Chapter 6

Flavonoid Phenolics in Red Winemaking

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Additional information is available at the end of the chapter

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Abstract

This chapter reviews the chemical diversity of flavonoid phenolics in grapes (*Vitis vinifera* L.) with impact on the sensory properties of red wines. Anthocyanins, flavan-3-ols, tannins, and polymeric pigments are discussed from a chemical, technological, and sensory perspective. Anthocyanins, responsible for the color of red wines, reach a peak of extraction after 4 or 5 days of maceration, followed by a decrease in their concentration as maceration progresses. Flavan-3-ols and oligomeric tannins from skins are responsible for bitterness and extracted within the first days of maceration, whereas extraction of seed-derived tannins requires longer maceration times. Matrix effects, including the presence of anthocyanins, polysaccharides, and other cell-wall components affect the rate of retention of tannins into wine. Polymeric pigments, bearing astringent and bitter properties different from those of intact tannins, are formed from covalent reactions between anthocyanins and tannins, putatively accounting for the changes in mouthfeel and textural properties of red wines during maceration and aging. Different maceration techniques applied during red wine production affect the rate, quantity, and the chemical composition of wine phenolics. Understanding of the factors that modulate phenolic retention into wine should allow the winemaker to adjust maceration variables to meet stylistic and/or commercial specifications.

Keywords: flavonoid, phenolic, anthocyanins, flavan-3-ols, tannins, polymeric pigments, maceration, sensory analysis

1. Introduction

The term "phenolics," however overarching, generally bears a positive connotation for grape growers and winemakers alike. In spite of the use (and abuse) of the concept that touts phenolics as naturally occurring, health-promoting compounds in plant-derived food and beverages, it is in wine, like in perhaps no other beverage, where this term has been so widely

discussed both in the popular press and in academia [1–4]. So this begs the question: why all the hype surrounding phenolics in wine? Perhaps the first reason stems from the fact the most phenolics bear color. Color in food and beverages have always captivated human beings. Louis Pasteur, the prominent French chemist and microbiologist, used the term "wine color" a whopping 119 times in its seminal treatise "*Etudes sur le vin*" [5]. Pigments were among the first organic compounds studied in wine, perhaps as an unconscious acknowledgement to the fact that it is color, through the sense of vision, the first attribute human beings appraise when approaching a glass of wine. The term "*oenin*" to characterize the grape anthocyanin malvidin-3-glucoside, one of the main pigments found in grapes and wines, was first used in 1915 [6]. The amphoteric nature of anthocyanins and their pH-dependent colored forms was recognized as early as 1806: "The colouring matter of the Alicant raisin is the same as that of the red fruits and common red wines; it has the singular property of becoming red by the acids; although blue by nature, it becomes green with the alkalis…" [7]. Tannins, which contribute indirectly to wine color, were also discussed by Pasteur [5] and later in 1895, by E. Manceau, then a scientists at Möet et Chandon (France) who published a method to study tannins in, expectedly, Champagne wines [8].

Another possible explanation for the early interest in the study of phenolics in wine is the fact that specific phenolic compounds were early recognized as determinants of the flavor and mouthfeel properties of red wines. The tactile sensation of astringency and the taste sensation of bitterness in red wines were recognized, again, by Pasteur [5], but no link to phenolics was made at the time. Later in 1958, E.C. Bate-Smith, a prominent British phytochemist, stated that tannins in wines were responsible for the "liquoring properties and body" of wines and were "intimately" concerned with the perception of quality [9]. Interest on the sensory aspects of phenolic compounds in wines quickly sparkled a series of studies on how to maximize the extraction of phenolics into wine during winemaking.

Indeed, Eugene W. Hilgard, a German-born UC-Berkeley professor, conducted perhaps the earliest studies on the effect of different processing techniques during the fermentation of red grapes in California (USA). His findings, though made between 1885 and 1890, were accurate and were confirmed decades later using much more advanced analytical tools. Hilgard noted that, for example, during red winemaking "maceration[1] of the wine on the pomace after fermentation increases tannins but adds nothing to color" [10]. Hilgard also noted that "it is quite certain that, according to the method of fermentation used, the extraction of the pomace and the consequent tint of the wine may seriously differ" [11]. This chapter expands on these later thoughts, i.e., the factors that underpin the extraction and retention of phenolic compounds into wine, along with the chemical and sensory implications they brought about to the finished wines. Before discussing how phenolics are extracted during red winemaking, it is

[1]Maceration is a crucial step during red winemaking whereby the fermentation solids (skins, seeds, lees, and eventually stems) are kept in contact with the fermenting must/wine. It is during maceration that phenolic compounds, free aroma, and aroma precursors are extracted into wine. Winemakers also refer to maceration as "skin contact time" or "maceration time."

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pertaining to present a succinct classification and occurrence of phenolic compounds in grapes and wines as well as an overview of the factors that underpin phenolic reactivity in wines.

2. Classification, occurrence, and general reactivity

The term "polyphenol," sometimes also referred to as phenol or *flavonoid*, encompasses about 4000 bioactive compounds of plant and fungal origin which have more than one aromatic phenol ring within the structure as opposed to *nonflavonoids* or simple phenols [12, 13]. Phenolic compounds are very reactive in wines due to the high electron density of the aromatic ring (s), which is enhanced by the presence of one or more electron-donating hydroxyl groups [12, 14]. As a result, the electron density at the *-ortho* and *-para* positions activates the carbons on those sites with partial negative charges, thus enabling the structure to undergo electrophilic substitutions at these positions (**Figure 1**). The reactivity of the phenol ring is further increased by the weak acidic character of the hydroxyl group that can donate a proton (H^+) due to the partial migration of electrons from the oxygen in the hydroxyl to the aromatic ring [14]. In addition, the pK_a of the most acidic phenolic hydrogen is above 7, which explains why polyphenols are easily deprotonated forming phenolate ions at physiological pH. Phenolate ions can donate electrons directly to oxygen, thereby forming reactive semiquinones [15]. However, at pH values between 3 and 4, such as the ones normally encountered in red wines, phenols are largely protonated and as such cannot directly donate electrons to oxygen [15–17].

Phenols can also readily participate in noncovalent interactions with various components of the wine matrix. The hydroxyl groups can act both as H-bond donors or acceptors. The phenol group(s) are also amphiphilic agents, as the hydroxyl groups are hydrophilic but the aromatic ring is hydrophobic, allowing the structure to become engaged in hydrophobic interactions as well [12]. Another distinctive property of phenolics is that, upon variation in the matrix

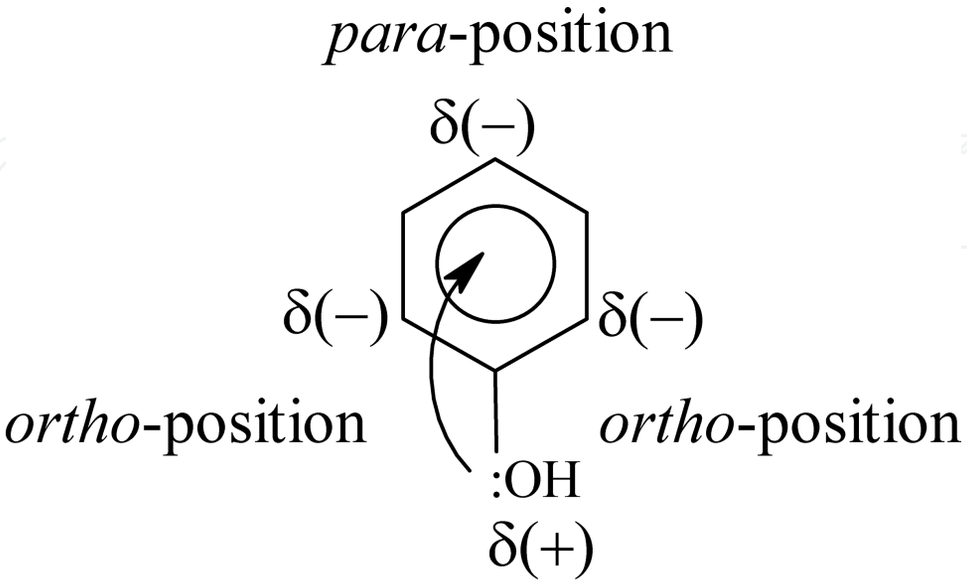

Figure 1. Activated sites of the phenol ring. Redrawn from Ref. [14].

conditions such as pH [14] or oxidation-reduction potential [18], phenolics can act both as electrophiles (i.e., electron-loving molecules) and nucleophiles, thus readily reacting with electron-rich or electron-deficient compounds, respectively (**Figure 2**).

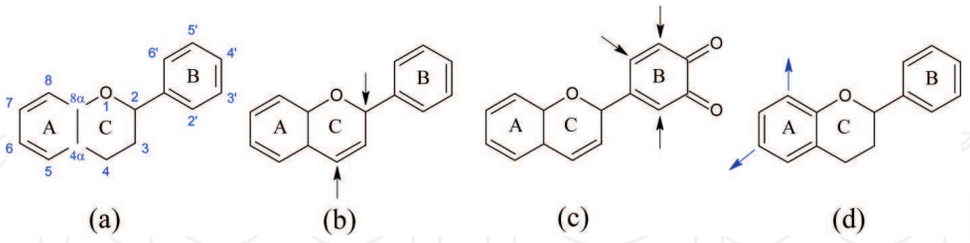

Figure 2. (a) Basic *flavonoid* ring structure and numbering, (b and c) sites for electrophilic substitutions, and (d) sites for nucleophilic substitutions. Redrawn from Refs. [13, 25].

For the purpose of this chapter, occurrence, reactivity, chemical structure, and sensory properties of phenolic compounds are discussed exclusively in the context of *Vitis vinifera* L. grapes and wines. Also, only the most important polyphenols of the *flavonoid* group, namely anthocyanins, flavan-3-ols and tannins, as well as their reaction products, will be discussed. Phenolics belonging to the *nonflavonoid* class include benzoic (e.g., gallic, hydroxibenzoic, protocatechuic, vanillic, and syringic acids) and cinnamic acid derivatives (*p*-coumaric, cafeic, ferulic, and sinapic acids). Also, the hydroxylated stilbenes are included in the *nonflavonoid* class, of which *trans*- and *cis*-resveratrol (3,5,4-trihydroxystilbene), as well as their glucose derivatives (*trans*- and *cis*-piceids), have all been identified in grapes and wines [19, 20]. Although quantitatively much less important than the *flavonoid* class, *nonflavonoids* phenolics play a direct role in both chemical and coupled-enzymatic oxidation reactions in white and red wines [21, 22]. Cinnamic acids can act as copigments,[2] inducing changes in color through the phenomenon of copigmentation[3] [23] and can also impact wine aroma when metabolized by yeast of the genera *Brettanomyces/Dekkera* to generate volatile ethyl-phenols [24].

Phenolic *flavonoids* possess a three-ring system, composed of 15 carbon atoms in the form C6-C3-C6 [12, 13]. The central C ring contains oxygen forming a pyran ring that can adopt various oxidation states, and it is fused to two aromatics rings, termed A and B (**Figure 2a**). In the *flavonoid* family, the B-ring is fused to the pyran ring at position 2. The A ring is derived from the phloroglucinol structure and is the most conserved portion of the C6-C3-C6 backbone. Furthermore, different substitutions in the B ring define different compounds within a family. The members of the *flavonoid* class found in grapes and wines all have the same substitution pattern of hydroxyl groups at carbons 5 and 7 of the A ring. On the other hand, differences in the oxidation state and substitution on the C ring define the different *flavonoid* classes [13]. Thus,

[2]Copigments are typically noncolored phenolic and/or nonphenolic compounds able to engage in copigmentation reactions with anthocyanins.

[3]As defined by Boulton [34], the phenomenon of copigmentation is due to molecular associations between pigments and other (usually non-colored) organic molecules in solution. These associations cause the pigments to exhibit far greater color than would be expected from their concentration. When anthocyanins engage in copigmentation, both hyperchromic (increase of absorbance) and bathochromic (shift of absorbance toward blue hues) are normally observed.

anthocyanins represent the highest oxidation state of the C ring, and on the other extreme, flavan-3-ols represent the most reduced state of the said ring.

From a chemical and sensory standpoint, the three most relevant phenolic classes within the *flavonoid* family are flavan-3-ols (the "building blocks" of tannins), anthocyanins, and tannins. A heterogeneous family of reaction products resulting from the reaction between anthocyanins and tannins, the so-called polymeric pigments, are not originally present on grapes but formed during winemaking operations and wine aging (Section 3.4). This chapter will focus on the chemical and sensory aspects of flavan-3-ols, anthocyanins, tannins, and polymeric pigments in red wines, providing along the way an overview of their extraction patterns during red winemaking.

