00	0.9902	**F**	**αβ**	−0.56 ± 0.00	0.3213	**A**	**α**	0.49 ± 0.09	0.5663	**E**	**β**	−5.53 ± 0.02	0.8155	**C**	**α**	0.30 ± 0.06	0.4808	**D**	**β**	0.64 ± 0.17	0.8378	**B**	**α**
	Grape−skin	0.08 ± 0.00	0.8542	**E**	**β**	−1.59 ± 0.00	0.9498	**C**	**αβ**	0.87 ± 0.05	0.7500	**DE**	**β**	−5.26 ± 0.04	0.7940	**B**	**α**	1.35 ± 0.05	0.9959	**C**	**αβ**	−0.63 ± 0.07	0.8009	**C**	**α**
	Quebracho	0.22 ± 0.00	0.9554	**B**	**α**	−3.24 ± 0.00	0.9672	**E**	**β**	0.98 ± 0.27	0.9600	**D**	**α**	−9.77 ± 0.02	0.7591	**D**	**αβ**	1.10 ± 0.19	0.9568	**C**	**αβ**	6.46 ± 0.12	0.7987	**A**	**α**
	Gallotannin	0.38 ± 0.00	0.9999	**A**	**αβ**	−5.22 ± 0.00	0.9994	**F**	**αβ**	6.88 ± 0.04	0.9945	**A**	**α**	−19.07 ± 0.02	0.9829	**F**	**α**	6.39 ± 0.08	0.9990	**A**	**αβ**	−4.09 ± 0.09	0.9986	**E**	**α**
	Ellagitannin	0.16 ± 0.00	0.9893	**C**	**αβ**	−2.72 ± 0.00	0.9840	**D**	**αβ**	2.80 ± 0.14	0.9932	**B**	**α**	−16.45 ± 0.07	1.0000	**E**	**αβ**	2.05 ± 0.10	0.9590	**B**	**β**	−2.25 ± 0.46	0.9998	**D**	**α**
EtOH 14%	**(−)-Epicatechin**	0.09 ± 0.00	0.9980	**C**	**β**	−1.17 ± 0.00	0.9937	**B**	**α**	1.75 ± 0.02	0.9977	**C**	**α**	−6.97 ± 0.00	0.9963	**B**	**αβ**	1.60 ± 0.02	0.9972	**C**	**β**	−1.08 ± 0.02	0.9967	**B**	**αβ**
	Grape-seed	0.06 ± 0.00	0.9954	**E**	**β**	−0.83 ± 0.00	0.9868	**A**	**αβ**	1.52 ± 0.02	0.9834	**D**	**αβ**	−35.41 ± 0.00	0.8453	**F**	**β**	1.02 ± 0.02	0.9999	**E**	**αβ**	−4.28 ± 0.03	0.8536	**D**	**β**
	Grape-skin	0.09 ± 0.00	0.9133	**C**	**αβ**	−1.44 ± 0.00	0.9651	**D**	**α**	1.32 ± 0.01	0.8271	**E**	**αβ**	−5.70 ± 0.02	0.9218	**A**	**αβ**	1.20 ± 0.01	0.8127	**D**	**β**	−0.87 ± 0.06	0.9251	**A**	**αβ**
	Quebracho	0.06 ± 0.00	0.7889	**D**	**β**	−1.35 ± 0.00	0.7311	**C**	**α**	1.07 ± 0.03	0.9791	**F**	**α**	−9.21 ± 0.01	0.9875	**C**	**α**	0.87 ± 0.02	0.9530	**F**	**β**	−1.14 ± 0.04	0.9588	**B**	**αβ**
	Gallotannin	0.34 ± 0.00	0.9982	**A**	**β**	−4.52 ± 0.00	0.9993	**F**	**α**	6.91 ± 0.03	0.9981	**A**	**α**	−20.86 ± 0.02	0.9644	**E**	**αβ**	6.11 ± 0.03	0.9974	**A**	**β**	−4.23 ± 0.08	0.9969	**D**	**αβ**
	Ellagitannin	0.14 ± 0.00	0.9328	**B**	**β**	−2.23 ± 0.00	0.9516	**E**	**α**	2.89 ± 0.01	0.9764	**B**	**α**	−16.15 ± 0.03	0.9745	**D**	**α**	2.44 ± 0.00	0.9680	**B**	**αβ**	−2.47 ± 0.10	0.9898	**C**	**α**

All data are the mean ± SD of three replicates. L*: Lightness; C^*_{ab}: Chroma; h_{ab}: hue; a*: red-greenness; b*: yellow-blueness; A_{520nm}: absorbance at 520 nm. Different capital caster letters indicate the existence of significant differences between tannins for the same pH level ($p < 0.05$). Different Greek letters indicates the existence of significant differences between pH level for the same tannin ($p < 0.05$).

In 89% of the cases (16/18 possibilities), gallotannin yielded the highest hyperchromic (increasing values of A_{520nm} and a* and decreasing values in L*) and bathochromic (decrease in b* and increase in C^*_{ab}) effect and ellagitannin was the second most efficient of oenological tannins in color stabilization. The difference in effectiveness between hydrolysable tannins and condensed tannins were highly notable. In fact, compared to condensed tannins, gallotannin and ellagitannin reached Cp values four and two times higher, respectively. Additionally, significant differences were observed between the different ethanol contents. At 10% vol. of ethanol, the greatest color stabilization effect was observed for (-)-epicatechin and all tested oenological tannins, except for quebracho tannin. Quebracho tannin reached its maximum effect on color stabilization at 12% vol. of ethanol. Moreover, 14% vol. of ethanol appeared to be the least appropriate condition for color stabilization between oenological tannins and malvidin-3-*O*-glucoside. For all tested oenological tannins except quebracho tannin, the increase in ethanol content resulted in a decrease in the Cp. It should be highlighted that the hyperchromic effect decreased with the augmentation of the ethanol content while the bathochromic effect decreased between 10 to 12% vol. of ethanol and remained quite stable between 12 and 14% vol. of ethanol. These results are in accordance with those obtained previously, which demonstrated that the presence of ethanol can disrupt copigmentation complexes [248]. Independently of ethanol content, only hydrolysable tannins were able to improve the color stabilization compared to (-)-epicatechin. Nevertheless, at 12% vol. of ethanol content, quebracho tannin presented a higher color stabilization effect than (-)-epicatechin. This means that in general, only the hydrolysable tannins present a higher capacity to stabilize the color of wines than (-)-epicatechin when the ethanol content changes. Independently of ethanol content, hydrolysable tannins, especially gallotannins, provided the best color stabilization effect. These results are in accordance with those previously obtained for the pH effect (3.1.2).

3.2. Influence of time contact between oenological tannins and malvidin-3-*O*-glucoside on color stabilization

In this part, the influence of the time contact between oenological tannins and malvidin-3-*O*-glucoside will be discussed. According to this, time contact influence will be estimate regarding the color stabilization with the analysis of color component and the determination of the copigmentation index. In addition, malvidin-3-*O*-glucoside and its degradation products were quantified during the different days of the experimentation, to follow its evolution.

3.2.1. Analysis of color component

As explained previously in the 3.1.2 and 3.1.3, the copigmentation effectiveness (Cp) was calculated using the values of the slopes (as shown as an example in **Figure 67** and **Figure 68**) since they indicate the effectiveness of (-)-epicatechin and oenological tannins in improving the color.

Regarding A_{520nm}, C^*_{ab} and a* parameters, a higher Cp indicates a greater impact while for b*, L* and h_{ab} parameters, a lower Cp indicates a greater impact.

In this part, the determination of the Cp and r^2 were made for A_{520nm} and CIELAB color space for each day (1, 7, 14 and 21) in the enriched model wine solutions of malvidin-3-*O*-glucoside by (-)-epicatechin or oenological tannins. In this way, **Table 24** presents the different results obtained for each copigment (oenological tannins and (-)-epicatechin) at the different days.

First, it should be highlighted that in the case of ellagitannin, changes during the time are really marked and obvious. Indeed, ellagitannin is the only one to present a diminution of his hyperchromic and bathochromic effect throughout the days. The diminution of the hyperchromic effect is induce by a decrease of the A_{520nm} from the 14th day, a decline of parameter a* from the 7th day and an increase of parameter L* from the 14th day. The diminution of the bathochromic effect is inducing by both a decrease of the parameter C^*_{ab} and a gain of the parameter b* from the 7th day. Concerning the others copigments tested it can be noted a slight decrease of the hyperchromic effect in the case of the grape-seed and grape-skin tannin. Indeed, for grape-seed tannin a diminution of L* and an augmentation of a* parameters were noted from the 7th day. Nevertheless, no significant differences were noted concerning the A_{520nm} which explained also the hyperchromic effect. Regarding grape-skin tannin, an increase of A_{520nm} and decrease of L* parameter were noted from the 7th day. In the case of (-)-epicatechin, quebracho and gallotannin, no significant differences were highlighted to explain changes in their hyperchromic and/or bathochromic effect.

Secondly, independently of the days, gallotannin was the most efficient one regarding color stabilization, since it presents the highest hyperchromic and bathochromic effect, reaching the uppermost values of Cp for A_{520nm}, a* and C^*_{ab} and the lowest values of Cp for L* and b* parameters. Additionally, on the first day ellagitannin and (-)-epicatechin presented great effect followed by grape-seed, grape-skin and quebracho tannins. On the seventh day, grape-seed and grape-skin tannins start to rich similar efficacy than ellagitannin and (-)-epicatechin meanwhile quebracho tannin remain the lowest efficient one. On day 14th, grape-skin tannin was most efficient than (-)-epicatechin and grape-seed tannin with ellagitannin beginning the lowest efficient one with quebracho tannin. Finally, on day 21, gallotannin was still the most efficient followed by grape-seed and grape-skin tannin, (-)-epicatechin and quebracho tannin and finally ellagitannin.

At the end, during the time of the experiment, gallotannin still remain the most efficient tannin to improve color stabilization by having a great hyperchromic and bathochromic effect. On the contrary, ellagitannin appears at the beginning as a great candidate to improve the color stabilization but its effect during the time change conducting to a poor efficiency at the end. Grape-seed and grape-skin tannins contrary to ellagitannin see their effects increase with the time of contact with the malvidin-3-*O*-glucoside. Finally, quebracho tannin, independently of the time contact remains the lowest efficient one.

Table 24: Copigmentation effectiveness (Cp) of model wine solutions of malvidin-3-O-glucoside (50 mg/L), supplemented with increasing concentrations of (−)-epicatechin and oenological tannins after 1, 7, 14 and 21 days of experimentation

		A_{520nm}				L*				C^*_{ab}				h_{ab}				a*				b*			
Tannins	**Days**	**Cp**	**r²**			**Cp**	**r²**			**Cp**	**r²**			**Cp**	**r²**			**Cp**	**r²**			**Cp**	**r²**		
(-)-Epicatechin	**1**	0.003 ± 0.000	0.8596	**A**	χ	-0.034 ± 0.007	0.5143	**AB**	α	0.082 ± 0.012	0.8993	**A**	β	-0.268 ± 0.014	0.8427	**B**	αβ	0.070 ± 0.007	0.8982	**A**	β	-0.038 ± 0.003	0.8760	**D**	βχ
	7	0.003 ± 0.001	0.8628	**A**	β	-0.040 ± 0.001	0.9426	**B**	αβ	0.061 ± 0.009	0.9182	**A**	β	-0.119 ± 0.007	0.4996	**A**	α	0.059 ± 0.009	0.9203	**A**	β	-0.017 ± 0.003	0.7889	**C**	α
	14	0.003 ± 0.001	1.0000	**A**	βχ	-0.031 ± 0.009	0.8954	**AB**	α	0.041 ± 0.051	0.3699	**AB**	β	-0.037 ± 0.109	0.1586	**A**	α	0.041 ± 0.052	0.3516	**AB**	β	-0.002 ± 0.007	0.1158	**B**	α
	21	0.003 ± 0.002	0.3015	**A**	αβχ	-0.020 ± 0.010	0.7651	**A**	α	-0.027 ± 0.001	0.3956	**B**	δ	0.020 ± 0.000	0.6336	**A**	β	-0.025 ± 0.001	0.4317	**B**	δ	0.024 ± 0.005	0.4118	**A**	α
Grape-seed	**1**	0.001 ± 0.000	1.0000	**A**	δ	-0.030 ± 0.007	0.7714	**A**	α	0.017 ± 0.004	0.9932	**B**	χ	-0.340 ± 0.026	0.9508	**B**	β	0.013 ± 0.006	0.2621	**B**	χ	-0.032 ± 0.002	0.9620	**BC**	αβ
	7	0.003 ± 0.000	0.9590	**A**	β	-0.049 ± 0.003	0.9873	**B**	βχ	0.041 ± 0.001	0.7096	**A**	χ	-0.432 ± 0.039	0.9603	**C**	β	0.034 ± 0.001	0.6638	**A**	βχ	-0.039 ± 0.007	0.9369	**C**	β
	14	0.003 ± 0.001	0.9677	**A**	βχ	-0.058 ± 0.007	0.9936	**B**	βχ	0.033 ± 0.009	0.7532	**A**	β	-0.188 ± 0.008	0.5126	**A**	αβ	0.032 ± 0.009	0.7495	**A**	β	-0.018 ± 0.002	0.5447	**A**	αβ
	21	0.003 ± 0.002	0.7238	**A**	αβχ	-0.061 ± 0.007	0.9408	**B**	χδ	0.038 ± 0.001	0.3796	**A**	β	-0.199 ± 0.034	0.3265	**A**	χ	0.035 ± 0.001	0.3681	**A**	β	-0.024 ± 0.006	0.3391	**AB**	βχ
Grape-skin	**1**	0.001 ± 0.000	0.0048	**B**	δ	-0.023 ± 0.005	0.2217	**A**	α	0.020 ± 0.006	0.3850	**B**	χ	-0.212 ± 0.005	0.9761	**B**	α	0.027 ± 0.010	0.3216	**A**	χ	-0.024 ± 0.001	0.9134	**C**	α
	7	0.003 ± 0.000	0.9554	**AB**	β	-0.052 ± 0.003	0.9711	**B**	χ	0.040 ± 0.008	0.5185	**A**	χ	-0.191 ± 0.001	0.9947	**B**	α	0.030 ± 0.003	0.4811	**A**	βχ	-0.024 ± 0.003	0.9233	**C**	α
	14	0.004 ± 0.000	0.9765	**A**	β	-0.076 ± 0.004	0.9908	**C**	χ	0.028 ± 0.003	0.4418	**AB**	β	0.050 ± 0.035	0.4954	**A**	α	0.027 ± 0.003	0.4267	**A**	β	0.010 ± 0.004	0.4908	**A**	α
	21	0.004 ± 0.002	0.8185	**A**	αβ	-0.080 ± 0.009	0.9914	**C**	δ	0.022 ± 0.006	0.3160	**B**	χ	0.050 ± 0.000	0.0264	**A**	β	0.013 ± 0.003	0.3283	**B**	χ	-0.015 ± 0.001	0.0649	**B**	β
Quebracho	**1**	0.001 ± 0.001	0.2316	**A**	δ	-0.033 ± 0.002	0.5738	**A**	α	0.026 ± 0.007	0.7175	**A**	χ	-0.540 ± 0.055	0.9884	**A**	χ	0.013 ± 0.005	0.5919	**A**	χ	-0.047 ± 0.009	0.9779	**A**	χ
	7	0.001 ± 0.000	0.5979	**AB**	χ	-0.036 ± 0.001	0.9823	**A**	α	0.012 ± 0.005	0.1192	**AB**	δ	-0.499 ± 0.045	0.9654	**A**	β	0.006 ± 0.006	0.0485	**AB**	χ	-0.043 ± 0.002	0.9571	**A**	β
	14	0.002 ± 0.001	1.0000	**A**	βχ	-0.051 ± 0.001	0.5717	**A**	β	-0.004 ± 0.009	0.1011	**B**	βχ	-0.636 ± 0.199	0.7143	**A**	χ	-0.011 ± 0.008	0.0036	**B**	βχ	-0.054 ± 0.022	0.6283	**A**	χ
	21	-0.001 ± 0.001	0.0448	**B**	βχ	-0.048 ± 0.016	0.9255	**A**	βχ	-0.036 ± 0.008	0.4569	**C**	δ	-0.549 ± 0.001	0.6672	**A**	δ	-0.093 ± 0.014	0.4678	**C**	ε	-0.036 ± 0.004	0.5319	**A**	χ
Gallotannin	**1**	0.010 ± 0.000	0.9501	**A**	α	-0.145 ± 0.001	0.9736	**AB**	χ	0.217 ± 0.003	0.9844	**A**	α	-0.954 ± 0.057	0.9703	**B**	δ	0.190 ± 0.005	0.9812	**A**	α	-0.144 ± 0.006	0.9988	**C**	ε
	7	0.011 ± 0.000	0.9559	**A**	α	-0.158 ± 0.008	0.9916	**B**	ε	0.222 ± 0.007	0.9487	**A**	α	-0.885 ± 0.020	0.9634	**AB**	χ	0.199 ± 0.007	0.9401	**A**	α	-0.130 ± 0.003	0.9983	**B**	δ
	14	0.009 ± 0.002	1.0000	**A**	α	-0.157 ± 0.008	0.9946	**B**	δ	0.168 ± 0.002	0.9740	**B**	α	-0.849 ± 0.034	0.9925	**A**	χ	0.154 ± 0.004	0.9710	**AB**	α	-0.110 ± 0.004	0.9880	**A**	δ
	21	0.008 ± 0.004	0.9756	**A**	α	-0.141 ± 0.000	0.9864	**A**	ε	0.146 ± 0.003	0.9290	**C**	α	-1.065 ± 0.020	0.9936	**C**	ε	0.126 ± 0.010	0.9209	**B**	α	-0.120 ± 0.004	0.9817	**AB**	δ
Ellagitannin	**1**	0.004 ± 0.001	0.7622	**A**	β	-0.079 ± 0.002	0.9498	**C**	β	0.086 ± 0.007	0.9366	**A**	β	-0.862 ± 0.063	0.9691	**C**	δ	0.067 ± 0.007	0.9055	**A**	β	-0.097 ± 0.004	0.9902	**D**	δ
	7	0.003 ± 0.000	0.9892	**A**	β	-0.086 ± 0.001	0.9983	**C**	δ	0.041 ± 0.007	0.8722	**B**	χ	-0.864 ± 0.040	0.9936	**C**	χ	0.030 ± 0.002	0.7806	**B**	βχ	-0.078 ± 0.005	0.9868	**C**	χ
	14	0.001 ± 0.000	0.9011	**B**	χ	-0.053 ± 0.008	0.9607	**B**	β	-0.037 ± 0.006	0.3786	**C**	χ	-0.373 ± 0.021	0.6794	**B**	β	-0.048 ± 0.001	0.4793	**C**	χ	-0.024 ± 0.003	0.4892	**B**	β
	21	-0.003 ± 0.001	0.9858	**C**	χ	-0.024 ± 0.001	0.5290	**A**	αβ	-0.100 ± 0.002	0.9597	**D**	ε	0.219 ± 0.006	0.7702	**A**	α	-0.101 ± 0.003	0.9806	**D**	ε	0.018 ± 0.009	0.5527	**A**	α

All data are the mean ± SD of three replicates. L*: Lightness; C^*_{ab}: Chroma; h_{ab}: hue; a*: red-greenness; b*: yellow-blueness; A_{520nm}: absorbance at 520 nm. Different capital caster letters indicate the existence of significant differences between days for the same tannin ($p < 0.05$). Different Greek letters indicates the existence of significant differences between tannins for the same day ($p < 0.05$).

3.2.2. Copigmentation index

The Copigmentation Index (**Figure 61)** proposed, allowed to measure the real effectiveness of commercial tannins and proposed a new tool for tannin manufacturers and potential consumers (winemakers) [250]. This index considers the total color difference (ΔE_{ab}) between an oenological tannin solution and a pure white color solution (L* = 100.00, a* = 0.00, b* = 0.00). Indeed, this is the best parameter to determine the real differences between the color of two solutions since it reflects the Euclidian distance between two point in the CIELAB space. This index considers also, the percentage of increase of ΔE_{ab} originated by the supplementation of the highest dose of tannins (0.4 g/L) to facilitate its quantification.

Table 25 shows the results obtained for the copigmentation index of the different commercial tannins and (-)-epicatechin at 1, 7, 14 and 21 days. This copigmentation index indicate in a simple way the effectiveness of the different commercial tannins and (-)-epicatechin (reference) to improve wine color stabilization. This improve is mainly due to the copigmentation phenomenon, even if the formation of polymerized pigments can also take place.

Table 25: Copigmentation index (%) of the oenological tannins and (-)-epicatechin at 1, 7, 14 and 21 days

	Copigmentation index (%)											
Copigments	**Day 1**			**Day 7**			**Day 14**			**Day 21**		
(-)-Epicatechin	11,1 ± 0,6	**C**	α	9,2 ± 0,7	**BC**	α	2,9 ± 1,3	**D**	β	0,0 ± 0,1	**C**	χ
Ellagitannin	15,2 ± 1,3	**B**	α	10,6 ± 1,1	**B**	β	0,5 ± 0,0	**D**	χ	0,0 ± 1,0	**C**	χ
Gallotannin	35,4 ± 1,2	**A**	α	37,0 ± 0,4	**A**	α	34,4 ± 2,2	**A**	α	27,4 ± 2,3	**A**	β
Grape-skin	3,9 ± 1,2	**D**	β	7,1 ± 0,2	**C**	α	8,6 ± 1,1	**B**	α	7,1 ± 1,1	**B**	α
Grape-seed	3,1 ± 0,8	**D**	β	7,4 ± 0,6	**C**	α	8,1 ± 1,9	**BC**	α	6,5 ± 0,2	**B**	α
Quebracho	3,9 ± 0,2	**D**	α	2,6 ± 2,0	**D**	α	3,8 ± 2,3	**CD**	α	0,0 ± 1,1	**C**	α

All data are the mean ± SD of three replicates. Different capital caster letters indicate the existence of significant differences between days for the same tannin ($p < 0.05$). Different Greek letters indicate the existence of significant differences between tannins for the same day ($p < 0.05$).

