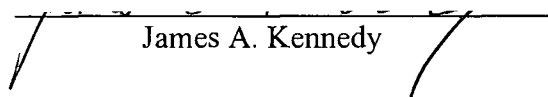


## AN ABSTRACT OF THE THESIS OF

Jose L. Pastor del Rio for the degree of Master of Science in Food Science and Technology presented on July 9, 2004.

Title: Development of Anthocyanins and Proanthocyanidins in Pinot noir Grapes and Their Extraction into Wine

Abstract approved:

James A. Kennedy

Color stability and mouth feel quality are two of the most important aspects of red wine quality. Anthocyanins and proanthocyanidins are responsible for these attributes and it has been shown that weather conditions during the growing season and grape maturity can effect these components in wine. However, investigations into proanthocyanidin development are for the most part incomplete. Although it is known that weather affects vine metabolism, it has not clearly understood how phenolics are affected by temperature and heat summation. It is generally believed that the wines made with riper grapes improve in flavor and mouth feel as a result of an “improvement” in skin tannin “ripeness”. The idea of “tannin ripeness” is usually used in the wine industry to explain this phenomenon, however, no scientific explanation for this concept has been given.

The objective of this project was threefold: I) Monitor phenolic development in Pinot noir grapes over three consecutive growing seasons and determine how anthocyanin and proanthocyanidin development in grapes was affected by heat

summation, II) investigate the transfer of grape phenolics into wine during fermentation and maceration and III) understand how grape maturity affected wine composition with a specific focus on proanthocyanidin structure.

In this study, anthocyanin and proanthocyanidin development in *Vitis vinifera* L. cv. Pinot noir grapes (Pommard clone) were monitored for three consecutive vintages (2001-2003). Five cluster samples (x5 replicates) were collected for analysis each week beginning approximately 4 weeks prior to véraison and continued through commercial harvest. Weather information (temperature and heat summation) showed that the growing seasons became increasingly warmer from 2001 to 2003. By harvest time, 2003 had the highest concentration of proanthocyanidins in seeds (per berry weight) in comparison with the other two vintages. Similarly, proanthocyanidins in skins had the highest concentration in 2003 (per berry weight). However, there was not difference in the concentration of flavan-3-ol monomers in seeds (per berry weight) between the three vintages. Anthocyanins were not significantly different over the three vintages. There was some relationship between the concentration of some proanthocyanidin components in grape seeds and fresh seed weight.

Information of grape and wine phenolics was compared with each year's temperature. The results suggested that changes in temperature and heat summation between vintages are associated with changes in proanthocyanidin content in grapes and wine. The data indicates that it is possible to predict proanthocyanidins in wine based upon early grape analysis. However, the anthocyanin content in grapes did not correlate with either weather or the anthocyanin content in wine. The concentration of seed and skin proanthocyanidins in grapes were compared with the proportions of

seed and skin proanthocyanidins found in wine. Based upon proanthocyanidin extraction from seeds and skin during winemaking, a formula to predict proanthocyanidin content in wine based upon grape analysis at harvest and véraison was developed. From this formula, Pinot noir wine contained 7.8% of the proanthocyanidins from seed and 19% of the proanthocyanidins from skin analyzed from grapes at harvest, and 3.6% of the seed proanthocyanidin and 9.7% of the skin proanthocyanidin present in grapes analyzed at véraison.

Based upon the analyses of this study, coupled with several informal sensory studies conducted on wine, the results of this thesis do not support the notion that “tannin ripeness” is due to structural changes in proanthocyanidin that occur during fruit ripening. Furthermore, this thesis suggests that “tannin ripeness” is not due to differential extraction of seed and skin proanthocyanidins as a result of fruit ripening.