3. Chemical, sensory aspects, and extraction of phenolic flavonoids during red winemaking

3.1. Anthocyanins

3.1.1. Occurrence, general chemistry, and sensory aspects

Anthocyanins occur as vacuolar components in the berry skin tissue (and in the mesocarp of the so-called teinturier varieties) and are present as monomers of six glycosylated forms, namely malvidin, cyanidin, petunidin, peonidin, delphinidin, and pelargonidin [26, 27]. Glycosylation occurs at the C3 position and renders the molecule water-soluble [28]. Acylation in turn occurs in the C6 position of the glucose moiety by esterification with an aromatic (*p*-coumaric, caffeic, ferulic, and sinapic acids) or an aliphatic acid (acetic, malic, malonic, oxalic, and succinic acids) (**Figure 3**). The acylation of the sugar might promote the chemical stability of the anthocyanin molecule [29], possibility through stacking of the acyl groups with the pyrilium ring of the flavylium cation, thereby reducing the susceptibility to the nucleophilic attack of water [30].

Anthocyanins are red pigments responsible for the color of red wines and owe their spectral properties to the resonant structure given by a 10-electron system, partially delocalized between the pyran C and A rings, as well as to the extended conjugated system of unsaturated bonds in the structure [13, 31]. As a result, the maximum absorbance (λ_{max}) of these compounds varies between 475 and 540 nm depending upon the aglycone (known as anthocyanidin) and substitution patterns of the C ring [26]. The λ_{max} between 475 and 540 nm confers anthocyanins blue to red color hues. *O*-methylation in aglycones such as malvidin, petunidin, and peonidin causes a slight reddening effect (**Figure 4**) and reduces the reactivity of the nearby phenolic hydroxyl groups, thereby increasing the stability of the molecule [32]. On the other hand, the increase in the number of free hydroxyl groups in the B ring increases blueness (i.e., bathochromic shift), which in turn renders the structure more polar but less stable. Acylation has also been suggested to produce a bathochromic shift, giving more purple tones, possibly as a result of intramolecular copigmentation reactions [30]. These and other features can be observed in **Figure 4** for purified anthocyanins isolated from Cabernet Sauvignon grapes after preparative HPLC fractionation of anthocyanins and confirmation by mass spectroscopy.

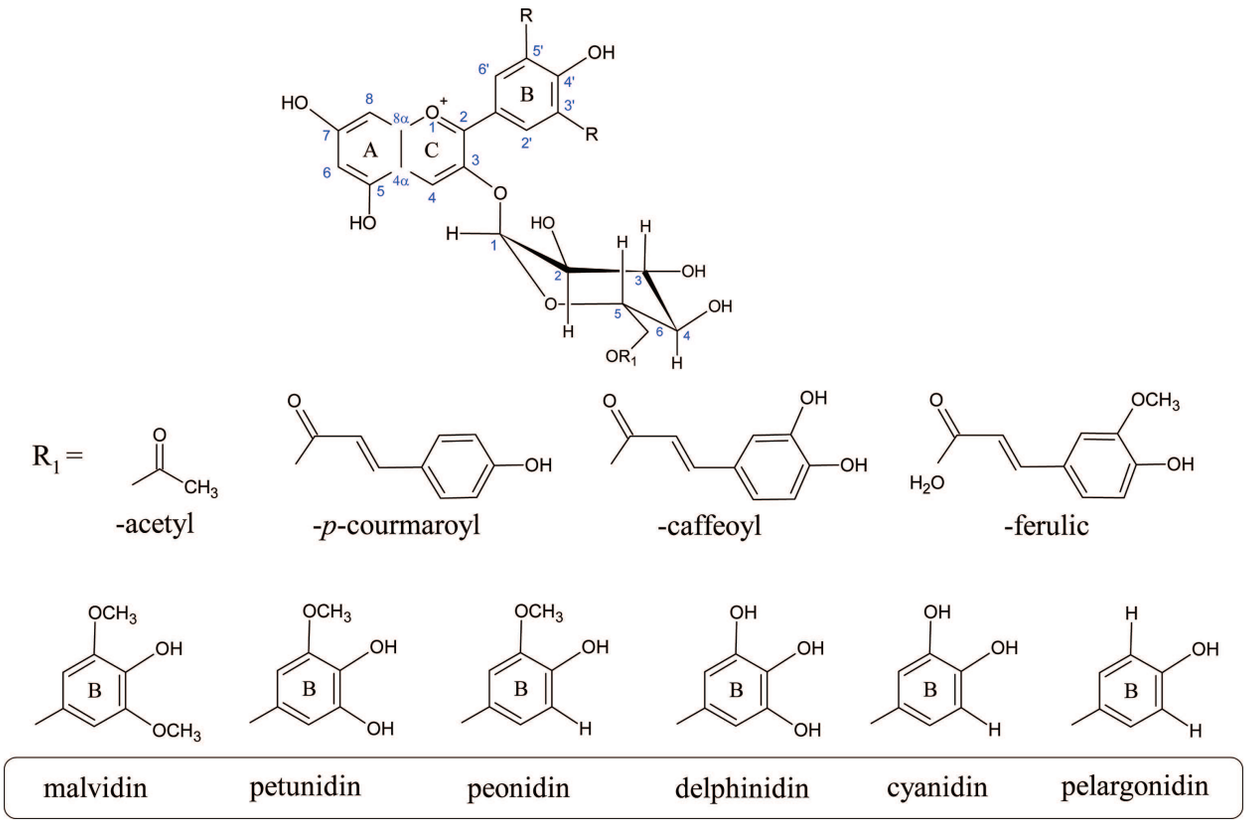

Figure 3. Basic anthocyanin structure, acyl groups, and the main six anthocyanindins found in *V. vinifera* grapes and wines.

Figure 4. Preparative HPLC chromatogram showing separation of anthocyanin monoglucosides and acyl-glucosides of Cabernet Sauvignon grapes from Washington State (USA). Anthocyanin fractions are shown approximately above of each major peak and were collected directly from the elutant at pH ~ 1.8. Peak assignment: (1) delphinidin-3-*O*-glucoside, (2) cyanidin-3-*O*-glucoside, (3) petunidin-3-*O*-glucoside, (4) peonidin-3-*O*-glucoside, (5) malvidin-3-*O*-glucoside, (6) delphinidin-3-*O*-acetyl-glucoside, (7) petunidin-3-*O*-aceyl-glucoside, (8) peonidin-3-*O*-acetyl-glucoside, (9) malvidin-3-*O*-acetyl-glucoside, (10) malvidin-3-(6-*O*-caffeoyl)-glucoside, (11) petunidin-3-(6-*O*-coumaroyl)-glucoside, (12) peonidin-3-(6-*O*-coumaroyl)-glucoside (shoulder), and malvidin-3-(6-*O*-coumaroyl)-glucoside.

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The flavylium cation of the anthocyanins is responsible for the chromatic properties of young red wines, with a molar extinction coefficient (ε) of 29,500 M^{-1} cm^{-1} for malvidin-3-glucoside in 0.1% HCl methanolic solution [33]. As a result of this, some of the color observed in aged red wines can still be attributable to the flavylium cation. However, upon crushing and during winemaking, anthocyanins undergo a variety of electrophilic and nucleophilic substitutions giving rise to cycloaddition and condensation products, and oligomeric and polymeric pigments (Section 3.4 and **Figure 13**). These transformations invariably have an impact on wine color, and it is usually during maceration and postmaceration when the most noticeable changes in wine color take place (**Figure 5**). For example, at the beginning of maceration, the intense purple-red color and hyperchromic shift (i.e., increase in absorbance) observed in red wines is the result of intra- and intermolecular and self-association copigmentation reactions [34]. Subsequently, with the disruption of copigmentation mediated by increasing ethanol levels, perceived color decreases and shifts toward more red tones [35]. As maceration winds up and aging ensues, the incorporation of anthocyanins into vitisins A, B, vinyl-catechol derivatives, and xanthylium salts, to name some possible reactions that lead to the formation of the so-called pyranoanthocyanins (**Figure 13**), causes a shift in color from deep-red to orange or brick-orange hues [36]. It is also expected that the incorporation of anthocyanins into

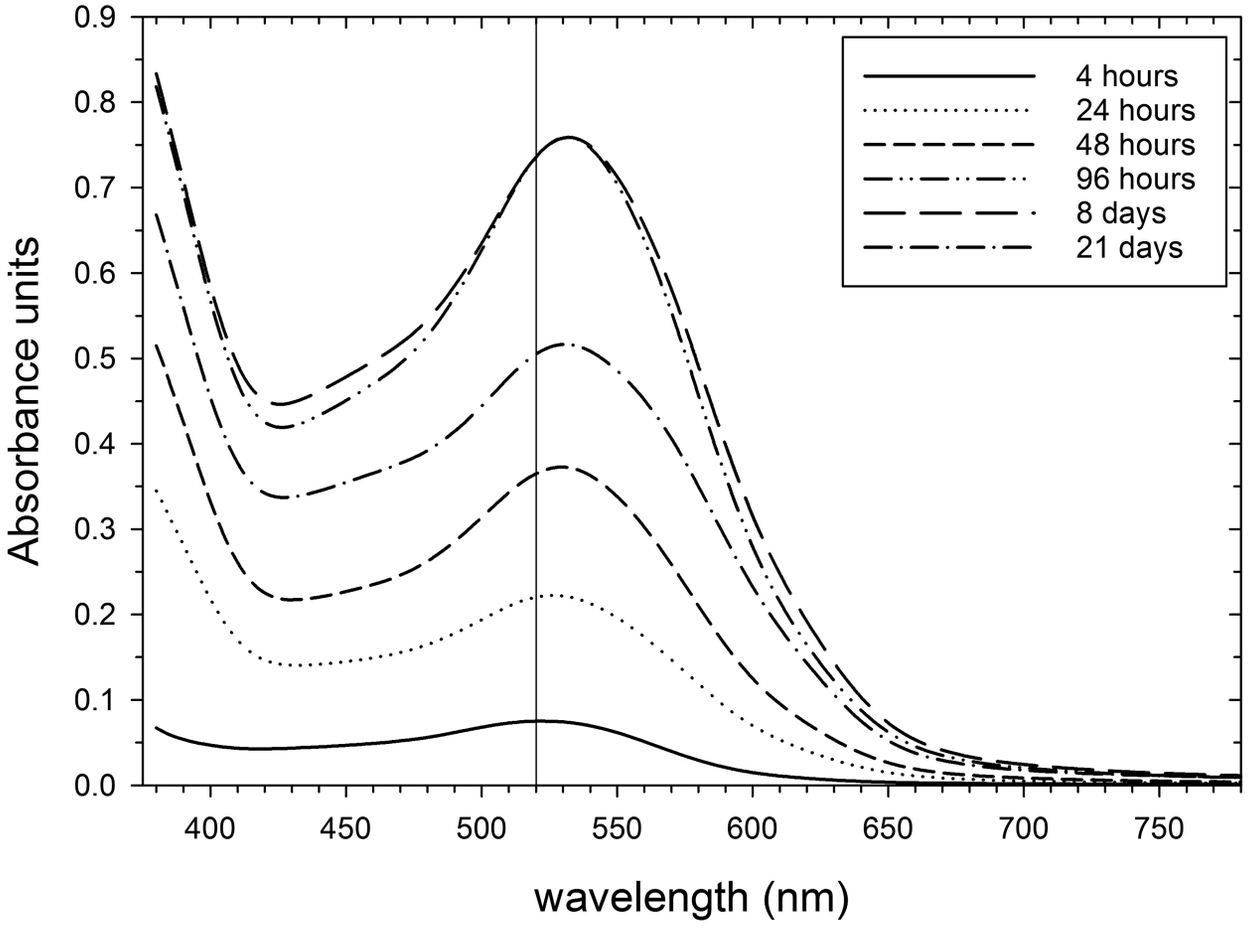

Figure 5. Evolution of the full-length visible spectrum of a Malbec wine produced with different maceration times, ranging from 4 hours to 21 days. The vertical line indicates the 520-nm wavelength, which is approximately the wavelength of maximum absorbance for most anthocyanins. Notice the drop in the absorbance values of the full spectrum in the wine produced with 21 days maceration relative to the wine produced with 8 days of maceration.

oligomeric and polymeric tannins (to form the so-called polymeric pigments) should cause a reduction in the molar extinction coefficient of the flavylium form, thus leading to a decrease in color saturation as winemaking progressed [37]. In the case of pyranoanthocyanins, these do not necessarily appear to have lower molar extinction coefficients relative to the native anthocyanins [38], and, in fact, their molar extinction coefficient is much more stable toward pH swings [39].