At day 1, according to this index, the best copigment was the gallotannin (35.4 ± 1.2%) followed in decreasing order by ellagitannin (15.3 ± 1.3%), (-)-epicatechin (11.1 ± 0.6%) and then grape-skin (3.9 ± 1.2%), grape-seed (3.1 ± 0.8%) and quebracho tannins (3.9 ± 0.2%). Then on day 7, only the copigmentation index of (-)-epicatechin, gallotannin and quebracho tannin remain stable, since no significant differences were observed. On the contrary, in the case of ellagitannin, grape-skin and grape-seed tannin considerable differences were observed. Concerning ellagitannin, the copigmentation index decreases meanwhile for grape-skin and grape-seed tannins the copigmentation index increase. Nevertheless, the order of classification of the tannins as copigments was not changed by this decline or augmentation of the values. Indeed, gallotannin still remain the most efficient one followed by ellagitannin, (-)-epicatechin and then grape-skin, grape-seed and quebracho tannins. On day 14, only gallotannin and quebracho tannin remain stable compared to day 1 and 7.

Grape-seed and grape-skin tannins, stand constant compared to day 7 with an increase of the index compared to day 1. To the opposite copigmentation index of ellagitannin continue to decrease on day 14, becoming close to 0%. In the case of the (-)-epicatechin, used as reference, on day 14, a diminution of the copigmentation index start to be observed. According to this, on day 14, the effectiveness of the commercial tannins was changed, and the classification removed. Indeed, gallotannin remain the most efficient one, but this time followed by grape-skin and grape-seed tannins, quebracho tannin and finally (-)-epicatechin and ellagitannin. On day 21, for the first-time significant differences were observed for the gallotannin, with a slow decrease of the copigmentation index. Ellagitannin, grape-skin, grape-seed and quebracho tannin remain stable compared to day 14 meanwhile, (-)-epicatechin copigmentation index continue to decrease. At the end on day 21, gallotannin (27.4 ± 2.3%) stay the most efficient one, followed by grape-skin (7.1 ± 1.1%) and grape-seed tannins (6.5 ± 0.2%). Ellagitannin (0.0%), (-)-epicatechin (0.0%) and quebracho (0.0%) tannin were the lowest efficient ones.

These results are in accordance with the previous ones obtained for analysis of color component in which it has been shown that throughout the time gallotannin outstanding the most efficient one meanwhile ellagitannin presented a decrease of his hyperchromic and bathochromic effects.

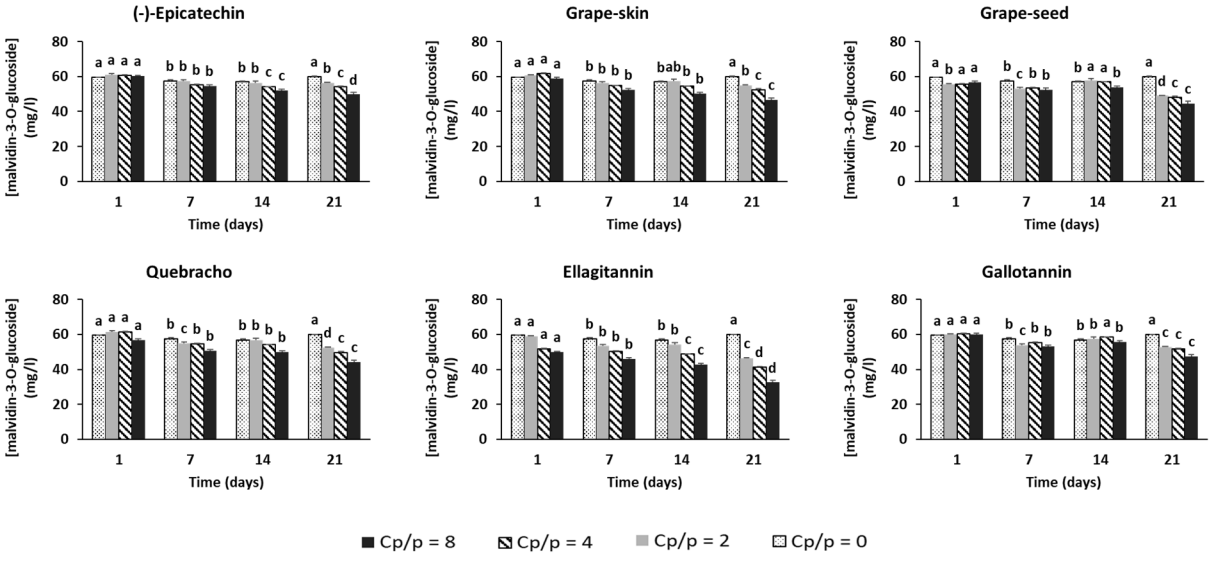
The fact, that the copigmentation index of (-)-epicatechin and ellagitannin decrease significantly during the time from day 1 to day 21 can be explained by different phenomenon. Indeed, this decrease suggests that the copigmentation effectiveness of these tannins decline with the time or that the malvidin-3-*O*-glucoside has reacted with tannins to form new polymerized pigments with a lower contribution to color. In this regard, it has been reported that colorless pigments were formed in solutions containing malvidin-3-*O*-glucoside and oak extract and that the ellagitannin concentration decreases over time [260]. Even more so, malvidin-3-*O*-glucoside could be degraded with the time and formed degradation product colorless. In contrast, the copigmentation of grape-seed and grape-skin tannins increase significantly during the time. This could be explained by the fact that, the stacking structures responsible of copigmentation needs some time to be completely formed or, because of polymerized pigments with a higher contribution to the color have been formed [256,261]. Regarding quebracho tannin, it appears to be weak copigment since the copigmentation index was stable during the time but in any case, low. Concerning gallotannin, it is possible that the complex formed between them and the malvidin-3-*O*-glucoside is enough stable to be conserved during the time.

In order to confirm these hypotheses, in the next part, the formation of degradation products of malvidin-3-*O*-glucoside will be studied.

3.2.3. Quantification of malvidin-3-*O*-glucoside by HPLC-DAD

Figure 69 shows the concentration in malvidin-3-*O*-glucoside of malvidin-3-*O*-glucoside solution (control) and malvidin-3-*O*-glucoside solution added by oenological tannins or (-)-epicatechin (samples).

The oenological tannins and (-)-epicatechin were added at different concentration to reach a ratio copigment/pigment (Cp/p) of 0, 2, 4 and 8. The experiment also elucidate how the solutions of malvidin-3-*O*-glucoside behave during the time (1, 7, 14 and 21 days) when the copigments, (-)-epicatechin and oenological tannins, were present in the media.



All data are the mean ± SD of three replicates. Different lower-case letters indicate the existence of significant differences between days at the same Cp/p ratio ($p < 0.05$).

Figure 69: Malvidin-3-O-glucoside concentration of the model wine solution supplemented with increasing concentrations of (-)-epicatechin and oenological tannins

First of all, the concentration in malvidin-3-*O*-glucoside at day 1 of the control was 59.5 ± 0.3 mg/L. On day 7 and 14 a slow decrease is noted but on day 21 the concentration (59.9 ± 0.3 mg/L) was not significantly different from day 1. The slight reduction of malvidin-3-*O*-glucoside on day 7 and 14 in the control is probably due to a quite oxidation of the malvidin-3-*O*-glucoside. Indeed, as described previously, this anthocyanin in a model wine solution without the presence of any other phenolic compounds can be oxidized [260].

In general, from day 7, the presence of all copigments brought about a significant decrease in malvidin-3-*O*-glucoside. Additionally, this decrease tended to be higher when the copigment/pigment ratio increased. Nevertheless, it should be highlighted that the comportment of the different copigments differs. These results are in accordance with previous ones obtained in a preliminary study conducted by our research group [217].

Indeed, (-)-epicatechin and ellagitannin caused higher decrease in the concentration of the anthocyanin when their copigment/pigment ratio effect was higher. Truly, for (-)-epicatechin at Cp/p = 2, significant differences were observed between day 1 and the other days. At Cp/p = 4, compelling disparities were observed between day 1 and day 7 and day 14, 21. At Cp/p = 8, significant differences were observed between all the days.

Concerning ellagitannin, at Cp/p = 2 important contrasts were observed between day 1 and day 7,14 and day 21. At Cp/p = 4 and Cp/p = 8, significant differences were observed between all the days.

Furthermore, ellagitannin was also the copigment which caused the higher decrease in the concentration of malvidin-3-*O*-glucoside during the time. Indeed, between the first day and the last day (day 21), at Cp/p = 2 and 4, 21% of the anthocyanin have been lost. Even more so at Cp/p = 8, 35% of the anthocyanin have been lost meanwhile for the other copigments tested only 20% was lost. In the case of the gallotannin, only 11% of the malvidin-3-*O*-glucoside has disappears.

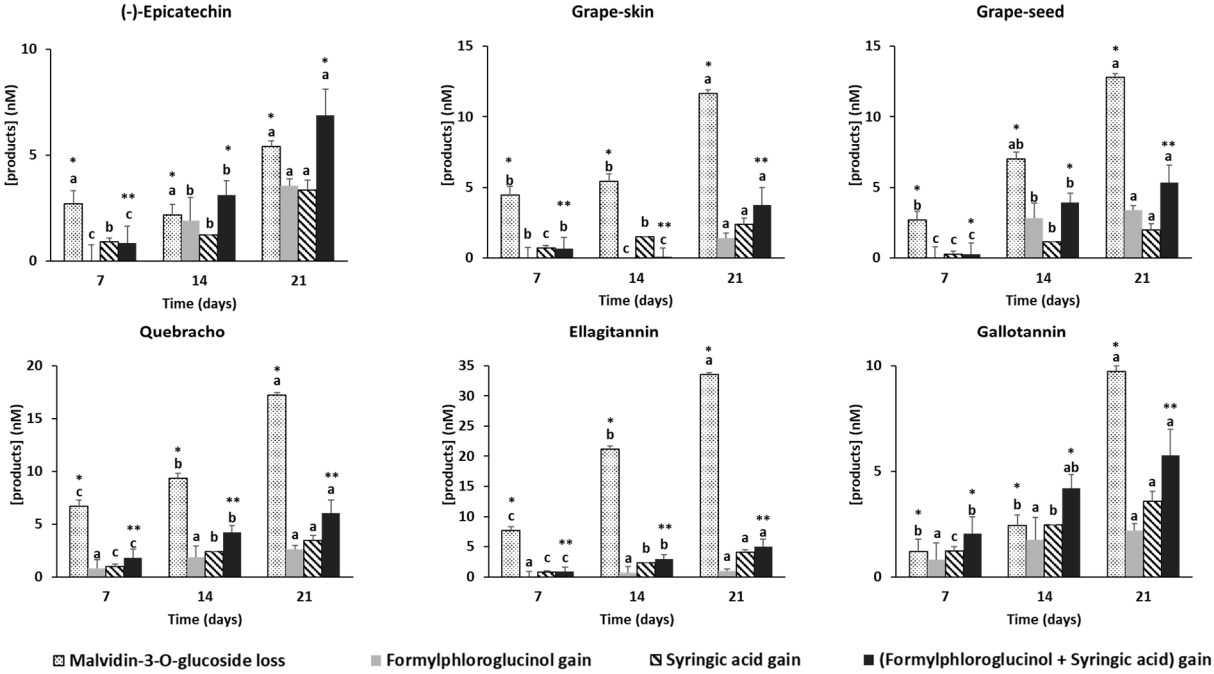
These results are in accordance with the previous ones obtained for the copigmentation index, in which ellagitannin presented during the time a higher decrease than the rest of the copigments. In any case, except for gallotannin, this reduction in the concentration of malvidin-3-*O*-glucoside is enough important (loss of 20%) to be elucidated and discussed. Obviously, the major part of the differences observed in the absorption spectrum will be mainly attributed to the copigmentation phenomenon, but it is necessary to consider this lost in anthocyanin.

As explained in the copigmentation index, this decrease may be related to the oxidation of this anthocyanin or its transformation, since it has been reported that proanthocyanidins and even ellagitannins can react with anthocyanins to form polymerized pigments. The absorption spectra of these polymerized pigments are somewhat different from that of malvidin-3-*O*-glucoside and differ mainly according to the nature of the new pigment. In fact, direct unions between malvidin-3-*O*-glucoside and flavanol induce a red hue meanwhile the same unions by the intermediate of an ethyl bridge induce a purple hue. Moreover, the different products resulting of a cycloaddition (pyranoanthocyanins) induce an orange hue [262].

3.2.4. Characterization of malvidin-3-*O*-glucoside degradation products

As demonstrated in the previous parts, some changes were observed regarding the ability of the oenological tannins to improve color stabilization during the time. In this way, the characterization and quantification of malvidin-3-*O*-glucoside degradation products was achieved, in order to understand and better explained the previous results obtained. Indeed, the degradation of the anthocyanin could be a part of the explication and even more so explained the differences between the oenological tannins, regarding color stabilization. All the samples were analyzed as described previously (2.3.2) and two products corresponding to the degradation of the malvidin-3-*O*-glucoside were found. These products were identified as the syringic acid and the formylphloroglucinol (also called 2,4,6-Trihydroxybenzaldehyde or phloroglucinol aldehyde). Various authors have also found these two compounds as degradation products of the malvidin-3-*O*-glucoside under different conditions (thermal degradation or enzymatic degradation mainly) [263–265].

According to this, **Figure 70** shows the concentration of malvidin-3-*O*-glucoside, formylphloroglucinol and syringic acid in the solution of malvidin added by the different copigments. In addition, to better visualize the results, the concentration of formylphloroglucinol and syringic acid were summed as the total concentration of degradation products. Only the results with the ratio Cp/p = 8 are presented, since as shown previously in the quantification of the malvidin-3-*O*-glucoside, it's the most significant conditions to observe the differences between the different copigments.



All data are the mean ± SD of three replicates. Different lower-case letters indicate the existence of significant differences between days for the same compounds ($p < 0.05$). Different lower-case asterisks indicate the existence of significant differences between compounds for the same day ($p < 0.05$).

Figure 70: Concentration in malvidin-3-O-glucoside loss and products of degradation gain in model wine solution with 50 mg/L added by different copigments (Cp/p =8) at different days of the experimentation

First, the malvidin-3-*O*-glucoside in model wine solution without any presence of copigments, present also a slight decrease of its concentration associated to the formation of degradation products. Additionally, the first day of the experiment was used as the control for determine the lost in malvidin-3-*O*-glucoside and the gain in the formation of degradation products compared to the other days of the experimentation.

First of all, in all cases, as expected, the concentration in the anthocyanin decrease and the total concentration in products of degradation increase during the experimentation. Additionally, it should be highlighted that in general, syringic acid is preferentially formed than the formylphloroglucinol. Nevertheless, in the case of the (-)-epicatechin, both products are formed in the same quantities and in the case of grape-seed tannin, the formylphloroglucinol was formed preferentially.

Concerning grape-skin tannin, quebracho tannin and ellagitannin, difference between the loss of the anthocyanin and the gain in products of degradation was significant since the seventh day of the experimentation until the last day.

Regarding (-)-epicatechin the difference was significant only at the day 7 meanwhile for gallotannin the difference was significant only on the last day of the experimentation.

Finally, the concentration loss of the anthocyanin differs according to the copigment present in solution and the day of the experimentation. Nevertheless, the total concentration in the degradation products formed was similar for all the copigments tested. These results mean, that the differences noted in the loss of malvidin-3-*O*-glucoside in presence of the different copigment cannot be attributed to the degradation products. Indeed, the formation of these products explained a part of the loss of the anthocyanin but less than 33% and even more so, less than 70% in presence of ellagitannin. According to this, the rest of the anthocyanin loss can be involved in other reactions as the formation of polymerized pigments.

The only exception, is the gallotannin which present significant differences only the last day, meaning that probably no other reactions additionally to copigmentation come into play. Indeed, most of the malvidin-3-*O*-glucoside loss is explained by the formation of the degradation products meaning that malvidin-3-*O*-glucoside cannot be involved in other mechanisms.

Finally, it should be highlighted that the maximum absorption of these two compounds (syringic acid and formylphloroglucinol) are 278 and 294 nm respectively, corresponding to colorless products [265]. This explained the changes noted in the color analyzes in which it has been observed in all cases a slight diminution of the color parameters. Indeed, the formation of this compounds, even if in few quantities, induce a quite diminution of the color of the solutions containing malvidin-3-*O*-glucoside and oenological tannins.

4. Conclusion

These results allowed us to conclude that botanical origin of oenological tannins influence their effectiveness regarding color stabilization. Indeed, hydrolysable tannins and more specifically gallotannins seem to be the most efficient compounds to stabilize the color of red wines during the aging by copigmentation. In contrast, condensed tannins seem to be weaker regarding the protection of the color by copigmentation even if procyanidins/prodelphinidins tannins appear to be more efficient than profisetinidins/prorobitenidins tannins.

Nevertheless, the experimentation conducted at different time of contact between oenological tannins and malvidin-3-*O*-glucoside (1, 7, 14 and 21 days), enlighten us about the real effectiveness over time of the oenological tannins.

Indeed, during all the time of the experiment, gallotannin still remain the most efficient one to improve color stabilization by having a great hyperchromic and bathochromic effect which is in accordance with the previous results obtained. On the contrary, ellagitannin appears at the beginning as a great candidate to improve the color stabilization but its effect during the time change conducting to a poor efficiency at the end. Grape-seed and grape-skin tannins, contrary to ellagitannins see their effects increase with the time of contact with the malvidin-3-*O*-glucoside. Finally, quebracho tannin, independently of the time contact remains as the lowest efficient one.

Likewise, the importance of the pH level and ethanol content of the wine in presence of oenological tannins has been elucidated. The obtained results shown that, regardless of the oenological conditions, hydrolysable tannins (gallotannin and ellagitannin) were the most efficient oenological tannins regarding color improvement as copigments. However, the pH level and ethanol content play a certain role, since they can increase or decrease the effect of the oenological tannins as copigments. Generally, the higher the pH and ethanol content, the lower the color stabilization effect. This could be explained by the fact that anthocyanins are more stable at lower pH levels, allowing for greater color improvement [94]. Alcohol also works against copigmentation by destabilizing the hydrogen bonding between anthocyanin aggregates, because it can disrupt the lattice like interaction of water molecules and destroy the molecular stacking of the anthocyanins [94]. Additionally, an increase in ethanol concentration reduces the extent of copigmentation of anthocyanin in ethanol solution kept at a constant pH and releases more flavylium cations. Therefore, the lower stability of malvidin-3-*O*-glucoside at an elevated ethanol concentration can be attributed to a higher content of free flavylium cations in the solution [254]. Nevertheless, the augmentation or decrease of these two parameters influence all the oenological tannins effects in the same way. Independently of the intrinsic wine conditions, hydrolysable tannins remain the most effective in stabilizing red wine color by copigmentation.

To conclude, oenological tannins appear as great candidate to stabilize the color of red wines and help to their improvement. More specifically, gallotannins are the candidate who should be used in priority, since its effect remain constant during the time and with the highest efficiency whatever the oenological conditions (changes in pH and ethanol content).

Chapter 6: Antioxidasic properties of oenological tannins against *Botrytis cinerea* strain 213 laccases

1. Introduction

Botrytis cinerea, is a ubiquitous, filamentous and necrotrophic fungus. Under particular environmental and grape growing conditions, *B. cinerea* can affect positively the grapes by causing "noble rot" leading to high-priced, natural sweet white wines, such as Tokaji Aszú, Sauternes or Passito wines for example [266]. This particular infection pathway, associated with noble rot, promotes favorable biochemical changes in grape berries, notably by the accumulation of secondary metabolites enhancing the grape composition [267]. Nevertheless, *B. cinerea*, can also cause grey mould or botrytis bunch rot (BBR), responsible for huge economic losses each year for the wine industry.

Infection of bunch by the pathogen provokes serious biological and chemical changes impacting negatively organoleptic qualities of wines [268]. Indeed, an important feature of laccases is to be very stable under wine conditions [269]. When climatic conditions are wet under mild weather conditions, *B. cinerea* can infect directly grape berries from veraison onwards [270]. A vintage contaminated by the pathogen at the rate of 5% in severity already shows irreversible consequences on the organoleptic features of a qualitative red wine [203]. The infection of grape berries by *B. cinerea* is accompanied by the excretion of metabolites (glycerol, gluconic acid, β-glucans) and enzymes (pectinases, proteases, tyrosinases and laccases) in the host cells [271]. Laccases (EC 1.10.3.2) are *o*-diphenol and *p*-diphenol: dioxygen oxidoreductases. These multi-copper glycoproteins use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism [272]. Generally, in *B. cinerea*, two genes have been found encoding laccases with molecular weights of about 60 kDa [273] and the molecular mass of the monomer ranges from about 50 to 100 kDa [272].

The oxidation of the polyphenols, and thus the alteration of the color, begins in the grape berry and continues in the grape juice. In skins from infected grape berries and in musts derived from botrytized grapes, the laccases produced by the fungus oxidizes the polyphenols and leads to the formation of quinones. These quinones will polymerize and form brown compounds, which is called browning in white wines and oxidasic casse in red wines, and leads to color degradation and instability [274]. This oxidase breakage phenomenon takes place in red and white grape juices, giving brick and brown tints, respectively. Moreover, changes in color due to the laccases produced by the pathogen are accompanied by others modifications in the organoleptic wine qualities which are impacted by changing their equilibrium, body and/or mouthfeel [273]. Several studies have shown that grey mold leads to the development of organoleptic deviations in grapes and wines and depreciation of botrytized wines have been attributed to off-flavors such as “damp earth”, “vegetal/herbal like” and “mushroom” [203,275]. Geosmin and 1-octen-3-one have been also identified in musts and wines made from botrytized grapes [276,277]. Regarding mouthfeel, botrytized red wines were described as less astringent, according with a decrease of the mDP of their tannins [203].

