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INTEREFERENTS IN CONDENSED TANNINS QUANTIFICATION BY THE VANILLIN ASSAY

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Ioanna Mavrikou: Inteferents in condensed tannins quantification with vanillin assay

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Key Words: Proanthocyanidins; Interference substances; Phenols; Vanillin assay

Abstract

Different methods have been established in order to perform accurately the quantification of the condensed tannins in various plant products and beverages. The method of reaction in acid medium has been widely used for the quantification of condensed tannins. This method is based on the reaction of vanillin with the phenolic rings of condensed tannins and more specifically with the fusel aromatic rings of their flavan-3-ol units. In a previous study (Sun *et al*., 1998), several parameters that can affect the accuracy of the determination of condensed tannins have been examined by this method, and among them the influence of phenolic compounds other than tannins, in particular non-flavonoids such as phenolic acids (cinnamic acid, *p*-hydroxybenzoic acid, caffeic acid, gallic acid, *p*-coumaric acid, syringic acid), but also flavonols (quercetin dihydrate, kaempferol, myricetin, rutin) and the anthocyanin malvidin-3-glucoside; that may interfere the reaction of proanthocyanidins with vanillin assay. According to this analytical procedure proposed by Sun *et al*. (1998), other phenolic compounds of oenological interest not tested so far were analyzed to assess their possible interference with the reaction of proanthocyanidins in its quantification by the vanillin assay. In details, the phenolic compounds that have been studied were flavonols and flavones, stilbenes, various volatile phenols, and other phenols from wood such as ellagitannins, coumarins, aldehydes and still other compounds such as tyrosol and 2-phenylethanol. The chemical compounds examined at different concentrations did not produce any reaction with the vanillin. Therefore, the modified vanillin assay can be interpreted as a method for quantification of condensed tannins in grape and wine samples without any important analytical interference from other compounds not condensed tannins.

Palavras-chave: proantocianidinas, substâncias de interferência; fenóis; ensaio vanilina.

Resumo

Diferentes métodos têm sido usados para se realizar com rigor a quantificação dos taninos condensados em diversos produtos vegetais e bebidas. O método da reação com a vanilina em meio ácido tem sido bastante usado para a quantificação de taninos condensados. Este método baseia-se na reacção da vanilina com os anéis fenólicos de taninos condensados e, mais especificamente, com os anéis aromáticos de fusel das suas unidades de flavan-3-ol . Em estudo anterior (Sun *et al.*, 1998), vários parâmetros que podem afectar a exactidão da determinação dos taninos condensados por este método foram examinados e, entre eles a influência de compostos fenólicos que não os taninos, em particular não-flavonóides, como sejam alguns ácidos fenólicos (ácido p-hidroxibenzóico, ácido cafeico e ácido gálico , ácido p-cumárico, ácido siringico), flavonóis (quercetina, kaempferol, miricetina, rutina), a antocianina malvidina-3-glucósido; e que possam interferir na reacção das proantocianidinas com a vanilina. Segundo este procedimento analítico proposto por Sun et al., 1998, outros compostos fenólicos com interesse enológico não testados até agora foram analisados para avaliar a sua eventual interferência com a reacção de proantocianidinas, na sua quantificação pelo ensaio de vanilina. Em detalhe, os compostos fenólicos que foram estudados foram outros flavonóis e flavonas, estilbenos, diversos fenóis voláteis, e outros fenóis da madeira, tais como elagitaninos, cumarinas, aldeídos e ainda outros compostos, como o tirosol e o 2-fenil-etanol. Os compostos químicos examinados em diferentes concentrações, não originaram qualquer reacção com a vanilina. Portanto, o ensaio modificado de vanilina pode ser interpretado como método de quantificação de taninos condensados em amostras de vinho e uva sem qualquer interferência analítica importante, por parte de outros compostos que não os taninos condensados.

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1. INTRODUCTION

1.1 PHENOLIC COMPOUNDS

Phenolic compounds make up one of the major families of secondary metabolites in plants and they represent a diverse group of compounds.

They are important constituents of woody plants, contributing to many aspects from wood quality to disease resistance. The term 'phenolic compound' encompasses all aromatic molecules from the simple aromatic amino acids to the most complicated condensed tannins. All these compounds are products of the plant aromatic pathway, which consists of three main sections, the *shikimate*, *phenylpropanoids*, and the *flavonoid* routes.

The major phenolics found in wine are either members of the diphenylpropanoids (**flavonoids**) or phenylpropanoids (**nonflavonoids**). The nonflavonoid compounds family includes hydroxycinnamic acids (e.g. cafeic acid), benzoic acids (e.g., gallic acid), hydrolyzable tannins (e.g. vescalagin) and stilbenes (e.g., resveratrol).

The wine flavonoids are all polyphenolic compounds, having multiple aromatic rings possessing hydroxyl groups. A specific three-ring system (Figure 1) defines flavonoids there being a central oxygen-containing pyran ring, C-ring, of different oxidation states. It is fused to an aromatic ring (A-ring) along one bond and attached to another aromatic ring with a single bond (B-ring). The flavonoids found in grapes and wine all have the same hydroxyl substitution groups on ring A, at positions C_5 and C_7. Differences in the oxidation state and substitution on ring C define the different *classes* of flavonoids. For instance, a saturated C-ring defines the flavans, a keto at position C_4 (and unsaturation between C_2 and C_3) defines the flavones, and the fully aromatic ring, which also has a positive charge, defines the anthocyanidins. The -ol ending further specifies an alcohol substituent on the C-ring, as in flavan-3-ol, where the position is distinguished because it could alternatively exist in the C_4 position. The substitution pattern on ring B defines the member of the class. Normal substitution patterns are a hydroxyl at the C_4 position with additional oxygen substitution at C_3 and/or C_5. Those oxygens can be hydroxyls (phenols) or methoxyls at positions C_3 and/or C_5. Thus, the number of class members is relatively short, however, the "free" flavonoid structure can also be substituted further (usually with sugar conjugation on the oxygens); this gives rise to many additional compounds. The major classes of wine flavonoids are flavan-3-ols (e.g. epicatechin), anthocyanins (e.g. malvidin), and flavonols (e.g., quercetin and rutin).

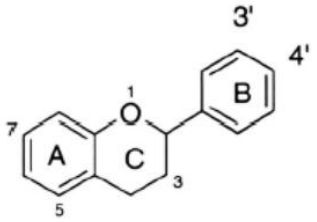

Figure 1. Flavonoid ring system

Tannins are highly hydroxylated molecules and can form insoluble complexes with carbohydrates and protein. This function of plant tannins is responsible for the astringency of tannin-rich foods, because of the precipitation of salivary proteins. The term “tannin” comes from the tanning capacity of these compounds in transforming animal hides into leather by forming stable tannin-protein complexes with skin collagen.

Plant tannins are conveniently subdivided into condensed tannins (proanthocyanidins), which are of flavonoid origin, and hydrolyzable tannins. Condensed tannins only occur in grapes, but hydrolyzable tannins can also be found in the wine extracted from oak cooperage.

1.1.1 HYDROLYZABLE TANNINS

As their name indicates, these tannins are easily hydrolyzed with acid, alkali, and hot water and by enzymatic action, which yield polyhydric alcohol and phenylcarboxylic acid. According to the nature of the latter, hydrolyzable tannins can be further subdivided into gallotannins, which are derived from gallic acid [Fig.2, (1)], or ellagitannins, which are derived from hexahydroxydiphenic acid and which take their name from the lactone ellagic acid [Fig.2, (3)]. Maximal substitution is reached with 1,2,3,4,6-pentagalloylglucose [Fig. 3, (2)], which is considered the immediate precursor of both classes of hydrolyzable tannins (i.e., gallotannins and ellagitannins). Gallotannins consist of gallic acid and its dimeric condensation product, hexahydroxydiphenic acid [Fig.2, (2)], esterified to a polyol, which is mainly glucose. These metabolites can oxidatively condense to other galloyl or hexahydroxydiphenic molecules and form high-molecular-weight polymers.

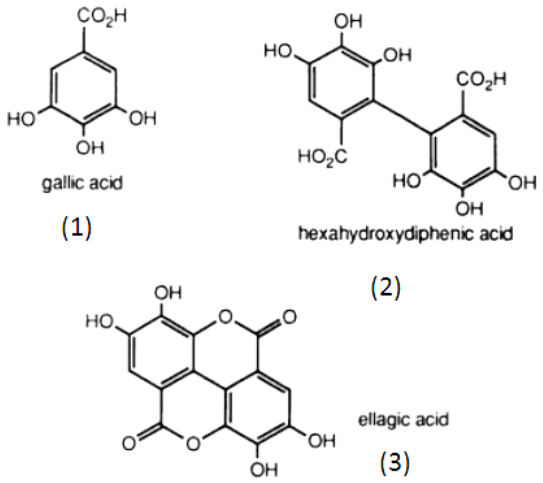

Figure 2. Structures of gallic acid, hexahydroxydiphenic acid and ellagic acid

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Gallotannins result from the attachment of additional galloyl units to the pentagalloylglucose [Fig.3, (2)] core via meta-despite bonds [Fig. 3, (3)].

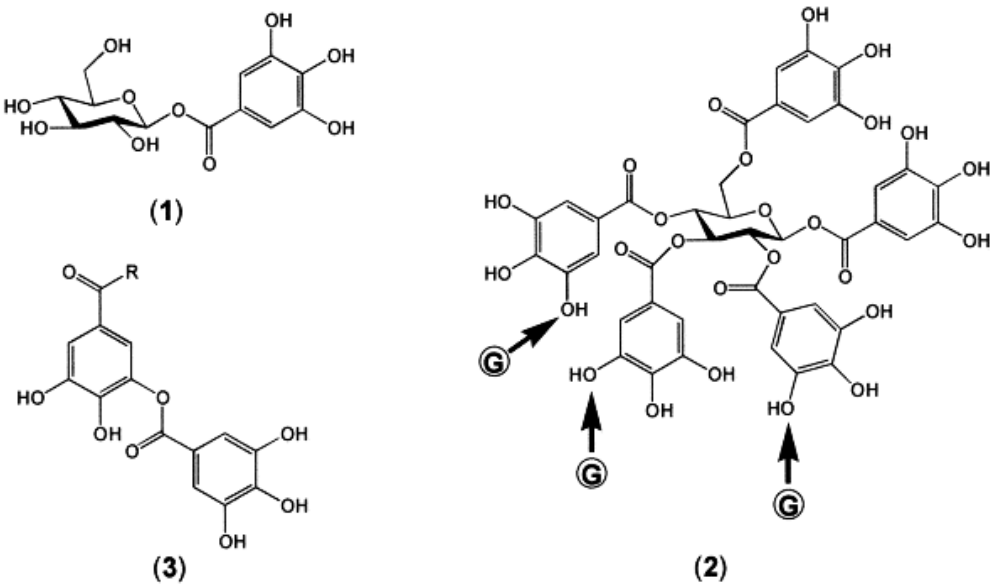


Figure 3. Characteristics of gallotannins (1) β-Glucogallin (1-O-galloyl-β-d-glucose), the principal galloyl donor; (2) 1,2,3,4,6-penta-O-galloyl-β-d-glucose, the basic galloyl acceptor in the biosynthesis of gallotannins; (3) meta-Digalloyl residue

Ellagitannins have a tendency to undergo further intermolecular oxidation reactions affording dimeric, and eventually oligomeric, derivatives in which the monomers are interconnected either through the aryl C-C linkages or via aryl C-O-C bonds whose formation involves dehydrogenation of a galloyl-OH group (Fig. 4). The two main ellagitannin isomers in oak used for cooperage are vescalagin (Fig.5) and castalagin *(*M = 934*)*, as well as two less important compounds, grandinin and roburin (Ribéreau-Gayon *et al*., 2006). The ellagitannin composition of extracts from the duramen depends on the species of oak. All four monomeric and four dimeric (roburin A, B, C, and D) ellagitannins are present in the three species of European oak, while the American species have practically no dimers. Hydrolyzable tannins are not naturally found in grapes. On the other hand, they are the main commercial tannins legally authorized as wine additives.