Isolated anthocyanins are tasteless or indistinctly flavored [40]; however, upon reaction with oligomeric or polymeric tannins during winemaking, oligomeric and polymeric pigments are formed (**Figure 14**) and these can in turn modulate astringency (Section 3.4).

3.1.2. Extraction during winemaking

The diffusion of anthocyanins into the must requires the breakdown of two biological barriers, namely the cell wall, including the degradation of the pectic substances in the middle lamella, and the tonoplast of the vacuoles of the skin subepidermal cells [41]. Normal operations during crushing ensure the breakdown of cellular walls, and native enzymatic reactions allow for the degradation of pectic substances and polysaccharides in the middle lamella. The diffusion process is favored by the water-soluble nature of anthocyanins, resulting in maximum extraction rates and a peak of extraction within the first 3 to 7 days of maceration [35, 42–48] as observed in **Figure 6**. The rate of extraction of the different anthocyanins seems to be similar [45, 47] and proportional to their original concentration in grapes. However, some studies have found that wines have a relative higher amount of malvidin-3-glucoside than that originally present in the grapes [49, 50]. Establishment of unequivocal extraction and retention patterns of different anthocyanin forms may be complicated by the fact that acylated anthocyanins may undergo acid hydrolysis upon extraction into the fermenting must/wine [50], thereby releasing the monoglucoside forms and/or by the fact that, for example, acylated derivatives are preferentially adsorbed by wine lees [51].

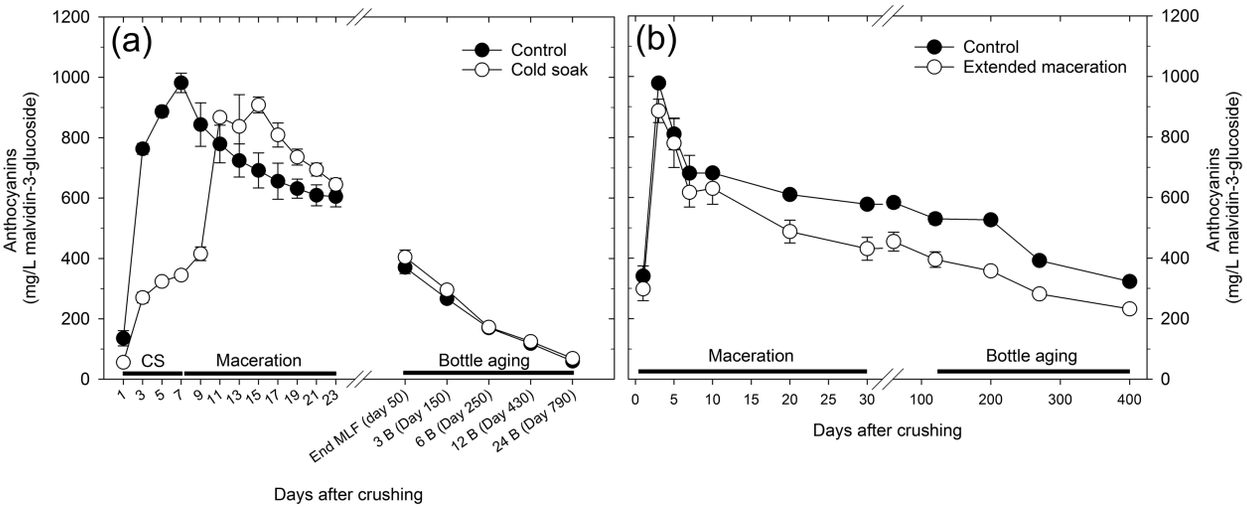

Figure 6. Overview of the extraction of and evolution of anthocyanins during (a) prefermentative cold soak (CS), maceration, end of malolactic fermentation (end MLF), and up to 24 months of bottle ageing (B) of Malbec wines processed with a maceration length of 15 days (control) and with cold soak for 7 days + 15 days of maceration (cold soak), and (b) maceration and bottle aging of Cabernet Sauvignon wines processed with a maceration length of 10 days (control) and 30 days (extended maceration). Adapted from Refs. [43, 55].

The early extraction of anthocyanins may also influence the solubility and retention of oligomeric and polymeric tannins through the formation of polymeric pigments [52–54]. Following the peak of anthocyanin extraction, a variable drop in concentration, which can be as high as 60% from peak concentration, is typically observed [42, 43, 55] (**Figure 6a**). Loss of anthocyanins during maceration in fermenting must and wines has been attributed to a variety of factors, including ionic adsorption by the negatively charged yeast cell walls and yeast lees during postmaceration [56, 57], adsorption onto bitartrate crystals and particulate matter [35], incorporation into small and large polymeric pigments [43, 58], formation of pyranoanthocyanins [56, 59], and oxidative cleavage of the heterocyclic C-ring leading to direct anthocyanin degradation [60]. A decrease in copigmentation as a result of an increasing concentration of ethanol in the fermenting must (which increases the hydrophobic character of the medium thereby disrupting the copigmentation complex) also contributes to both the loss of anthocyanins and a decrease in wine color [34, 35, 61]. At the end of maceration, the levels of anthocyanins recovered in the wine, relative to the grape initial content, have been reported to be around 40% [26].

Counter to what intuition may suggest, there appears to be a negative relationship between maceration length and anthocyanins retained in the resulting wine [43, 47, 58, 62, 63] (**Figure 6b**). Moreover, analysis of anthocyanins recovered in the pomace hardly increased the recovery yield, suggesting that a major proportion of anthocyanins were converted to other species or were irreversibly adsorbed on the solid material during maceration [26, 47]. This discrepancy was originally attributed to the enhanced formation of polymeric pigments in wines undergoing extended maceration. One study found that Merlot wines produced with extended maceration were lower in anthocyanins but higher in polymeric pigments relative to control wines [58]. However, the formation of polymeric pigments during extended maceration cannot fully explain the observed anthocyanin loss [47]. This suggests that, in addition to anthocyanin losses by adsorption, degradation reactions may be at play during extended maceration. Ultimately, the extraction patterns during winemaking and aging and thus the final concentration of anthocyanins are both modulated by the variety and the maceration technique the winemaker decides to put in place, which can be as simple as delaying the onset of alcoholic fermentation (e.g., the technique known as cold soak[4]) or increasing maceration time (e.g., extended maceration) (**Figure 6a** and **b**, respectively).

3.2. Flavan-3-ols

3.2.1. Occurrence, general chemistry, and sensory aspects

In *V. vinifera* grapes and wines, flavan-3-ols occur both in seeds and skins as five monomers: (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (+)-epigallocatechin, and (-)-epicatechin-3-*O*-gallate [64–66] (**Figure 7**). The glycosyl derivatives of four of these monomers have also been

[4]Cold soak, also known as prefermentative cold soak, is a winemaking technique in which the onset of alcoholic fermentation is delayed by keeping the must (and thus the fermentation solids) at temperatures ranging from 5 to 15°C by means of a cooling system in jacketed fermenters or by the use of solid CO_2 (dry ice). During cold soak, the extraction of water-soluble compounds is sought, as opposed to the extraction that takes place in the presence of ethanol during alcoholic fermentation. The duration of cold soak is defined by the winemaker and it can be as short as 2 days and as long as 14 days.

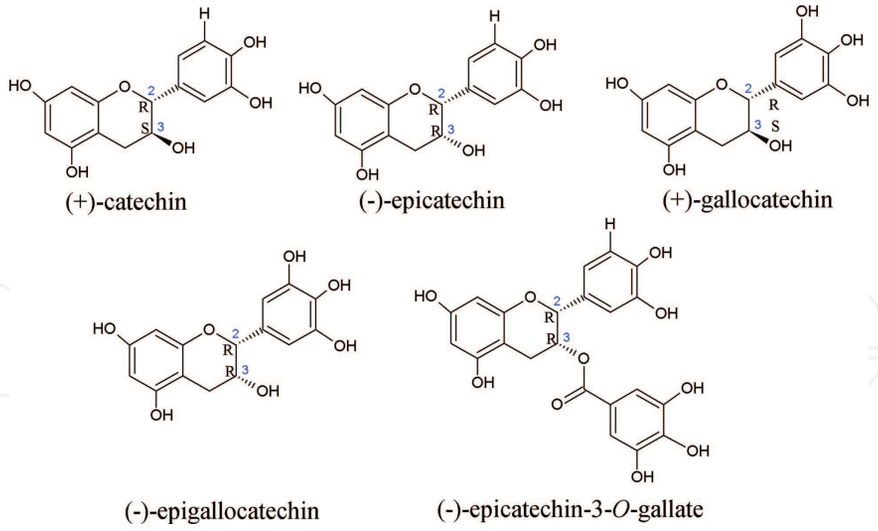

Figure 7. Monomeric flavan-3-ols found in *V. vinifera* grapes and wines.

recently reported in Merlot grapes and wines [67]. Flavan-3-ols occur in several isomeric forms. The carbons at the C2 and C3 positions of the flavan-3-ol backbone are two asymmetric centers, such as the five monomeric flavan-3-ols are grouped into two diastereomers pairs, with configurations 2*R*:3*S* for (+)-catechin and (+)-gallocatechin and 2*R*:3*R* for (-)-epicatechin, (-)-epigallocatechin, and (−)-epicatechin-3-*O*-gallate [68]. These different isomeric configurations, in turn, have an impact on bioavailability [69], antioxidant and radical scavenging properties [70], and, ultimately, on sensory properties [71, 72], as further discussed later.

In seeds, flavan-3-ols are located in thin-walled cells between the external cuticle and the inner lignified layers. The (sometime observed) seed browning during berry ripening is thought to be the result of both monomeric flavan-3-ols and tannins undergoing oxidation [73]. Seeds contain only (+)-catechin, (−)-epicatechin, and (-)-epicatechin-3-*O*-gallate [64, 74]. In the skins, flavan-3-ols occur in the subepidermal cell as shapeless or spherical inclusions free in the vacuoles but also associated with the tonoplast [75]. Skins contains (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, and, additionally, (-)-epigallocatechin [76], as well as trace amounts of (+)-gallocatechin and (-)-epigallocatechin gallate [66, 77].

Quantitative differences also occur within the berry tissues. Seeds concentrate the vast majority of flavan-3-ols of the berry [66, 75–79]. For example, flavan-3-ol concentrations of about 179 mg/kg fresh weight (FW) have been found in Cabernet Sauvignon seeds whereas skins of the same variety only have 4.8 mg/kg FW [80]; similar results are reported for other varieties [81, 82]. In wines, the content of monomeric flavan-3-ols varies from 29 to 41 mg/L in Tempranillo and Graciano wines [78], 107 to 176 mg/L in Pinot Noir wines [83], 182 mg/L in Tannat wines [84], and up to 288 mg/L in Cabernet Sauvignon wines [43].

Due to the reduced state of the C ring of the flavan-3-ol structure, and thus favorable one-electron donation properties, flavan-3-ols can react with several wine electrophiles. The condensation of monomeric flavan-3-ols with anthocyanins either by a direct covalent reaction between them [85, 86] or mediated by acetaldehyde [87, 88] is one of the main reactions with impact on color during wine aging and is further addressed in Section 3.4.

Flavan-3-ols are colorless and do not absorb light in the visible spectrum, but have a peak of UV absorbance between 270 and 280 nm [89, 90]. Catechin and epicatechin are both susceptible to enzymatic [91] and nonenzymatic oxidation [92, 93], with both mechanisms resulting in a change in spectral properties from colorless to yellow hues [89, 94]. The end products of these reactions are quinones. Quinones are powerful electrophiles that can readily react with wine nucleophiles such as sulfur compounds (e.g., volatile thiols, common in Sauvignon Blanc wines) and hydrogen sulfide, thus decreasing their volatility and their influence on wine aroma [95, 96].

Flavan-3-ols have defined taste attributes. Flavan-3-ols have a marked bitter taste, and their influence on the development of the bitterness sensation was recognized as early as 1966, when Rossi and Singleton isolated an ether-soluble fraction from grape seeds containing (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate [97]. Addition of this fraction at 200 mg/L to a white wine showed no contribution to astringency but significantly increased bitterness. Later, it was found that the chiral difference between the two main wine flavan-3-ols produces a significant difference in temporal perception of bitterness: (-)-epicatechin is significantly more bitter and had a significantly longer duration of bitterness than (+)-catechin [71, 98, 99]. The more planar conformation of the C ring of (-)-epicatechin compared with the less planar (+)-catechin may facilitate the diffusion of the molecule to the gustative receptor; in addition, the higher lipophilicity of epicatechin (relative to catechin) could also explain its higher comparative bitterness [71, 100].