In the vineyard, until now, the preventive means used are fungicides. Essential oils, mineral oils, plant hormones, plant extracts, are also tested as preventatives [278]. In the cellar, browning and oxidasic casse are prevented by sorting healthy grapes from contaminated grapes and by adding SO_2 at doses up to 50 mg/L which has antioxidasic activity as well as antioxidant properties [171,279]. Grape juice can also be protected against oxidation using inert gas or ascorbic acid [280]. Alternatives are developing such as biocontrol, involving inoculation of one or more microorganisms as yeasts or bacteria strains on the vine [281,282]. Indeed, the microorganisms can colonize the environment, leaving no space for the development of *B. cinerea* which is harmful to the quality of the grapes. Winemakers can also use thermovinification, which allows the laccase enzyme to be denatured by heat, rendering it inactive [171,271]. Nevertheless, this process involves a significant energy expenditure and may affect certain thermolabile compounds such as aroma compounds for example and thus reduce the final quality of the wine.

In our days, the consumer wants healthier and eco-friendlier products and for this reason, the wine industry is searching for alternative products for reducing or even eliminating the use of sulfur dioxide. Nevertheless, as shown previously, until now, there are currently few alternatives to them to treat musts from botrytized grape harvests. In this way, oenological tannins could be an alternative since they present various properties, and they are yet used for other purposes. Nevertheless, although these properties of enological tannins have been widely described in the literature, the antioxidasic properties (anti-laccase) are not yet well documented.

Thus, the aim of this chapter was first to determine the ability of enological tannins to reduce the laccase activity produced from the pathogenic *B. cinerea*. Consequently, their ability to protect the wine color against enzymatic browning and oxidasic haze was also determined. Additionally, to the impact on wine color, the organoleptic impact was also evaluated.

The second aim was to understand the mechanisms of action of oenological tannins against laccases produced by *B. cinerea* (strain 213) in grapes and musts. In this way, enzymatic kinetic parameters were determined, and electrophoresis characterization was achieved.

Finally, the objective was also to compare the effects of different types of oenological tannins against laccases as an alternative of the two main usual oenological additives used in the wine industry nowadays: ascorbic acid and sulfur dioxide.

2. Materials and methods

The method to obtain all the musts and wines used in this chapter has been previously described in the general experimentation section (Chapter 2).

Five oenological tannins were used to facilitate the experimentations and winemaking: 1 grape-seed tannin, 1 grape-skin tannin, 1 quebracho tannin, 1 ellagitannin and 1 gallotannin were used as representative of specific botanical origin.

2.1. Laccase activity measurements

2.1.1. Laccase activity and residual laccase activity

For all the experimentations, laccase activity was determined using an adaptation of the syringaldazine test method [283]. 5 mL of the different samples were added with 0.8 g of PVPP (to remove phenolic compounds that can cause interference), stirred and centrifuged for 10 minutes at 8,500 rpm. 1 mL of the supernatant was introduced into a plastic spectrophotometer cuvette (10 mm path length) to which were also added: 1.4 mL of buffer solution (8.2 g/L of sodium acetate in deionized water, pH 5.5) and 0.6 mL of syringaldazine solution (60 mg/L of syringaldazine in ethanol 96% vol.). The solution was then homogenized by inverting the cell and the absorbance was measured at 530 nm every minute for 5 minutes (including time measurement at 0 minute). A laccase unit (UL) corresponds to the amount of enzyme catalyzing the oxidation of a micromole of syringaldazine per minute per liter. The following equation was used for the calculation of laccase activity by using the slope from a calibrating linear regression (ΔA) expressed in absorbance units/minute:

$$Laccase\ activity = 46.15\ x\ \Delta A\ \mu mol.\ L^{-1}.\ min^{-1} = 46.15\ x\ \Delta A\ UL$$

Moreover, the residual laccase activity was determined for each sample using the following equation:

$$\%\ of\ residual\ activity = (laccase\ activity_{sample}\ /\ laccase\ activity_{control})\ x\ 100$$

2.1.2. Influence of time contact between oenological tannins and laccases on residual laccase activity

Tannins solutions were prepared in model wine solution (12% vol. of ethanol, 4 g/L of tartaric acid with a pH of 3.5) at 40 g/hL. Then, different solutions were prepared by adding 4 mL of botrytized must to 1 mL of each tannin solutions (samples) or 1 mL of model wine solution (control). The estimation of the influence of time contact between oenological tannins and laccases on laccase activity was carried out at 0, 1, 2, 3, 4, 5 or 10 minutes of incubation before adding the PVPP, stirred and centrifuged for 10 minutes at 8,500 rpm. Laccase activity was determined using the syringaldazine test method as previously described. All analyses were performed in triplicate.

These data were used to determine the time needed to reach the maximal inhibition of laccase activity for all the next experimentations. All analyses were performed in triplicate.

2.2. Impact of tannin addition in botrytized musts and wines

2.2.1. Impact of oenological tannins on the laccase activity of botrytized red wines

The laccase activity was measured for all the samples at 20% and 50% of *B. cinerea* contamination at different times of the winemaking. Indeed, laccase activity was measured on vatting (VAT), after post-alcoholic fermentation maceration (MAC) and on the 3 months after bottling.

2.2.2. Influence of oenological tannins on the color of botrytized wines

After the infection by *B. cinerea*, color parameters are among the most impacted. In this way for each winemaking, different color components were analyzed to determine the ability of the oenological tannins to protect wine color against *B. cinerea* damages.

2.2.2.1. Influence on botrytized white wine

Once the fermentations were completed, the impact of *B. cinerea* on the color of the white wines was determined by measuring the absorbance at 420 nm (yellow). Additionally, the total color difference (ΔE_{ab}) between samples (with and without botrytization) was obtained using the CIELAB coordinates [249] and was calculated using the following equation:

$$\Delta E_{ab} = \sqrt{(L_2{}^* - L_1{}^*) + (a_2{}^* - a_1{}^*) + (b_2{}^* - b_1{}^*)}$$

The ΔE_{ab} represents a measure of the difference between two colors. ΔE_{ab} is used to know whether the difference between two samples can be detected visually by the human eye. Generally, it is considered that the difference is visible to the human eye when $\Delta E_{ab} > 3$ [284].

2.2.2.2. Influence on botrytized “rosé” wine

In the case of the “rosé” wines, the absorbance at 520 nm (pink-red) was measured as well as the total color difference (ΔE_{ab}). Moreover, the total anthocyanin (TA) concentration was also analyzed [206]. A solution A containing 250 µL of sample, 250 µL of ethanol acidified with 0.1% HCl and 5 ml of 2% HCl (v/v) was previously prepared. Then, 1 mL of solution A was put in 2 different tubes. In the first one, 400 µL of distillated water was added to solution A meanwhile in the second tube, 400 µL of 15% potassium bisulfite was added. After 20 minutes, the absorbance at 520 nm from both tubes was measured in a 1 cm optical path cuvette. TA was calculated as follow:

$$\text{TA (eq mg of malvidin-3-}O\text{-glucoside/L)} = 875 \text{ x } \Delta A_{520\text{nm}}$$

2.2.2.3. Influence on botrytized red wine

Concerning red winemaking, the absorbance and CIELAB parameters were determined as well as the ΔE_{ab}. These measures were made as well as for white and “rosé” wines. Additionally, to this, the total anthocyanin concentration was measured as explained for the “rosé” wines.

2.2.3. Influence of oenological tannins on the sensory properties of red botrytized wines

Sensory analyses were carried out 3 months after bottling the wines. The wines were evaluated by 20 judges, considered as "expert" from the Oenology department of the University of Bordeaux. They were all selected based on interest and availability as well as their experience in red wine sensory analysis. All analyses were performed in a specific room at 20 °C with controlled hygrometry (ISO 8589:2007) and isolated tasting stations. Regardless of the type of test, 20 mL of wines were presented in clear glasses to evaluate color or in black glasses to evaluate the olfactory and taste compounds (NF V 09 110). Samples were presented randomly numbered with 3-digit codes and balanced (triangular tests) or randomized (ranking tests and profiles).

2.2.3.1. Triangle tests

Several triangle tests (ISO4120: 2007) on the 3-months bottling red wines were carried out to highlight significant differences.

A first serie of triangle tests was realized in order to evaluate possible significant differences between the healthy control wines [0% Botrytis, Ø tannins (without tannins)] and the botrytized control wines [20% and 50% Botrytis, Ø tannins]. In fact, theses triangle tests, allowed to determine if the judges were able to discriminate the contaminated wines from the wines made only with healthy grapes.

A second serie of triangle tests was proposed in order to evaluate possible significant differences between each botrytized control wines [20% and 50% Botrytis, Ø tannins] and its corresponding wines with addition of the diverse oenological tannins [20% and 50% Botrytis, tannins addition]. These triangle tests, allowed to determine if the judges were able to discriminate the contaminated control wines from the same wines added by oenological tannins.

During the sessions, it was asked to the judges, to observe, smell and/or taste the samples in the defined order (1 order by taster). Then, they had to indicate which glass seemed different to them from the two other ones, even if they were not sure of their answer. Finally, it was also asked to the panel, to note one or more descriptor(s) that allowed it to differentiate the samples. The descriptors were retained only in the case of a good answer from the judge, concerning the different glass. For triangle tests, the number of correct answers were summed and compared to the data of the binomial table (minimum number of correct responses needed to conclude that a perceptible difference exists based on a triangle test (ISO-4120, 2004)). If the number of correct responses was greater or equal to the number given in the table (corresponding to the number of assessors and the a-risk level chosen for the test), it was concluded that a perceptible difference existed between the samples.

2.2.3.2. Ranking tests

Once the triangular tests done, ranking tests (ISO8587: 2007) were also conducted during three different sessions containing each one six wines. Before beginning the ranking test, it was only notified to the judges, that the samples were red young wines made from Merlot grape variety. Then, for the ranking tests, it was asked to the panel to classify six wines according to their "overall quality", including the visual, aromatic and taste quality. In order to make this, judges had to assign rating of 1 for the most qualitative wine and to 6 for the less qualitative.

The results were finally, processed by the Friedman test on the sum of the ranks assigned by the judges. Then, the smallest significant difference was calculated and compared to the absolute value of the difference in the sum of the ranks (for 2 wines). This calculation/comparison allowed determining significant differences between products from each other.

2.3. Comprehension of action mechanisms of oenological tannins on laccases produced by *B. cinerea*

2.3.1. Enzymatic kinetics characterization: determination of the residual laccase activity, V_{max} and K_M parameters

Tannin solutions were prepared in model wine solution (12% vol. of ethanol, 4 g/L of tartaric acid and adjusted to pH 3.5) at different doses: 10, 20, 30 and 40 g/hL. Then, five different solutions were prepared by adding 4 mL of botrytized white must to 1 mL of each tannin solutions (samples) or 1 mL of model wine solution (control). The mix was left four minutes in contact before being added by 0.8 g of PVPP, stirred and centrifuged for 10 minutes at 8,500 rpm. Laccase activity was determined using the syringaldazine test as previously described.

After this, the enzyme kinetic of the *B. cinerea* laccase was studied using the previous solutions and different concentrations of syringaldazine as the substrate. The Michaëlis-Menten plot was represented for each sample allowing us to represent the Lineweaver-Burk curve (double reciprocal plots). Then, the values of K_M and V_{max} were determined from the linear regression of double reciprocal plots obtained by varying the final concentration of syringaldazine in the reaction medium from 0 to 33 µM.

All the analyses were performed in triplicate.

2.3.2. Influence of bentonite treatment

Increasing volumes of a 200 g/hL bentonite (Natural sodium bentonite, micro-granulated) suspension were added to 3 mL of botrytized white must to reach final bentonite concentration between 0 and 100 g/hL. The samples were left 20 minutes in contact before being added by 0.5 g of PVPP, stirred and centrifuged for 5 min at 8,500 rpm. Laccase activity was then determined as previously described.

2.3.3. Electrophoresis SDS-PAGE of laccase produced by *B. cinerea* supplemented by oenological tannins

In order to conduct the electrophoresis SDS-PAGE in presence of oenological tannins, extracellular laccases were produced to facilitate the assay with specific steps. Indeed, it was easier to work on a liquid medium containing only extracellular laccases excreted by *B. cinerea* and supplemented it with oenological tannins to see the interactions than work in botrytized must.

2.3.3.1. Extracellular laccase production

Extracellular laccases were produced from the strain 213 of *B. cinerea*, strain previously described in chapter 2. The strain was kept as a stock suspension of spores in 20% of glycerol and stored at -80 °C.

B. cinerea laccases were produced as previously described with some modifications [285]. Briefly, cultures on YPD medium were kept for 1 week at 20 °C in incubator to induce growth and sporulation. The spores were then gently scraped from one 55 mm diameter Petri dish to inoculate 125 mL of sterilized culture medium in an Erlenmeyer flask of 500 mL. Sterilized culture medium was composed as followed (in g/L): glucose, 40; glycerol, 7; L-histidine, 0.5; $CuSO_4$, 0.1; $NaNO_3$, 1.8; NaCl, 1.8; KCl, 0.5; $CaCl_2.2H_2O$, 0.5; $FeSO_4.7H_2O$, 0.05; KH_2PO_4, 1.0; and $MgSO_4.7H_2O$, 0.5. After 3 days of incubation (20 °C, 180 dark, 140 rpm), 100 mL of these pre-cultures were transferred into an Erlenmeyer flask of 5 L containing 1.4 L of the culture medium. After 2 days of growth (20 °C, dark, 140 rpm), gallic acid was added at 2 g/L and cultures were maintained 5 more days under the same conditions. Then, the liquid medium was filtered through a filter paper and kept until use at -80 °C. Fresh fungal biomass was lyophilized and dried biomass weighed. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard [286]. Initial laccase activity of the liquid medium was also determined as described previously.

2.3.3.2. Enzyme electrophoresis

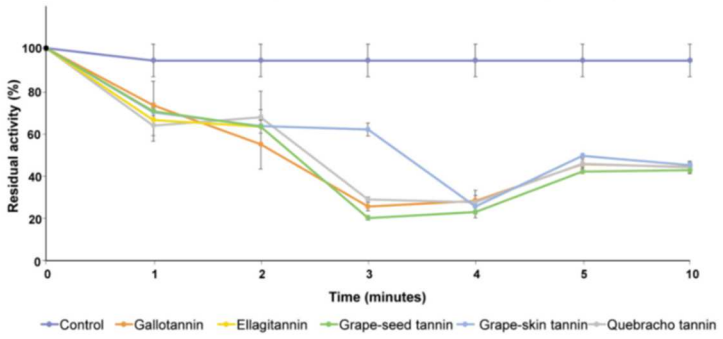
B. cinerea laccase was analyzed by SDS-PAGE as previously described [287] with some modifications. Briefly, 10 µL of the enzyme solution was mixed with Laemmli buffer: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 25% glycerol and 0.01% bromophenol blue. The gel was a 10% Mini-PROTEAN® TGX™ Gel (Bio-Rad Laboratories, Hercules, CA, USA) and the electrophoresis was carried out at 120 V, using the Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA). A Spectra™ Multicolor Broad Range Protein Ladder (10-260 kDa, Thermo Fisher Scientific Inc., Waltham, MA, USA) was included in each electrophoresis run as standards for molecular weight estimation. The gel was stained with a Pierce™ Silver Stain Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). A digitized image of the gel was obtained by photography. The molecular weight, intensity and relative quantity in proteins of the bands were determined with GeneTools analysis software (Syngene, Cambridge, UK).

3. Results and discussion

3.1. Laccase activity measurements

3.1.1. Influence of time contact between oenological tannins and laccases on residual laccase activity

The inhibition kinetics of laccase activity exerted by the different oenological tannins at the dose of 40 g/hL is presented in **Figure 71**. The results clearly indicated that all the tannins reached their maximal inhibitory effect against laccases after 3 minutes, with the only exception of grape-skin tannin that needed 1 minute more. This data indicated that all enological tannins can inhibit laccase activity in a very short time. Thus, after 4 minutes, all tannins allowed a decrease of 70%. In this way, before measuring laccase activity, for the next experimentations, tannins will be stayed in contact 4 minutes with the botrytized samples.

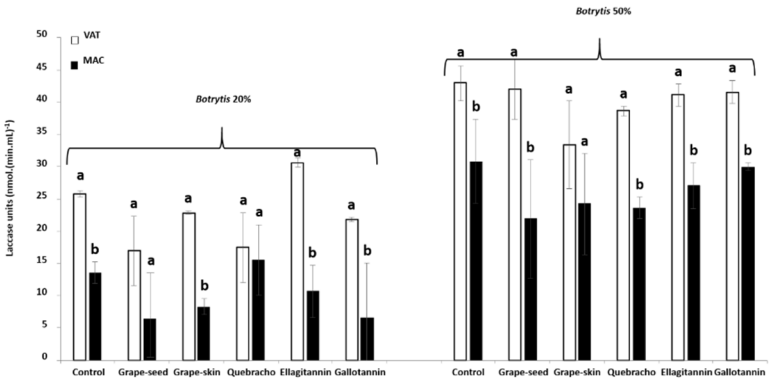


All data are the mean ± SD of three replicates.

Figure 71: Influence of time contacts between oenological tannins at 40 g/hL and laccases on residual laccase activity (%)

3.1.2. Impact of oenological tannins on the laccase activity of botrytized red wines

This part concerns the micro winemaking of red Merlot grapes. Laccase activities were measured on botrytized red must after vatting (VAT) and on botrytized red wines after the maceration following the alcoholic fermentation (MAC). These measurements were achieved for both 20 and 50% botrytization rates and with or without tannin additions. Results are presented in **Figure 72**.



All data are the mean ± SD of three replicates. Different lower-case letters indicate the existence of significant differences between VAT and MAC laccase units ($p < 0.05$).

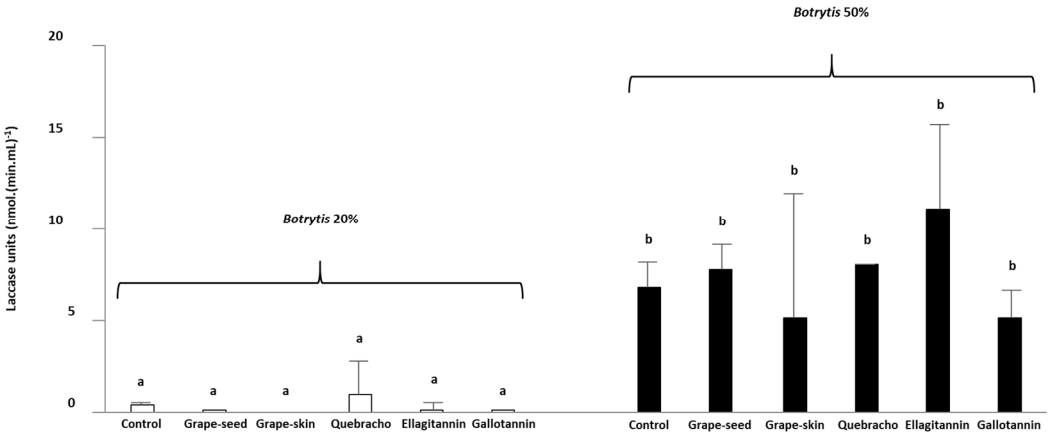
Figure 72: Laccase activity in botrytized (20 and 50 %) of musts (VAT) and post-alcoholic fermentation maceration wines (MAC) in laccase units

First of all, concerning VAT samples, the control botrytized at 50% reached values of laccase activity 1.65-fold-times higher than the control botrytized at 20%. Indeed, the laccase activity of the botrytized control at 20% and 50% were 26 and 43 laccase units respectively. Additionally, in general, independently of the commercial tannin used, laccase activities were also lower in the modalities at 20% than at 50% of *Botrytis cinerea*. Nevertheless, at this moment of the experimentation (VAT) none of the tannins seems to reduce significantly the laccase activity compared to the control at 50% of contamination. Concerning samples at 20% of contamination, no significant differences were highlighted but the tendency showed that laccase activity was slightly reduced when tannins were added (except for ellagitannins) in the VAT.

Then, after the post-alcoholic fermentation (MAC), control at 20% and 50% saw their laccase activity fall by 47% and 28% respectively. However, laccase activity of the control at 50% of contamination was still 2-fold-times higher than the laccase activity of the control botrytized at 20%. At this moment, the laccase activity was now around 30 and 15 laccase units respectively. The same tendency, as in the controls, was observed in presence of the diverse oenological tannins, as the laccase activity between VAT and MAC samples also decrease. Some differences, regarding the comportment of the different tannins can be highlighted. Concerning, the modality of 50%, grape-skin tannin, ellagitannin and gallotannins induce the same decrease in activity between VAT and MAC than the control (decrease of 30-35%). This meaning that these three kinds of tannins seem not to be so efficient during winemaking at this botrytization rate. On the contrary, grape-seed tannin and quebracho tannin, seems to present inhibitory effect during winemaking, since they caused higher decrease in laccase activity between VAT and MAC than the control (40-50%).

For the modality 20% of botrytization, the effects of the tannins are more efficient in laccase reduction activity than the 50% of botrytization modality. For 20% of botrytization, quebracho tannin present the lowest ability to reduce laccase activity (10%) between VAT and MAC, including lower than the control one (50%). At the opposite, grape-skin, grape-seed, gallotannin and ellagitannin present higher ability than the control to reduce laccase activity (60-70%). Regarding these results, grape-seed tannin seemed to be the more efficient at 20% and 50% botrytization rate.

In order to follow the laccase activity during time, the laccase activity after 3-month of botrytized wines was also determined and presented in **Figure 73**.



All data are the mean ± SD of three replicates. Different lower-case letters indicate the existence of significant differences between 20% and 50% of botrytis for the same tannin ($p < 0.05$).

Figure 73: Laccase activity in botrytized (20 and 50 %) of 3-months wine bottling in laccase units

Before bottling after the malolactic fermentation, sulfur dioxide (SO_2) was added at 20 mg/L in all the modalities. Just after this addition, laccase activity was measured, and no residual activity was remaining in all the samples.