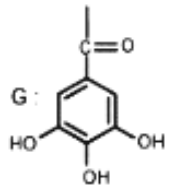


Figure 4. Galloyl residue

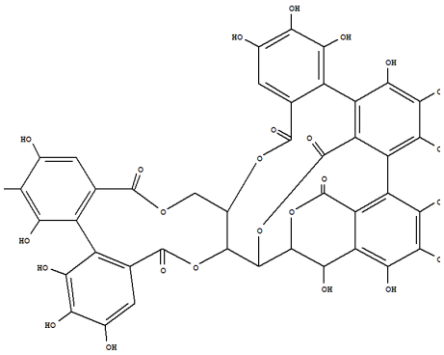


Figure 5. Vescalagin

1.1.2 CONDENSED TANNINS – PROANTHOCYANIDINS

Condensed tannins are synonymous with the proanthocyanidins (PAs), one of the major groups of phenolic compounds, which belong to the flavonoid class.

Proanthocyanidins are flavanol oligomers and polymers consisting of chains of polyhydroxyflavan-3-ol monomer units. The monomer units are linked through C_4-C_6 or C_4-C_8 interflavanoid bonds. The term “proanthocyanidins” was defined due to the fact that these molecules release red anthocyanidin pigments, when heated under acidic conditions. Flavonoids may exist free or in polymers with other flavonoids, sugars, nonflavonoids, or a combination of these. Those esterified to sugars and nonflavonoids are called glycosides and acyl derivatives, respectively.

Generally, the structure variability of PAs depends upon the nature (the stereochemistry at the chiral centers and the hydroxylation pattern) of the flavan-3-ol extension and end units, the location and stereochemistry of the interflavan linkage between the monomeric units and the degree of polymerization. According to their increasing degree of polymerization, proanthocyanidins are termed to dimmers, trimmers, oligomers and condensed tannins.

The chemical structure of proanthocyanidins is a flavan-3-ol unit. The flavan-3-ol units have the typical C_6-C_3-C_6 flavonoid skeletons. The heterocyclic benzopyran ring is referred to as the C-ring, the fused aromatic ring as the A-ring, and the phenyl constituent as the B-ring (Fig.6).

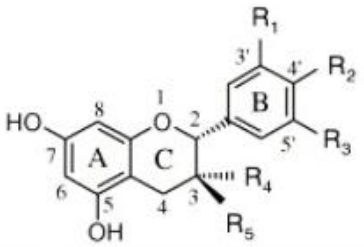


Figure 6. Chemical structure of flavan-3-ol unit

The skeleton of the flavan-3-ol nucleus can be hydroxylated at the C_5 and C_7 on A-ring, in C_3 on heterocycle and in C_3, C_4 and/or C_5 on B-ring. According to the hydroxylation form, proanthocyanidins can be distinguished to several classes. The two major classes are the procyanidins, which release cyanidins, and the prodelphinidins, which release delphinidins, when both hydrolyzed. Procyanidins are constituted from catechin and epicatechin. The

carbon atoms (C_2 and C_3) in the pyran ring allow of stereoisomerism, so that we have catechin (Trans) and epi-catechin (cis); and they are also asymmetric, resulting in optical activity with + and - variants in each. Catechin and epicatechin differ only in their stereochemistry. Catechin possesses its C_3 hydroxyl group in a plane opposite the B-ring, whereas epicatechin possesses both C_3 hydroxyl groups in the plane of the B-ring. On the other hand, prodelphinidins are constituted from gallocatechin and epigallocatechin. Epigallocatechin differs from epicatechin in possessing a third hydroxyl group in its B-ring. The most common monomer units are listed below (Fig. 7).

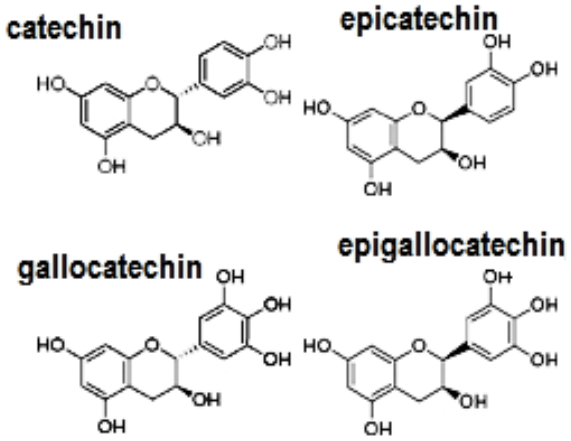


Figure 7. Monomer units of Proanthocyanidins

The flavan-3-ol unit can be substituted by gallic acid, in the C_3 form 3-O-esters of flavan-3-ols. Various flavan-3-ol glycosides have been isolated from plant tissues and the 3-, 5- and 7-O and 6- and 8-C-glycosides of flavan-3-ols have already been known (Porter, 1988). (-)-epicatechin gallate has a gallic acid esterified to C_3 (Fig.8).

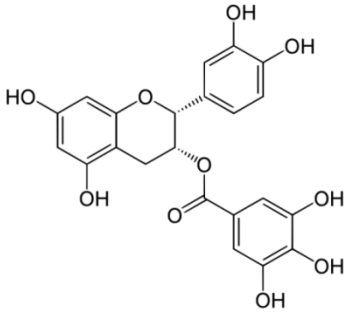


Figure 8. (-)-epicatechin 3-O-gallate

Proanthocyanidins can also be divided into two types based on intermonomeric linkages; A-type and B-type. In B-type proanthocyanidins, the flavanol constitutive units are linked by C_4-C_8 and/or C_4-C_6 bonds, opening the possibility for branched structures. Proanthocyanidin B1 to B4 dimers differ only in the arrangement of the initial and terminal epicatechin and catechin units. Bonding of flavonoids between C_4 and C_6 sites permits branching of the normally linear, proanthocyanidin polymer.

The A-type proanthocyanidins present double linkages, with C_2-O-C_7 or C_2-O-C_5 bond in addition to the C_4-C_6 or C_4-C_8 bond.

The Fig. 9 gives some examples of B-and A-type proanthocyanidins.

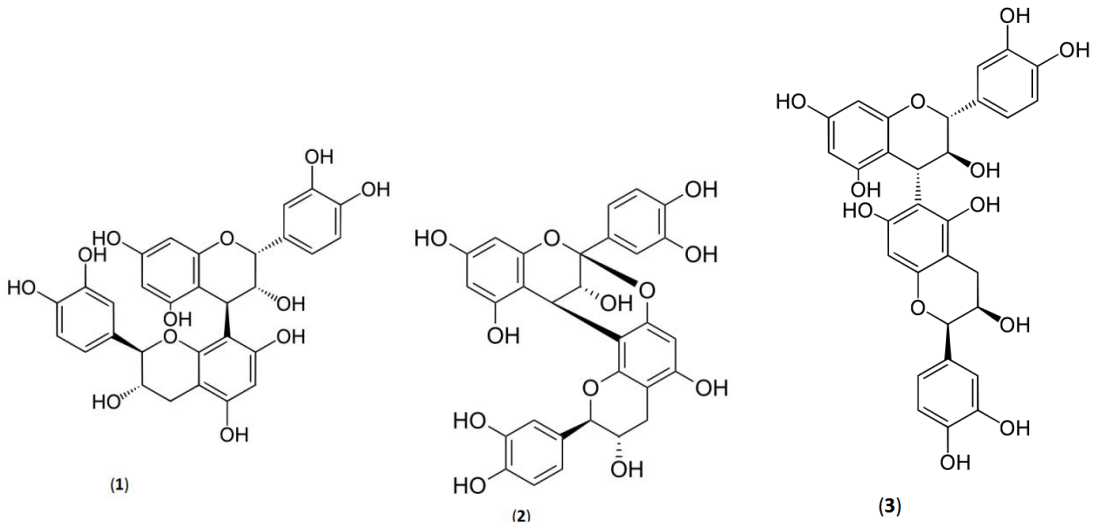


Figure 9. (1) Proanthocyanidin B1 (epicatechin-(4β→8)-catechin); (2) Proanthocyanidin A2 (epigallocatechin-(2β→7,4β→8)-epicatechin); (3) Proanthocyanidin B8 (catechin-(4α→6)-epicatechin)

In plant tissues, proanthocyanidins are found in higher oligomers and polymers and the polymerization degree may vary. For example, the DP of PAs in cider apple skin and pulp range from 7 to 190, in brown or black soybean coat it can be up to 30 and even more (Guyot S. *et al*., 2001; Takahata Y. *et al*., 2001). The figure 10 demonstrates an example of a polymer proanthocyanidin. Monomeric flavan-3-ols and oligomeric procyanidins found in wines Tempranillo, Graciano, and Cabernet Sauvignon were also present in seeds, although differences in their relative abundance were seen (Monagas *et. al*., 2003).

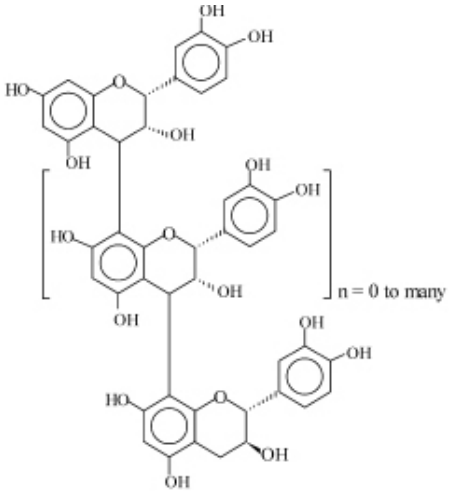


Figure 10. General structure of proanthocyanidin

The quantity, structure, and degree of polymerization of grape proanthocyanidins differ, depending on their localization in the grape tissues (Ricardo da Silva *et al*., 1991; Prieur *et al*., 1994). While seed tannins are oligomers and polymers composed of the monomeric flavan-3-ols (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate linked by C_4−C_8 and/or C_4−C_6 bonds (B type) (Prieur *et al*., 1994), skin tannins also contain (−)-epigallocatechin and trace amounts of (+)-gallocatechin and (−)-epigallocatechin gallate (Souquet *et al*., 1996). Therefore, wine contains both procyanidins and prodelphinidins (Fulkrand *et al*., 1999). The seeds contain higher concentrations of monomeric, oligomeric, and polymeric flavan-3-ols than the skins (Ricardo da Silva *et al*., 1992). However, the skin tannins have a much higher degree of polymerization than that from the seeds (Souquet *et al*., 1996) and are more easily transferred into wine (Sun, 1999). Whereas it is known that the proanthocyanidin concentration of wines is mainly determined by the grape proanthocyanidin content and by other factors such as the extraction or winemaking techniques and the aging conditions (Ricardo da Silva *et al*., 1992; Santos-Buelga *et al*., 1995) the structural features (composition, degree of polymerization, galloylation, cis/trans ratio, etc.) of wine proanthocyanidins have been less studied than those of the solid parts of the grapes (Monagas *et al*., 2003).