Development of Anthocyanins and Proanthocyanidins in Pinot noir Grapes and Their  
Extraction into Wine

by  
Jose L. Pastor del Rio

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degree of

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## **CHAPTER 1**

### **Introduction**

Color stability and mouth feel quality are two of the most important aspects of red wine quality. Anthocyanins and proanthocyanidins are the phenolic compounds responsible for these attributes. Anthocyanins are responsible for the color in red wines while proanthocyanidins provide bitterness and astringency. These compounds are present in grapes and are extracted into the wine during fermentation and maceration. The differences in quantity and composition of these compounds have been associated with overall differences in wine quality. Winemaking practices (i.e. length of maceration, sulfur dioxide addition, cap management practices, pressing) clearly affect the amount and composition of these compounds in wine, and are easily controlled. Conditions in the vineyard during berry development (i.e. sun exposure, temperature, precipitation), are also thought to play a role in the presence of these compounds in wine. Unfortunately, a lack of knowledge necessary to influence their presence in wine currently exists.

Limited information on the environmental factors affecting anthocyanin and proanthocyanidin biosynthesis in grapes exists. Many studies identified variables affecting anthocyanin accumulation in grapes. While some authors suggest that light is involved in anthocyanin biosynthesis (Kliewer, 1977), others have contradicted these findings (Price et al., 1995). Low temperatures have been identified as having a positive effect on anthocyanin biosynthesis (Kliewer and Torres, 1972). Recent

studies analyzing the combined effects of light and temperature have indicated that low temperatures and high levels of sun exposure enhanced the accumulation of anthocyanins in grapes (Spayd et al., 2002). Much more limited is information regarding proanthocyanidin biosynthesis. Few authors have studied the effects of environmental factors on proanthocyanidin biosynthesis in grapes. Current information about this suggests the effect of light on skin proanthocyanidin biosynthesis (Downey, 2003), however, additional information is scarce and inconclusive.

Flavonoid diffusion into wine is not well understood. Some research has been conducted on the rate of extraction of anthocyanins and proanthocyanidins during maceration. According to Ribéreau-Gayon (1982), anthocyanin extraction is maximal during the days of maceration while proanthocyanidin extraction increases constantly according to the time of pomace contact. Moreover, there have been reports that grapes with higher anthocyanin content do not always produce wines with high anthocyanin content (Ribéreau-Gayon et al., 2000B). These differences in extractability have been attributed to differences in ripening conditions (Ribéreau-Gayon et al., 2000B). However, further research attempting to explain this phenomenon as related to proanthocyanidin diffusion has been incomplete. There are two common questions among winemakers regarding the proanthocyanidin content in grapes and wine: I) is there a relationship between proanthocyanidin content in grapes and proanthocyanidin content in wine, and II) is it possible to predict proanthocyanidin content in wine based on early grape analysis?

Investigation into proanthocyanidin diffusion into wine is needed to answer these questions.

In the wine industry, it is generally accepted that wines made with more mature grapes will improve in quality as a result of an increase in skin proanthocyanidin proportion. The expression of “tannin ripeness” is generally applied to the improvement in astringency quality. However, no scientific explanation for this observation has been provided. While some authors have studied the evolution of compounds that provide organoleptic quality to the wine (Perez-Magariño and Gonzalez-San José, 2004; Miranda-Lopez et al., 1992) including phenolics, no studies have been conducted to understand how the maturity of the grapes affects the proanthocyanidin structure and perception of the wines.

The purpose of the present study was to improve our understanding of the relationship between weather, grape maturity, wine quality and proanthocyanidin structure. The objectives of this project were threefold: I) Monitor phenolic development in Pinot noir grapes over three consecutive growing seasons and determine how anthocyanin and proanthocyanidin development in grapes is affected by heat summation, II) investigate the transfer of grape phenolics into wine and III) understand how grape maturity affects wine quality as related to astringency perception and proanthocyanidin structure.

## CHAPTER 2

### Literature Review

**Phenolic compounds.** The terms phenolic or polyphenolic can be defined chemically as a substance which possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives (i.e. esters, ethers, glycosides, etc.) (Ho, 1992). While a small number of phenolics compounds occur in animals, many plant phenolics have been identified. Indeed, the presence of a “phenolic fraction” is a characteristic feature of all plant tissues (Mann et al., 1996). The phenolic compounds, which occur commonly in food materials, can be classified into three groups: simple phenols and phenolic acids, hydroxycinnamic acid derivatives and flavonoids (Ho, 1992).