However, as shown in **Figure 73**, in the wines containing 50% of botrytization after 3-months in bottles, a recovery of laccase activity was observed. On the contrary, with 20% of botrytization, the high residual laccase activity presents at 50% of botrytization was not observed in the samples after 3-months in bottles. In fact, statistical tests indicate a "botrytization level" effect (p-value=$7.931*10^{-7}$) without highlighting a "tannin" effect (p-value = 0.2342). This means that this high residual laccase activity at 50% of botrytization after the 3-months was due to the high level of contamination. Additionally, this high residual laccase activity can be explained by the fact, that the quantity of SO_2 added before the bottling (20 mg/L) was active to stop and/or reduce laccase activity after few days as long as free SO_2 still remain present. However, if the sulfur dioxide combines with other compounds present in wine, affecting the SO_2 efficacy to destroy laccase enzymes. Indeed, the sulfur dioxide in its combined form does not present the antioxidasic properties attributed to the free form [2].

In fact, at so high level of botrytization (50%), the quantity of free sulfur dioxide added before bottling is not enough to inhibit and destroy completely the laccases. In this way, the botrytized wines still contained between 5 and 11 laccase unit (without significant differences between wines: p-value = 0.2206) which is enough to present significant risk for the wine to have oxidasic casse. It was reported that a wine containing more than 3 UL/mL presented an instability of its color evolving into casse [288]. On the contrary, the wines from botrytized grapes at 20% present slight or no laccase activity meaning that the dose of sulfur dioxide added was enough to completely stop laccases. No significant differences were noted between the wines at 20% of botrytization. However, the trend showed that grape-skin tannin, grape-seed tannin and gallotannin seems to be the most efficient ones to decrease the laccase activity compared to the control (decrease of 90-100%).

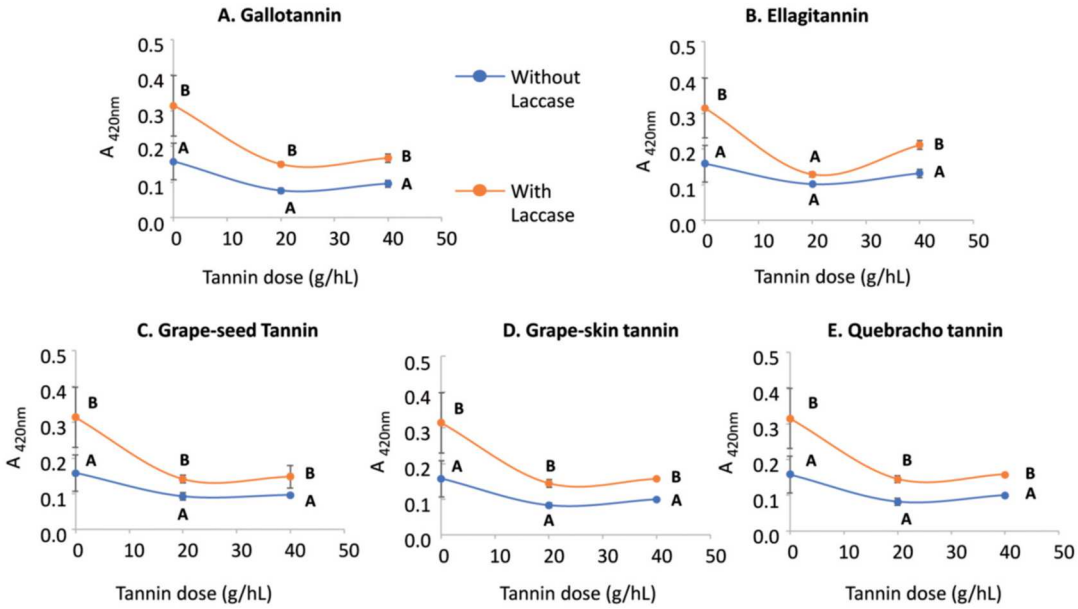
These results are in accordance with the previous ones obtained at MAC stage for the same level of botrytization.

3.2. Influence of oenological tannins on the color of botrytized wines

Color of the wine is one of the important parameters for the winemaker and wine consumers. In this way, the color parameters of botrytized white, "rosé" and red wines with addition of oenological tannins, have been determined in order to show the ability of tannins to protect wine color.

3.2.1. Influence on botrytized white wine

The intensity of changes of yellow color (A_{420nm}) in the white micro-fermentations has been studied and the results are presented in **Figure 74**.



All data are the mean ± SD of three replicates. The capital letters indicate the existence of significant differences between with and without laccase for the same samples and the same doses ($p < 0.05$).

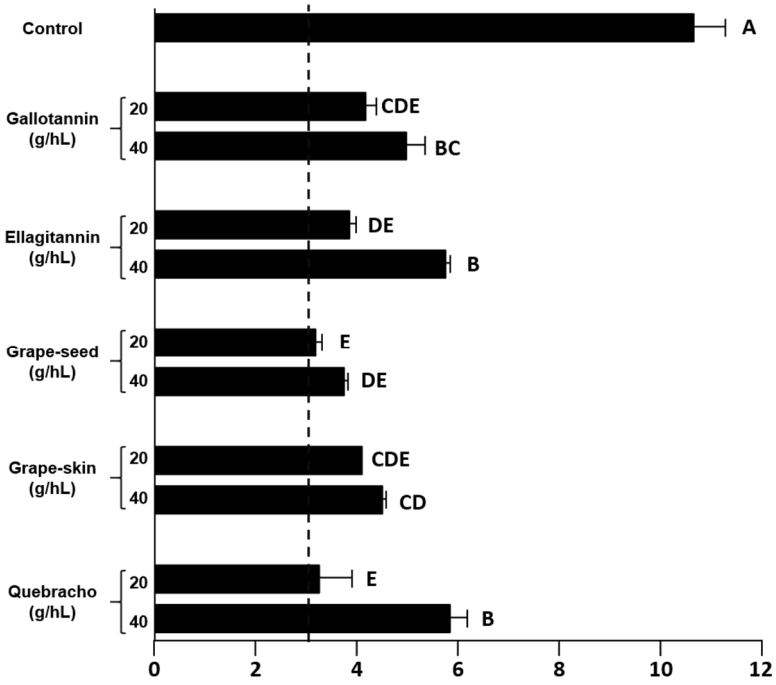
Figure 74: Influence of the supplementation with enological tannins on the changes in the yellow component of the color ($A_{420\,nm}$) of wines made with healthy white grape juice with addition or not of 1.5 units of laccase activity

As expected, the A_{420nm} of the control (without tannins) supplemented with laccases was significantly higher than in the control (without tannins) without laccases. This data confirmed that laccases, as it is well known, caused browning [268]. No significant differences were found in A_{420nm} when oenological tannins are added in control wines (without addition of laccase), despite a certain decreasing tendency. This trend suggests that the supplementation with enological tannins may play a certain role in protecting the wine against oxidation. In fact, it has been reported previously that oenological tannins were able to consume oxygen [164,289].

The decrease in A_{420nm} following addition of oenological tannins was quite more important when laccases were inoculating, approaching the values of the control wines (without laccases).

This decrease in A_{420nm} reached its maximal effect at 20 g/hL, being similar at 40 g/hL for all oenological tannins except for ellagitannin, for which the effect was, surprisingly, significantly lower at the high dose. This effect was specially marked and constant for 20 and 40 g/hL in the case of the grape-seed tannin. This data supports the previous one obtained for the red winemaking in which grape-seed appears as one of the best oenological tannin to reduce laccase activity. Protection of white wine color appears possible with oenological tannins by their abilities to reduce laccase activity.

Additionally, the total color differences (ΔE_{ab}) between each one of the white wines supplemented with laccases and the control wine without laccases have been calculated in **Figure 75**. This value is a good indicator to assess whether the color of the wines was affected by the presence of laccases and degraded enough to be distinguished by the human eye. The human eye can theoretically distinguish two samples when $\Delta E_{ab} \geq 1$ unit [290]. However, it is also generally accepted that tasters can only distinguish the color of two wines through the glass when $\Delta E_{ab} \geq 3$ units [284].



All the data are the mean ± SD of three replicates. The capitals letters represent the significate differences between the different samples ($p < 0.05$).

Figure 75: Impact of enological tannins added in botrytized white wines on color visible to the human eye

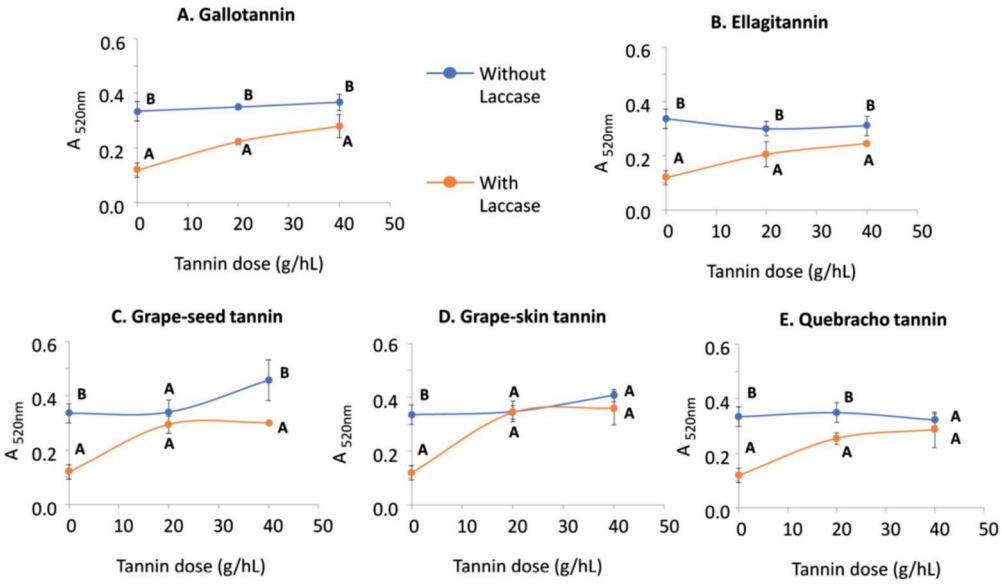
The comparison between both control white wines (with and without laccases) showed a ΔE_{ab} reaching around 10 units. Since this value is much higher than 3 units, this result confirms that the presence of laccases drastically affected the quality of the wine color.

The total color difference between the wines affected by the presence of laccases and the control wine without laccases was also, in all cases, greater than 3 units. However, it must be highlighted that these ΔE_{ab} values were found, much lower in all cases, than those observed between both control wines: with and without laccases.

Even in the case of the lower dose of grape-seed tannin the corresponding ΔE_{ab} values were very close to 3 units. Additionally, independently of the dose added of oenological tannin, grape-seed, grape-skin and gallotannin seems to present the greatest ability to protect white wine color. Once again, these results are in accordance with previous ones obtained for the laccase activity.

3.2.2. Influence on botrytized "rosé" wine

As for white wine, **Figure 76** shows the intensity of red color (A_{520nm}), in this case of the "rosé" wines (micro-fermentations supplemented with 50 mg/L of malvidin-3-*O*-glucoside).



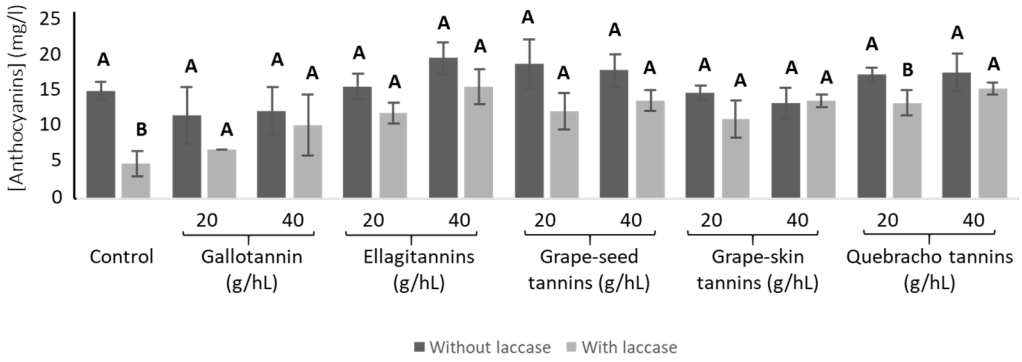
All data are the mean ± SD of three replicates. The capital letters indicate the existence of significant differences between with and without laccase for the same samples and the same doses ($p < 0.05$).

Figure 76: Influence of the supplementation with enological tannins on the changes in the red component of the color ($A_{520\,nm}$) of wines made with healthy white grape juice (supplemented at 50 mg/L of malvidin-3-O-glucoside) with addition or not of 1.5 units of laccase activity

As expected, the intensity of the red color of the control wine with laccases was significantly lower than in the control wine without laccases. In fact, red color intensity was decreased almost by half, confirming that the presence of this enzyme seriously affects the color of rosé wines. This decrease in the A_{520nm} of the wines supplemented with laccases was mitigated by the presence of all the oenological tannins and this effect was in general greater for the highest dose (40 g/hL) (except for grape-seed tannin). Condensed tannins protected better the red color than hydrolysable tannins because the differences in A_{520nm} with the wines obtained without laccase were lower and even sometimes not significant. Our data, demonstrated that oenological tannins addition, really protects the color of red wine against the presence of laccases, preventing the oxidasic haze in red wines caused by the presence of *B. cinerea*.

Additionally, to the evaluation of the red component (A_{520nm}), the concentration of the malvidin-3-*O*-glucoside was determined to better visualize the loss of the anthocyanin in presence of laccases and oenological tannins.

In this way, the concentration of anthocyanins in the different "rosé" wines elaborated is presented in **Figure 77**.

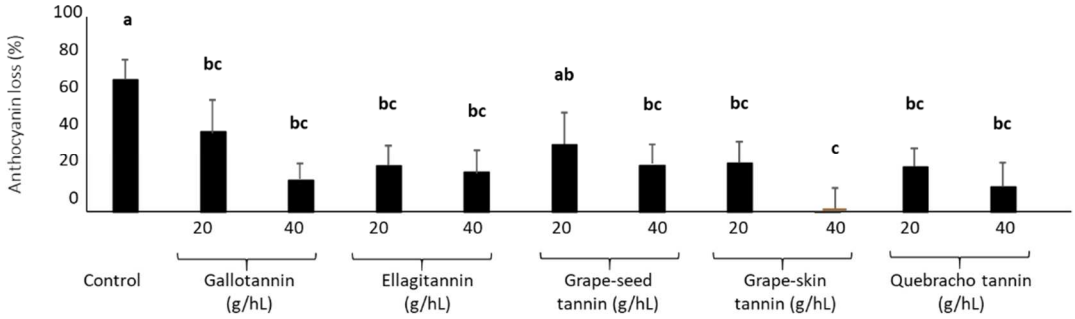


All data are the mean ± SD of three replicates. The capital letters indicate the existence of significant differences between with and without laccase for the same samples and the same doses ($p < 0.05$).

Figure 77: Influence of the supplementation with enological tannins on the malvidin-3-O-glucoside concentration of wines made with healthy white grape juice (supplemented with 50 mg/L of malvidin-3-O-glucoside) with addition or not of 1.5 units of laccase activity

The total concentration of anthocyanins in the control wine without addition of laccases was 15 mg/L. This concentration was quite low in comparison with the concentration of malvidin-3-*O*-glucoside added to the grape juice (50 mg/L) probably due to oxidation and/or absorption by the yeast surface and/or formation of new pigments. In any case, the total concentration of anthocyanins in the control wine with laccases inoculation was significantly lower (p-value < 0.05) than in the control wine without laccase. Anthocyanins pigments are strongly affected by laccases as anthocyanins concentration was found to decrease to less than one third. The inhibitory effect of oenological tannins on laccase activity was noticeable since the concentration of anthocyanins was significantly higher (p-value < 0.05) when oenological tannins were added in the presence of laccases.

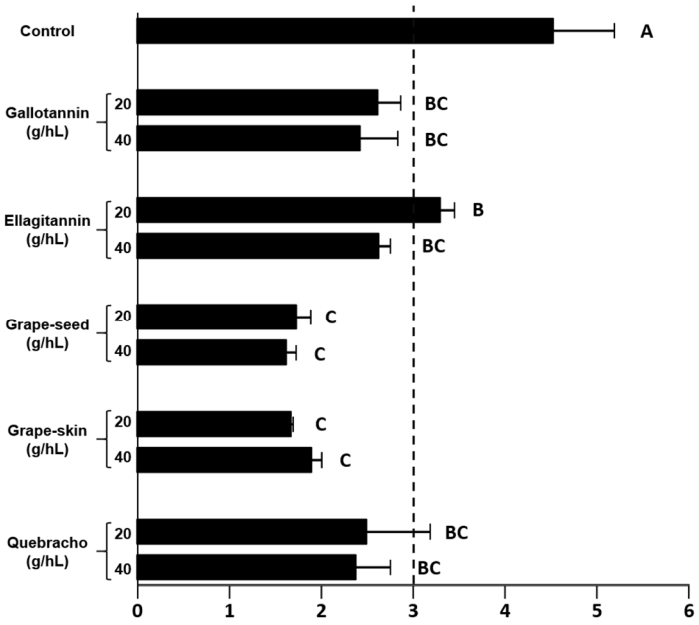
Figure 78 shows the protective effect of oenological tannins in terms of percentage of anthocyanin loss originated by the presence of laccases according to the tannin dose.



All the data are the mean ± SD of three replicates. The lower-case letters indicate the existence of significant differences between the samples ($p < 0.05$).

Figure 78: Influence of the supplementation with enological tannins on the malvidin-3-O-glucoside loss of wines made with healthy white grape juice (supplemented with 50 mg/L of malvidin-3-O-glucoside) with addition or not of 1.5 units of laccase activity

The results clearly confirmed that the presence of all type of oenological tannins significantly protected anthocyanins against the laccases effect and that this protection tended to be greater when the dose of tannins was highest. As for the winemaking in white, to complete the previous results, the total color differences in the "rosé" winemaking (white wines supplemented with malvidin-3-*O*-glucoside) are presented in **Figure 79**. The general trend was very similar to that observed for the white wines.



All the data are the mean ± SD of three replicates. The capitals letters represent the significate differences between the different samples ($p < 0.05$).

Figure 79: Impact of enological tannins added in botrytized "rosé" wines on color visible to the human eye

The ΔE_{ab} comparing the two control red wines, with and without laccase, was around 5 units. This value being greater than 3 units, the presence of laccase deteriorated sufficiently the color of the "rosé" wine to be distinguished by the human eye. Moreover, the supplementation with every oenological tannin significantly decreased the ΔE_{ab} compared to the control wine without laccase. The corresponding ΔE_{ab} values in most of the cases were lower than 3 units. These results confirmed again that enological tannins protected the color of the "rosé" wines, since in most of the supplemented wines the human eye cannot distinguish between the healthy wine and the wines affected by the presence of laccases. Once again grape-seed, grape-skin and gallotannin reached the greatest ability to protect "rosé" wines color as well as for white wines.

3.2.3. Influence on botrytized red wine

Concerning the influence of oenological on the color of botrytized 3-months bottling wines, first the color component of these wines was analyzed. For this purpose, the absorbance at 420, 520 and 620nm (A_{420nm}, A_{520nm} and A_{620nm}) were measured, and then the colorant intensity (IC), percentage of yellow (% yellow), percentage of red (% red), percentage of purple (% purple) and the tint were calculated. The obtained results are presented in **Table 26**.