1.1.3 PROANTHOCYANIDINS IN VINE PLANTS

A lot of researches have been made in order to find the localization of the proanthocyanidins in the vine and more specifically they can be found in leaf, shoot, seeds, skin, stem and very little in pulp.

1.1.3.1 LOCALIZATION

The vast majority of proanthocyanidins in wine are derived from grapes, with only trace amounts possibly being extracted from oak cooperage. Structural differences exist among skin, stem, and seed proanthocyanidins. There is also considerable variation in the types and concentrations among cultivars.

In grapes, flavonoids are primarily synthesized in the skins and seeds. They are produced in smaller amounts in the stems. Flavan-3-ols and their proanthocyanidin polymers are synthesized primarily in seeds and stems (about 60 and 20% respectively), with skins producing about 15–20% (Bourzeix *et al*., 1986). Procyanidin dimers and trimmers were first identified in seeds but they are also present in skins and stems with different distributions (Ricardo da Silva *et al*., 1991a) and trace amounts of B1 through B4 have been detected in pulp (Bourzeix *et al*., 1986).

Flavanol monomers and oligomers have been found in small amounts (a few mg/L) in white wines made without maceration (Ricardo da Silva *et al*., 1993).

Stem and seed (seed coat) flavan-3-ols occur primarily as catechin, epicatechin, epigallocatechin and the ester, epicatechin-gallate, as well as proanthocyanidin oligomers and polymers (condensed tannins). Until oxidized, grape tannins are colorless. Seed tannins are primarily smaller than those found in the skins (mean of 10 units vs. 30 for skin tannins), possess significantly greater epigallocatechin content, and are less galloylated (esters of flavanols with gallic acid) (Souquet *et al*., 1996; Downey *et al*., 2003b). Stem tannins have a size distribution between those of skin and seed tannins, and consist principally of epicatechin extension units (Souquet *et al*., 2000). Some varieties, for example ‘Pinot noir,’ appear to produce no condensed tannins in the skin (Thorngate, 1992). This may partially explain the poor color intensity typical of ‘Pinot noir’ wines, as well as why stems have often been added to the ferment. ‘Pinot noir’ also appears atypical in its low proportion of seed tannins, the majority of flavonoids occurring as monomers. During fermentation, skin tannins are extracted earlier than seed tannins, but this tends to change as fermentation progresses (Peyrot des Gachons & Kennedy, 2003).

1.1.3.2 BIOSYNTHESIS

Proanthocyanidins are synthesized as oligomeric or polymeric end products of one of several branches of the flavonoid pathway. The first committed step of the pathway is the condensation and subsequent intramolecular cyclization of three malonyl-CoA molecules with one 4-coumaroyl-CoA molecule to produce a naringenin chalcone (Kreuzaler & Halhbrock, 1972). This process is catalyzed by the ubiquitous plant enzyme chalcone synthase, which possesses extensive biological functions. Additionally, the two kinds of precursors are derived from phenylalanine and acetyl-CoA, respectively. The second step of the pathway is the isomerization of the naringenin chalcone to the naringenin, which can occur spontaneously, without enzymatic activity. However, chalcone isomerase stereospecifically directs and greatly accelerates the intramolecular cyclization of chalcones to form the flavanones, which serve as exclusive substrates for downstream reactions (Cain, 1997). Hither, the basic skeleton of all flavonoids which consist of three C_6-C_3-C_6 aromatic rings has been generated through the catalysis of chalcone synthase and chalcone isomerase. The following steps are related to the “Gird” section with the B-ring hydroxylation. The possibility of a “Grid” system permitting multiple pathways to 3-hydroxyflananone is due to the fact that the P-450 hydroxylases of the B-ring can function at either the flavanones or 3-hydroxyflavanone level of oxidation; orientation of the pathway enzymes in a linear array, however, could limit the pathway to one route (Stafford, 1990). To produce the 5-deoxy A-ring of oligomeric proanthocyanidins, a NADPH-dependent reductase coats with chalcone synthase, producing a 6’-deoxychalcone which is isomerized to 5-deoxynaringenin(Ayabe et al., 1988; Welle and Grisebach, 1988). A first NADPH-depedent reductase step forms flavan-3,4-diol. A second NADPH-depedent reductase reduces flavan-3,4-diol intermediates to a flavan-3-ol, forming catechin and epicatechin and their respective trihydroxy forms, gallo- and epigallo-catechin (Stafford, 1990).

(3-hydroxy)proanthocyanidins are oligomers and polymers of flavan-3-ols. They are formed by the sequential addition of flavan-3,4-diol (or their rearrangements to quinone methides, etc.) to a flavan-3-ol. The chain is shown in the figure 9 and grows only in one direction and nothing is added to the terminal (or initiating) flavan-3-ol (Stafford, 1990).

However, many enzymatic steps involved in the biosynthesis of proanthocyanidins are still unclear and further studies need to elucidate certain aspects of proanthocyanidin biosynthesis.

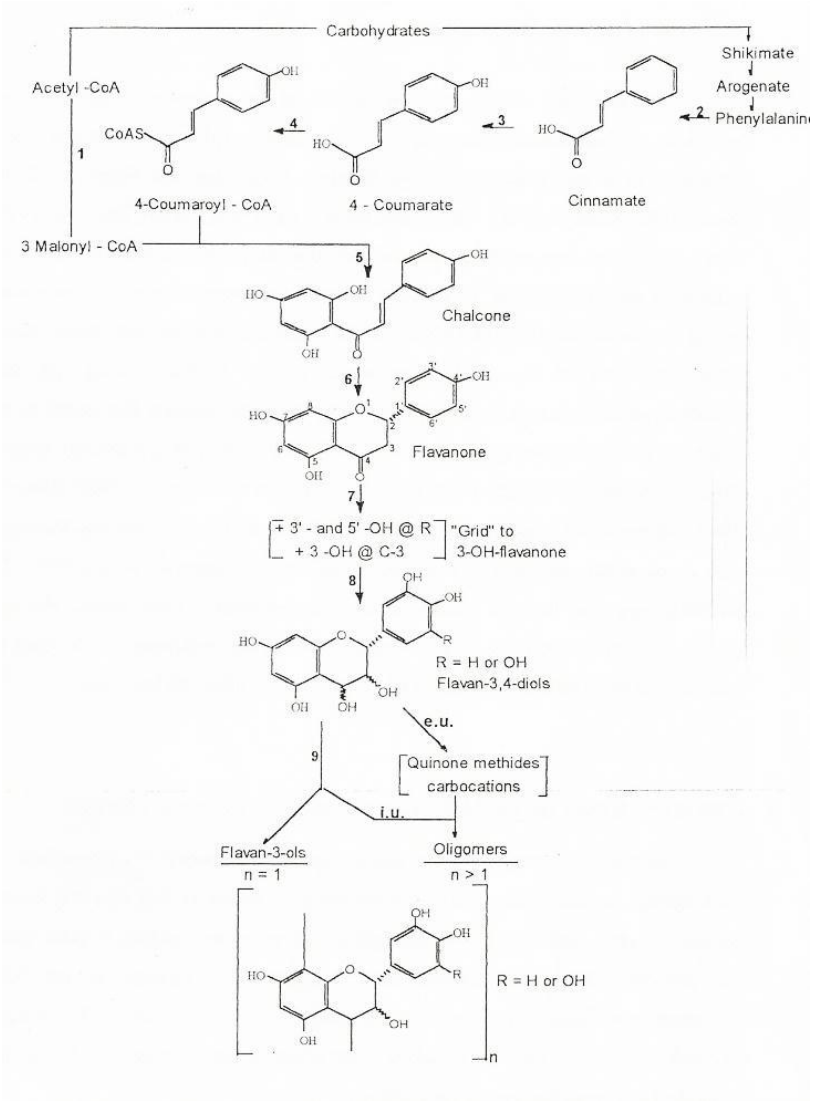


Figure 11. Biosynthesis of condensed tannins (according to Stafford, 1990). (1) Acetyl-Coa carboxylase; (2) Phenylalanine ammonia-lyase; (3) Cinnamate 4-hydroxylase; (4) Coumarate: CoA ligase; (5) Chalcone synthase; (6) Chalcone isomerase; (7) Hydroxylases; (8,9) NADPH reductase; i.u. initiating unit; e.u. extension unit.

1.1.4 CHEMICAL REACTIONS AND PROPERTIES OF PROANTHOCYANIDINS

Proanthocyanidins are unstable in aqueous solution and disproportionate in mild acids or bases. The monomer units rearrange in strong acidic or basic solutions. The reactions are largely radical-mediated in basic solutions to form highly rearranged and oxidatively coupled products.

Proanthocyanidins are very reactive compounds due to their acidic character of the hydroxyl groups and their nucleophilic properties of the phenolic rings. Their reactivity of proanthocyanidins with other compounds depends mostly on the hydroxylation pattern of proanthocyanidin molecules (Mc Graw, 1989). These reactions occur in the A-ring and B-ring, as well as in the interflavan bond and maybe in the C-ring.

According to the reactivity of the phenolic rings, there are three types of reactions that can occur on the **A-ring**:

- Aromatic substitution or proton shift (at C_6 and C_8 position). The resonance between the free electron pair of phenolic oxygen and the benzene ring enhances electron delocalisation and confers the position adjacent to the hydroxyl a partial negative charge and thus a nucleophilic character (showing an excess of electrons and thus prone to react with electrophiles, showing an electron deficiency). Such nucleophilic sites are encountered on the phloroglucinol A-ring of flavonoids, in C_6 and C_8 due to their meta hydroxyl substitution pattern.
- Degradation and condensation of proanthocyanidins.
- Oxidation with the formation of free radicals.

All these reactions occur at carbon or OH groups of the A-ring, which presents a strong nucleophilicity. The hydroxylation of the A-ring is in form of resorcinol, phloroglucinol or pyrogallol. In acidic conditions, the reactions of the aromatic electrophile substitution of A-ring are strong owing to carbocation formation. An example of this reaction can be shown with vanillin, phloroglucinol and acetaldehyde.

The reactions that occur on the **B-ring** of proanthocyanidins are similar to that of simple phenols and they consider being esterification, etherification and electrophilic addition for all types of B-rings. Moreover, other reactions include metal chelation, oxidation, properties of free radical scavenge and ketal formation for catechol or pyrogallol B-rings. In addition, intermolecular coupling via the B-ring would be expected to occur, leading to coupled and o-quinonoid products (Waters, 1964). The latter is an electrophilic species and thus prone to suffer nucleophilic addition (Moreno-Arribas & Polo, 2009).

Reactions can also occur in the **C-ring**. In the presence of alkali and traces of oxygen or a sulfur nucleophile, the tertiary carbon at C_2 is particularly susceptible to attack by singlet oxygen to form radicals; it could take part to the “aging” process of the condensed tannins.

The most important reaction of proanthocyanidins is their ready cleavage of **interflavan bond** in mild acid solutions to form a flavan-3-ol and a quinone methide, which is in equilibrium with the carbocation in stronger acid solutions. The carbocation is converted to an anthocyanidin on heating in alcoholic solutions in the presence of traces of oxygen. The quinone methide may be captured by a suitable nucleophile and thus, this reaction has been used widely for proanthocyanidin synthesis and structural determination (Matsuo *et al*., 1981).

Proanthocyanidins complex strongly with metal ions, carbohydrates and proteins. Under the chemical reactions, proanthocyanidin polymers have the power to form strong and often insoluble complexes with metal ions. For example, they form strong complexes with iron and aluminium, which is of importance due to its usage in technology (traditional tannin/iron inks, recovery of minerals from water, etc.).