Although it is assumed that both (+)-catechin and (-)-epicatechin cannot precipitate proteins, protein-induced precipitation of these flavan-3-ols has been confirmed by peptide models of salivary proline-rich-proteins (PRPs) whereby PRPs did indeed interact with flavan-3-ols having masses below 500 Da [101, 102]. Further confirmation was reported in model wine studies whereby a time-intensity sensory procedure found both (+)-catechin and epicatechin to elicit astringency [98, 103]. Also, (-)-epicatechin-3-*O*-gallate and (+)-catechin were found to precipitate PRPs when the molar ratio of flavan-3-ols to protein exceeded 27 [104]. In Cabernet Sauvignon wines, the perception of astringency was at least partially explained by the simultaneous occurrence of a comparatively higher concentration of flavan-3-ols and tannins [105]. Astringency of monomeric flavan-3-ols may be the result of cooperative precipitation of, or cooperative binding with, proteins due to the presence of one 1,2-dihydroxy or 1,2,3-trihydroxy groups [103, 106], and this may be enhanced by the presence of tannins [105]. These studies highlight the potential role of flavan-3-ol monomers on astringency perception in red wines.

3.2.2. Extraction during winemaking

Release of flavan-3-ols from skins occurs early during winemaking, within the first 2 or 3 days of skin contact [107–110]. For example, levels of (+)-catechin and (-)-epicatechin after 5 days of skin contact represented between 80 and 85% of the maximum content attained later at pressing in Grenache wines [60]. In Tempranillo and Grenache-Graciano blends, the release of flavan-3-ols occurred early, between days 3 and 5 of maceration, and remained unchanged during a postmaceration period of 1 week followed alcoholic fermentation [109]. In this same study, (-)-epigallocatechin, only located on the skins, was extracted rapidly, suggesting that the

extraction of flavan-3-ols and small oligomers from skins occurs during the first days of maceration [109].

Release of flavan-3-ols from seeds requires longer maceration times. For example, the maximum extraction of flavan-3-ols from seeds occurred after 2 to 3 weeks of maceration in model wines, and under those extended conditions, the seeds contributed almost 90% of the total flavan-3-ol content of the final wines [108]. Also, in model wines containing only seeds of the variety Monastrel, the levels of (+)-catechin and (-)-epicatechin increased from 5 mg/L at day 2 to 27 mg/L at day 10 [111]. This represents a slower rate of extraction relative to that of flavan-3-ols from skins. Moreover, longer maceration times, favored by the winemaking technique known as extended maceration, enhance the overall extraction of flavan-3-ols and, more specifically, that of epicatechin-3-*O*-gallate from seeds [43] (**Figure 8b**). Other studies have also reported that the percentage contribution of galloylated subunits increases along with maceration time [109, 111–113], and this, together with the content of epigallocatechin (exclusive of the skins), has been used as a surrogate to estimate the percentage contribution of seeds (and skins) to the overall wine's flavan-3-ol and tannin content [114].

Since extraction of flavan-3-ol from seeds occurs toward the end of a regular maceration period (e.g., 15 days), the extraction of both flavan-3-ols and tannins from seeds was assumed to be mediated by the dissolutive effect of ethanol on the lipidic outer coat of the seeds, which will typically occur toward the end of the fermentation process [115]. From this perspective, higher ethanol levels should selectively favor the extraction of seed phenolics in general and that of flavan-3-ols in particular [48, 110]. However, model wine experiments have shown that extraction of both flavan-3-ols and tannins can occur in the absence of ethanol, and that the role of ethanol may be to solely increase the rate of extraction [111]. Current evidence supports the notion of maceration length, and not alcohol, as being one of the main drivers of seed tannin extraction, at least under typical final ethanol concentrations (13–14.5%, v/v) [47, 116]. Thus, an alternative explanation for the late release and extraction into wine of seed flavan-3-ols and tannins is that extraction from the seeds may only occur after the seeds had attained maximal hydration. In studying seed extraction in model wine solutions, and noticing a lag phase

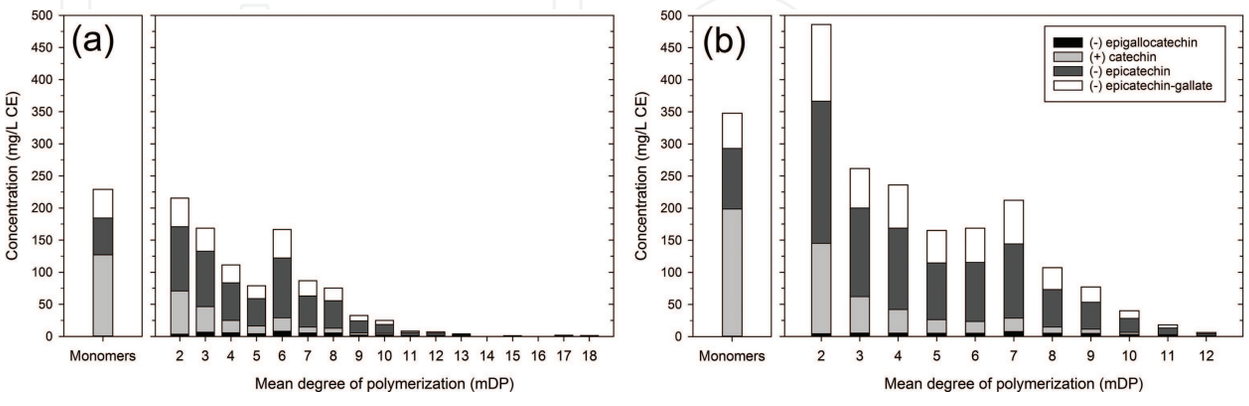

Figure 8. Monomeric flavan-3-ol concentration and composition and tannin size distribution by concentration and composition in Cabernet Sauvignon wines grouped as a function of the maceration length treatment. (a) Control wines (10 days of maceration) and (b) extended maceration wines (30 days of maceration). CE: catechin equivalents. Adapted from [43].

during the time course of maceration, Singleton and Draper noted that "…The lag may be an expression of the fact that most cells do not surrender their constituents for extraction while they are still living. Swelling and osmotic-pressure phenomena may also be involved…" [117]. If this is accepted, then the onset of seed tannin and flavan-3-ol extraction will occur once seeds have reached full hydration, whereby the leakiness of the parenchyma cells outside the true seed coat would allow the prompt release of flavan-3-ols [111, 118].

3.3. Tannins

3.3.1. Occurrence, general chemistry, and sensory aspects.

Tannins, also known as proanthocyanidins, and/or *condensed* tannins,[5] encompass oligomeric (degree of polymerization ≤ 2 and < 5) and polymeric flavonoids (degree of polymerization ≥ 5) made up by the five monomeric flavan-3-ols shown in **Figure 7** [12, 119]. As defined by Bate-Smith and Swain (1962), tannins are "water soluble compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins" [120]. Haslam [12] further noted that tannins could also form complexes with polysaccharides and that molecular weights as high as 20,000 can be found in nature. These annotations certainly pertain to grapes and wines. Wine tannins readily interact with native or yeast-derived polysaccharides [121, 122], and this interaction can modulate sensory properties such as astringency [121, 123] (**Figure 9**). Furthermore, based on the degree of polymerization, molecular weights as high as 5000, 5200, and 22,000 have been reported in seed, wine, and skin extracts, respectively, from *V. vinifera* [124], highlighting the polymeric nature of grape and wine tannins.

Oligomeric and polymeric tannins are defined by the connectivity and nature of the interflavanic bond. This refers to a covalent connection between two flavan-3-ols, also referred as "subunits"; the *average number* of constitutive subunits in the oligomer or polymer (assuming a normal distribution of tannin sizes as a function of molecular weight) is referred to as degree of polymerization. A technique based on HPLC analysis, known as phloroglucinolysis, allows for the calculation of the so-called mean degree of polymerization (mDP) [125].

Two types of interflavanic bonds have been observed. Interflavanic bonds connecting either the carbon at position 4 in the extension subunit and the carbon at position 8 in the terminal subunit (C4 → C8), or the carbon at position 4 in the extension subunit and the carbon at position 6 in the terminal subunit (C4 → C6), are collectively known as B-type linkages [12]. The flavan-3-ol subunits can also be connected by two single bonds, one between C4 and C8, and another between the hydroxyl groups at C7 and C2, forming an ether bond known as A-type linkage [126, 127]. The occurrence of these three types of bonds is not uniformly distributed within grape and wine tannins. The C4 → C8 bonds are found in skins, seeds, and wines [68]; however, C4 → C6 and A-type linkages are formed as a result of rearrangements and intra and/or intermolecular oxidation reactions during winemaking [128].

[5]The relevant literature reviewed here uses several terms to refer to red wine tannins, including "tannin," "condensed tannin," and "proanthocyanidin." The term "tannin" will be used throughout this review.

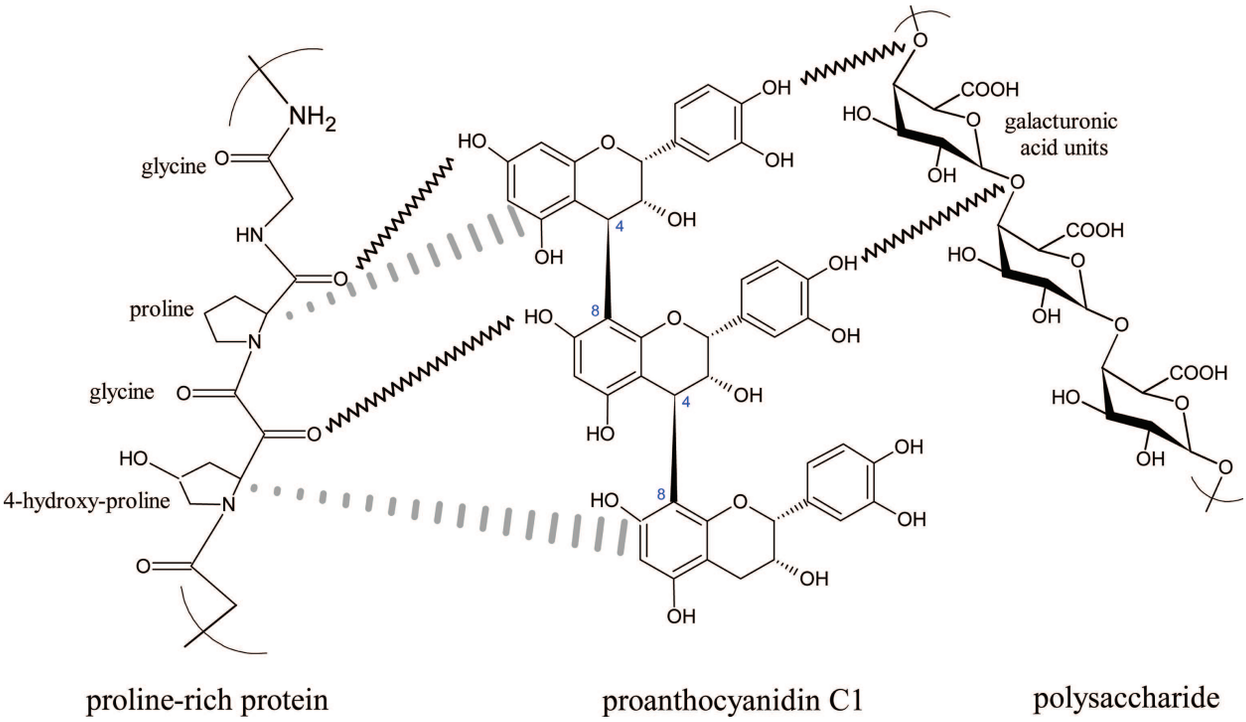

Figure 9. Interaction between proline-rich proteins, a tannin trimer, and a polysaccharide fragment. Narrow black saw-like lines are suggested sites of hydrogen bonding. Wide gray lines are suggested regions of hydrophobic interactions. Redrawn and modified from Ref. [123].

The three connectivities described (C4 → C8, C4 → C6, and C7 → C2) can, in turn, display the usual two stereochemistries of monomeric flavan-3-ols, with the *R* stereochemistry referred to as α and the *S* stereochemistry as β [129]. As a result of the enantiomeric and conformational properties of the flavan-3-ols subunits, the number of possible structures increases in a factorial fashion as the number of subunits in the oligomer or polymer increases. For example, considering only the C4 → C8 linkages, about 10^5 unique tannin structures have been estimated to exist.