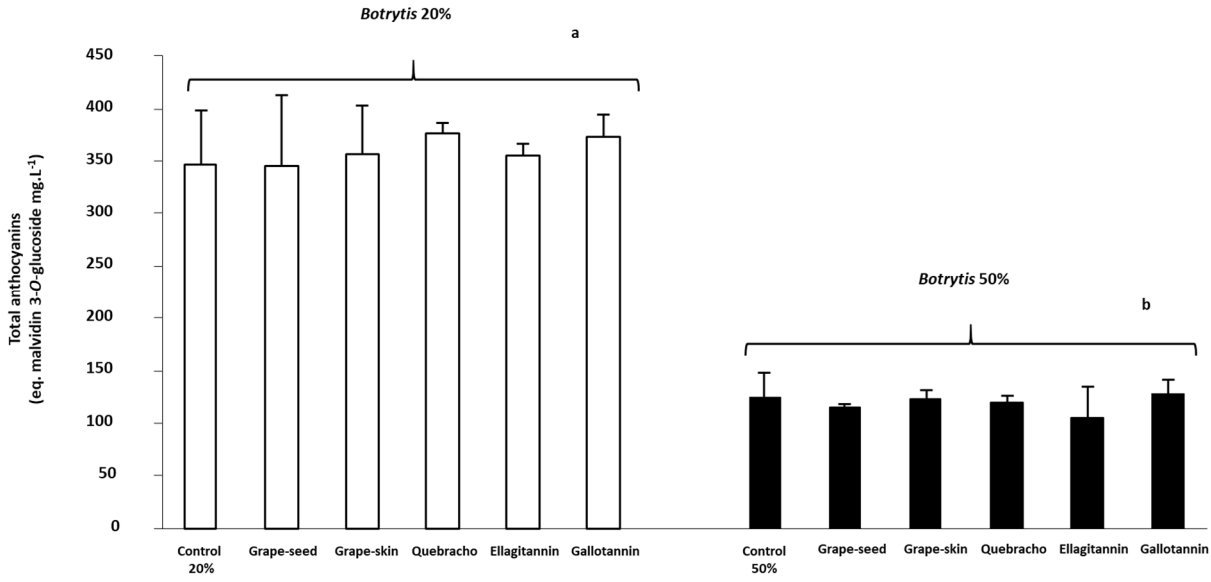
Table 26: Analysis of the color component (A_{420nm}, A_{520nm}, A_{620nm}, IC, % yellow, % red, % purple and tint) of 3-months bottling red wines botrytized

Modalities	Samples	A_{420nm}			A_{520nm}			A_{620nm}			IC			% yellow			% red			% purple			Tint		
Botrytized red wines at 20%	Control	0.300 ± 0.015	A	α	0.412 ± 0.094	A	α	0.088 ± 0.001	A	α	7.9 ± 0.1	B	α	38.1 ± 2.2	A	β	50.6 ± 2.2	A	α	11.2 ± 0.0	A	α	0.8 ± 0.1	A	β
	Grape-seed	0.302 ± 0.016	A	α	0.428 ± 0.009	A	α	0.095 ± 0.008	A	α	8.3 ± 0.3	B	α	36.5 ± 0.5	A	β	51.9 ± 1.0	A	α	11.5 ± 0.5	A	α	0.7 ± 0.0	A	β
	Grape-skin	0.304 ± 0.005	A	α	0.415 ± 0.012	A	α	0.096 ± 0.003	A	α	8.1 ± 0.2	B	α	37.3 ± 0.3	A	β	51.0 ± 0.2	A	α	11.7 ± 0.1	A	α	0.7 ± 0.0	A	β
	Quebracho	0.321 ± 0.020	A	α	0.431 ± 0.031	A	α	0.100 ± 0.007	A	α	8.5 ± 0.6	B	α	37.8 ± 0.5	A	β	50.5 ± 0.5	A	α	11.7 ± 0.1	A	α	0.7 ± 0.0	A	β
	Ellagitannin	0.335 ± 0.018	A	α	0.466 ± 0.025	A	α	0.105 ± 0.008	A	α	9.0 ± 0.5	A	α	37.0 ± 0.1	A	β	50.5 ± 0.5	A	α	11.6 ± 0.3	A	α	0.7 ± 0.0	A	β
	Gallotannin	0.322 ± 0.010	A	α	0.426 ± 0.021	A	α	0.095 ± 0.006	A	α	8.4 ± 0.4	B	α	38.2 ± 0.5	A	β	50.5 ± 0.2	A	α	11.3 ± 0.2	A	α	0.8 ± 0.0	A	β
Botrytized red wines at 50%	Control	0.267 ± 0.005	B	β	0.226 ± 0.030	A	β	0.056 ± 0.001	A	β	5.5 ± 0.0	B	β	49.0 ± 0.8	A	α	40.8 ± 0.7	B	β	10.2 ± 0.2	A	β	1.2 ± 0.0	A	α
	Grape-seed	0.253 ± 0.010	B	β	0.222 ± 0.043	A	β	0.056 ± 0.008	B	β	5.3 ± 0.6	B	β	47.8 ± 3.6	A	α	41.6 ± 3.3	AB	β	10.6 ± 0.3	A	β	1.2 ± 0.2	A	α
	Grape-skin	0.249 ± 0.005	BC	β	0.222 ± 0.001	A	β	0.054 ± 0.000	B	β	5.2 ± 0.0	B	β	47.4 ± 0.6	A	α	42.3 ± 0.5	A	β	10.3 ± 0.1	A	β	1.1 ± 0.0	A	α
	Quebracho	0.254 ± 0.013	B	β	0.214 ± 0.007	A	β	0.053 ± 0.002	B	β	5.2 ± 0.3	B	β	49.0 ± 0.2	A	α	40.8 ± 0.0	AB	β	10.2 ± 0.0	A	β	1.2 ± 0.0	A	α
	Ellagitannin	0.238 ± 0.001	C	β	0.184 ± 0.023	B	β	0.048 ± 0.003	C	β	4.7 ± 0.3	C	β	50.6 ± 2.6	A	α	39.1 ± 2.7	AB	β	10.3 ± 0.1	A	β	1.3 ± 0.2	A	α
	Gallotannin	0.310 ± 0.005	A	α	0.251 ± 0.010	A	β	0.063 ± 0.002	A	β	6.2 ± 0.1	A	β	49.7 ± 1.3	A	α	40.2 ± 1.1	AB	β	10.0 ± 0.2	A	β	1.2 ± 0.1	A	α

All data are the mean ± SD of three replicates. Different capital letters indicate the existence of significant differences between samples for the same modality ($p < 0.05$). Different Greek letters indicate the existence of significant differences between modalities for the same sample ($p < 0.05$).

The results showed a clear effect of the botrytization rate, since significant differences were observed between the two modalities (20% and 50%) for all the color components evaluated. Indeed, if a contamination at 20% induce changes in the color of the wines corresponding to a browning, the contamination at 50% completely change the color with a hard decrease in % red and an increase in % yellow. Concerning the modality at 20% of botrytization, no significant differences were found between the control wine and the wines with the different oenological tannins addition. This was true for all the parameters determined. Similar results were obtained for the modality at 50% of botrytization rate, even if some significant differences were present. Nevertheless, it should be highlighted that in the case of the ellagitannin (at 50%), significant differences were obtained for the absorbances at 420, 520 and 620nm. According to this, the colorant intensity of the wine added by ellagitannin (at 50% of botrytization) was also significantly lower than the control wine of the same modality.

Additionally, to the evaluation of the color component (A_{420nm}; A_{520nm}; A_{620nm}), the concentration in total anthocyanins was determined to better visualize the variation in the concentration of the anthocyanins in presence of *B. cinerea* and the oenological tannins. The concentration of total anthocyanins in the different red wines after 3-months of bottling is presented in **Figure 80**.



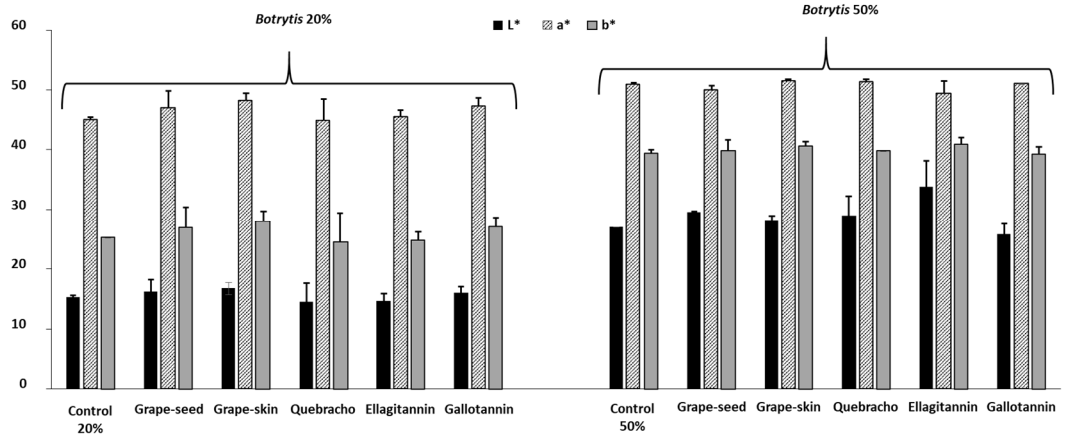
All the data are the mean ± SD of three replicates. The lower-case letters indicate the existence of significant differences between the samples at 20% and 50% of botrytization ($p < 0.05$).

Figure 80: Total anthocyanins concentration of 3-months bottling red wines at 20% and 50% of botrytization supplemented by oenological tannins

Concerning the total anthocyanins concentration, no significant differences were observed between the control and the wines supplemented by oenological tannins for both condition (20 and 50% of botrytization). This mean that at this high level of botrytization rate, the presence of oenological tannins is not enough to avoid the degradation of the anthocyanins. Nevertheless, it should be highlighted that the conditions chosen for this experimentation were extreme.

Additionally, the initial total anthocyanins concentration (wine containing 0% of botrytization) was 547.4 ± 1.1 mg/L eq. malvidin-3-*O*-glucoside. In the wines containing 20% and 50%, the total concentration in anthocyanins felled by 35% and 70% respectively and independently of the condition (addition or not of oenological tannins). Nevertheless, it should be highlighted and remember that the conditions choose for this experimentation were drastic (25 UL and 45 UL for the initial musts at 20% and 50% respectively). In fact, this significant degradation of the anthocyanins in wines made from botrytized grape has been also previously demonstrated at *B. cinerea* rates ranging from 5 to 20% [203]. Finally, regarding total anthocyanins concentration, a "botrytis" effect was observed meanwhile the "tannin" effect was not present or enough compared to the botrytization rate.

In order to verify the previous results obtained, CIELAB (L*, a* and b*) parameters were also determined to evaluate possible hyperchromic or bathochromic effects not highlighted before. **Figure 81** show the results obtained for these parameters of the wines contaminated at 20 and 50%.



All the data are the mean ± SD of three replicates.

Figure 81: CIELAB parameters (L, a*, b*) of 3-months wines bottling with or without additions of oenological tannins*

As for total anthocyanins, the results of the ANOVAs obtained for the CIELAB parameters showed an effect of the "botrytization level" meanwhile no "tannin" effect or "botrytization level/tannin" interaction effect was observed. These results mean that all the modalities botrytized at 20% and 50% were respectively similar between them from the point of view of the CIELAB parameters (L*, a* and b*).

Additionally, regarding the parameter a*, no differences were highlighted between wines botrytized at 20% and at 50%. These results are quite curious, since the total anthocyanins concentration present a high decrease between 20 and 50% of contamination. Since, the parameter a*, represent the displacement towards red component, it could be expected a decrease of this parameter. However, concerning the parameters L* and b*, a significant increase is observed between the modality at 20% and the modality at 50%. For both parameters in wines contaminated at 50%, values were higher and involved a displacement of the color of these wines toward yellower and lighter color.

Moreover, the color differences between 20% and 50% botrytized wines, ΔE_{ab}, were also calculated from the parameters L*, a* and b* in **Table 27**. In general, values greater than or equal to 3 indicate that the differences between the two wines considered are perceptible to the naked eye [284]. Thus, 20% botrytized and 50% botrytized wines appeared different with ΔE_{ab} between 14 and 26 meaning that it is really easy to distinguish a wine contaminated at 20% from a wine contaminated at 50%.

Table 27: Impact of enological tannins added in botrytized wines after 3-months of bottling on color visible to the human eye

ΔE_{ab}	Control 20%	Grape-seed 20%	Grape-skin 20%	Quebracho 20%	Ellagitannin 20%	Gallotannin 20%	Control 50%	Grape-seed 50%	Grape-skin 50%	Quebracho 50%	Ellagitannin 50%
Grape-seed 20%	2.7	-	-	-	-	-	-	-	-	-	-
Grape-skin 20%	4.4	1.7	-	-	-	-	-	-	-	-	-
Quebracho 20%	1.0	3.6	5.3	-	-	-	-	-	-	-	-
Ellagitannin 20%	0.8	2.9	4.6	0.7	-	-	-	-	-	-	-
Gallotannin 20%	2.9	0.4	1.5	3.8	3.1	-	-	-	-	-	-
Control 50%	19.3	16.9	15.5	20.3	19.8	16.9	-	-	-	-	-
Grape-seed 50%	16.9	18.9	17.6	22.1	21.6	18.9	2.7	-	-	-	-
Grape-skin 50%	15.5	18.9	17.4	22.2	21.7	18.8	1.9	2.1	-	-	-
Quebracho 50%	20.3	18.7	17.3	22.0	21.5	18.7	2.0	1.5	1.1	-	-
Ellagitannin 50%	19.8	22.6	21.4	25.7	25.2	22.7	7.1	4.4	6.0	5.4	-
Gallotannin 50%	16.9	16.2	14.7	19.5	19.0	16.1	1.2	3.9	2.8	3.2	8.3

$\Delta E_{ab} < 3$ | $3 < \Delta E_{ab} < 10$ | $\Delta E_{ab} > 10$

Additionally, although no tannin effect or Botrytis/tannin interaction were demonstrated, the calculation of ΔE_{ab} showed differences among wines botrytized at 50%. The wines with addition of ellagitannin were significantly different from the 50% control wine and even more so from the wines with addition of other commercial tannins. In fact, the wine with ellagitannin addition presented more oxidized wine color characteristic meaning that this type of tannin possesses less ability to protect wine color component against browning induce by *B. cinerea*.

Concerning the wines botrytized at 20%, only the wine with grape-skin tannin was noticeably different from the control (ΔE_{ab} = 4.4). Regarding color, wines with quebracho and ellagitannin additions were similar, but different from the wines with grape-skin, grape-seed and gallotannin additions. These three last wines presented closer color with a $\Delta E_{ab} < 2$.

These results indicate differential effects on the botrytized wines in function of the type of oenological tannins added. Grape-skin tannin, grape-seed tannin and gallotannin addition induce an improvement of the color of the wines botrytized at 20%. On the contrary, the addition of ellagitannin, causes a slight degradation of the color in the wines botrytized at 50%, wines which are already drastically degraded.

3.3. Influence of oenological tannins on sensorial properties of red botrytized wines

Triangular tests and ranking tests (1 for the most qualitative and 6 for the less qualitative) were carried out to highlight significant visual, olfactory and gustative differences.

These differences were looking on one hand between the healthy control (0%) and the botrytized controls (20% and 50%) and on another hand, between the botrytized controls and the corresponding wines supplemented by oenological tannins (**Annex 3**).

3.3.1. Impact of the botrytization level on the visual, aromatic and gustative quality of the red wines

In order to evaluate, the impact of the botrytization level on the visual, aromatic and gustative quality of the red wines, each botrytized control (20% and 50% of botrytization) was compared to the healthy control (0% of botrytization). The results are presented in **Table 28** and highlight the significant differences.

Visually, judges were all able to differentiate the botrytized wines from the healthy wine independently of the contamination level (excepted one wrong answer for 20% of botrytization). In both cases (20% and 50%), the panels describe the wines as more orange than the healthy wine. Additionally, they also described the wine contaminated at 20% as less purplish than the control wine. Concerning the wine with 50% of botrytization, it was described as browner than wine without any presence of *B. cinerea*.

Regarding aromatic and gustative quality, significant differences were also observed between both botrytized control and healthy control. Nevertheless, it should be highlighted that 4 judges between 18 (22.0%) do not perceive differences between the 0% and 20% of botrytization. On the contrary, only 1 judge on 18 (5.5%) do not perceive the difference between the 0% and 50% of botrytization. It appears, easier to differentiate the 0% from the 50% of botrytization, than the 0% from the 20% of botrytization. Concerning the descriptors associated, oxidized and jammed have been attributed to the wine botrytized at 20% meanwhile acescent, dust, old and oxidized have been attributed to the wine botrytized at 50%.

3.3.2. Impact of the tannin addition on the visual quality of the red wines botrytized at 20 and 50%

Each control at different level of botrytization (20% and 50%) was compared visually, with their respective wines added by oenological tannins.

Concerning wines botrytized at 50%, significant visual differences appears between the control and some corresponding wines supplemented by oenological tannins as shown in **Table 28**. Indeed, the triangular tests highlight significant differences ($\alpha = 0.001$) between the control (botrytized at 50%) and the wines added by grape-skin tannin (50% grape-skin) and ellagitannin (50% ellagitannin). Nevertheless, even if 50% grape-skin and 50% ellagitannin wines were both significantly different from the control (botrytized at 50%), the descriptors attributed to each one were not the same. In fact, the descriptions made by the panel were at the opposite.

50% botrytized wines with grape-skin was described as less orange, more red, limpid and intense than the control (botrytized at 50%), meanwhile, 50% botrytized wines with ellagitannin was described as more orange, less red, limpid and intense than the control.

Additionally, the ranking tests presented in **Table 29**, revealed that, an addition of gallotannin in 50% botrytized would have to tend to slightly intensify the color. The addition of grape-seed tannin, quebracho tannin and ellagitannin in 50% botrytized must produce the opposite effect. In the case, of addition of grape-skin tannin, no significant effect compared to the control will be highlighted. The obtained results, of the ranking test for the oak tannin, agreed with the descriptors cited by the panel in the triangular tests.

Regarding wines botrytized at 20%, triangular tests (**Table 28**) highlighted the same significant differences than for the wines botrytized at 50%. Indeed, significant differences ($\alpha = 0.001$) were obtained between the control (20%) and the wines added by grape-skin tannin (20% grape-skin) and ellagitannin (20% ellagitannin).

The addition of grape-skin tannin induces a darker color of the wine compared to the control. This result was in agreement with the result obtained in the triangular test for higher level of botrytization (50%) and also with the results obtained for the calculation of the ΔE_{ab} (**Table 27**). The corresponding ranking test, on the overall visual quality also showed, that the wine botrytized at 20% supplemented by grape-skin tannin have been ranked in first best position by the judges.

Moreover, the wine at 20% of botrytization added with ellagitannin, seems to induce a positive impact on the color, since the panel described it as more intense, purple and less orange than the control. Nevertheless, this difference in color noted by the judges, was not perceptible in the calculation of the total color difference, with a $\Delta E = 0.8$. Furthermore, this result was also not confirmed by the ranking test, since the wine at 20% of botrytization added by ellagitannin was not significantly different from the control wine (botrytized at 20%).

Table 28: Triangle tests from 0%, 20% and 50 % botrytized 3-month wines with or without tannins

Triangle test (A vs B)		Number of judges	Number of correct answers		Comments from correct answers: B compared to A	
A	**B**		**Visual test**	**Aromatic/gustative test**	**Visual aspect**	**Olfactory/gustative description**
0%	**20%**	18	17***	14***	+ orange, - purple, + evolve	Oxidized, jammy fruits
0%	**50%**	18	18***	17***	Orange and brown	Ethyl acetate, old, undergrowth, dust, oxidized
20%	**20% grape-seed**	18	6	7	+ purple	+ ample
20%	**20% grape-skin**	18	10*	13***	+ dark	sour
20%	**20% quebracho**	18	6	6		
20%	**20% ellagitannin**	18	14***	8	+ intense, - orange, + purple	+ vegetal
20%	**20% gallotannin**	18	4	9		Mushroom, earthy
50%	**50% grape-seed**	18	9	12**	- intense, - dark	+ acidic, + astringent, + fruity, pepper
50%	**50% grape-skin**	22	18***	12*	- orange, + red, + intense, + limpid	Dried prune, + acidic, + astringent, - bitter, - mushroom
50%	**50% quebracho**	22	5	12*	- red, - intense	
50%	**50% ellagitannin**	18	17***	14***	- red, - intense, + evolve, + orange, - limpid	Isoamyl acetate, candy, nougat, rose
50%	**50% gallotannin**	22	11	9	+ intense, + dark, - yellow	+ vegetal, + astringent, dried prune

All the data are the mean ± SD of three replicates. The asterisk indicates the existence of significant differences between sample A and sample B (* α= 0.05; ** α= 0.01; *** α= 0.001).

Table 29: Ranking of 20% and 50 % botrytized 3-month wines with or without tannins regarding visual, aromatic and gustative quality

Ranking test	Number of judges	Control	Grape-seed	Grape-skin	Quebracho	Ellagitannin	Gallotannin	F[a]	Significance
					Botrytization rate 50 %				
Visual quality	**20**	**49**	**93**	**58**	**66**	**113**	**38**	**51.2**	**0.1%[b]**
Differences between wines		a, b	c	a, b	b	c	a		5%
Aromatic quality	**20**	**72**	**59**	**76**	**84**	**50**	**71**	**-5.2**	**not**
Differences between wines		a	a	a	a	a	a		
Gustative quality	**20**	**77**	**55**	**52**	**72**	**70**	**88**	**1.2**	**not**
Differences between wines		a	a	a	a	a	a		
					Botrytization rate 20 %				
Visual quality	**20**	**93**	**58**	**40**	**50**	**77**	**60**	**29.0**	**0.1%**
Differences between wines		c	a, b	a	a	b, c	a, b		**5%**
Aromatic quality	**20**	**69**	**63**	**56**	**73**	**50**	**67**	**5.9**	**not**
Differences between wines		a	a	a	a	a	a		
Gustative quality	**20**	**54**	**56**	**50**	**66**	**49**	**82**	**13.3**	**5%**
Differences between wines		a	a	a	a, b	a	b		**5%**

All the data are the mean ± SD of three replicates. [a]: F was calculated as described in ISO 8587:2007 for Friedman test. $F = (12 \times \sum pi = 1\ Ri^2) / (n \times p \times (p + 1)) - 3 \times n \times (p + 1)$ → n represents the number of tasters, p represents the number of modalities and Ri represents the sum of the ranks for the modality. [b]: The test was significant at 0.1% when F > 20.52.

3.3.3. Impact of the tannin addition on the aromatic and gustative quality of the red wines botrytized at 20% and 50%

In addition, to the evaluation of the visual quality, it was also asked to the panel to evaluate the aromatic and gustative quality of the different botrytized wines supplemented or not by oenological tannins.

For the 20% botrytized wines, triangular tests showed that only the 20% grape-skin wine was perceived significantly different from the corresponding control. Although this difference was not significant for the ranking test, in general 20% grape-skin was better punctuated than the control.

According to the triangular tests (**Table 28**), all the wines botrytized at 50% supplemented by oenological tannins, were significantly different from the control, excepted for the wine added by gallotannin. Nonetheless, when the panel had to classify the wines botrytized at 50%, no significant differences were highlighted between them and with the control (**Table 29**). This means that from the point of view of the overall aromatic and gustative qualities (ranking tests), judges were not able to define one wine greater or worsted than another one. This might probably due to the fact, that at this level of botrytization, wines are so deteriorated that it is impossible to protect their aromatic and gustative quality. Indeed, when the 50% control was compared to the 0% control, almost all the judges were able to discriminate them, describing the 50% as old, undergrowth dust or oxidized.

Finally, the botrytized wines supplemented by gallotannin (20% gallotannin and 50% gallotannin) were always ranked in the last position regarding the global aromatic and gustative quality. The wines were described as earthy with mushroom odor for the 20% and more vegetal and astringent with dried prune aromas for the 50%.

3.4. Comprehension of action mechanisms of oenological tannins on laccases produced by *B. cinerea*

3.4.1. Enzymatic kinetics characterization: determination of the residual laccase activity, V_{max} and K_M parameters

The impact of oenological tannins on botrytized white, “rosé” and red wines has been evaluated regarding residual laccase activity, color and organoleptic (gustative and olfactory) parameters. Additionally, to this, it was also important to observe more specifically the enzymatic kinetics of the laccases produced by *B. cinerea* in order to better understand their mechanisms of action. This kinetics were also conducted in presence of oenological tannins, ascorbic acid and sulfur dioxide in the medium to see their inhibition type against laccases. Ascorbic acid and sulfur dioxide were used as a comparison with oenological tannins since they are the two most antioxidasic compounds used in winemaking.