Another property of proanthocyanidins is their ability to strongly complex to carbohydrates and proteins; the origin of astringency (Haslam, 1989). The complexation with saliva proteins is the origin of astringency. However, the binding is powerful and difficult to reverse (Rahman, 1988). The complex formation is probably the origin of the difficult extraction or apparent solubility of proanthocyanidins from many hard plant tissues.

Finally, the most important reaction of proanthocyanidins is their condensation reaction with anthocyanins during the storage and ageing of red wine. This reaction leads to new-colored combination products, which are more stable than free anthocyanins, less sensitive to SO_2, and less sensitive to pH alterations.

1.2 QUANTITATIVE METHODS OF PROANTHOCYANIDINS

Information as to the actual content of the polyphenolic compounds in grapes, musts and wines (both young and aged), including monomeric and polymeric forms, is important to flavour/colour assessment. There is clearly a considerable problem in defining all these compounds that can be present, in devising methods of analyzing them separately, and in determining content in real weight/volume units. Similarly, determining threshold values for their sensory properties is a large task.

Many assays have been used for the estimation of proanthocyanidins. They can be classified into three groups according to the type of reaction involved:

- Precipitation of proteins or alkaloids
- Reaction with phenolic rings
- Depolymerization

Some of these assays give a reaction with all kinds of tannins and others with a specific class of tannins, such as proanthocyanidins, gallotannins or ellagitannins.

1.2.1 METHODS BASED ON PHENOLIC RINGS

There are many assays based on reagents that exploit the particular reactivity of different phenolic rings found in the tannin molecules. In hydrolyzable tannins, there are mainly pyrogallol rings, whereas the proanthocyanidins contain phenolic rings based on phloroglucinol, resorcinol, catechol, pyrogallol, or phenol. Some of these reagents are chosen for their ability to react at a more or less equal stoichiometry with any type of phenolic group, whereas, others will react specifically with only some of them, thus allowing the estimation of a particular group of tannins.

In the present work, we will only base on the quantitave methods that involve reaction with phenolic rings in order to estimate the proanthocyanidins with the vanillin assay.

Vanillin, the most widely used aldehyde, has been developed as a quantitative assay for proanthocyanidins as their A-rings usually have either phloroglucinol or resorcinol functionality. It gives a red chromophore with proanthocyanidins (λmax is about 500nm) (Swain *et al*., 1959). According to Pew (1951), m-Diphenols are known to react with aldehydes in acid solution and the methylene-ols resulting from the condensation form a colored carbonium anion in concentrated acid. It is a typical condensation between aldehyde and A-ring of flavanols. The reaction is demonstrated in figure 12. Another aldehyde, p-dimethylaminocinnamaldehyde, gives a green chromophore (λmax=640nm), which may be useful for the estimation of proanthocyanidins in colored extracts absorbing about 500nm (McMurrough *et al*., 1978). Even though this reagent offered the same specificity with vanillin, the color formed was not stable (Sun *et al*., 1998, Sun, 1999).

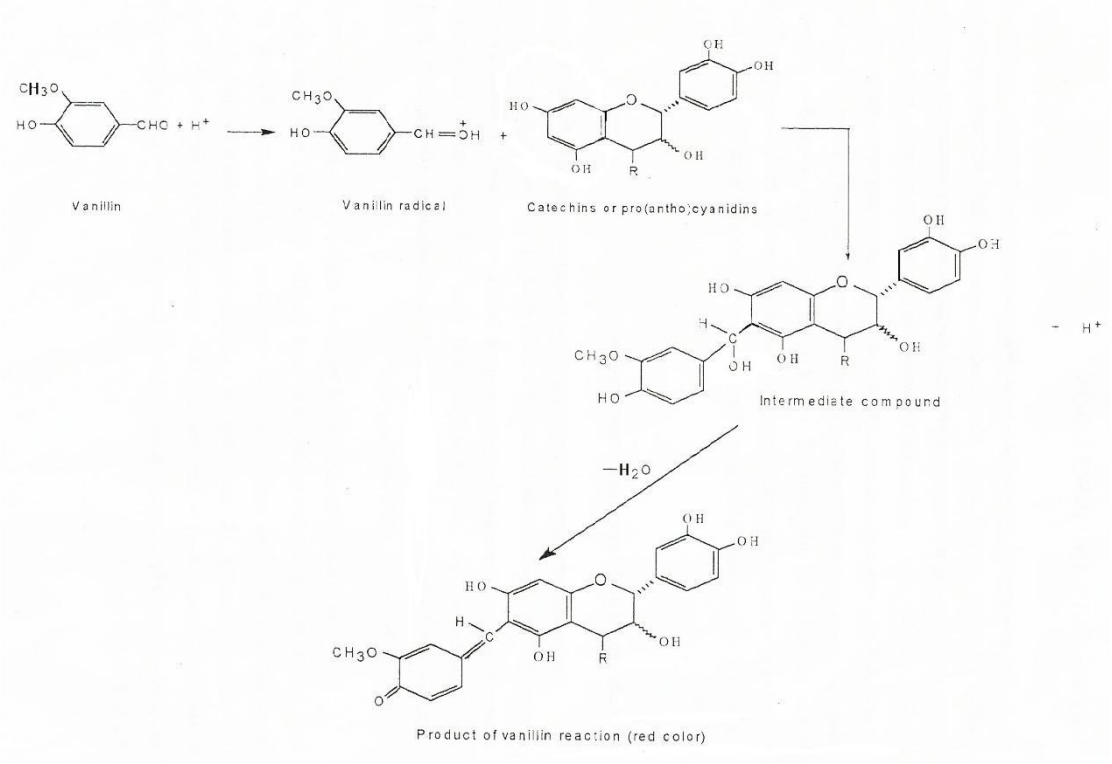


Figure 12. Vanillin reaction with catechins (according to Ribéreau-Gayon, 1966)

Another method used for total phenol determination is based on the reduction of a phosphotungstic-phosphomolybdic reagent in slightly alkaline medium and thus, on the reducing power of phenolic hydroxyl groups. The Folin-Denis method was later modified by the addition of lithium sulfate to the reagent to avoid the casual formation of precipitates, called now Folin-Ciocalteu method (Folin & Ciocalteu, 1927). The Folin-Ciocalteu method is slightly more sensitive and the absorbance maximum not as broad as that obtained with the Folin-Denis method. However, the main disadvantage is the low specificity of the method which depends upon the reduction of an aqueous solution of sodium tungstate, phosphomolybdic acid and phosphoric acid by phenolic compounds in the presence of an excess of sodium carbonate acting as an alkali (Puech *et al*., 1999).

A different method based on the depolymerization treatments has been widely applied to the estimation of proanthocyanidins. The method of depolymerization with HCL/BuOH is based on the transformation of proanthocyanidins into anthocyanidins in hot mineral acid solutions (Porter *et al*., 1986). The formed anthocyanidins take a red color (λmax around 500nm). However, according to Scalbert, the transformation of proanthocyanidins into anthocyanidins is not complete; the yields of colored anthocyanidins depend on the structure and the polymerisation degree of proanthocyanidins. Moreover, side reactions are common during the transformation, and lead to the formation of red-brown polymers absorbing around 450nm (Scalbert, 1992) and, thus, this method can lead to an estimation error and its application has been limited.

Vanillin assay for proanthocyanidins is more attractive and preferred due to its sensitivity, specificity and simplicity (Desphande *et al*., 1986). The chief advantage of this method is its specificity to a narrow range of flavanols (monomers and polymers) and dihydrochalcones, which have a single bond at 2,3 position and free meta-orientated hydroxyl groups on the B-ring (Sarkar & Howarth, 1976). It was used for many years as a colorimetric method, but due to its lack of reproducibility many researchers tried to improve it (Broadhurst & Jones, 1978; Price *et al*., 1978). The latest attempt to improve this quantitative method was done by Sun and published in 1998.

Sun optimized the vanillin assay and its application to grape and wine proanthocyanidins. He studied the influence of various factors, such as acid nature and concentration, reaction time, temperature, water content, vanillin concentration, diffuse sunlight, reference standard and presence of interfering substances on reaction of vanillin with (+)catechin, (-)epicatechin, oligomeric and polymeric proanthocyanidins (Sun *et al*., 1998). The results gave a critical evaluation of vanillin assay for plant proanthocyanidins. They were described by Sun, as follows:

- Separation of catechins from proanthocyanidins in the sample and quantifying each of them separately.

- Use of absolute methanol as solvent for the sample and for the reagents.

- Use of H_2SO_4 (instead of HCl) as reagent b and well controlling its concentration.

- Use of purified proanthocyanidins issue of the source itself and (+)catechin or (-)epicatechin as reference standards, for proanthocyanidin estimation and catechin estimation.

- Elimination of interfering substances (chlorophyll, ascorbic acid,..) and correction of anthocyanins (in the cases of red wine or extract of red grape skins) by suitable blank.

- For catechin estimation the reaction temperature (25-30°C) should be well controlled and reaction time is fixed at 15 min; proanthocyanidin estimation can be performed at room temperature and the maximum ΔA500 should be taken as a measured value.

Vanillin assay under this proposed analytical procedure can be considered as a valid method for quantification of total catechins, total oligomeric and total polymeric proanthocyanidins in grape and wine samples (Sun *et al*., 1998; Sun, 1999).

1.3 INTERFERENCE SUBSTANCES

Several parameters mostly affecting the precision and accuracy of vanillin assay were optimized. Interference substances have shown that they affect the sensitivity of the vanillin assay. Chlorophyll and ascorbic acid or ascorbate can interfere with vanillin assay. Chlorophyll exist in some plant samples like grape stem interfere, but it can be easily extracted by hexane without modifying catechins and proanthocyanidins (Sun *et al*., unpublished data). Ascorbic acid, which also interferes in the vanillin assay (Broadhurst & Jones, 1978), has to be separated from catechins and proanthocyanidins and it can be easily done by fractionation with C18 Sep-Pack cartridges (Sun *et al*., 1998; Sun, 1999).

Anthocyanins belong to the flavonoids and are the red pigments in the grapes. It has been published that anthocyanins can react with vanillin assay, and thus the latter can be used as a method for the quantification of anthocyanins (Khoshayand *et al*., 2012). However, Broadhurst & Jones (1978) reported that anthocyanins do not react with vanillin at low concentration. Moreover, Sun (1998) proved that vanillin does not react with malvidin 3-glucoside and thus, confirmed that anthocyanins do not interfere in the vanillin assay. As a conclusion, we assume that the study of Khoshayand (2012) has been mistaken on the use of the term “anthocyanins”, as well as due to the fact that catechin (monomer of proanthocyanidins or condensed tannins) was used as a standard solution.

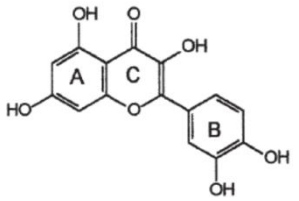
The reactivity of vanillin with other non-flavanol compounds is often encountered in plant tissues, such as acids, flavonols, and volatile phenols. The phenolic acids (cinnamic acid, p-hydroxybenzoic acid, cafeic acid, gallic acid, p-coumaric acid and syringid acid) and the flavonols (quercetin dehydrate, kaempferol, myricetin, rutin) do not react with vanillin (Sun *et al*., 1998; Sun, 1999). However, there are several phenolic compounds from the flavonoid, non-flavonoid families and other phenols that are categorized to different groups, such as volatile phenols, wood phenols originated from the wood including the ellagitannins, furanic aldehydes, coumarins, and others like tyrosol, tryptophol and even the 2-phenylethanol, that have not been tested yet and they can be considered as interference substances in the vanillin assay.