Oligomeric tannins include tannin dimers, trimers and tetramers of different connectivities. B-type dimers isolated from grapes and wines are composed of (+)-catechin and (-)-epicatechin and include the dimers B1 (epicatechin-(4β→8)-catechin), B2 (epicatechin-(4β→8)-epicatechin), B3 (catechin-(4α→8)-catechin), and B4 (catechin-(4α→8)-epicatechin) (**Figure 10**). Moreover, dimers having a C4 → C6 connectivity (B5 to B8 series) and epigallocatechin have also been observed [130]. Likewise, the α and β dimers B1, B2, B3, and B4 of epicatechin-3-*O*-gallate and either catechin or epicatechin have also been identified in seeds, skins, and wines [131–133]. Dimers, trimers, tetramers, and pentamers containing A-type bonds were recently found in grape seeds of both white and red *V. vinifera* varieties [127]. Tannin trimers found in grapes and wines consist of three flavan-3-ol units linked by two C4 → C8 interflavan bonds (C-type) or one C4 → C6 interflavan bond in the terminal or extension subunits, and one C4 → C6 interflavan bond in the remaining subunit (T-type) [130, 134, 135]. A nonexhaustive account of the most common connectivities in dimers and trimers observed in *V. vinifera* grapes and wines is shown in **Figure 10**.

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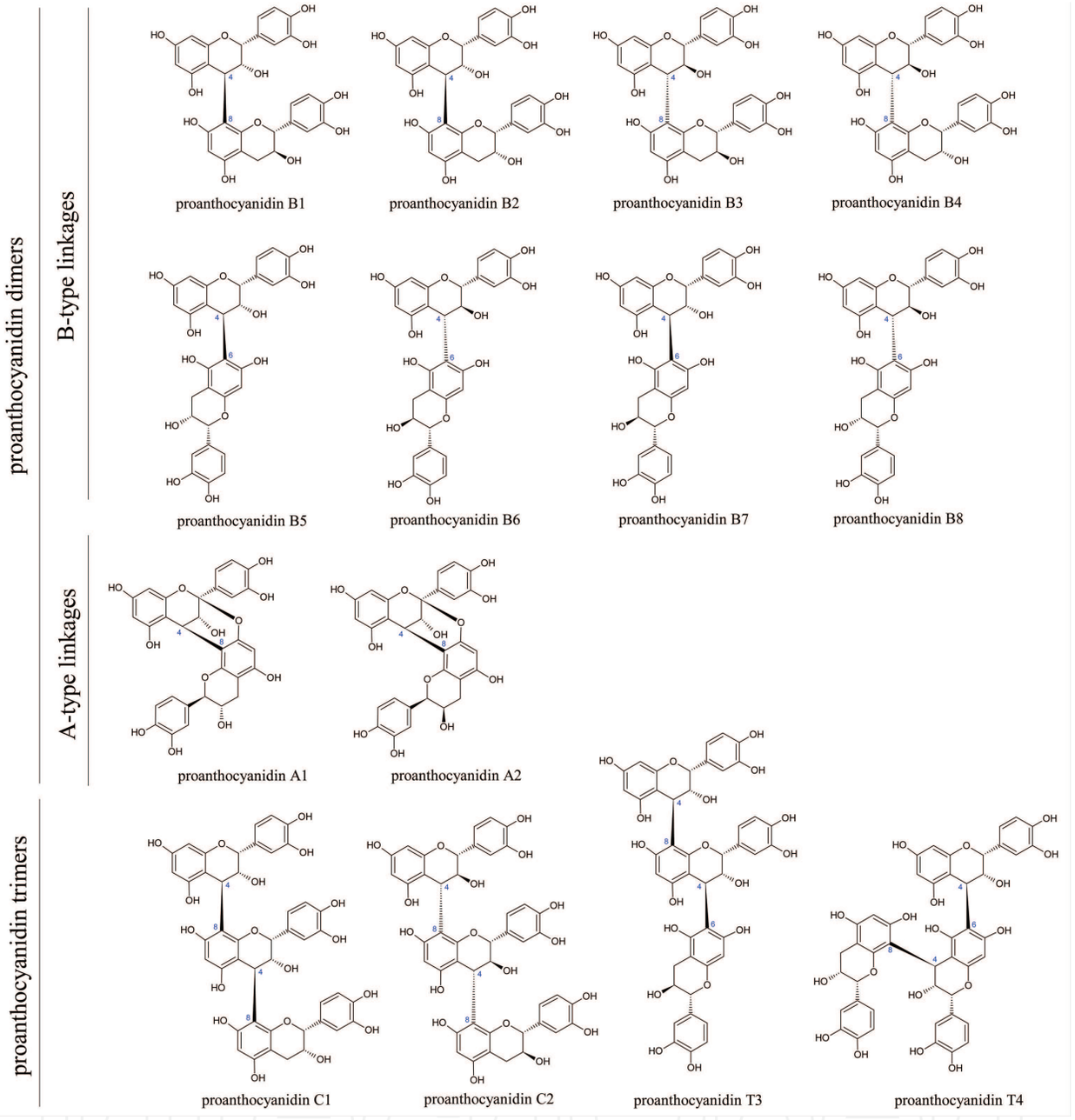

Figure 10. Overview of some of the most abundant tannin dimers and trimers observed in *V. vinifera* grapes and wines. Only tannins based on (+)-catechin and (-)-epicatechin are shown; however, dimers and trimers containing (-)-epigallocatechin and (-)-epicatechin-3-*O*-gallate also occur in *V. vinifera*.

Polymeric tannins have an mDP ≥ 5 subunits. Early studies by Czochanska and colleagues on tannins from 22 plant sources with molecular weights ranging from 1500 to 5000 elucidated the structure and stereochemistry of the interflavanic bond in these polymers by ^{13}C NMR and confirmed the presence of C4 → C8, C4 → C6, and A-type (C7 → C2) linkages [136]. Therefore, the observations described previously for oligomers also apply to polymeric tannins.

The term "proanthocyanidin," commonly used in the literature to refer to tannins, stems from the ability of these compounds to release anthocyanins upon cleavage of the interflavanic bond

under heat and acidic conditions. Based on the last reaction, tannins are classified as *procyanidins*, when they release cyanidin (characteristic of tannins containing (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-*O*-gallate), and *prodelphinidins*, when they release delphinidin (as is the case of tannins containing (-)-epigallocatechin) [137]. The spectral features mentioned for monomeric flavan-3-ols also apply for oligomeric and polymeric tannins.

Oligomeric and polymeric tannins occur both in skins and seeds, although their quantitative and qualitative composition, as well as their molecular weight and distribution, differ within these tissues and also in the resulting wines. On a whole berry basis, up to 80% of the total extractable tannin pool of the berry is located in the seeds [12]. In seeds at physiological maturity, seed tannins measured by protein precipitation vary from 2.15 to 4 mg/g FW for varieties such as Cabernet Sauvignon, Merlot, and Syrah [43, 47, 138–140]. Conversely, skin tannins vary between 0.35 and 1 mg/g FW [43, 47, 139, 140]. In wines, total tannins (by protein precipitation) measured in 1325 commercial red wines ranged from 30 to 1895 mg/L, with a mean concentration of 544 mg/L [141].

Seed-derived polymeric tannins are co-located with the flavan-3-ols in the cells below the external cuticle [142]. Seed tannins associated with cell walls reportedly have a higher mDP than their cellular counterparts [143]. The mDP of seed tannins of different grape varieties vary between 2 and 22 [73, 78, 134, 143, 144–146], which represents a 11-fold variation. Seed tannins are composed of (+)-catechin, (-)-epicatechin, and epicatechin-3-*O*-gallate, with monomeric flavan-3-ols, dimers (a portion of them bearing A-type linkages), and trimers being the predominant species [74, 130, 134]. The percentage of galloylation of seed tannins has been estimated to be around 30% [26]. In the skins, tannins occur as vacuolar components in subepidermal, thick-walled cells, but they are also found in the tonoplast and cell walls [75, 147]. Skin tannins associated with cell walls have a higher mDP than those found as free cytoplasmic components; however, tannins found in the cytoplasm are more abundant [147]. Skin tannins typically have the highest molecular weight within the berry, varying from 6 up to 85 subunits in varieties such as Cabernet Franc, Cabernet Sauvignon, Graciano, Merlot, Pinot Noir, Syrah, Carmenère, and Tempranillo [78, 124, 134, 144–148]. This represents a 14-fold variation. Skin tannins are composed primarily of (-)-epicatechin, (-)-epigallocatechin, and (+)-catechin. However, trace amounts of epicatechin-3-*O*-gallate have also been found [147], giving a percentage of galloylation estimated to typically be <5% [26].

Wine tannins are composed of all the five monomeric flavan-3-ol subunits, with (-)-epicatechin, followed by (+)-catechin and (-)-epigallocatehin being the major constituents [78]. Moreover, the proportion of each subunit is affected by both grape variety and winemaking technique, and, like with flavan-3-ols, a relationship between an increased percentage of galloylated subunits and maceration length has been observed [43, 113, 135]. For wines, the reported mDP values vary between 2 and 17 subunits [47, 124, 148–151]. However, oligomeric tannins with an mDP < 5 are the major constituents of wine tannins [43, 109, 124, 152] (**Figure 8a** and **Figure 8b**). Taking into consideration only the reported mDP values and subunit composition of seeds, skins, and wines, it seems as though tannin composition and distribution in wine resembles more of that of the seeds than that of the skins and that this feature is amplified as maceration time increases (**Figure 8b**).

As noted previously, upon crushing and during winemaking, tannins not only undergo modifications in their original molecular weight, but they can also undergo further reactions and re-arrangements with anthocyanin, to form the so-called polymeric pigments (Section 3.4), as well as with other flavan-3-ols and/or tannins dimers and trimers [153].

The ability of wine tannins to interact with proteins provides the physiological basis of the sensation of astringency, of paramount importance in red wines. Astringency is a tactile (not a taste) sensation with a marked temporal aspect that appears as a pucker feeling (typically in the upper lip) and/or feeling of dryness in the palate, which arises from a sudden loss of lubrication of the oral epithelium [154–157]. Astringency is not confined to a particular region of the mouth but is a diffuse surface phenomenon, which typically develops between 15 and 20 s [158, 159].

The development of astringency necessitates the presence of both tannins and proteins capable of interact with each other. The wine provides the tannins. The proteins are provided by the subject's saliva. The saliva of humans and other mammals contains proline-rich proteins (PRPs) [160]. Salivary PRPs constitute 70% of the proteins in saliva and are made up of three types: the acidic, glycosylated, and basic proteins, which comprise roughly 30, 23, and 17% of unstimulated saliva, respectively [161]. While acidic- and glycosylated proline-rich proteins have specific biological roles, basic PRPs display high affinity for tannins [162, 163]. Basic PRPs have a relatively preserved sequence across a wide range of species of mammals, composed of a 19-aminoacid sequence dominated by proline, glutamine, and glycine [164]. This sequence is repeated with variations between 5 and 15 times to result in a protein of ~150 amino acids in length that is extended and random-coiled in confirmation [164, 165]. The flexibility of the protein is further improved by the existence of several low-energy conformations [166, 167]. The biological role of astringency in mammals has been intensively debated but there is now evidence that astringency may be an evolutionary defense mechanism against dietary anti-feeding factors [168]. Tannins can have a variety of harmful effects on animals, including sequestration of iron and inhibition of digestive enzymes [160]. The role of PRPs may be to bind to the tannins and precipitate them, thereby preventing harmful effects in the gastrointestinal tract [169].

The interaction between PRPs and tannins results from hydrogen bonding between the tertiary amide or carbonyl groups of the proline subunits and the hydroxyl groups of the tannin [167, 170]. However, hydrophobic interactions whereby the hydrophobic face of the aromatic ring of the phenol interacts with the pyrrolidine ring of the protein may also cooperatively aid in the complexation process [163, 170, 171] (**Figure 9**). Likewise, the galloyl ring in epicatechin-3-*O*-gallate and tannins containing this flavan-3-ol provides supplementary aromatic surfaces that may engage in hydrophobic complexation with the proline ring as well [163, 172]. From this perspective, increase in the percentage of galloylation in a given tannin should result in enhanced perceived astringency.

The development of astringency follows a three-stage process, consistent with the in-mouth temporal development of this sensation [173, 174]. In the first stage, the binding of multidentate tannins to several sites on the protein causes the randomly coiled protein to coil around the phenol, compacting the protein. In the second stage, the tannin fractions of the protein-tannin complexes cross-link forming polyphenol bridges and creating protein dimers.