3.4.1.1. Determination of the inhibition type

As expected, laccases showed a classical Michaëlis-Menten kinetics since the reaction rate shows a first-order kinetics behavior when the substrate concentration was low and became a zero-order kinetics when the substrate concentration was saturated. Supplementation with increasing concentration of oenological tannins, sulfur dioxide and ascorbic acid in botrytized must induced a diminution of the maximal reaction rate (V_{max}) (**Figure 82**).

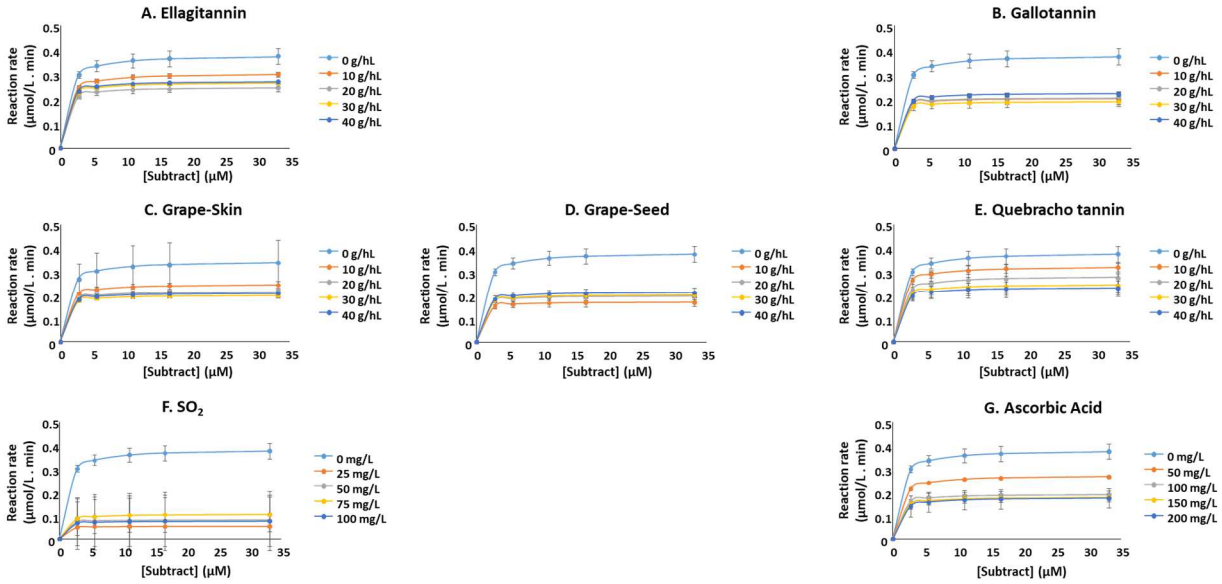


Figure 82: Michaëlis-Menten plots to visualize the kinetics of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO_2 (F) or ascorbic acid (G)

This confirm that all these additives exert an inhibitory effect on laccase activity, even if this effect does not seem to be similar for all these products. Indeed, three different types of inhibitory effect could take place, which are competitive inhibition, non-competitive inhibition and uncompetitive inhibition (**Figure 83**).

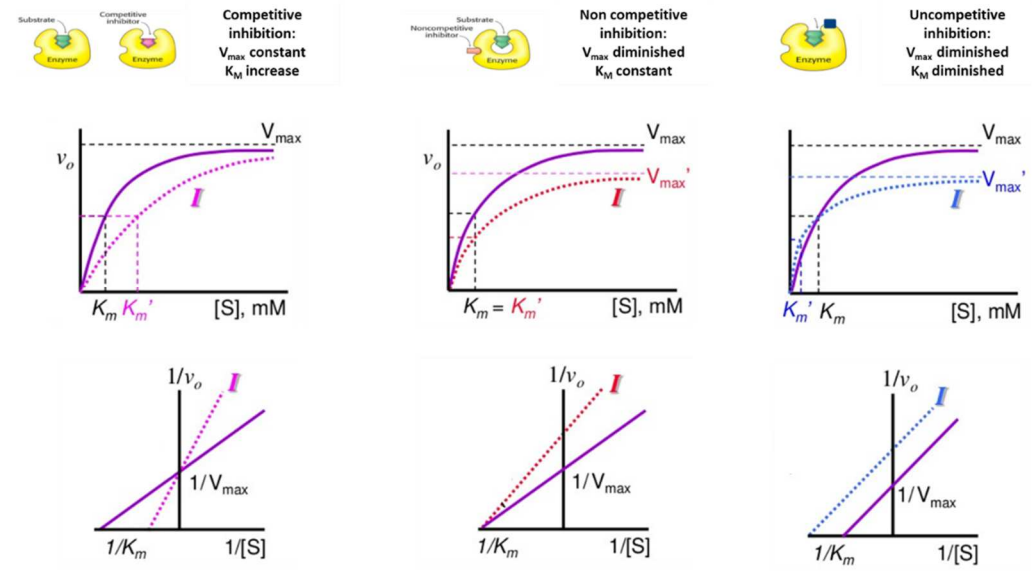


Figure 83: Example of the three different types of inhibition (competitive, non-competitive and uncompetitive)

According to this, oenological tannins and sulfur dioxide seems to exert uncompetitive inhibition since the Lineweaver-Burk plots of laccases kinetics in the presence of these additives were parallel with that obtained without any addition (**Figure 84**). An uncompetitive inhibition takes place when an enzyme inhibitor (I) binds only to the complex "ES" formed by the enzyme (E) and the substrate (S). This phenomenon induced the reduction of the concentration of ES complex which can be explained by the fact that ES complex may be essentially converted into ESI complex ("enzyme-substrate-inhibitor"), thus considered as a separate complex altogether. This reduction in ES complex concentration is accompanied by a decrease of V_{max}, since it takes longer for the substrate or product to leave the active site. The reduction of the binding affinity (K_M) can also be linked back to the decrease in ES complex concentration.

In contrast, ascorbic acid seems to exert a non-competive inhibition since the x intercepts of the Lineweaver-Burk plots of laccase kinetics added by ascorbic acid or not (control) stay constant (**Figure 84**). In a non-competitive inhibition, the inhibitor binds to an enzyme at its allosteric site; therefore, the K_M of the substrate with the enzyme remains the same. On the other hand, the V_{max} decrease relatively to an uninhibited enzyme.

In this way, Michaëlis-Menten (**Figure 82**) and Lineweaver-Burk plots (**Figure 84**) allowed to qualify oenological tannins and sulfur dioxide as uncompetitive inhibitors, whereas ascorbic acid can be rather qualified as non-competitive inhibitor. To confirm this graphical statement, kinetics parameters were calculated as follows.

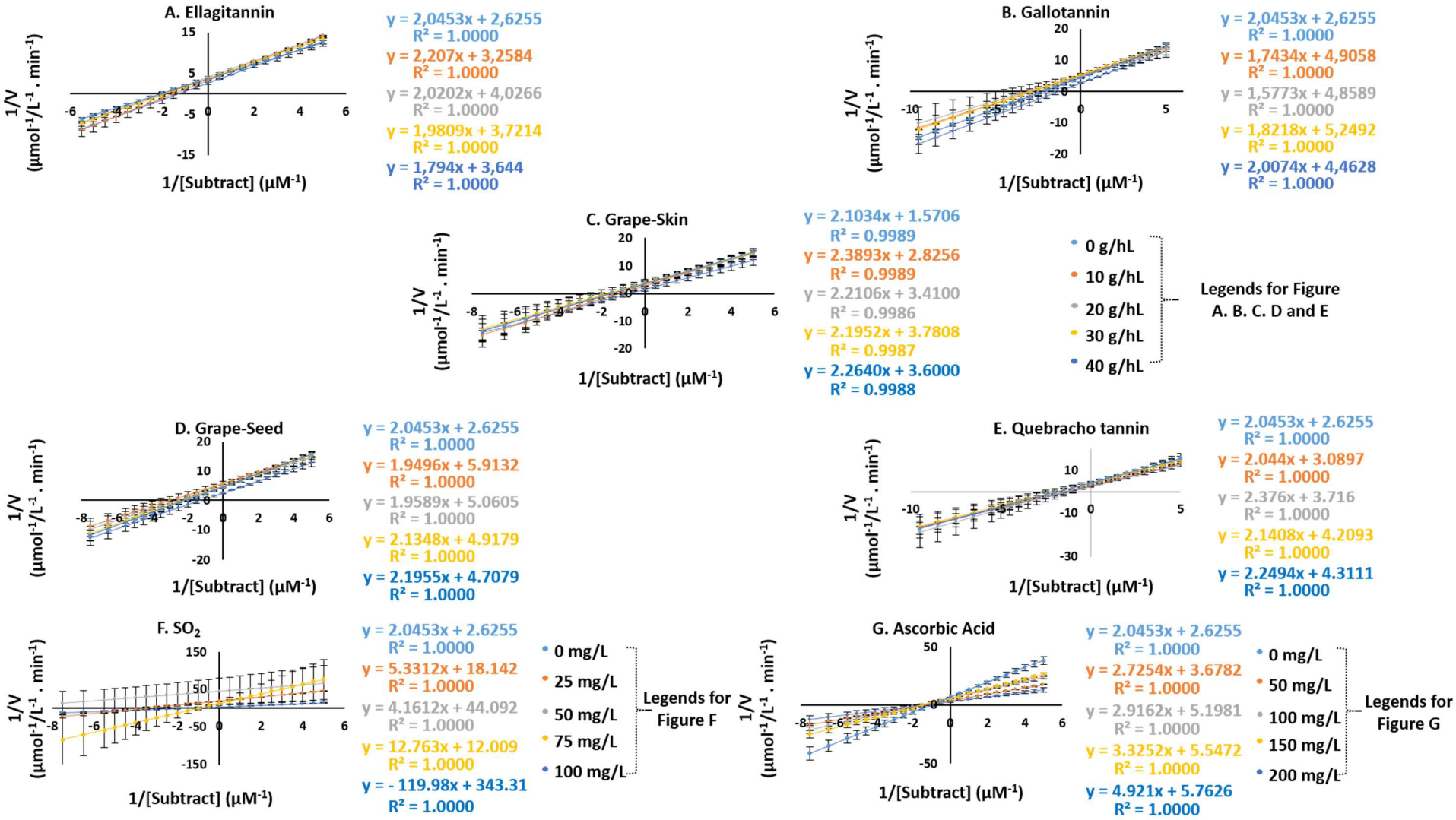


Figure 84: Lineweaver-Burk plot for determining the kinetic constants of laccase and the inhibitory effect of oenological tannins (A, B, C, D, E), SO_2 (F) or ascorbic acid (G)

3.4.1.2. Determination of kinetics parameters and residual laccase activity

The values of V_{max} and K_M were calculated by the extrapolation of the curve obtained from the Lineweaver-Burk plot. The enzyme obtained from the *B. cinerea* strain 213 in this experimentation, had a V_{max} of 0.364 ± 0.026 µmol/L.min and a K_M of 0.697 ± 0.133 µmol/L.

Table 30: Kinetics parameters (V_{max} and K_M) and residual laccase activity of botrytized white must added by oenological tannins or SO_2 or ascorbic acid at different concentration

Samples	Doses	V_{max} (µmol/L.min)			K_M (µmol/L)			Residual activity (%)		
Ellagitannin (g/hL)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	10	0.307 ± 0.010	**B**	**α**	0.678 ± 0.024	**A**	**α**	78.4 ± 6.4	**B**	**α**
	20	0.257 ± 0.011	**C**	**β**	0.534 ± 0.042	**AB**	**αβ**	80.4 ± 3.2	**B**	**α**
	30	0.269 ± 0.006	**C**	**α**	0.525 ± 0.011	**AB**	**β**	76.0 ± 3.9	**B**	**α**
	40	0.274 ± 0.002	**C**	**α**	0.506 ± 0.019	**B**	**β**	81.8 ± 3.2	**B**	**α**
Gallotannin (g/hL)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	10	0.212 ± 0.010	**B**	**γ**	0.396 ± 0.053	**B**	**β**	41.5 ± 3.9	**B**	**γ**
	20	0.221 ± 0.019	**B**	**βγ**	0.407 ± 0.100	**B**	**αβ**	39.9 ± 6.4	**B**	**γ**
	30	0.202 ± 0.015	**B**	**β**	0.364 ± 0.020	**B**	**β**	45.6 ± 3.9	**B**	**γ**
	40	0.220 ± 0.006	**B**	**β**	0.417 ± 0.048	**B**	**β**	44.6 ± 7.8	**B**	**γ**
Quebracho (g/hL)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	10	0.334 ± 0.013	**A**	**α**	0.663 ± 0.037	**A**	**α**	73.6 ± 3.2	**B**	**αβ**
	20	0.318 ± 0.043	**AB**	**α**	0.571 ± 0.032	**B**	**αβ**	80.4 ± 8.5	**B**	**α**
	30	0.245 ± 0.051	**BC**	**αβ**	0.411 ± 0.088	**B**	**β**	73.6 ± 17.8	**B**	**αβ**
	40	0.217 ± 0.010	**C**	**β**	0.462 ± 0.015	**B**	**β**	67.9 ± 11.7	**B**	**αβ**
Grape-skin (g/hL)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	10	0.248 ± 0.015	**B**	**β**	0.620 ± 0.061	**AB**	**α**	65.6 ± 3.2	**B**	**β**
	20	0.215 ± 0.015	**BC**	**βγ**	0.386 ± 0.070	**C**	**αβ**	61.5 ± 3.2	**BC**	**β**
	30	0.200 ± 0.010	**C**	**β**	0.386 ± 0.052	**C**	**β**	53.7 ± 3.9	**BC**	**βγ**
	40	0.201 ± 0.007	**C**	**β**	0.426 ± 0.036	**BC**	**β**	50.7 ± 5.5	**C**	**βγ**
Grape-seed (g/hL)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	10	0.180 ± 0.013	**B**	**δ**	0.295 ± 0.057	**B**	**β**	13.5 ± 3.2	**B**	**δε**
	20	0.209 ± 0.001	**B**	**βγ**	0.343 ± 0.009	**B**	**αβ**	16.2 ± 0.0	**B**	**δ**
	30	0.210 ± 0.005	**B**	**β**	0.481 ± 0.025	**B**	**β**	15.6 ± 3.2	**B**	**δ**
	40	0.214 ± 0.019	**B**	**β**	0.470 ± 0.072	**B**	**β**	12.8 ± 6.4	**B**	**δ**
SO_2 (mg/L)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ±6.4**	**A**	**α**
	25	0.055 ± 0.003	**B**	**ε**	0.274 ± 0.031	**B**	**β**	4.1 ± 0.0	**B**	**δε**
	50	0.016 ± 0.001	**C**	**δ**	0.237 ± 0.141	**B**	**β**	2.0 ± 0.0	**B**	**ε**
	75	0.065 ± 0.000	**B**	**γ**	0.349 ± 0.000	**B**	**β**	1.4 ± 1.0	**B**	**δ**
	100	0.003 ± 0.002	**C**	**δ**	0.451 ± 0.000	**B**	**β**	0.9 ± 0.4	**B**	**δ**
Ascorbic acid (mg/L)	**0**	**0.364 ± 0.026**	**A**	**α**	**0.697 ± 0.133**	**A**	**α**	**100.0 ± 6.4**	**A**	**α**
	50	0.272 ± 0.003	**B**	**β**	0.741 ± 0.030	**A**	**α**	21.3 ± 3.9	**B**	**δ**
	100	0.203 ± 0.007	**C**	**γ**	0.667 ± 0.121	**A**	**α**	12.8 ± 0.0	**B**	**δε**
	150	0.193 ± 0.001	**C**	**β**	0.724 ± 0.153	**A**	**α**	19.6 ± 3.2	**B**	**δ**
	200	0.156 ± 0.004	**D**	**γ**	0.996 ± 0.137	**A**	**α**	11.5 ± 3.2	**B**	**δ**

All data are the mean of triplicate. Mean ± Standard deviation. Capital letters indicate significant differences between the different doses for the same sample ($p < 0.05$). Greek letters indicate significant differences between samples for the same doses ($p < 0.05$).

According to the previous statement, oenological tannins and sulfur dioxide presented, a significant decrease in their V_{max} and K_M compared to the control (without any inhibitor) (**Table 30**).

Likewise, except for quebracho tannin, the significant differences of V_{max} were already shown at the lowest dose of inhibitor compared to the control (**Table 30**). Nonetheless, at the highest dose, sulfur dioxide presented the greatest ability to reduce the V_{max} (0.003 ± 0.002 µmol/L.min), while ellagitannin (0.274 ± 243 0.002 µmol/L.min) had the lowest ability. The other oenological tannins presented similar effects (0.201 ± 244 0.007 µmol/L.min to 0.220 ± 0.006 µmol/L.min), since no significant differences were observed between them (**Table 30**). All the inhibitors tested, i.e. oenological tannins and sulfur dioxide, at the highest dose showed the same ability to decrease K_M without any significant difference. The initial K_M was diminished from 0.697 ± 0.133 µmol/L (control) at 0.417 ± 0.048 µmol/L (gallotannin) to 0.506 ± 0.019 µmol/L (ellagitannin). Nevertheless, for both ellagitannin and quebracho tannin, the lowest dose was not enough to decrease significantly the enzyme's K_M compared to the control. To obtain this significant decrease of K_M in comparison with the control, 40 g/hL and 20 g/hL of ellagitannin and quebracho were respectively necessary. However, 10 mg/L of sulfur dioxide and 25 g/hL of the others oenological tannins were enough to reach the same results. Higher is the decrease of the K_M constant and higher is the increase of the affinity between the enzyme and the subtract.

In the case of ascorbic acid, as it was a non-competitive inhibitor, the enzyme's V_{max} decreased significantly with addition of the lowest dose (50 mg/L) compared to the control (0 mg/L). Moreover, at the highest dose (200 mg/L), V_{max} reached values three-fold time lower than control, ranking from 0.364 ± 0.026 µmol/L.min to 0.156 ± 0.004 µmol/L.min. However, K_M values remained constant, since no significant differences were observed with control, even if values were ranked between 0.667 ± 0.121 µmol/L (100 mg/L) and 0.996 ± 0.137 µmol/L (200 mg/L).

Based only on the determination of V_{max} and K_M parameters, it was not possible to compare the effect of enological tannins with ascorbic acid, since they are different type of inhibitors. It must also be considered that laccase enzyme, in our reaction conditions, had two subtracts, syringaldazine and oxygen. Oenological tannins, sulfur dioxide and ascorbic acid can react directly with oxygen, decreasing therefore its concentration in the medium [164,289]. Consequently, this reduced the V_{max} of the reaction. However, considering the oxygen consumption rate of oenological tannins and sulfur dioxide, as reported previously [164], the oxygen consumption by these compounds can be considered as negligible. Indeed, the oxygen consumption of oenological tannins and sulfur dioxide at normal concentrations (400 mg/L for oenological tannins and 50 mg/L for sulfur dioxide) represented less than 0.2% compared with the consumption capacity of laccases in our botrytized grape juice, for 30 UL. In contrast, ascorbic acid consumes oxygen much faster and can really compete with laccase [164]. Specifically, the oxygen consumption rate of 100 mg/L of ascorbic acid is around the 50% of the oxygen consumption rate of the botrytized must use with a laccase activity of around 30 UL. Thus, the inhibitory effect of ascorbic acid on laccase activity seems to result from a competition with laccase for the oxygen present in the medium.

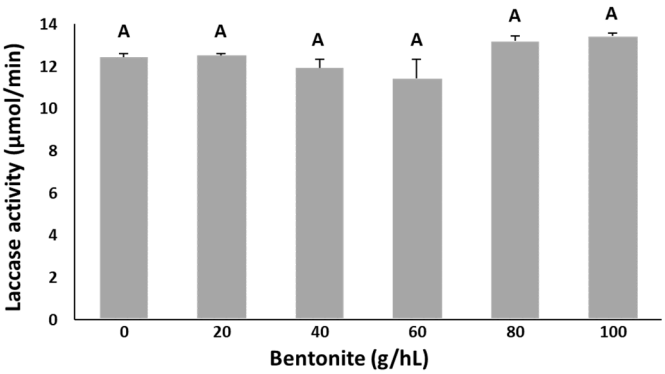
The residual laccase activity was additionally determined, for each sample, to make possible the comparison between the different oenological tannins.

Initial activity of the control was 27.3 ± 0.6 UL and was considered as the 100 ± 6.40% of residual activity (**Table 30**). All the samples tested, including oenological tannin, sulfur dioxide and ascorbic acid, decrease significantly the residual activity at the lowest dose tested. Sulfur dioxide clearly appeared as the most efficient to reduce laccase activity, since with 100 mg/L, no residual laccase activity was detected (0.9 ± 0.4%). Ascorbic acid and grape-seed tannin appeared also as great candidates to reduce laccase activity, since at the highest dose (200 mg/L and 40 g/hL respectively), only 11.5 ± 3.2% and 12.8 ± 6.4% of the laccase activity remained present, respectively (**Table 30**). Similarly, gallotannin and grape-skin tannin at the highest dose presented an equivalent effect, since they reduced by half the laccase activity: 44.6 ± 7.8% and 50.7 ± 5.5%, respectively (**Table 30**). Nevertheless, gallotannin reached this value (44.6 ± 7.8%) at the lowest dose used (10 g/hL), while grape-skin tannin only reduced by 35% the laccase activity at the lowest dose. Concerning ellagitannin and quebracho tannin, their ability to reduce laccase activity was clearly lower than the other oenological tannins. At the highest dose (40 g/hL), ellagitannin and quebracho tannin were able to reduce the activity only between 20 and 30%, respectively **(Table 30**).