1.3.1 FLAVONOIDS

1.3.1.1 FLAVONOLS

Quercetin

The principal flavonols are kaempferol (3,5,7,4'-tetrahydroxyflavone), quercetin (3,5,7,3',4-pentahydroxyflavone) (Fig.13) and myricetin (3,5,7,3',4',5'-hexahydroxyflavone). In wine, they may act as copigments with anthocyanins. Of grape flavonoids, flavonols occur in the lowest concentration, varying from 1–10% of the total phenolic content, depending on the cultivar and growing conditions. Synthesis is primarily active at fruit set, and subsequently during ripening. The synthesis of flavonols (and to a lesser degree anthocyanins – depending on the cultivar) is activated by exposure to UV and blue radiation (Jackson, 2008) due to the fact that they act as UV-B protectants. The concentrations of total flavonols ranged from 4.6 to 41.6 mg/L, whereas quercetin levels are between 0,3 - 21,8 mg/L. Moreover, quercetin levels in red wine found to be between 5 and 15mg/L (Hollman *et al*., 2000). High total flavonol levels appear to be associated with the use of thick-skinned grape varieties, such as Cabernet Sauvignon. The production of high-flavonol wines is linked not only with the use of very ripe, thick-skinned grapes but also with the application of modern methods of vinification (Mc Donald *et al*., 1998).



Quercetin
[3,3′,4′,5,7-pentahydroxyflavone]

Figure 13. Chemical structure of Quercetin

1.3.1.2 FLAVONES

Flavones are a large class of flavonoids and more than 4000 flavones have been identified and divided into several subclasses. Their structure is based on a 15-carbon skeleton, consisting of two phenyl rings and a heterocyclic ring. Most flavones are conjugated to a carbohydrate moiety, differing in hydroxylation, methoxylation, glycosylation, or acylation patterns. They are found in plants bound to sugars as O-glycosides. Flavones may also occur as C-glycosides. Natural flavones include Apigenin (4',5,7-trihydroxyflavone) (Fig.14), Luteolin (3',4',5,7-tetrahydroxyflavone) and Tangeritin (4',5,6,7,8-pentamethoxyflavone). Practical interest in flavones and related phenolic compounds is due to their properties, contributing not only to the taste and color of plants, but also to their positive role in a variety of biological activities, such as antioxidative (Moore *et al*., 2001), radical scavenging (Dugas *et al*., 2000; NG *et al*., 2000), anti-inflammatory (Crespo *et al*., 1999), anti-depressant (Butterweck *et al*., 2000), and assumed cancer-preventive effects (Marchand, 2002).

Apigenin

According to Gambelli (2003), apigenin found in the polyphenolic content of 10 different wines from different geographical origins of South Italy to range from 0,2 to 4,7 mg/L. However, apigenin seems to be found in such small quantity in wines that cannot be detected by HPLC (Fang *et. al*., 2007).

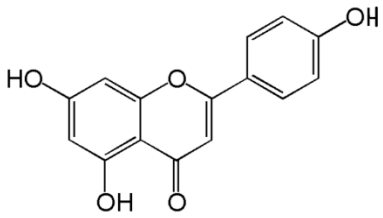


Figure 14. Chemical structure of Apigenin

1.3.2 NON-FLAVONOIDS

1.3.2.1 PHENOLIC ACIDS

Ellagic Acid

Ellagic acid (Fig. 15) is a dimeric derivative of gallic acid and is generally recognized as the hydrolytic byproduct following the release of a hexahydroxydiphenoyl (HHDP) ester group from ellagitannins, which spontaneously converts to its characteristic bislactone structure. Ellagic acid is present in many woody plants, fruits and nuts.

Ellagic acid has a highly complex molecular structure, and is essentially a dilactone of gallic acid, which is a trihydroxyl derivative that participates in the formation of hydrolysable gallotannins. The inner, older heartwood is characterized by higher levels of free ellagic acid and extracted wood residues of ellagic acid on acid hydrolysis than do residues of the outer heartwood (Peng *et al*., 1991). Ellagic acid in wine originates either from wooden containers or from the addition of enological tannins (Ribéreau-Gayon *et al*., 2006).

The concentration of ellagic acid varies among the cultivars and methods of extraction. According to Talcott (2002), the concentration of ellagic acid in juice and wine varied from 1,20 to 102 mg/L since the fermentation until storage for 60 days in temperature 20^{o}C and 37^{o}C.

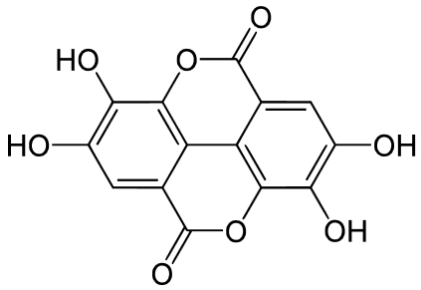


Figure 15. Chemical structure of Ellagic acid

1.3.2.2 STILBENES

Another family of more complex polyphenols is also present in grapes, wine and oak wood. Stilbenes have two benzene cycles, generally bonded by an ethane, or possibly ethylene, chain. Among these *trans*-isomer compounds, resveratrol, or 3.5,4- trihydroxystilbene (Figure 16), is thought to be produced by vines in response to a fungal infection (Langcake, 1981). Resveratrol, located in the skins, is mainly extracted during the fermentation of red wines and seems to have some healthful properties. Concentrations are on the order of 1–3 mg/l. Recent research (Jeandet *et al*., 1995; Bourhis *et al*., 1996) has identified many oligomers of resveratrol in *Vitis Vinifera* (Ribéreau-Gayon *et al*., 2006).

Resveratrol

trans-Resveratrol (3,5,4¢-trihydroxystilbene), a phytoalexin that belongs to the group of compounds known as stilbenes, is known to occur in grapes and consequently in grape products and in wine. It is abundant in grape skin and present in higher concentration in red grape varieties compared with white varieties (Sieman, 1992). *trans*-Resveratrol (Fig. 16) was originally identified as the active ingredient of an Oriental herb (Kojo-kon) used for treatment of a wide variety of diseases including dermatitis, gonorrhea, fever, hyperlipidemia, atherosclerosis, and inflammation (Careri *et al*., 2003). Several studies have shown its ability to prevent platelet aggregation in coronary arteries (Kimura *et al*., 1985) and lessen the incidence of cancer (Jang *et al*., 1997).

According to Flanzy (1998), the trans-resveratrol ranges between 0,6-10 mg/L and the cis-resveratrol ranges between 0,2-3 mg/L. However, Alonso (2002) studied the determination of antioxidant power in different wines by a new electrochemical method correlated to the polyphenolic content. According to his results, resveratrol reached the threshold of 18,92 mg/L.

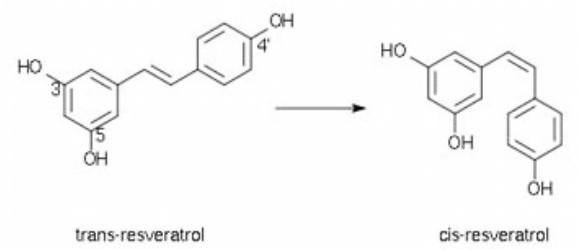


Figure 16. Chemical structure of trans-resveratrol and its isomer cis-resveratrol

1.3.3 VOLATILE PHENOLS

Phenolic acids are colorless in a dilute alcohol solution, but they may become yellow due to oxidation. From an organoleptic standpoint, these compounds have no particular flavor or odor. Coumaric and ferulic acids only are precursors of odorous volatile phenols in wine, coumaric acid generating (4-vinyl- and 4-ethyl)-phenols and ferulic acid, (4- vinyl- and 4-ethyl)-guaiacols. The vinyl derivatives, 4-vinyl-phenol and 4-vinyl-guaiacol, are formed during the alcoholic fermentation by decarboxylation of the free cinnamic acids with a cinnamate decarboxylase of *Saccharomyces cerevisiae* yeasts (Albagnac 1975; Chatonnet *et al*. 1993a,b). However, as this enzyme is inhibited by catechins and catechic tannins, abundant in red wines, the levels of volatile phenols formed in red wines are generally much lower than those in white and rosé wines, although the contents in hydroxycinnamic precursors in the corresponding red musts are higher (Chatonnet *et al*. 1993a,b).They are, however, precursors of the volatile phenols produced by the action of certain microorganisms (yeasts in the genus *Brettanomyces* and bacteria). Ethyl phenols, with animal odors, and ethyl guaiacols are found in red wines. In white wines, vinyl phenols, with an odor reminiscent of gouache paint, are accompanied by vinyl guaiacols. Thus, the vinylphenols may contribute to the aroma of white and rosé wines only. However, 4-vinylphenol seems to depreciate the aroma of white wine as soon as it is perceived by masking the fruity note, then at higher concentrations it is responsible for phenolic off-flavors (Chatonnet et al. 1993a,b).

Brettanomyces/*Dekkera* yeasts can be found in fermenting must and in wine. Typically they grow after alcoholic and malolactic fermentations during storage of wines in tanks, barrels or bottles. They contribute characteristic ‘bretty’ flavours which are described as smoky, plastic, burnt plastic, vinyl, band-aid and creosote (Chatonnet *et al*., 1992; Licker *et al*., 1999). Compounds which are responsible for ‘bretty’ flavour in a wine are mainly 4-ethylphenol, 4-ethylguaiacol and isovaleric acid (Chatonnet *et al*., 1995; Suarez *et al*., 2007). The 4-ethylphenol is present at trace quantities in oak wood but can reach values approaching their olfactory detection thresholds (605 µg/L for the former and 110 µg/L for the latter in red wines) in wines aged for lengthy periods, giving rise to unpleasant “horse stable” and “medicinal” aromas (Chatonnet *et al*. 1992b).

Guaiacol is one of the major volatile phenols that have a sensory impact on wines aged in the wood. When wines are aged in new oak barrels, the toasting of the wood involved in barrel manufacture causes the breakdown of lignin and the formation of various components in the same family, with a variety of smoky, toasty and burnt smells: gaiacol, methyl gaiacol,

propyl gaiacol, allyl gaiacol (isoeugenol), syringol and methyl syringol (Ribéreau-Gayon *et al*., 2006).

4-Ethylphenol

4-Ethylphenol is used by some wineries as an indicator compound for the activity of *Brettanomyces* (Licker *et al*., 1999). Preliminary studies show that 4-ethylphenol is formed during all the growth period of *Brettanomyces* and this compound can be used to confirm the presence of 'bretty' flavours. There are significant differences between strains of *Brettanomyces* in their ability to produce 4-ethylphenol (Fariña *et al*., 2007).

The average value of 4-Ethylphenol (Fig. 17) varies from 0,01 mg/l to 1mg/l (Flanzy, 1998). 4-Ethylphenol is present at trace quantities in oak wood but can reach values approaching their olfactory detection thresholds (605 µg/L in red wines) in wines aged for lengthy periods, giving rise to unpleasant "horse stable" aromas (Chatonnet *et al*. 1992b). Moreover, this can be confirmed from recent studies identifying a content of 4-ethylphenol at 720 µg/l (Fariña *et al*., 2007).