The most important group of phenolic compounds in foods are the flavonoids, which consist mainly of flavan-3-ol monomers, proanthocyanidins, anthocyaninins, flavones and flavonols (Das, 1994; Ho, 1992). Over 6000 naturally occurring flavonoids have been described (Harborne and Williams, 2000). Flavonoids are built upon a C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> flavone skeleton (Figure 2.1). Some flavonoids are glycosides of a relatively small number of flavonoid aglycones, which are generally water-soluble and accumulate in plant vacuoles (Koes et al., 1993). The non-flavonoids are represented mostly by phenolic acids (i.e.: benzoic acid) with C<sub>6</sub>-C<sub>1</sub> structures and

hydroxycinnamic acids (i.e. caffeic acid) with C<sub>6</sub>-C<sub>3</sub> structures (Cheynier et al., 1997).

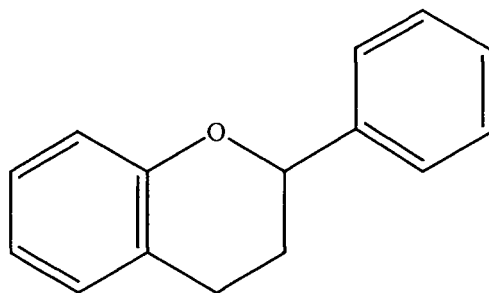


Figure 2.1. Flavone skeleton (structure C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub>).

Proanthocyanidins, condensed tannins, or simply tannins by many winemakers, are polyflavonoid in nature (Figure 2.2), consisting of chains of flavan-3-ol units such as (+)-catechin (Das, 1994; Ho, 1992). Proanthocyanidins are so named because under oxidative and acidic conditions, they are degraded to anthocyanidins (Figure 2.3). In a proanthocyanidin dimer, acid catalysed fission gives the flavan-3-ol from the “lower half” (terminal subunit) and the flavan-4-yl carbocation from the “upper half”. This extremely reactive intermediate, unless it is trapped (by phloroglucinol, for example), decays by loss of a proton and a hydride ion to give the anthocyanidin (Haslam, 1977). However, the carbocation may be trapped by a reactive nucleophile (such as thiol or phloroglucinol), and this reaction forms the basis of the acid-catalysis cleavage analysis, which is used for the identification of proanthocyanidin extension subunits (Haslam, 1977; Kennedy and Jones, 2001).

Two groups of proanthocyanidins exist in grapes. The first group, based solely upon (+)-catechin [2R:3S] and its diastereoisomeric epimer (-)-epicatechin [2R:3R],

are procyanidins as they consist of o-diphenols hydroxylated in the 3' and 4' positions in the B-ring. The second group, which also contain (-)-epigallocatechin and/or (-)-gallocatechin, are prodelphinidins and are trihydroxylated in the 3', 4' and 5' position (Figure 2.4). In addition, a single esterified derivative, (-)-epicatechin-3-O-gallate, has been frequently reported in grapes (Gawel, 1998). Within the proanthocyanidins terminated with flavan-3-ol units, (+)-catechin is the most common terminal subunit (Hemingway and Karchesy, 1989).