3.4.2. Influence of bentonite treatment

Bentonite is an inorganic clay finning agent, negatively charged, which is responsible for the binding of proteins who have a net positive charge at wine pH, resulting in their removal from the wine [291]. Bentonite is universally used in the wine industry to remove wine proteins by electrostatic adsorption, because it is efficient, with low economic cost, and because its use needs a simple batch process that does not require any specialized equipment or knowledge [292]. Laccases produced by *B. cinerea* are proteins which have an isoelectric point lower than usual wine pH and, consequently, they are charged negatively at wine pH [293]. Theoretically they cannot be removed with bentonite.

The influence of a bentonite treatment on botrytized white must is showed in **Figure 85**.



All data are the mean of triplicate. Mean ± Standard deviation.

Figure 85: Influence of bentonite treatment at different doses on laccase activity of botrytized white must

No significant differences were observed regarding laccase activity, by comparing the botrytized white must not treated and the botrytized must treated at different doses of bentonite. The initial laccase activity was around 12 µmol/L.min and remained stable when the botrytized must was previously treated with bentonite. According to these results, it was decided to use bentonite treatment for the enzyme electrophoresis when it was necessary to remove other proteins without affecting laccase activity.

3.4.3. Electrophoresis SDS-PAGE of laccase produced by *B. cinerea* supplemented by oenological tannins

Once having determined which type of inhibitor were the oenological tannins, it was necessary to determine if they can also precipitate the enzyme produced by *B. cinerea*.

3.4.3.1. Extracellular laccase production

The strain 213 produced extracellular enzymes when cultured under the conditions described in section 2.3.3.1. The amount of the dry fungal biomass was 0.0429 g/mL and the content of protein found in the culture medium was 0.15 mg/mL. The yield of protein per gram of dry fungal biomass was 3.49 mg/g for the strain 213. These results are in accordance with previous published ones in similar conditions for three other different *B. cinerea* strains, yields values ranging between 2 and 5 mg/g [285].

3.4.3.2. Enzyme electrophoresis

The SDS-PAGE of laccases sample showed the presence of only one band corresponding to the laccase proteins (**Figure 86** – well 1). The strain produced laccase enzymes with a molecular weight of 97 kDa, which is in accordance with previous results published [285,294]. Nevertheless, values ranging from 36 kDa to 97 kDa have been also reported, suggesting a great variability between the enzymes with laccase activity produced by different strains of the pathogen. When bentonite (200 g/hL) was added to laccase sample (**Figure 86** – well 2), a part of proteins was removed, since the band at 97 kDa appeared weaker suggesting that bentonite has eliminated other proteins of similar molecular weight but not laccase since its enzymatic activity remains stable. The SDS-PAGE of the white wine (**Figure 86** – well 3) showed the presence of two bands with a molecular weight of 57 kDa and 20 kDa, as previously reported [295]. When bentonite was added to the wine (**Figure 86** – well 4), these two proteins bands were not detectable. It has been previously shown that more than 50% of the whole protein content was removed with only 50 g/hL of bentonite, but around 15% of this protein content remained non-adsorbed even for a bentonite concentration as high as 150 g/hL [296]. This means that the dose of 100 g/hL could be the most appropriate in order to remove all the protein content, nevertheless such a high addition of bentonite can decrease the quality of the wines [296]. After bentonite treatment of laccase samples and white wine, 70% and 100% of the proteins were removed respectively (**Table 31**).

Therefore, at this dose, bentonite was able to remove all the proteins from wine. However, bentonite was not able to remove all proteins of laccase sample since 30% of them remained after the treatment. These results are not in accordance with other one in which laccases was not removed by bentonite fining [297]. Nevertheless, as it has been commented above, laccase activity is not impacted by bentonite treatment (**Figure 85**), meaning that, probably, bentonite has removed some other proteins of similar molecular weight but not laccases. Additionally, the SDS-PAGE of white wine supplemented by laccases sample (**Figure 86** – well 5) presented three band at 97 kDa, 57 kDa and 20 kDa corresponding to the bands obtained for laccases sample and white wine proteins in wells 1 and 3, respectively. When bentonite was added to the mixture made of the white wine and laccases (**Figure 86** – well 6), only the band corresponding to laccases remained visible. This result confirms once again the ability of bentonite to precipitate white wines proteins, but not laccases.

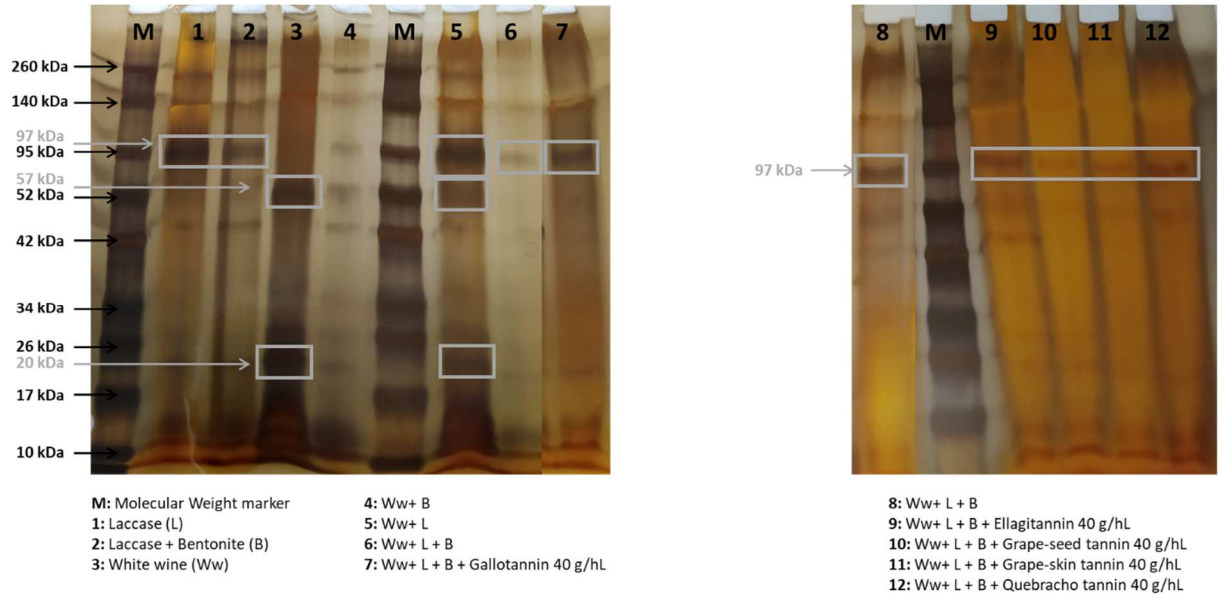


Figure 86: Electrophoresis gels of laccases produced by Botrytis cinerea and supplemented by oenological tannins (40 g/hL) with a bentonite treatment (200 g/hL)

Regarding the ability of oenological tannins to precipitate laccases proteins in white wine treated with bentonite, grape tannins (grape-seed and grape-skin) followed by gallotannin were the most efficient ones (**Figure 86** – well 7, 10 and 11). Grape-seed and grape-skin led to precipitate 77.8% and 74.5% of laccases proteins, respectively (**Table 31**). Gallotannin allowed precipitating almost 50% of laccase proteins (**Table 31**). In contrast, ellagitannin and quebracho tannin did not present any ability to precipitate laccases proteins (**Figure 86** – well 9 and 12), since 100% of laccases proteins remained present (**Table 31**). These results are in accordance with the previous ones obtained in **Table 30**, in which grape tannins and gallotannin were the most efficient products tested to reduce laccase activity. Nevertheless, gallotannin showed a lower ability to precipitate laccase proteins, but presented similar kinetics parameters and ability to reduce laccase activity than grape-skin tannin.

This could be explained by the fact that, on one hand, oenological tannins can precipitate laccases proteins, thus reducing laccase activity, or on the other hand, by inhibition of laccase activity without protein precipitation. Indeed, the binding of an inhibitor and its effect on the enzymatic activity are two distinct and quite different mechanisms. According to this hypothesis, grape tannins and gallotannin presented the ability to inhibit laccase activity and to precipitate (bounds) laccases proteins. Specifically, gallotannin was the most efficient to inhibit laccases produced by *B. cinerea*, whereas grape-skin tannin was most efficient regarding laccases proteins precipitation. Additionally, grape-seed tannin was the most efficient of the oenological tannins to inhibit laccase activity and precipitate laccases proteins. Quebracho tannin, possess an ability to inhibit laccase activity, but its ability to precipitate laccases proteins was almost non-existent. Ellagitannin presented the lowest ability to inhibit and to precipitate laccases from *B. cinerea*. Nevertheless, even though ellagitannin presented low ability to inhibit laccases, it can reduce the activity by 20%.

Table 31: Molecular weight and intensity of the bands of laccases produced by Botrytis cinerea; Influence of treatment with oenological tannins (40 g/hL) and with bentonite (200 g/hL)

Well		1	2	3	4	6/8	7	9	10	11	12
Band 1 (laccase)	**MW (kDa)**	97	97			97	97	97	97	97	97
	Quantity (%)	100.0	31.6			100.0	48.3	94.4	22.8	25.5	105.3
Band 2 (wine)	**MW (kDa)**			57	57	57					
	Quantity (%)			100.0	nd	nd					
Band 3 (wine)	**MW (kDa)**			20	20	20					
	Quantity (%)			100.0	nd	nd					

1: Laccase (L); 2: Laccase + Bentonite (B); 3: White wine (Ww); 4: Ww+ B; 6/8: Ww+ L + B 7: Ww+ L + B + Gallotannin 40 g/hL; 9: Ww+ L + B + Ellagitannin 40 g/hL; 10: Ww+ L + B + Grape-seed tannin 40 g/hL; 11: Ww+ L + B + Grape-skin tannin 40 g/hL; 12: Ww+ L + B + Quebracho tannin 40 g/hL; nd : not detectable; MW : Molecular Weight

4. Conclusion

In terms of winemaking process, the duration of contact needed to reach the maximal inhibition of laccases by the oenological tannins can be considered as very short (around 4 minutes).

The supplementation with all oenological tannins really mitigates the negative effect due to the presence of laccases by affecting the color of white and “rosé” wines. This effect seems to be more effective in the case of the protection of red color in “rosé” wines. Indeed, the presence of oenological tannins allows protecting anthocyanins from oxidation.

Concerning red winemaking, high botrytization rates (20 and 50%) were chosen in order to highlight sliced effects of the addition of these tannins. Results of this study initially revealed that at botrytization rates of 50%, laccase activity was so important that only the effect of botrytization was measurable. Indeed, this high level of contamination not allow demonstrating a tannin effect.

Additionally, all the wines produced presented a residual laccase activity greater than 5 UL/mL despite the sulfur dioxide addition (20 mg/L), making them unstable and assuredly candidates for oxidasic breakage. Regarding the addition of tannins on 20% botrytized musts, beneficial effects were obtained even if, this level of contamination remains high. Indeed, grapes tannins (grape-seed and grape-skin) presented an inhibitory effect on laccase activity, since the residual laccase activity was lower with their presence in the must. Concerning the organoleptic properties, wines coming from botrytized grapes (20% and 50%) appear visually different from the control coming from healthy grapes (0%). Indeed, they presented hues (h_{ab}) more orange and a less intense color, characteristic of the action of laccases. Differences of effect between tannins on the wines obtained were highlighted, as among others, the less important degradation of the color by the addition of grape-skin tannin. From the point of view of the olfactory/gustative quality, it was difficult to highlight significant differences.

Once the effects of the oenological tannins on laccases in winemaking studied, their mechanisms of action were also investigated. As expected, bentonite did not permit to reduce laccase activity in white must, even if it was responsible for precipitation of other proteins similar in molecular weight to laccase from *B. cinerea* strain 213. Oenological tannins were characterized by different abilities regarding prevention of *B. cinerea* damage, since they showed different ability to precipitate laccases produced and/or reduce their activity. They were all uncompetitive inhibitors, but gallotannin, grape-skin and even more so grape-seed tannin presented the greatest ability to reduce V_{max}, K_M and residual laccase activity. Addition of grape-seed tannin was efficient to reduce laccase activity with a similar level effect than that of ascorbic acid and sulfur dioxide at traditional doses employed in oenology.

These results confirmed the ones obtained in winemaking (white, “rosé” and red), and demonstrated that oenological tannins and more specifically, grape tannins and gallotannin, are excellent processing aids to prevent *B. cinerea* damage in grape musts. Oenological tannins used on botrytized grapes should permit also to decrease sulfur dioxide use during winemaking.

Further studies are required to study the laccases inhibitory mechanisms of oenological tannins, but the actual findings justify why the OIV has included this functionality in the OIV International Oenological Codex.

General conclusion

The main objective of this thesis was to shed light on the oenological tannins, a new tool commonly used in winemaking, but presenting a high lack of knowledge concerning their composition, their possible properties and potential functionalities in oenology. Indeed, at the beginning of this research, the use of oenological tannins was only authorized by the OIV in order to facilitate the finning of wines and musts. Nevertheless, winemakers used oenological tannins for other purposes and functionalities. Until now, this was based on empirical observations with none official experiments, and the use of oenological tannins for other functionalities than finning was not authorized. In addition, due to the wide range of oenological tannins origin and diversity present in the market, it is quite difficult for the winemakers to select the more adapted tannin according to the desired effect. Furthermore, the current OIV method to measure the tannin richness of oenological tannins, was not adapted in function of their type and origin, since it measures all polyphenols and not tannins specifically.

Therefore, the purpose of our PhD was to:

- Develop firstly, a method to determine quickly the tannic richness of oenological tannins, in order to help the winemakers to choose them.
- It was secondly, essential to develop a method allowing oenological tannins characterization more specifically, according to their different composition.
- Thirdly, a major goal was to explore and clarified new properties/functionalities attributed to the oenological tannins in accordance to their botanical origin.

As results, among the various methods used to characterize the thirty-six oenological tannins, precipitation with methyl-cellulose can be proposed as the best method to determine quickly the richness in tannins of the different extracts. Moreover, it has been demonstrated that no significant differences were observed between tannins of different botanical origins but that presents similar structure. In this way, a classification of the different commercial tannins, in four groups can be proposed including:

- ellagitannins (oak and chestnut)
- gallotannins (tara and nut gall)
- procyanidins/prodelphinidins (grape, grape-seed and grape-skin)
- profisetinidins/prodelphinidins (quebracho and acacia)

Then, once the characterization and classification of the different commercial tannins has been achieved, three main different properties/functionalities have been studied and elucidated.

Concerning the ability of oenological tannins to protect the musts and wines against oxidation, two different ways have been followed. First, the antioxidant capacity of the oenological tannins was studied.

Our results show that the combination of different antioxidant assays is necessary, in order to be closer as possible of the reality of the different oxidation mechanism/reaction that can happen in must and wine. According to the results, condensed tannins can be separate from hydrolysables tannins using principal component analysis (PC1 + PC2 = 77.80%) based on their antioxidant capacities with different validated methods. In general terms, hydrolysable tannins have a higher antioxidant capacity (1.5 fold-time higher) than condensed tannins according to all the analytical methods except for ORAC.

Secondly, we proposed a new method, to directly measured the oxygen consumed (OCR) by a luminescence non-invasive approach. Our results indicate that the new method (OCR) allowed to separate among the hydrolysable tannins, the gallotannins from the ellagitannins, the last one being the most efficient. This study therefore concludes that ellagitannins (0.84 mg of O_2/L.day.$g_{tannins}$) are the most effective to protect wine against chemical oxidation of the various oenological tannins, followed in decreasing order by condensed tannins (0.28 to 0.38 mg of O_2/L.day.$g_{tannins}$) and finally gallotannins (0.10 mg of O_2/L.day.$g_{tannins}$).

Regarding color stabilization, the thirty-six oenological tannins used permit to highlight the importance of the botanical origin since they do not present the same abilities. Indeed, gallotannins appears as the most efficient ones to stabilize the color of red wines. We determine then, the influence of the intrinsically incidence of the pH level and ethanol content on color stabilization. Results show that with increasing concentrations of ethanol and highest pH level, the efficiency of the oenological tannins as copigment decrease. Nevertheless, independently of the conditions, gallotannins remains as the most efficient ones. Finally, we follow the evolution of the color stabilization in function of time, and evidence changes in the comportment of the different oenological tannins. Some interesting modifications were noted, since ellagitannins present great ability to decrease their copigmentation index during the time (CI at day 1= 15.2% and CI at day 21 = 0%). These results, allowed concluding that gallotannins are the candidate who should be used in priority, since its effect remain constant during the time (CI at day 1= 35.4% and CI at day 21 = 27.4%) and with the highest efficiency whatever the oenological conditions (changes in pH and ethanol content).

For the antioxidasic capacity of the oenological tannins, our various and numerous experimentations conducted permit to achieved and evaluate the impact on residual laccase activity, color and organoleptic aspects. First of all, the syringaldazine test has been improved to measure quickly the enzymatic activity of the samples. Then, oenological tannins were supplemented on white, rosé and red winemaking. The supplementation with all enological tannins really mitigates the negative effect on color browning due to the presence of laccase and this effect was particularly efficient on rosé wines by protecting the anthocyanins from oxidation (diminution of 50% at least of the anthocyanins losses). For red wines, our results were impacted by the botrytization rate choose that can be qualified as high (20 and 50%).

From the point of view of the olfactory/gustative quality, it was difficult to highlight significant differences. Nevertheless, grapes tannins (grape-seed and grape-skin) presented an inhibitory effect on laccase activity, since the residual laccase activity were lower with their presence in the must (diminution of 60-70% of the laccase activity). Concerning the organoleptic properties, wines coming from botrytized grapes (20% and 50%) appear visually different from the control coming from healthy grapes (0%). Indeed, they presented hues (h_{ab}) more orange and a less intense color, characteristic of the action of laccases. Once the effects of the oenological tannins on laccases in winemaking were studied, their mechanisms of action were also investigated. Oenological tannins are all uncompetitive inhibitors, grape-seed tannin being the most efficient by reducing of 80% the laccase activity, followed by gallotannin and grape-skin tannin by reducing of 50% the laccase activity. Oenological tannins presented also different ability to precipitate laccases, grape tannins (seed and skin) being the most efficient one by precipitating 80% of the proteins, followed by gallotanins able to precipitate 50% of the laccases. Oenological tannins present different ability to precipitate and/or inhibit laccases, but grape tannin and gallotannin were the most efficient ones, being excellent processing aids to prevent some of the *B. cinerea* damages in grape musts.

To synthetize, it will be recommended to a winemaker to use ellagitannins regarding oxidation problems in musts and wines, meanwhile gallotannins will be recommended for color stabilization. In case of infection by *B. cinerea*, procyanidins/prodelphinidins tannins will be advised in priority even if gallotannins could be also used.

Finally, all the results obtained have led to the modification (functionalities addition) of the file 2.1.7 'Tannin addition' in part II, chapter 2 of the OIV International Code of Oenological Practices (**Annex 1**) and the file 3.2.6 'Tannin addition' in part II, chapter 3 of the OIV International Code of Oenological Practices (**Annex 2**). Both files have been adopted in the OIV General Assembly in July 2019, in Geneva. The modifications of the sheets were the main objective of our thesis and now it remains only to actualize the OIV Oenological Codex, which is in process.

In the future, it will also be interesting to evaluate the different properties using mixtures of different type of tannins to see the effect of the combination, on all the new functionalities that we found. Indeed, a commercial powder made of tannins appertaining to different groups can maybe enhanced the properties associated specifically to each one. For example, a mixture of a gallotannin and an ellagitannin maybe can present a good ability to stabilize the color and protect the wine against the oxidation meanwhile a simple powder of a gallotannin or ellagitannin cannot combine these two functions.

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Annexes

Annex 1: RESOLUTION OIV-OENO 612-2019

RESOLUTION OIV-OENO 612-2019

WARNING: This resolution amends the following resolution:
- OIV-OENO 16/70

UPDATE TO THE OENOLOGICAL PRACTICE ON TANNIN ADDITION IN MUSTS

THE GENERAL ASSEMBLY,

IN VIEW of article 2, paragraph 2 ii of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

CONSIDERING the work of the "Technology" expert group,

DECIDES, following a proposal made by Commission II "Oenology", to replace file 2.1.7 'Tannin addition' in part II, chapter 2 of the *International Code of Oenological Practices* with the following oenological practices and treatments:

Part II

Chapter 2: MUSTS

TANNIN ADDITION

Definition:

Addition of tannins to must.

Objectives:

a) To facilitate the subsequent stabilisation of wines by partial precipitation of excess proteinaceous matter in musts;
b) to facilitate the fining of musts in conjunction with protein-type fining agents and prevent over-fining;
c) to contribute to the antioxidant and antioxidasic protection of compounds of the must;
d) to promote the expression of colour in red wines obtained from musts to which tannins have been added.

Prescriptions:

a) To facilitate rapid incorporation into must, tannins may be added immediately after harvesting;
b) the tannins used should comply with the prescriptions of the *International Oenological Codex*.

Certified in conformity Geneva, 19th July 2019
The General Director of the OIV
Secretary of the General Assembly
Pau ROCA

OIV

OIV recommendation:
Accepted.

Certified in conformity Geneva, 19th July 2019
The General Director of the OIV
Secretary of the General Assembly
Pau ROCA

OIV

Annex 2: RESOLUTION OIV-OENO 613-2019

RESOLUTION OIV-OENO 613-2019

WARNING: This resolution amends the following resolution:
- OIV-OENO 16/70

UPDATE TO THE OENOLOGICAL PRACTICE ON TANNIN ADDITION IN WINES

THE GENERAL ASSEMBLY,

IN VIEW of article 2, paragraph 2 ii of the Agreement of 3 April 2001 establishing the International Organisation of Vine and Wine,

CONSIDERING the work of the "Technology" expert group,

DECIDES, following a proposal made by Commission II "Oenology", to replace file 3.2.6 'Tannin addition' in part II, chapter 3 of the *International Code of Oenological Practices* with the following oenological practices and treatments:

Part II

Chapter 3: WINES

TANNIN ADDITION

Definition:

Addition of tannins to wine.