During the third stage, the dimers aggregate to form larger complexes that eventually precipitate out of solution. In wines, the spectrum of subtle differences in astringency sensations was compiled in the "red wine mouthfeel wheel" in which astringency is categorized in 7 subqualities and 30 subattributes [175], which highlights the complex nature of this sensation in red wines.

Perhaps the most determining factor on astringency development in red wines is, simply, tannin concentration [105, 176–178]. However, additional effects include wine pH and ethanol content [179, 180], viscosity [181, 182], and the presence of sugars [183, 184] and polysaccharides [121, 185]. Also, the tannin stereochemistry, composition, and connectivity affect the perception of astringency. For example, the dimer (+)-catechin-(+)-catechin linked via a C4 → C8 interflavan bond has lower astringency than its C4 → C6 counterpart or than the C4 → C8 (+)-catechin-(-)-epicatechin dimer [103]. Second to tannin concentration, tannin size (molecular weight) also modulates the development of astringency [186–189]. Precipitation of salivary PRPs is enhanced by the presence of high molecular weight tannins that have some structural flexibility, as in the case of tannins containing freely rotating interflavan bonds and gallolyl groups, due to a larger number of available binding sites for interaction with the proline residues [102]. Larger tannins can also engage in self-association, thereby promoting complex aggregation. However, the relationship between polymer size and perceived astringency does not progress linearly. For example, at equimolecular concentrations, Vidal et al. found that a wine-like solution containing tannins with an mDP of 70 is only two times more astringent than one with an mDP of 3 [188]. Empirical evidence that astringency elicited by grape tannins is mostly concentration-driven can be found by comparatively assessing astringency in seeds (overall high tannin concentration, with tannins of low molecular weight) with that of skins (relative lower tannin concentration than seeds but with tannins of high molecular weight). Seeds typically elicit a much higher astringency sensation than skins in spite of being composed of low molecular weight tannins and this may be due to the fact that seed tannins are typically at much higher concentrations than skin tannins on a berry fresh weight basis.

Lastly, astringency perception in red wines is also influenced by factors extrinsic to the nature of the tannin and PRP's interactions, including salivary flow rate [159], sensitivity to the bitter agent 6-*n*-propylthiouracil (PROP), known as PROP status [190], and frequency of exposure [183, 191]. Sensitivity to PROP seems to have equivocal effects on astringency development; however, individuals with low salivary flow rate (1.92 g/min) perceive astringency later and with higher intensity than individuals with high salivary flow rate (3.73 g/min) [159].

Some oligomeric and polymeric tannins can also interact with gustatory receptors, and they can thus elicit a mild bitter response [26]. Time-intensity sensory studies have shown that bitterness perception decreases from flavan-3-ols monomers to trimers [103]. More generally, bitterness decreases as the polymer size increases, probably because of the difficulty of large polymers to diffuse inside the taste bud pores [26].

3.3.2. Extraction during winemaking

Tannin dimers and trimers follow extraction kinetics similar to those reported for flavan-3-ols, and as such these oligomers can be extracted in the absence of ethanol, for example during prefermentative cold soak [111]. In Tempranillo wines after a 2-day cold soak, the concentration

of tannin dimers, trimers, and tetramers increased from 22, 23, and 0 mg/L, respectively, to 27, 30, and 6 mg/L, respectively, after postfermentative maceration for 1 week [109]. In another study, the levels of the B2 dimer and the C trimer increased from about 6 mg/L at day 2 to about 22 mg/L (B2 dimer) and to 11 mg/L (C trimer) after 20 days of maceration in the white variety Viura [108]. These results suggests that as the oligomers increases in size, their extraction and retention into wine progresses more slowly.

Extraction of tannins (mDP ≥ 5) into wine during winemaking has been followed using different analytical approaches, ranging from protein precipitation [47, 58] to acid-catalyzed depolymerization followed by HPLC analysis [111, 146]. Expectedly, the comparison of extraction patterns established by both methods is tenuous at best, as protein precipitation and HPLC greatly differ in the amount, composition, and molecular weight of the tannins that each method quantify. Moreover, extraction patterns of skin and seed tannins are not necessarily similar and they certainly diverge from each other as maceration progresses; as such it is often difficult to unequivocally ascertain the source of tannin extraction during the time course of maceration. Using acid-catalyzed depolymerization and HPLC, the extraction of skin tannins into wine was found to follow a Boltzmann sigmoid model [146]. In this model, a lag phase of initially slow extraction is observed due to the period of time required for the tannins to diffuse out of the berry cells and into the fermentor. The extent of this lag phase may be modulated by the degree of berry crushing or the ethanol concentration. This lag phase is followed by a plateau concentration, which is reached when tannin concentration is at its apparent maximum. Likewise, the extraction of tannins from seeds has also been modeled using a Boltzmann sigmoid extraction pattern, but this model has only proved valid for the first 6 days of contact with the fermentation solids [111]. In model wine experiments containing only seeds with varying ethanol levels, these same authors observed an initial slow extraction of tannins, which was attributed to the period of time required for these tannins to diffuse out of the seed cells and into the solution. As with skins, this lag period was followed by a plateau, the value of which increased with ethanol content, suggesting an initial effect of the ethanol in the degradation of the outer protective layers of the seeds. However, from day 6 to day 10, tannin extraction into wine increased linearly. The authors attributed this observation to the hydration of seed cells and subsequent cellular leakiness, leading to a somewhat abrupt release of seed tannins.

In Merlot wines, tannins measured by protein precipitation increased almost linearly during the first 7 days of maceration. Moreover, extended maceration for 20 days increased tannin extraction from a mean of 469 mg/L in control wines to 985 mg/L in the extended maceration wines [58]. Also, in Merlot wines, protein precipitable tannins were followed at 2-day intervals during a 30-day maceration period. Tannin extraction occurred quickly and almost linearly from day 2 to day 10, then reached a plateau between day 10 and 22, and was followed by an almost linear increase up to the peak of extraction [47] (**Figure 11a**). While the observed plateau between day 10 and 20 is consistent with seed hydration, the ensuing linear increase is consistent with the extraction of seed tannins [111]. Indeed, after 30 days of maceration, the proportion of seed-derived tannins in finished wines was estimated to be about 73% in Cabernet Sauvignon [43] and between 73 and 80% in Merlot wines [47, 116]. It has also been suggested that postfermentation extraction of tannins could be the result of a desorption mechanism whereby ethanol may disrupt the noncovalent interactions of the previously extracted tannins that were bound to cell-wall material [192]. It is unclear if this linear increase is the result of one or a

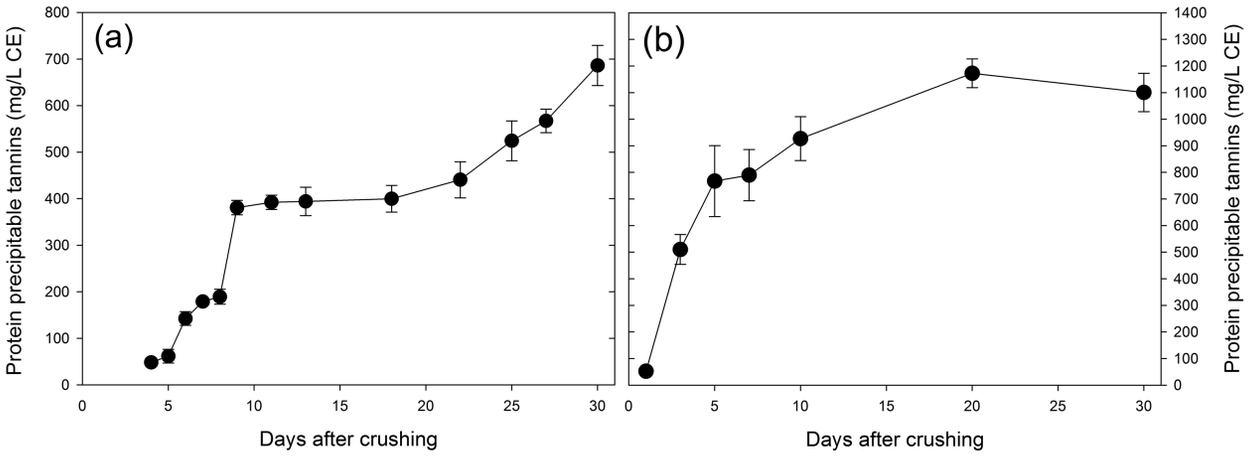

Figure 11. Extraction patterns of protein precipitable tannins during a 30-day maceration period in (a) Merlot and (b) Cabernet Sauvignon wines. CE: catechin equivalents. Adapted from Refs. [43, 47].

combination of both mechanisms, but in the range of 11–14.4% ethanol this supposed "desorptive" effect seems to be inexistent [47, 116]. It is also unclear if the extraction patterns shown in **Figure 11** will apply to other varieties with vastly different seed and skin phenolic composition, such as Pinot Noir, Sangiovese, Tempranillo, or Nebbiolo.

The extraction into wine and the ensuing fate of oligomeric and polymeric tannins during postmaceration and aging is also governed by the matrix composition, including the presence (or lack thereof) of anthocyanins and the presence of other compounds known to bind with wine tannins, primarily mannoproteins from yeast origin, polysaccharides, and other cell-wall components. Indeed, wine tannins can also react via noncovalent interactions with cell-wall material present during fermentation. These noncovalent interactions include hydrogen bonding and hydrophobic interactions. For example, the hydrogen-bonding-mediated interaction between tannins and polysaccharides is illustrated in **Figure 12**. As tannins increase in molecular weight, each additional monomer increases the number of sites that can form hydrogen bonds between the tannin and cell-wall components [192–195]. An increase in polymerization also increases the hydrophobic character of the tannin. The presence of galloylated units, more common on seed than in skins tannins, enhances the tannin-polysaccharide interaction as well, as highly galloylated tannins may be encapsulated by hydrophobic pockets and pores in the polysaccharide network [195, 196]. These findings had led to the hypothesis that the failure to recover high molecular weight tannins in wine is the result of tannin-cell-wall interactions occurring during winemaking and that the binding capacity of the cell walls is influenced by both tannins and polysaccharide structure and composition [192]. A series of studies conducted by Bindon and colleagues had confirmed this hypothesis. Bindon et al. found a significant relationship between the tannin molecular mass and the proportion of tannins adsorbed by skin cell-wall polysaccharides, with the end result being that high molecular weight tannins (>15,000 g/mol) are not extractable and/or removed from the wine by interaction with cell-wall components [194, 197–199]. In practical terms, the proportion of bound (and thus "missing") tannins during Cabernet Sauvignon winemaking have been estimated to range from 17 to 29% of those originally measured in the fruit [200, 201].

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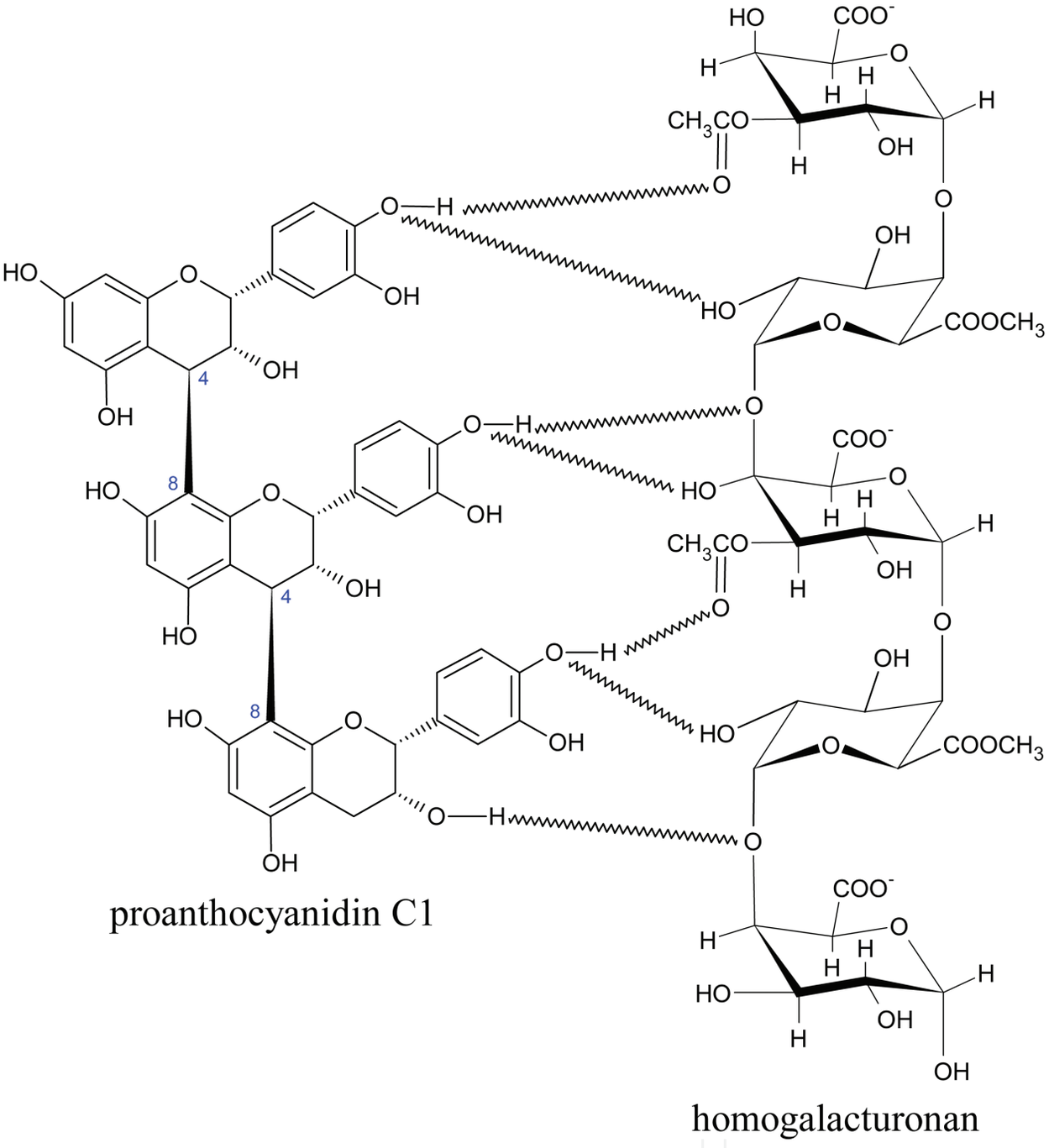