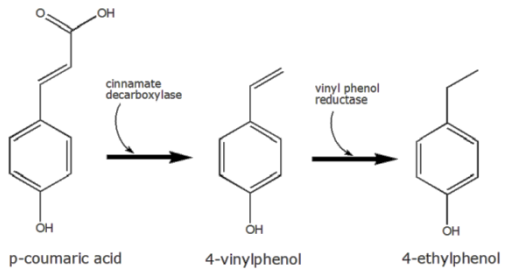


Figure 17. The production pathway of 4-Ethylphenol

Isoeugenol (Fig. 18)

The thresholds of the volatile phenols are between 420 µg/L for a 10/1 mixture of 4-vinlyphenol and 4-vinylguaiacol in white wine and 720µ g/L for a 1/1 mixture of the ethyl-phenols in red wine (Moreno-Arribas & Polo, 2009).

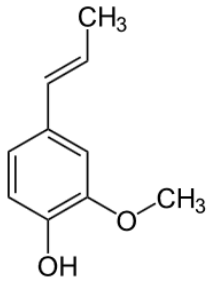


Figure 18. Chemical structure of Isoeugenol

Eugenol

Eugenol, with its characteristic odor reminiscent of cloves, is the main volatile phenol. In several studies, eugenol has been found to range between 0,02-0,03 mg/L (Diaz-Plaza *et al*., 2002; Garde *et al*., 2002). Commercial tannins, liquid flavoring and toasted chips lack almost all of the most volatile compounds, with the exception of eugenol (Fig. 19) and isoeugenol (Ribéreau-Gayon *et al*., 2006).

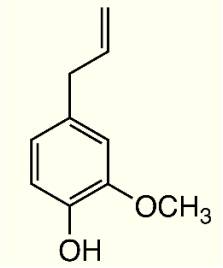


Figure 19. Chemical structure of Eugenol

Guaiacol

Eugenol and Guaiacol seem to be more stable than phenolic aldehydes, and to date no alterations during wine aging in oak barrels have been reported; hence the concentration of these compounds depends solely on the extent to which they are extracted (Pérez-Prieto et al. 2003; Gómez Plaza et al. 2004). Their concentration in wine increases over the initial months of aging and the levels off and remains virtually constant after 12 months in the barrel (Garde-Cerdan et al. 2002).

According to Flanzy (1998), the value of guaiacol (Fig.20) in wine is between 0,06 - 0,5 mg/L.

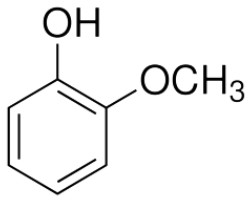


Figure 20. Chemical structure of Guaiacol

1.3.4 WOOD PHENOLS

1.3.4.1 HYDROLYZABLE TANNINS

As mentioned in 1.1.1., these tannins are easily hydrolyzed either enzymatically or in acid or base conditions to form free gallic or HHDP acid, the latter spontaneously lactonizing to give ellagic acid. Hydrolyzable tannins are classified to gallotannins and ellagitannins, according to the type of acid formed.

Ellagitannin

The ellagitannins may make up to 10% of heartwood dry weight (Scalbert *et al*., 1988) and the two most common ellagitannins in oak have been identified as vescalagin and castalagin (Mayer *et al*., 1967). Six additional water-soluble ellagitannins (roburins A-E and grandinin) have been identified (Herve du Penhoat, 1991) as they derived from vescalagin or castalagin and are either dimer and/or characterized by the addition of a pentose residue (Fig. 21). The Bate-Smith assay (Bate-Smith, 1954) is the most frequently followed method and has been shown to be specific to ellagitannins in wood extracts containing both ellagitannins and proanthocyanidins (Scalbert *et al*., 1989). The highest amounts of ellagitannins are extracted from the young outer heartwood of trees (Mayer *et al*., 1967).

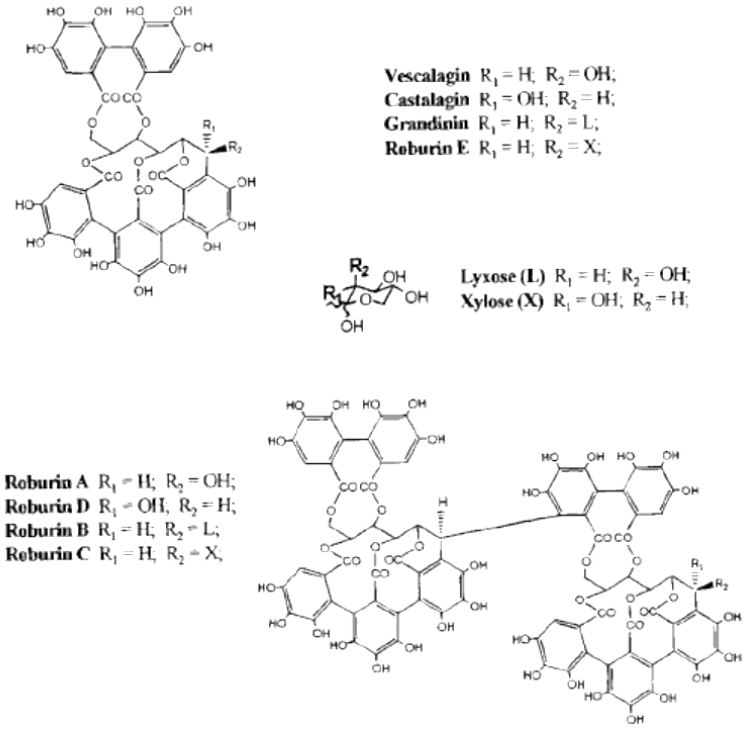


Figure 21. The structure of eight ellagitannins identified in oak heartwood (according to Puech, 1999)

In studies, it has been found that vescalagin values ranged from 6.1 to 39.9 mg/L and for castalagin the values varied from 9.7 to 45.6 mg/L, but in general, after between 16 and 40 extraction days, we observed an evident decrease of the compounds under all extraction conditions (Jordao *et al*., 2005). Moreover, ellagitannins have been found in highly variable concentrations in oak aged wines, with maximum reported values of 21 mg/L of castalagin and 7 mg/L of vescalagin (Moutounet *et al*., 1981). However, these levels are much lower than expected given the often high levels of these easily soluble compounds in oak wood due to the effect of wood treatment during the fabrication of barrels, the process of extraction from barrel staves, and the subsequent transformation of ellagitannins after or during their extraction into the wine.

The abundant ellagitannins of oak wood may increase the astringency and bitterness of wines matured in oak casks and also, they influence wine through the regulation of oxidative reactions occurring during wine aging.

1.3.4.2 AROMATIC ALDEHYDES

Guymon and Crowell (1968) and Baldwin *et al*., (1967) published work in the late 1960s which showed that aromatic aldehydes such as vanillin, syringaldehyde, coniferaldehyde, and sinapaldehyde resulted from degradation of lignin. Much research was generated which examined the content of these aldehydes and various phenolic acids in wines aged in oak (Guymon *et al*., 1968; Baldwin *et al*., 1967). Baldwin *et al*., (1967) proposed a pathway by which the byproducts of lignin ethanolysis are further oxidized to vanillin. Also, Litchev (1989) hypothesized that copper ions are involved in the non-enzymatic oxidation of lignin breakdown products and proposed a series of reactions to describe it. The conversion of aromatic acids to aldehydes with longer R groups to vanillin is very important process in the development of oak imparted flavors in wine. Therefore, several studies have been made over the different oak species.

According to several authors (Chatonnet *et al*., 1989; Artajona, 1991; Canas, 2003), untoasted wood has low contents of phenolic aldehydes (vanillin, syringaldehyde, coniferaldehyde, sinapaldehyde), arising from lignin degradation (Puech *et al*., 1989, 1990), with wood toasting being responsible for the increased amounts found (Chatonnet *et al*., 1989; Dubois, 1989; Canas, 2003). Phenolic aldehydes, however, are thermo degradable into phenolic acids or volatile phenols (Chatonnet, 1995). The toasting effect has not yet been explained fully. However, Chatonnet *et al.* (1989) claims that phenolic aldehydes reach

higher levels at medium toast, decreasing afterwards with toast intensity, but Artajona (1991) states that they continue to increase with increasing toasting level.

Syringaldehyde

Puech (1987) studied the evolution of aromatic aldehydes in wines aged in Bulgarian oak barrels, where he found that the best organoleptic result for the Cabernet Sauvignon was obtained after 18 months with Syringaldehyde content at 0.86mg/L. Martinez (2001) measured that the value of syringaldehyde in wine aged in American oak was from 9.42 to 17.26 mg/L. However, Herjavec (2005) studied the content of aromatic aldehydes in Chardonnay and Cabernet Sauvignon wine aged in Croatian oak barrels and he detected 1.97-2.96 mg/L and 0.89-2.90 mg/L of syringaldehyde (Fig. 22), respectively.

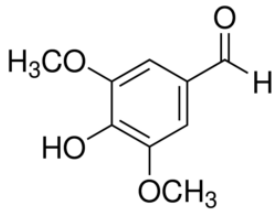


Figure 22. Chemical structure of Syringaldehyde

1.3.4.3 AROMATIC KETONES

Acetovanillone

Belongs to the phenolic ketones and is a cyclic compound containing a benzene ring. Acetovanillone (Fig. 23) has a vanilla aroma and is produced by the breakdown of lignin (Moreno & Peinado, 2012). Carillo (2005) studied 14 volatile compounds in aged wines of different origin, vintage year and maturation time in oak barrel, and he found that acetovanillone ranged between 0.07-3.8 mg/L. Whereas, Martinez-Gil (2012) studied if the extract's volatile components can be transferred to grapes and then to wines in petit Verdot vineyards that were treated at veraison with a commercial aqueous French oak extract. He measured that acetovanillone ranged from 0.009 to 0.2 mg/L (Martinez-Gil *et al*., 2012).

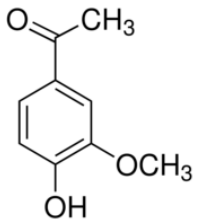


Figure 23. Chemical structure of Acetovanillone

1.3.4.4 FURANIC ALDEHYDES

Cellulose, hemicellulose and lignin (the most important polymers present in natural oak) are fragmented by the effect of the high temperatures, generating compounds with a toasty flavour, such as furfural derivatives (furfural, 5-methylfurfural and furfuryl alcohol) (Carillo *et al*., 2005).

Furfural

Carillo (2005) studied 14 volatile compounds in aged wines of different origin, vintage year and maturation time in oak barrel. He detected that furfural (Fig. 24) varied among 0.03-1.5 mg/L. Moreover, a new method was studied to determine the content of volatile compounds in wine by Diaz Plaza (2002). At this study, furfural was the second most abundant compound in these three wines, but it is not found above its olfactory perception threshold 14.1 mg/L (Chatonnet *et al*., 1992).

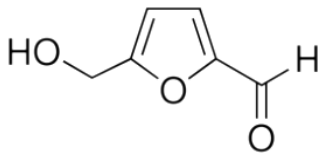


Figure 24. Chemical structure of 2-furfuraldehyde

1.3.4.5 COUMARINS

Coumarins may be considered derivatives of cinnamic acids, formed by the intramolecular esterification of a phenol OH into the *α* of the carbon chain. These molecules are components of oak, either in glycosylated form (esculin and scopoline) in green wood or in aglycone form (esculetin and scopoletin) in naturally seasoned wood. Although very small quantities (a few µg/L) of coumarins are found in wood-aged wine, they still affect its organoleptic characteristics, as glycosides are bitter and aglycones are acidic, with a detection threshold in red wine of 3 µg/L (Ribéreau-Gayon *et al*., 2006).