Objectives:

a) To facilitate the stabilisation of wines by partial precipitation of excess proteinaceous matter;
b) to facilitate the fining of wines in conjunction with protein-type fining agents and prevent over-fining;
c) to contribute to the antioxidant and antioxidasic protection of compounds of the wine;
d) to promote the expression, stabilisation and preservation of colour in red wines.

Prescriptions:

The tannins used should comply with the prescriptions of the *International Oenological Codex*.

OIV recommendation:

Accepted.

Certified in conformity Geneva, 19th July 2019
The General Director of the OIV
Secretary of the General Assembly
Pau ROCA

1/1

OIV

Annex 3: Tasting Sheets

1/ NOM, Prénom : ..... **Date : 16/03/2018**

TESTS VISUELS :

Pour chacun des 4 postes, 3 vins numérotés vous sont présentés, deux sont identiques. Observez les échantillons dans l'ordre proposé et entourez celui que vous percevez comme différent. **Veuillez donner une réponse même si vous n'êtes pas certain.**

Vous pouvez également ajouter un commentaire ou un descripteur qui vous a permis de faire la différence.

N° de poste	Ordre de dégustation			Commentaire(s), descripteur(s)
Poste n°1A	053	531	270	
Poste n°2B	261	768	281	
Poste n°3A	360	624	818	
Poste n°4B	789	001	328	

Autre(s) commentaire(s) :

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TESTS OLFACTIFS/GUSTATIFS :

Pour chacun des 4 postes, 3 vins numérotés vous sont présentés, deux sont identiques. Observez les échantillons dans l'ordre proposé et entourez celui que vous percevez comme différent. **Veuillez donner une réponse même si vous n'êtes pas certain.**

Vous pouvez également ajouter un commentaire ou un descripteur qui vous a permis de faire la différence.

N° de poste	N° d'échantillons			Commentaire(s), descripteur(s)
Poste n°5A	979	604	901	
Poste n°6B	900	408	619	
Poste n°7A	691	372	112	
Poste n°8B	259	691	903	

Autre(s) commentaire(s) :

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Formulaire de consentement

Promoteur : ISVV, unité d'œnologie, Laboratoire polyphénols (Villenave d'Ornon, France).

Investigateurs : Anne-Laure Gancel sous la responsabilité du Pr. Pierre-Louis Teissèdre

But de l'étude : Mettre en évidence un effet protecteur des tanins œnologiques contre l'oxydation des vins par *Botrytis cinerea*.

Engagement des investigateurs principaux : les investigateurs principaux s'engagent à mener cette recherche selon les dispositions éthiques et déontologiques, à protéger l'intégrité physique, psychologique et sociale des personnes tout au long de la recherche et à assurer la confidentialité des informations recueillies. Ils s'engagent également à fournir aux participants tout le soutien permettant d'atténuer les effets négatifs pouvant découler de la participation à cette recherche. Des crachoirs sont mis à disposition.

Liberté du participant : le consentement pour poursuivre la recherche peut être retiré à tout moment sans donner de raison et sans encourir aucune responsabilité ni conséquence. Les réponses aux questions ont un caractère facultatif et le défaut de réponse n'aura aucune conséquence pour le sujet.

Information du participant : le participant a la possibilité d'obtenir des informations supplémentaires concernant cette étude auprès des investigateurs principaux, et ce dans les limites des contraintes du plan de recherche.

Confidentialité des informations : toutes les informations concernant les participants seront conservées de façon anonyme et confidentielle. Le traitement informatique n'est pas nominatif, il n'entre pas de ce fait dans la loi Informatique et Liberté (le droit d'accès et de rectification n'est pas recevable).

Déontologie et éthique : le promoteur et les investigateurs principaux s'engagent à préserver absolument la confidentialité et le secret professionnel pour toutes les informations concernant le participant.

Villenave d'Ornon, le 16/04/18

Nom Prénom	Mention « lu et approuvé »	Signature

1/ NOM, Prénom **Date : 16 Avril 2018**

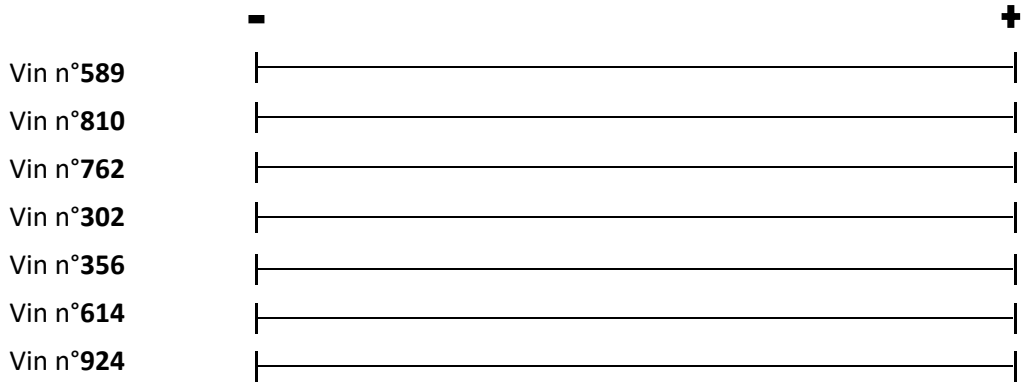
PROFILS Vins de 4 mois produits par micro-vinification à partir de Merlot

7 vins numérotés vous sont présentés.
Sentez-les, goûtez-les ou observez-les dans **l'ordre indiqué** sur cette feuille et notez l'intensité perçue pour chaque vin et chaque descripteur.
Veuillez donner une réponse même si vous n'êtes pas certain.

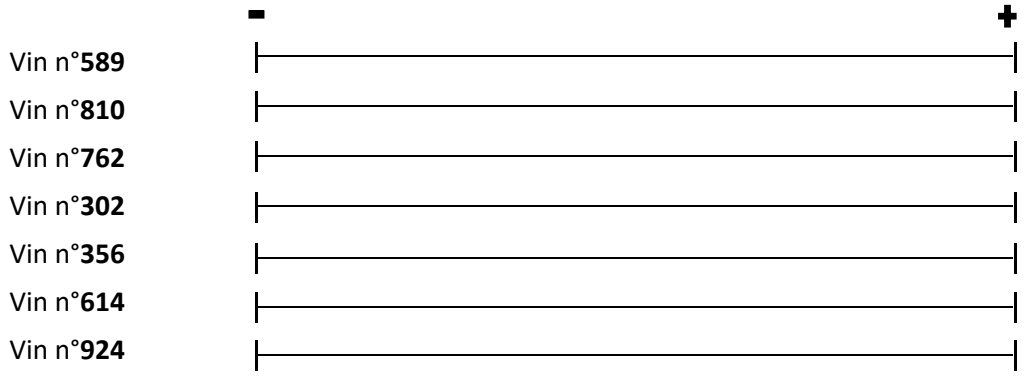
Poste 1, 2, 3 ou 4 : OLFACTIF/ GUSTATIF

Composante aromatique

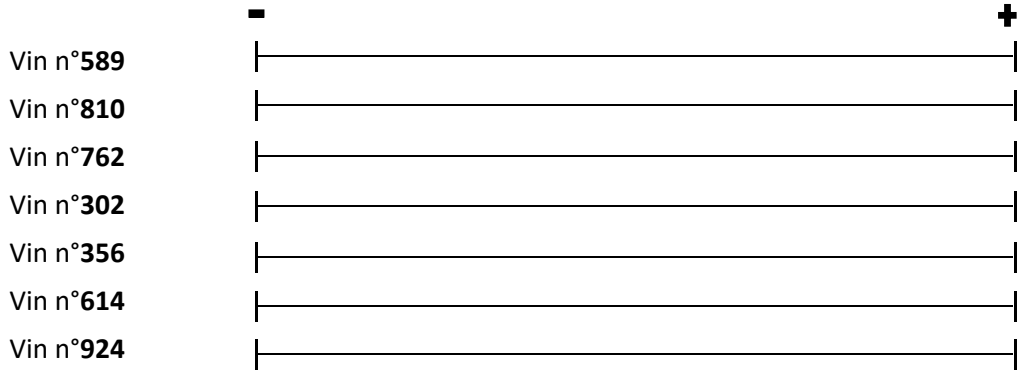
Fruits frais



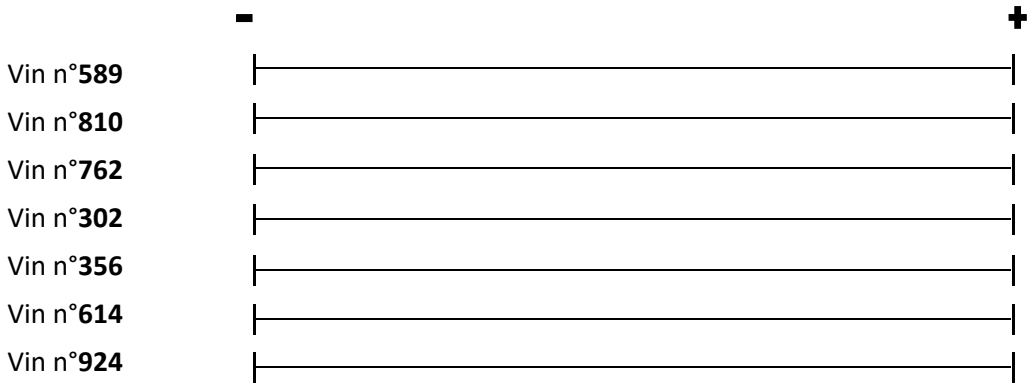
Fruits cuits/confiturés



Végétal

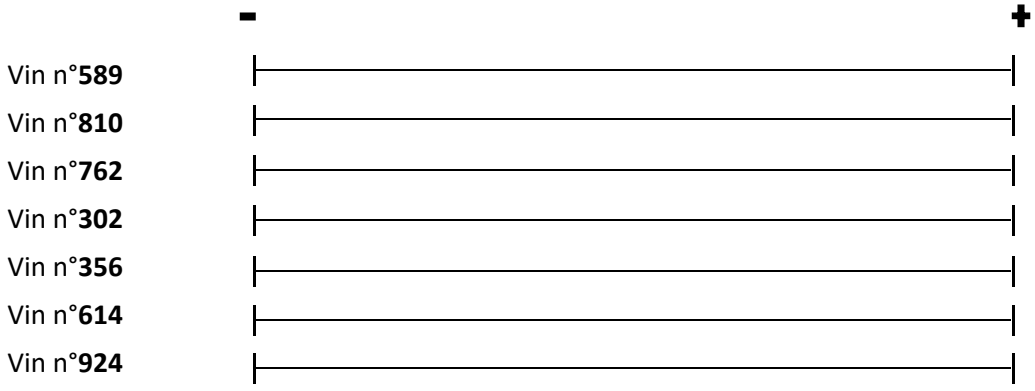


Moisi-Terreux



Acidité volatile

Oxydé



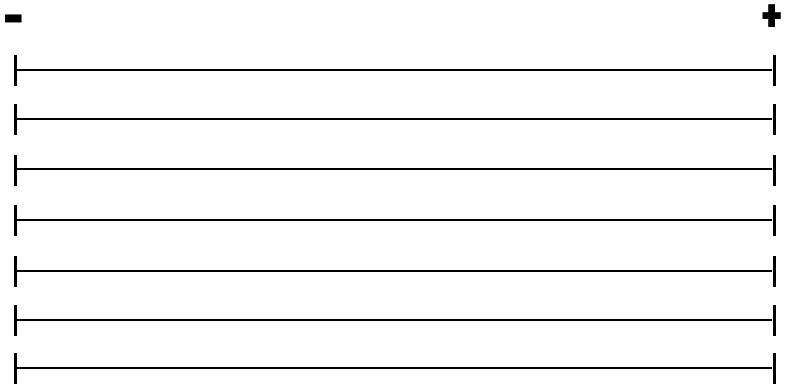
Veuillez classer les vins selon leur « Qualité olfactive globale » en leur attribuant une note de 1 à 7 sachant qu'il s'agit de vins micro-vinifiés de 4 mois, cépage Merlot :

1 pour le plus qualitatif et 7 pour le moins qualitatif

589	810	762	302	356	614	924

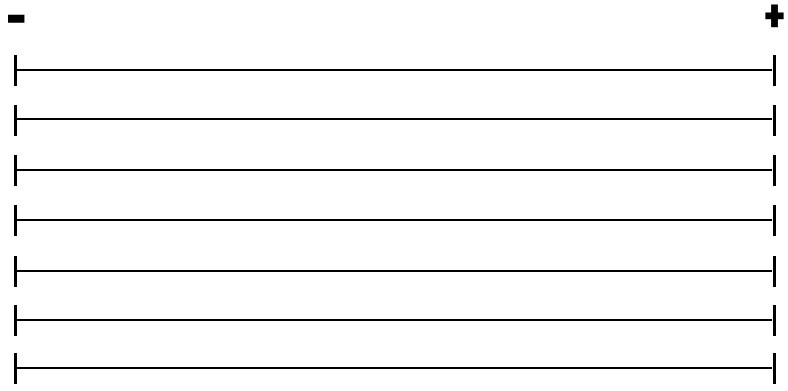
Composante gustative

Acidité



Amertume

Astringence



Veuillez classer les vins selon leur « Qualité gustative globale » en leur attribuant une note de 1 à 7 sachant qu'il s'agit de vins micro-vinifiés de 4 mois, cépage Merlot :

1 pour le plus qualitatif et 7 pour le moins qualitatif

589	810	762	302	356	614	924

Poste 5 ou 6 : VISUEL

Composante visuelle

Veuillez classer les vins selon leur « Qualité visuelle globale » en leur attribuant une note de 1 à 7 sachant qu'il s'agit de vins micro-vinifiés de 4 mois, cépage Merlot :

1 pour le plus qualitatif et 7 pour le moins qualitatif

589	810	762	302	356	614	924

Commentaire(s) éventuel(s) :

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Taninos enológicos: características, propiedades y funcionalidades.

Impacto sobre la calidad de los vinos

Los taninos enológicos son comúnmente utilizados durante la vinificación. Actualmente, la OIV sólo autoriza su uso para facilitar la clarificación de vinos y mostos. No obstante, dada la existencia de una amplia variedad de taninos enológicos en el mercado, es incuestionable que podrían utilizarse para muchos otros fines. Es por ello que hace unos años la OIV creó un grupo de trabajo que estudiara de forma detallada las características, propiedades y funcionalidades de los taninos enológicos. La presente tesis se enmarca dentro de esta línea de investigación, teniendo como primer objetivo la determinación de la composición química y la riqueza tánica de los taninos enológicos. Los principales monómeros, dímeros, trímeros y tetrámeros de los taninos comerciales fueron identificados y cuantificados por HPLC-MS. Por su parte, la riqueza en taninos fue estimada vía diferentes métodos: IPT, Bate-Smith, Metilcelulosa, Folin-Ciocalteu, Método oficial de la OIV y Floroglucinólisis. En una segunda fase, se estudiaron las posibles funcionalidades de dichos taninos enológicos, centrándose principalmente en su potencial efecto antioxidante, su capacidad para estabilizar el color de los vinos y su actividad antioxidásica. Se utilizaron diferentes métodos espectrofotométricos o fluorimétricos (ABTS, CUPRAC, DPPH, FRAP y ORAC) para determinar la capacidad antioxidante. El consumo directo de oxígeno se estimó utilizando un método no invasivo basado en la luminiscencia. La medición de la actividad lacasa, la determinación del tipo de inhibición y la caracterización de las lacasas por electroforesis SDS-PAGE, permitieron evaluar las propiedades antioxidásicas de los taninos enológicos. Finalmente, la capacidad de los taninos enológicos para estabilizar el color se evaluó por inducción del efecto batocromático e hipercrómico, así como por el cálculo del índice de copigmentación. También se estudió de manera exhaustiva la estabilización del color en función del tiempo, pH y concentración de etanol.

Los resultados obtenidos confirman que los taninos enológicos, principalmente los elagitaninos, protegen al vino de la oxidación, gracias a su capacidad antioxidante y su aptitud a consumir directamente el oxígeno. Además, los taninos comerciales, y más notablemente los galotaninos, permiten estabilizar el color de los vinos tintos actuando como copigmentos. Finalmente, los taninos enológicos, especialmente los taninos de uva, son una nueva herramienta para inhibir o precipitar las lacasas producidas por *B. cinerea*.

Como fruto de estos resultados, las resoluciones OENO-TECHNO 17-612 y OENO-TECHNO 17-613, recientemente aceptadas, proponen incluir dos aplicaciones adicionales de los taninos enológicos durante la vinificación:

- Contribuir a la protección antioxidante de los componentes del mosto y del vino.
- Promover la expresión, estabilización y conservación del color.

Palabras clave: taninos enológicos, composición química, riqueza tánica, capacidad antioxidante, consumo de oxígeno, estabilización del color, propiedades antioxidásicas, lacasa de *B. cinerea*

Tanins œnologiques : caractéristiques, propriétés et fonctionnalités

L'utilisation de tanins œnologiques en vinification relève d'une pratique courante, bien que ceux-ci ne soient à ce jour uniquement autorisés dans le but de faciliter la clarification des vins et des moûts par l'OIV. En revanche, les tanins œnologiques peuvent aussi être utilisés à d'autres fins, expliquant l'existence d'une grande variété sur le marché. En ce sens, l'OIV à créer il y a quelques années, un groupe de travail afin de conduire une vaste étude sur les tannins œnologiques. L'objectif de cette thèse était donc de mener une étude exhaustive afin de déterminer dans un premier temps leur composition chimique et leur richesse en tanins. Dans un second temps leurs potentielles fonctionnalités ont été étudiées, en mettant l'accent sur leur activité antioxydante, leur capacité à stabiliser la couleur des vins et, enfin, leur activité antioxidasique. Concernant, la composition chimique, les principaux monomères, dimères, trimères et tétramères des tanins ont été identifiés et quantifiés par HPLC-MS-UV et LC-QTOF. La richesse en tanins a été estimée par différentes méthodes : IPT, Bate-Smith, méthyl-cellulose, Folin-Ciocalteu, méthode officielle de l'OIV et phloroglucinolyse. La capacité antioxydante a été mesurée par 5 méthodes (ABTS, CUPRAC, DPPH, FRAP et ORAC), tandis que la consommation d'oxygène a été déterminée à l'aide d'une méthode non invasive basée sur la luminescence. La mesure de l'activité laccase, la détermination du type d'inhibition et la caractérisation des laccases par électrophorèse SDS-PAGE, ont permis d'évaluer les propriétés antioxidasique des tannins œnologiques. La capacité des tanins œnologiques à stabiliser la couleur a été déterminée par l'induction d'effet bathochrome et hyperchrome ainsi que par le calcul de l'indice de copigmentation. Cet effet a été évalué pendant plusieurs jours, à différentes concentrations d'ethanol et à différents pH. Les résultats obtenus confirment que les tanins œnologiques et plus particulièrement les ellagitannins protègent le vin de l'oxydation, de par leur pouvoir antioxydant et capacité à consommer directement l'oxygène. Les tanins œnologiques, et plus particulièrement les gallotannins, permettent de stabiliser la couleur des vins rouges, en jouant le rôle de copigments. Enfin, les tanins œnologiques, et en particulier les tanins du raisin, constituent un nouvel outil afin d'inhiber ou précipiter les laccases produites par B. cinerea. Fruit de ces résultats, les résolutions de l'OIV OENO-TECHNO 17-612 et OENO-TECHNO 17-613, récemment acceptées, proposent d'inclure deux nouvelles fonctionnalités d'utilisation des tanins dans les moûts et les vins :

• Contribuer à la protection antioxydante des composants du moût et du vin

• Promouvoir l'expression, la stabilisation et la préservation de la couleur

Mots clés : Tanins œnologiques, composition chimique, richesse en tannins, capacité antioxydante, consommation d'oxygène, stabilisation de la couleur, propriétés antioxidasique, laccase de *B. cinerea*

Oenological tannins: characteristics, properties and functionalities

The use of oenological tannins is a common practice worldwide in winemaking. However, up to date this use, is only authorized by the OIV to facilitate wines and must clarification. Nevertheless, oenological tannins are used for several reasons, explaining the existence of diverse tannins on the market. For all these reasons, OIV has begun few years ago to create a working group in order to conduct a large study on oenological tannins. Thus, the aim of this research was to carry out an exhaustive study to determine firstly the chemical composition and the richness in tannins of the oenological tannins. Secondly, their potential functionalities were studied, focusing on their antioxidant activity, their ability to stabilize and improve wine color and finally, their antioxidasic activity. Concerning chemical composition, the principal tannins monomers, dimers, trimers and tetramers were identified and quantified by HPLC-MS and LC-QTOF. The abundance in tannins was estimated by different methods: TPI, Bate-Smith, Methyl-cellulose, Folin-Ciocalteu, OIV official method and phloroglucinolisis. The antioxidant capacity was measured 5 methods (ABTS, CUPRAC, DPPH, FRAP and ORAC) meanwhile the oxygen consumption was measured using a non-invasive method based on luminescence. Antioxidasic property, was achieved by measuring first the laccase activity in winemaking, then by determined inhibition type and finally by laccase characterization precipitation by electrophoresis SDS-PAGE. The oenological tannins ability to stabilize the color was determined by their bathochromic and hyperchromic effect and by the calculation of the copigmentation index. Their color stabilization effect was evaluated during several days, different ethanol content and pH level. The results confirm that oenological tannins and specifically, ellagitannins, really protect wine against oxidation either because they exert antioxidant capacity and/or they consume directly oxygen. Moreover, oenological tannins can improve and stabilize red wines color acting as copigments, with gallotannins being the most efficient ones. In addition, oenological tannins, particularly grapes tannins, can be a new tool to inhibit the laccases produced by *B. cinerea*. As a fruit of these results, the OIV resolutions OENO-TECHNO 17-612 and OENO-TECHNO 17-613, just accepted, proposed to include two new functionalities of tannins uses in musts and wines:

- Contribute to the antioxidant protection of must and wine components
- Promote the expression, stabilization and preservation of color

Keywords: Oenological tannins, chemical composition, tannins richness, antioxidant capacity, oxygen consumption, color stabilization, antioxidasic properties, *B. cinerea* laccases

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