Figure 12. Hydrogen-bonding-mediated interaction between the hydroxyl groups of the tannin trimer C1 and the oxygen atoms and glycosidic linkages of the polysaccharide homogalacturonan. Redrawn and modified from Ref. [192].

3.4. Polymeric pigments and other anthocyanin-derived pigments

3.4.1. Occurrence, general chemistry, and sensory aspects

An overview of the variety of reactions anthocyanins can undergo during maceration and aging is presented in **Figure 13**. Upon crushing (and releasing from vacuoles) and during aging, anthocyanins can readily react with a variety of electrophiles and nucleophiles, including

other anthocyanins [202], flavan-3-ols via direct condensation [203–206], or acetaldehyde-mediated [204, 205, 207, 208], dimeric or trimeric tannins [86, 209], lactic acid [204], glyceraldehyde [210], acetaldehyde [211, 212], pyruvic acid [212, 213], glyoxylic acid [214, 215], and a number of other aldehydes, including furfural, 5 hydroxymethylfurfural, isovaleraldehyde, benzaldehyde, propionaldehyde, isobutyraldehyde, formaldehyde, and 2-methylbutyraledehyde [215, 216]. Aldehydes are formed either from the metabolism of *Saccharomyces cerevisiae* during alcoholic fermentation [212, 217] or from the metal-catalyzed oxidation of

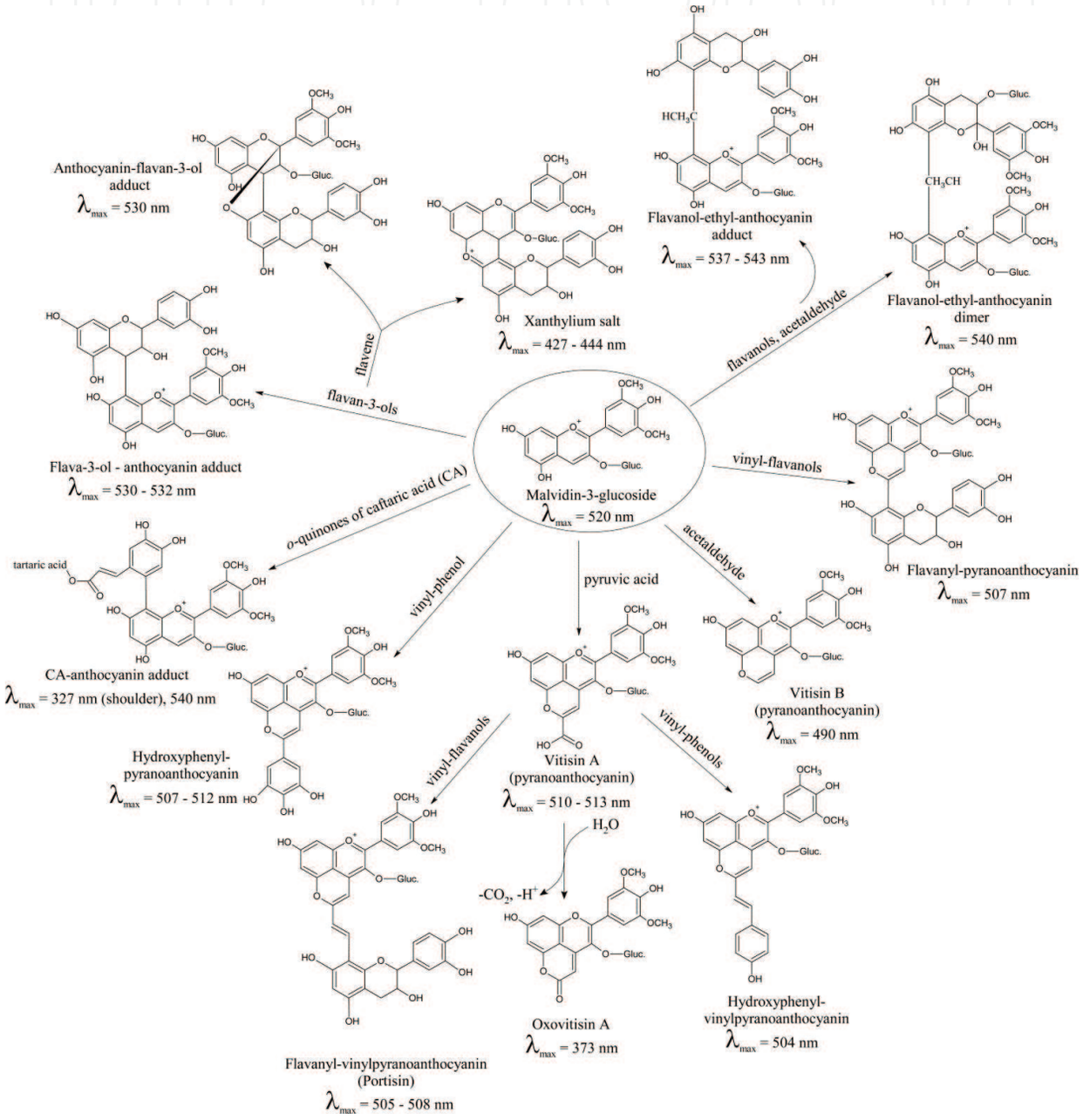

Figure 13. Main chemical pathways involving anthocyanins during winemaking and aging of red wines. Malvidin-3-glucoside is shown as the starting anthocyanin, but other anthocyanins may also participate in these reactions. Redrawn and adapted from Ref. [219] and complemented with data from Refs. [26, 204, 220, 221].

several wine substrates, especially ethanol (to acetaldehyde), most particularly thorough the Fenton Reaction [17, 218]. All these anthocyanin-derived products have spectral properties different from that of the native anthocyanins (**Figure 13**). Ultimately, these reactions together with the formation of oligomeric and polymeric pigments account for the evolution of wine color, from deep purple, due to monomeric anthocyanins and self-association copigmentation reactions in young wines, to orange, brick-red tones as the wine ages.

Polymeric pigments encompass a variety of winemaking artifacts formed by the covalent association, either direct or mediated by an aldehyde (e.g., acetaldehyde, glyceraldehyde, and glyoxilic acids) between anthocyanins and tannins. Relative to intact anthocyanins, polymeric pigments share two fundamental features: they are (1) partially resistant to bisulfite bleaching and (2) more resilient to pH changes [222, 223]. This is because polymerization protects by steric hindrance of the chromophore of the anthocyanin from the attack of water and other nucleophiles, oxidation, or the bleaching effect of SO_2 [222, 224]. The presence of anthocyanins during maceration increases the solubility and retention of tannins [52–54], an observation classically attributed to the formation of polymeric pigments. Singleton hypothesized that the glucose moiety in the anthocyanin and the polarity of the flavylium cation may decrease the precipitability of the resulting polymeric pigment [225]. In a subsequent experiment in which white wines were produced with different portions of added tannins and anthocyanins, the retention of tannins and formation of polymeric pigments increased in the presence of added anthocyanins [53]. However, this later study also found that the stoichiometric addition of anthocyanins relative to tannins approached an ideal proportion, where excessive anthocyanins did not increase pigmented polymer formation. This finding suggests that the proportion of anthocyanins and tannins during maceration can condition anthocyanin and tannin stability and the subsequent formation of polymeric pigments.

Polymeric pigments modulate wine color, long-term color stability, and possibly astringency changes during winemaking and aging. From the perspective of color, the UV-visible spectra of anthocyanin-flavan-3-ol adducts (i.e., dimers) resulting from condensation with aldehydes is bathochromically shifted (i.e., bluish color) compared to those of their precursors, with a shift in absorbance of 10 nm for linear substituents and of 20 nm for branched substituents [26, 207, 216] (**Figure 13**). However, the chromatic properties of polymeric pigments are less clear. Indeed, a numerical value for the molar extinction coefficient of polymeric pigments at wine pH is not yet available, but (indirect) experimental evidence suggests that it should be comparatively lower than that of the intact anthocyanins [37, 43, 226]. This, together with the disappearance and/or transformation of anthocyanins, may in turn explain why the color of red wine not only changes in hue but also decreases in saturation during aging.

Evidence for the existence of polymeric pigments in wine is abundant [37, 43, 143, 227–229], and some possible structures are shown in **Figure 14**. In Pinot Noir wines, pigmented polymers were isolated and characterized as mixtures of dimers to octamers in which the anthocyanin moiety was linked to the flavan-3-ol by B-type and A-type linkages [227]. In a separate study, ultrafiltration and gel adsorption chromatography combined with ^{1}H, ^{13}C, and 2D-NMR were used to characterize a high molecular weight tannin polymer (>5 kDa) isolated from a Bordeaux red wine [229]. The structural backbone of this polymer consisted of a tannin chain with (-)-epicatechin,

(+)-catechin, and (-)-epicatechin-3-*O*-gallate as extension and terminal subunits. The presence of acetaldehyde bridges was also observed in the A ring of some subunits as well as that of pyranoanthocyanins linked to the backbone via C4 → C6 or C4 → C8 linkages. Interestingly, polysaccharides were also found to be present within the structure, although these were not covalently linked to the tannin backbone. Organic and phenolic acids as well as aminoacids were also found to be part of the polymeric fraction structure. This appears to be the first report to completely elucidate the heterogeneous structure of these compounds as they occur in red wines. Lastly, in Cabernet Sauvignon wines, pigmented material was isolated by preparative HPLC [43]. The spectral features of the pigmented material featured a comparatively higher 280 to 520 nm absorbance ratio compared to that of intact anthocyanins (thus indicating the presence of flavan-3-ols) and an mDP between 5 and 10 units. The tannin component of this polymeric material was also put in evidence by subjecting the polymer to protein precipitation with bovine serum albumin (BSA), further indicating the potential astringent properties of this material.