Scopoletin

The coumarins not present in grapes may be present in wines on account of ageing in oak barrels. Coumarins are benzo-pyr-2-ones, present in either free or glycosylated form. The aglycone basic portion (3.I) is either (a) umbelliferone, which has R Ό H or (b) aesculetin, has R Ό OH; as (c) scopoletin, OCH_3. The glycosides are named aesculin at C_6 and scopolin at C_7 (Clarke & Bakker, 2004). According to Tricard (1987), scopoletin was tested as an indication of storage in oak barrels. In all wines examined, scopoletin (Fig. 25) content increased with time storage from 9.5 µg/L at 3 months to 33 µg/L at 9 months in red wine stored in a new barrel , and from 7.5 to 27 µg/L in the same wine but in a 2 years-old barrel. In most of the samples scopoletin increased with the period of storage Tricard *et al*., 1987).

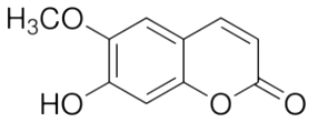


Figure 25. Chemical structure of Scopoletin

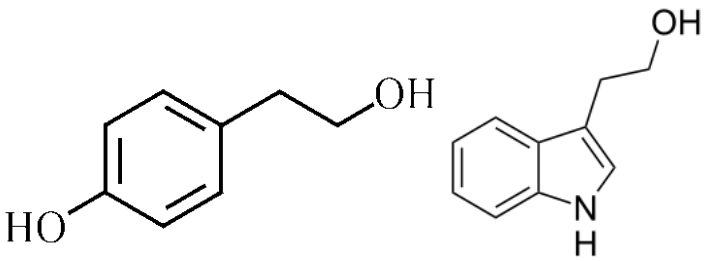
1.3.5 OTHER PHENOLS

1.3.5.1. NON-VOLATILE AROMATIC ALCOHOLS

The amino acid profile may influence the order in which the different amino acids are taken by the yeast, which in turn would influence the ratio of secondary metabolites produced and, thus can be related to the aromatic profile of the wine.

Tyrosol & **Tryptophol**

Tyrosol (Fig. 26) is included in the group of non-volatile compounds (Ribéreau-Gayon & Sapis, 1965). It is always present in both red and white wine and it ranges between 20–30 mg/L. The concentration of the phenolic alcohol tyrosol and of the non-phenolic alcohol tryptophol, compounds formed during yeast fermentation from tyrosine [3-(4-hydroxyphenyl)-alanine] and tryptophan [2-amino-3-(3-indolyl)- propionic acid], respectively (Barcenilla *et al*., 1989) presented a slight decrease during the first months of aging in the bottle, as shown by Monagas (2005). However, this compound remains at relatively constant concentrations throughout aging, is accompanied by other non-phenolic alcohols like tryptophol (0–1 mg/L) (Ribéreau-Gayon *et al*., 2006). According to Flanzy (1998), the mean concentration of tyrosol is between 1.7-80 mg/L and for tryptophol is between 0.9-18 mg/L.



(1) (2)

Figure 26. Chemical structure of (1) tyrosol and (2) tryptophol

1.3.5.2 VOLATILE AROMATIC ALCOHOLS

Alcohols with more than two carbon atoms are commonly called higher or fusel alcohols. They may be present in healthy grapes, but seldom occur in significant amounts. Significant higher alcohols from grapes that survive fermentation are 2-ethyl-1-hexanol, benzyl alcohol, 2-phenylethanol, 3-octanol, and 1-octen-3-ol. However, most higher alcohols found in wine are byproducts of yeast fermentation. Their synthesis closely parallels that of ethanol production. They commonly account for about 50% of the aromatic constituents of wine, excluding ethanol.

Higher alcohols may originate from grape-derived aldehydes, by the reductive denitrification of amino acids, or via synthesis from sugars. The relative importance of those sources appears to vary with the specific fusel alcohol. Amino acid deamination is especially important in the generation of longer chain higher alcohols (Chen, 1978). Additional higher alcohols may come from the metabolic activity of spoilage yeasts and bacteria. Occasionally, pleasant smelling higher alcohols may be produced by microorganisms.

Higher alcohols also play an indirect role in the development of an aged wine bouquet. By reacting with organic acids, they add to the number of esters found in wine. During fermentation, the production of esters occurs rapidly under the control of yeast enzymes. Certain esterification reactions continue during aging, but at a much slower, nonenzymatic pace (Rapp & Güntert, 1986). However, the level of 2-phenylethanol has been reported to be related both to grape variety and yeast metabolism (Gomez-Plaza *et al*., 1999).

Several researches have indicated the presence of higher alcohols on Muscat varieties (Dieguez *et al*., 2003). 2-phenylethanol and benzyl alcohol have been identified in the glycosidically-bound franction of terpenes. Even though, according to Fenoll *et al*. (2009) bound benzyl alcohol was more abundant than bound 2-phenylethanol, both were present at higher levels than their free forms.

2-phenylethanol (phenethyl alcohol)

This compound is the most important phenol-derived higher alcohol. It is produced during fermentation and gives a rose odor. According to Flanzy (1998), the range of the 2-phenylethanol is between 10-180 mg/L.

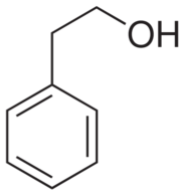


Figure 27. Chemical structure of 2-phenylethanol

Benzyl alcohol

Benzyl alcohol (Fig. 28) is used as a solvent in the resin base (epichlorhydrine and bisphenol A) and hardeners (aromatic amine) incorporated in epoxy resin linings (Blaise, 1986). If the vat lining is not correctly applied, residual benzyl alcohol from the resin may migrate after polymerization and penetrate into the wine, where it is oxidized to form the benzoic aldehyde responsible for this organoleptic defect of “bitter almond” flavor (Ribéreau-Gayon *et al*., 2006).

In wines produced by Baga variety was detected 0.71 mg/L (Rocha *et al*., 2004). Furthermore, Guedes Gomes (1974) reported benzyl alcohol content in samples of port wine ranging from 0.25 to 3.2 mg/L.

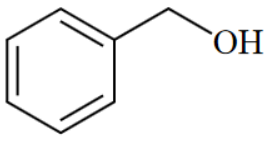


Figure 28. Chemical structure of Benzyl alcohol

1.4 PURPOSE OF THE PRESENT STUDY

Chemical compounds in the wine may interfere to the estimation of proanthocyanidins by the vanillin assay. Ascorbic acid and chlorophyll can interfere, but they can be easily extracted ((Broadhurst & Jones, 1978; Sun *et al*., 1998; Sun, 1999). However, Sun proved that vanillin does not react with anthocyanins, phenolic acids and flavonols at λmax 500nm. However, there are several substances that exist in grapes and wine without being tested for a possible reaction with the vanillin assay. Therefore, our goal is to verify our hypothesis that no interference substances can show reaction with vanillin and to ensure the accuracy of the modified vanillin assay. Thus, it can be used as a quantitative estimation method limitating the parameter interference substances.

2. MATERIALS AND METHODS

2.1 MATERIALS

Vanillin was obtained by ACROS ORGANICS (New Jersey, USA) with purity 99%. Methanol used as a solvent was obtained by FISHER SCIENTIFIC UK Limited (Leicestershire, UK). Sulfuric acid H_2SO_4, used as an acid solution, was furnished by SIGMA-ALDRICH (St Luis, USA). The interference substances tested with the vanillin assay are demonstrated on the table 1.

Table 1. Interference Substances and their origin

Chemical Compounds	Company	Origin
Quercetin	Merck	Darmstadt, Germany
Apigenin	Sigma Chemicals C.O.	India
Ellagic acid	Fluka Chemie AG	Buchs, Switzerland
Resveratrol	Sigma Chemicals C.O	Germany
4-Ethylphenol	Fluka Chemie AG	Buchs, Switzerland
Isoeugenol	Fluka Chemie AG	Buchs, Switzerland
Eugenol	Fluka Chemie AG	Buchs, Switzerland
Guaiacol	Merck	Darmstadt, Germany
Commercial Tannins	Enartis	San Martino, Italy
Syringaldehyde	Acros Organics	New Jersey, USA
Acetovanillone	Safc	USA
Furfural	Acros Organics	New Jersey, USA
Scopoletin	Acros Organics	New Jersey, USA
Tyrosol	Extrasynthese	Genay, France
Tryptophol	Sigma Chemicals C.O.	St. Luis, USA
2-Phenylethanol	Acros Organics	New Jersey, USA
Benzyl Alcohol	Carlo Erba Reagenti	Milano, Italy

UV/VIS Spectrophotometer UNICAM was used to read the possible reactions with vanillin at the wavelength of 500nm due to the fact the colored product formed from the reaction with vanillin absorbs at 500 nm.

2.2 METHOD

The total volume of reaction was fixed at 6 ml in a tube. It was composed by 1 ml of sample, 2.5 ml of reagent a (1% w/v vanillin solution in methanol) and 2.5 ml reagent b (sulfuric acid solution 25% v/v in methanol). Two tubes were used, one with the sample and one as a blank, which prepared on the same way except of the addition of reagent a (1% w/v vanillin solution in methanol).

The sample (or standard) was prepared in methanol due to the fact that the color is more stable and the sensibility is higher in a medium free of water (Sun *et al.,* 1998; Sun, 1999). For each interference compound, there were taken three different concentration values and each one measured twice, and thus we had two replicates for each concentration value. The concentration decided to be tasted in low, medium and high rates (Table 2.), according to several studies that have been determined the range of each compound in the wine (Section 1.3). Commercial tannins used instead of ellagitannins. For this reason, the concentration rates multiplied 10 times more than the normal value of ellagitannin found in wine.

Table 2. Concentration rates A, B, C for each interference substance

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Flavonols	Quercetin	1	10	25
Flavones	Apigenin	0.05	1.5	5
Phenolic Acids	Ellagic Acid	1	50	150
Stilbens	Resveratrol	0.1	5	15
Volatile Phenols	4-Ethylphenol	0.005	0.1	5
	Isoeugenol	0.005	0.02	1
	Eugenol	0.005	0.02	1
	Guaiacol	0.01	0.1	2
Hydrolyzable Tannins	Ellagitannins	5	50	100
Aromatic Aldehydes	Syringaldehyde	0.05	1.5	5
Aromatic Acetones	Acetovanillone	0.005	1	5
Furanic Aldehydes	Furfural	0.005	1	15
Coumarins	Scopoletin	0.005	0.025	0.05
Non-Volatile Aromatic Alcohols	Tyrosol	1	40	85
	Tryptophol	0.5	10	20
Volatile Aromatic Alcohols	2-Phenylethanol	1	50	200
	Benzyl Alcohol	0.05	1	5

After the preparation of the reagents and the final sample, the blank and the sample were pured in two cells and put in the UV/VIS Spectrophotometer. Due to the fact that methanol is easily volatile; the cells used for measuring absorbance of vanillin reaction were the ones with stoppers. The possible reaction of vanillin was measured at the 500nm in the UV/VIS Spectophotometer under room temperature 30 °C.

3. RESULTS

The presence of interfering substances was studied on their reaction with vanillin. Interference substances consider the representative compounds of classes and/or families that are present in grapes and wine. The UV/Vis spectrophotometer measures the intensity of light passing through the sample and compares it to the intensity of light passing through the blanc at λmax equal to 500nm. The absorption rate was shown on graphs, which indicates if there is a reaction or not.

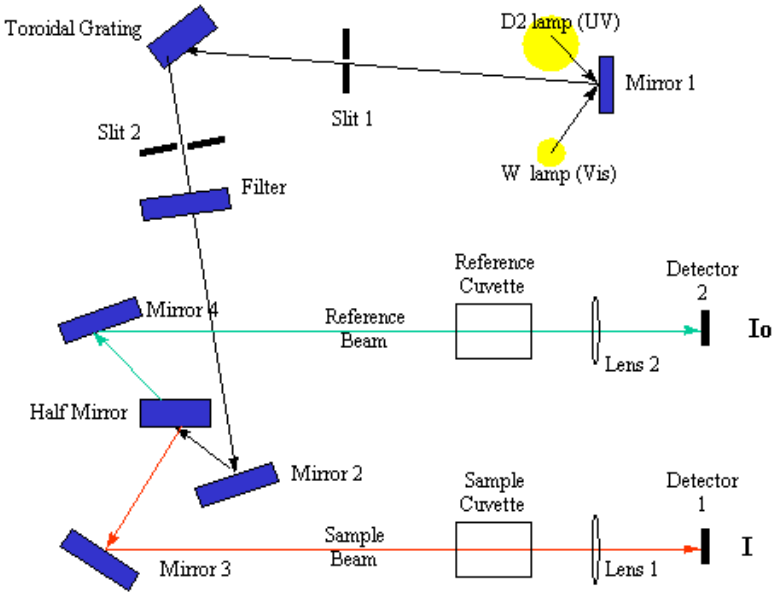


Figure 29. Mechanism of the UV/VIS Spectophotometer

3.1 FLAVONOIDS

FLAVONOLS

Quercetin was used as a control for the vanillin assay (Table 3). According to Sun (1999), quercetin does not react with the vanillin. In the present work, we used the quercetin to verify the non-reaction and the correct application of the process based on the modified vanillin assay, which was proposed by Sun (1999).

Table 2. Quercetin verified no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Flavonols	Quercetin	1	10	25
	Results	**No reaction**	**No reaction**	**No reaction**

FLAVONES

Apigenin is the major representative compound of flavones. In the present work, it showed no reaction with the vanillin at any concentration rate (Table 4).

Table 3. Apigenin showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Flavones	Apigenin	0.05	1.5	5
	Results	**No reaction**	**No reaction**	**No reaction**

3.2 NON-FLAVONOIDS

PHENOLIC ACIDS

Ellagic acid, which is converted to ellagitannin, was measured for a possible reaction. The results taken form the UV/Vis spectrophotometer showed no reaction with the vanillin (Table 5).

Table 4. Ellagic acid showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Phenolic Acids	Ellagic Acid	1	50	150
	Results	**No reaction**	**No reaction**	**No reaction**

STILBENES

Resveratrol represented the class of stilbenes. It did not illustrate any reaction with vanillin at any concentration rate (Table 6).

Table 5. Resveratrol did not illustrate any reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Stilbenes	Resveratrol	0.1	5	15
	Results	**No reaction**	**No reaction**	**No reaction**

3.3 VOLATILE PHENOLS

4-Ethylphenol, Isoeugenol, Eugenol and Guaiacol are the major representative compounds of volatile phenols in the wine. They showed no reaction with the vanillin at any concentration rate.

Table 6. Volatile phenols did not illustrate any reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Volatile Phenols	4-Ethylphenol	0.005	0.1	5
	Results	**No reaction**	**No reaction**	**No reaction**
	Isoeugenol	0.005	0.02	1
	Results	**No reaction**	**No reaction**	**No reaction**
	Eugenol	0.005	0.02	1
	Results	**No reaction**	**No reaction**	**No reaction**
	Guaiacol	0.01	0.1	2
	Results	**No reaction**	**No reaction**	**No reaction**

3.4 WOOD PHENOLS

HYDROLYZABLE TANNINS

The commercial tannins used to represent the ellagitannin seemed to have no reaction with vanillin at 500nm (Table 8).

Table 7. Ellagitannin showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Hydrolyzable Tannins	Ellagitannins	5	50	100
	Results	**No reaction**	**No reaction**	**No reaction**

AROMATIC ALDEHYDES

Syringaldehyde is a major compound of aldehyde found in wine. Syringaldehyde did not seem to react with the vanillin (Table 9).

Table 8. Syringaldehyde showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Aromatic Aldehydes	Syringaldehyde	0.05	1.5	5
	Results	**No reaction**	**No reaction**	**No reaction**

AROMATIC KETONES

Acetovanillone is a representative compound of aromatic ketones. It showed no reaction with the vanillin at any concentration rate (Table 10).

Table 9. Acetovanillone showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Aromatic Ketones	Acetovanillone	0.005	1	5
	Results	**No reaction**	**No reaction**	**No reaction**

FURANIC ALDEHYDE

The furanic aldehyde class is represented by the furfural. The results of the UV/Vis Spectrophotometer showed no reaction with vanillin at any concentration rate (Table 11).

Table 10. Furfural showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Furanic Aldehydes	Furfural	0.005	1	15
	Results	**No reaction**	**No reaction**	**No reaction**

COUMARINS

Scopoletin present in wines belongs to the coumarins. It was observed no reaction with vanillin at any concentration rate (Table 12).

Table 11. Scopoletin showed no reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Coumarins	Scopoletin	0.005	0.025	0.05
	Results	**No reaction**	**No reaction**	**No reaction**

3.5 OTHER PHENOLS

NON-VOLATILE AROMATIC ALCOHOLS

Tyrosol and Tryptophol are chemical compounds that represent the aromatic alcohols. Tyrosol seems to give no reaction with vanillin, whereas tryptophol showed a possible reaction with vanillin on the higher concentrations (Table 13). The reaction might have occurred due to the fact that the tryptophol used for the trial was an old compound kept in the laboratory and it might have been contaminated form previous uses.

Table 12. Tyrosol showed no reaction and tryptophol shows possible reaction with vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Non-Volatile Aromatic Alcohols	Tyrosol	1	40	85
	Results	**No reaction**	**No reaction**	**No reaction**
	Tryptophol	0.5	10	20
	Results	**No reaction**	**Possible Reaction**	**Possible Reaction**

VOLATILE AROMATIC ALCOHOLS

2-Phenylethanol and Benzyl alcohol represent the volatile aromatic alcohols. These chemical compounds seemed to have no reaction with the vanillin at any concentration rate (Table 14).

Table 13. 2-Phenylethanol and Benzyl Alcohol did not seem to react with the vanillin at 500nm

Chemical Class	Chemical Compounds	Concentration A (mg/L)	Concentration B (mg/L)	Concentration C (mg/L)
Volatile Aromatic Alcohols	2-Phenylethanol	1	50	200
	Results	**No reaction**	**No reaction**	**No reaction**
	Benzyl Alcohol	0.05	1	5
	Results	**No reaction**	**No reaction**	**No reaction**

These findings suggest that almost all the above chemical compounds showed no reaction with vanillin at any concentration level and at the wave length of 500nm. Nevertheless, it was also shown that tryptophol seemed to have a possible reaction with vanillin. Due to the fact that it is difficult to find a scientific chemical explanation of the reaction, we reached the conclusion that this reaction could be caused by a contamination of the chemical compound, which had been used several times in the past during its keeping in the laboratory.

4. DISCUSSION

Vanillin assay is widely used for the quantification of condensed tannins or its monomer units in sorghum grains (Burns, 1971 from Price, 1978). This method is based on the ability of flavanols to react with vanillin in the presence of mineral acid to produce a red pigment (Awika *et al*., 2005). On the other hand, redox-based colorimetric methods such as the Folin-Denis or Prussian blue methods detect any phenol content (Price *et al*., 1978) and thus, are less specific. In 1978, Price, van Scoyoc and Butler proposed the vanillin-HCl method, which used the hydrochloric acid as the acidic medium, in sorghum grains. However, Sun *et al*. (1998) examined in grapes and wine, the parameters to optimize the vanillin assay and among them the influence of acid nature and concentration due to the fact that the vanillin reaction with catechins or proanthocyanidins should be carried out at acid medium. The evidence from this study suggested the use of sulfuric acid, which gave higher sensibility than using the hydrochloric acid and thus, Sun *et al*. (1998) reached a conclusion to a modified vanillin assay (Chapter 1.2). Moreover, according to Sun *et al*. (1998), the influence of interfering non-flavanol compounds, and more specifically phenolic acids (cinnamic acid, *p*-hydroxybenzoic acid, caffeic acid, gallic acid, *p*-coumaric acid, syringic acid), flavonols (quercetin dihydrate, kaempferol, myricetin, rutin) and the anthocyanin malvidin-3-glucoside, were examined at the same experiment and the results showed that neither of them interfered to the vanillin reaction with catechins or proanthocyanidins.

The present study was designed to determine the reactivity of vanillin with other possible interfering substances, which represent major families and/or classes present in wine. Non-flavanol phenolic compounds were examined, and in particular compounds belong to the class of phenols. The phenolic compounds tested are flavonols and flavones from the flavonoids, stilbenes and phenolic acids from the non-flavonoids, volatile phenols, wood phenols such as ellagitannins, coumarins, aldehydes and other compounds such as tyrosol and 2-phenylethanol.precisely flavonols and flavones from the flavonoids, stilbenes from the non-flavonoids, volatile phenols, wood phenols such as ellagitannins, coumarins, aldehydes and other compounds such as tyrosol and 2-phenylethanol (Table 2). The results of this study indicate that none of the phenolic compounds did react with vanillin; apart from tryptophol. The reaction vanillin-tryptophol is considered as a possible contamination of the chemical compound used. The method followed the proposal of the modified vanillin assay (Sun *et al*., 1998) and was applied separately for every chemical compound (Table 1). On the other hand, the chemical compounds represent wider chemical groups of families present in grapes and/or in wine. According to Sun *et al*. (1998), one sample may contain some unpredictable interfering substances. Our answer to this doubt is related with the major

chemical compounds that can be found in wine. We may reach the conclusion that their families, do not interfere to the vanillin assay, and nor interfered with vanillin reaction with catechins or proanthocyanidins. The present study confirms previous findings and contributes additional evidence that ensures the accuracy of the modified vanillin assay.

The vanillin assay is used for the estimation of condensed tannins and its reaction is based on the reaction with phenolic rings; phloroglucinol, resorcinol, catechol, pyrogallol, or phenol (Scalbert, 1992). Vanillin has the ability to react with A-ring of catechins or proanthocyanidins, which have a phloroglucinol or resorcinol ring, in an acidic medium (Fig. 12) and forms a colored product. This is a typical condensation reaction between aldehyde and A-ring of flavanols. Therefore, vanillin reacts with the specific chemical structure of flavan-3-ol (Fig. 6), but not with different chemical structures such as flavones (apigenin Fig. 14) or volatile phenols (4-Ethylphenol Fig. 18) despite the fact that all belong to the major class of phenols.

This research has thrown up many questions in need of further investigation. The current study has only examined the hypothesis of interference substances to react with the vanillin assay and was proved to be true as none of the substances react with vanillin. However, the chemical explanation for the non-reaction between these chemical compounds with the vanillin at the acid medium and wavelength 500nm has remained unknown. More information on the chemical field would help us to establish a greater degree of accuracy on this matter.

Taken together, the findings of the present work helped to enhance the reliability of the modified vanillin assay (Sun *et al*., 1998) and its use as a quantitative method of total catechins, total oligomeric and total polymeric proanthocyanidins in grape and wine samples without any major analytical interference.

5. BIBLIOGRAPHY

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