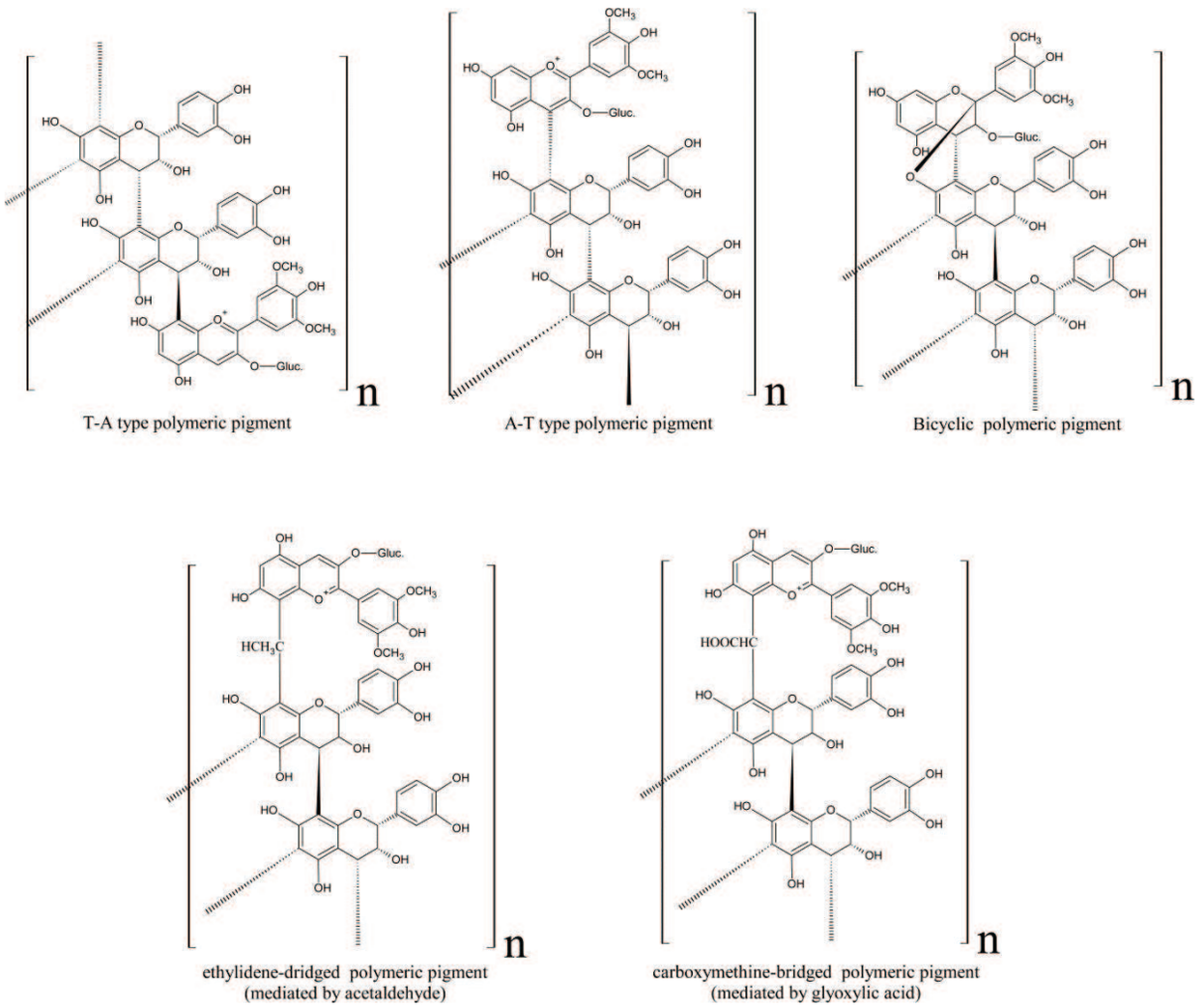

Figure 14. Potential structures of polymeric pigments resulting from the covalent reaction between anthocyanins (A) and tannins (T). Only C4 → C8 and A-type interflavanic bonds are depicted but C4 → C6 interflavanic bonds can also occur.

From a sensory standpoint, polymeric pigments affect two key aspects in red wines: color development and mouthfeel modification. Direct sensory evidence of the role of polymeric pigments in astringency changes during aging is, however, relatively recent. Indeed, the observed "lessening" of astringency during aging was thought to be the result of the reaction of anthocyanins with tannins of various sizes to give rise to polymeric pigments [222, 230]. However, the structural complexity and heterogeneity of these pigments prevented their isolation and subsequent chemical and sensory characterization. With the advent of new analytical, semipreparative, and preparative HPLC techniques, the characterization of these compounds has become possible. Work by Vidal and colleagues in 2004 found that polymeric pigments with mDP of ~3 and 9 and bearing an anthocyanin moiety were less astringent than apple tannins with the same mDP but deprived of anthocyanins [40]. Moreover, these authors showed that modifying the molecular structure by introducing an acetaldehyde bridge decreased astringency but also increased bitterness. An explanation for the comparatively lower astringency of polymeric pigments relative to that of intact tannins is that the incorporation of an anthocyanin moiety with its glycoside portion increases the polarity of the polymer [225]. As the development of astringency is partially governed by hydrophobic interactions between salivary PRPs and phenols, the higher hydrophilic character of the pigmented polymer would decrease the interaction of this polymeric material with salivary PRPs and thus reduce perceived astringency.

Recently, Weber et al., using size-exclusion chromatography on Sephadex resin, isolated 14 tannin fractions from the 2005 Cabernet Sauvignon wine [37]. Anthocyanins, mainly malvidin-3-glucoside, were found in the first 10 fractions, indicating the pigmented nature of the polymeric fraction. Fractions 1 to 3 were composed of large polymeric pigments as measured by protein precipitation, with low anthocyanin and tannin content; fractions 4 to 7 consisted of anthocyanin-rich pigmented polymers with medium tannin content; and fractions 8 to 14 consisted of small-sized, tannin-like oligomers with very low anthocyanin content but very high tannin content. Upon sensory evaluation of each fraction dissolved in model wine at iso-concentrations of 500 mg/L, fractions with a low amount of anthocyanins elicited higher astringency, suggesting that further incorporation of anthocyanins into polymers should result in a decrease in astringency. In another report, a pigmented polymer isolated from a Bordeaux red wine was fractionated into eight fractions of different molecular weights by gel permeation chromatography [229]. Upon dissolution of these fractions in 1% ethanol at iso-concentrations, astringency was found no to vary in seven of these fractions in spite of differences in mDP and degree of galloylation. However, one fraction consisting of 50% polysaccharides was found to be less astringent. Overall, current evidence suggests that the lessening of astringency along with red wine aging may not be related to a drastic change in the total amount of tannin present. Rather, the structural modification of wine tannins, primarily resulting from the incorporation of anthocyanins, and, secondarily, from the addition of other metabolites such as carbohydrates, proteins, and polysaccharides, may drive changes in perceived astringency during aging.

3.4.2. Formation during winemaking

Polymeric pigment formation increases progressively during maceration and aging (**Figure 15**) ultimately leading to color changes, modification of mouthfeel properties, and,

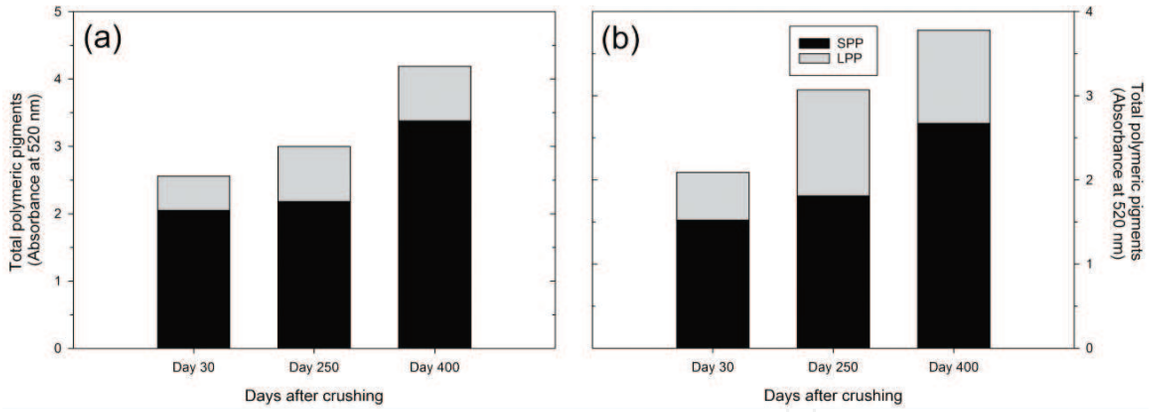

Figure 15. Overview of the formation of polymeric pigments during maceration and bottle aging of Cabernet Sauvignon wines processed with a maceration length of 10 days (control) (a) and 30 days (extended maceration) (b). SPP: small polymeric pigments; LPP: large polymeric pigments. Adapted from Ref. [43].

eventually, precipitation. Singleton and Trousdale reported that white wines produced with added tannins and anthocyanins showed a linear increase in polymeric pigment content after addition of seed tannins in the range of 0 to 1000 mg/L (gallic acid equivalents) and anthocyanins in the range of 0 to 500 mg/L [53]. Using protein precipitation, Harbertson et al. found that large polymeric pigments (LPP), which precipitate BSA, increased by 70% between pressing and 185-day postpressing in Merlot wines [58]. Small polymeric pigments (SPP), which do not precipitate BSA, and are assumed to be composed of tannin-anthocyanin dimers, either of direct condensation or mediated by acetaldehyde [231], comparatively increased 30% from pressing to 185-day postpressing. In this same experiment, wines produced with extended maceration and saignée[6] and containing a higher concentration of tannins gave rise to an enhanced formation of LPP; however, this occurred with a decline in the anthocyanin content of 43% relative to its peak concentration [58]. A similar trend was observed in Merlot wines obtained with extended maceration (30 days), in which a two-fold increase of the total polymeric pigments was observed from day 4 to day 30, along with significant losses of malvidin, delphinidin, petunidin, and peonidin anthocyanin derivatives [47]. Furthermore, this later work demonstrated that the formation of polymeric pigments alone during extended maceration was only partially responsible for the observed anthocyanin loss because an increase in the polymeric pigment content of 13 mg/L from day 4 to day 30 occurred along with a drop in wine anthocyanins of 231 mg/L in this same time frame. In summary, these results suggest a complex relationship between tannin content, anthocyanin extraction (or loss), and polymeric pigment formation during maceration. As shown in **Figure 15**, a common feature of extended maceration seems to be the formation of polymeric pigments with the ability to precipitate BSA (and by a similar mechanism to elicit astringency), but this occurs at the expense of anthocyanin loss (and, consequently, of wine color saturation) (although this anthocyanin loss is generally not fully explained by the formation

[6]The practice of saignée consists of taking a portion of the must from the bottom of the tank before the onset of alcoholic fermentation with the aim of increasing the solid to volume ratio of the remaining must and then furthering the extraction of phenolics and aroma compounds from seeds and skins.

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of polymeric pigments). Altogether, these findings suggest that the presence of anthocyanins invariably leads to the formation of polymeric pigments; yet, the proportion of anthocyanins and tannins during maceration, which is expected to differ widely depending on variety, clone, and viticultural and climatic conditions, as well as winemaking technique, will condition the amount of pigmented tannins that are effectively formed during winemaking and aging.

4. Concluding remarks

The above literature review was undertaken with the aim to highlight the remarkable chemical diversity of flavonoid phenolic compounds in grapes and wines. This diversity is furthered from the very first moment the grapes are crushed, thereby allowing vacuolar and pulp components to be released into the fermenting must and wine. This chemical diversity further increases during winemaking and bottle aging, thus adding to the already present chemical diversity, a variety of new sensory dimensions, ranging from changes in wine color and aroma to modification of astringency.

For a wide number of red grape varieties, the extraction of anthocyanins peaks during the first 4 or 5 days of maceration, which is followed by a decrease in concentration along with the lengthening of maceration. This decrease in anthocyanin concentration is typically accompanied by the formation of polymeric pigments, by which formation is modulated, among others, by the molar proportion of anthocyanins and tannins. Flavan-3-ols and small oligomeric tannins from skins are extracted within the first days of maceration, whereas the extraction of seed-derived tannins requires longer maceration times. It also seems that high molecular weight tannins are not retained into wine, probably due to interactions with polysaccharides and other nonphenolic materials during winemaking. Indeed, specific matrix effects affect the rate of retention of tannins into wine, particularly at the latter stages of maceration. These include (but are not limited to) the presence of anthocyanins, polysaccharides, and other cell-wall components such as structural proteins. As phenolic and nonphenolic compounds are extracted and/or formed during maceration and aging, a dynamic set of chemical and biochemical reactions occurs, resulting in the formation of new structures not previously found in grapes. Some of these new phenolic classes, which may also contain nonphenolic material of yeast and/or grape origin, are responsible for a variety of new sensory attributes. Polymeric pigments, bearing astringent and bitter properties different from those of intact tannins of equivalent molecular weight, are candidates for the changes in the mouthfeel and textural properties of red wines during maceration and aging. Although the taste and mouthfeel attributes of polymeric pigments are starting to be clarified, their interaction with other phenolic and nonphenolic materials and the volatile fraction of the wine matrix remain to be explored.

Different maceration techniques applied during red wine production affect the rate, quantity, and sometimes the chemical composition of the phenolic compounds that end up in the wine. Control and understanding of the factors that modulate phenolic extraction and retention into wine during maceration should ultimately allow the winemaker to adjust maceration variables to meet a given wine style sought and/or to comply with commercial specifications.

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