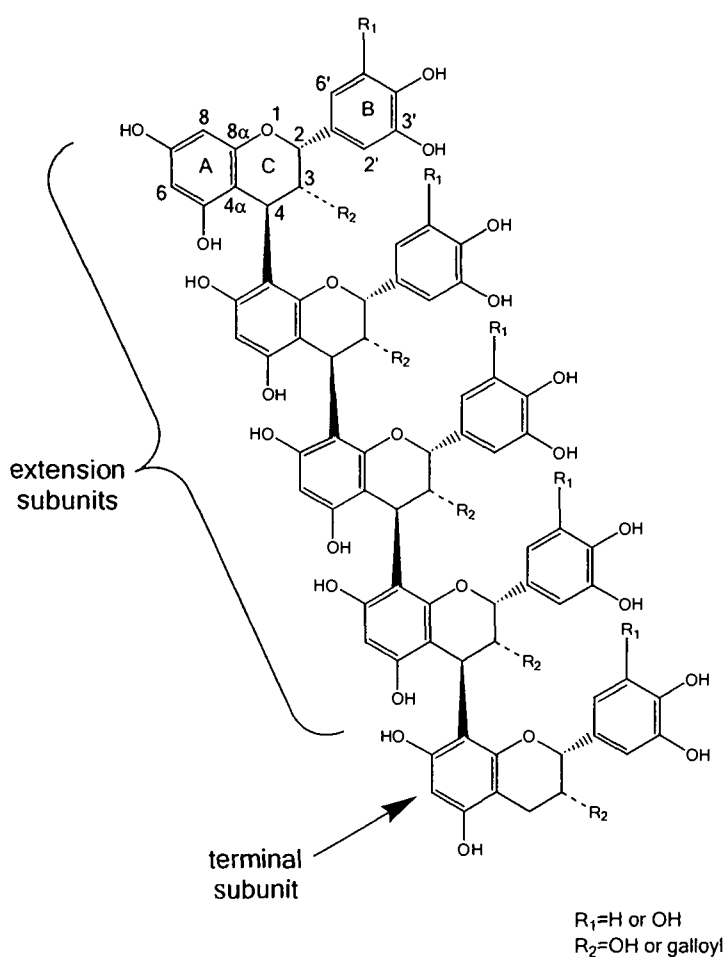


Figure 2.2. Generalized skin proanthocyanidin structure found in *Vitis vinifera* L. berries (Kennedy et al., 2001)

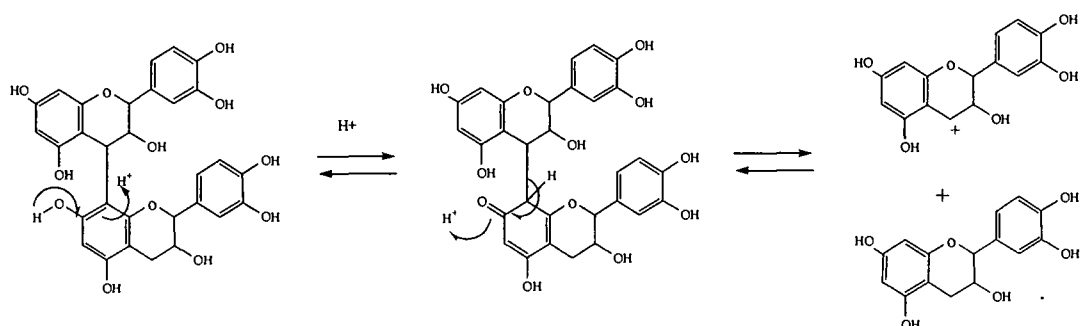


Figure 2.3. Mechanism of acid-catalyzed fission in a proanthocyanidin dimer (Kennedy and Jones, 2001).

Flavan-3-ol monomers and proanthocyanidins are important sensory components, providing red wine with bitterness and astringency. Sensory studies indicate that small molecular weight proanthocyanidins are dominated by the perception of bitterness, and secondarily by astringency. Degrees of polymerization as high as 80 have been claimed for some proanthocyanidins (Souquet et al., 1996). As the molecular weight of the proanthocyanidins increases, astringency becomes the dominant perception. Bitterness is a taste sensation, most commonly perceived at the back of the tongue. Astringency is a tactile sensation in which a drying, puckering feeling is perceived throughout the oral cavity. This phenomenon occurs as a result of the interaction between proteins and glycoproteins in our mouth and proanthocyanidins from wine (Robichaud and Noble, 1990). At the molecular level, the phenolic groups of the tannins bind with the  $-NH$  group of glycoproteins (Heldt, 1997). Evaluating in more detail the proanthocyanidin interactions with salivary proteins, it was observed that (+)-catechin had a higher proanthocyanidin specific activity for proline-rich proteins than (-)-epicatechin (De Freitas and Mateus, 2001). Similarly, larger molecular weight proanthocyanidins interact more readily with

proteins, thus protecting dimers and trimers. In addition, it has been reported that when small amounts of proanthocyanidins are present, the lower molecular weight proline-rich proteins and other low molecular weight proteins had the highest ability to precipitate with proanthocyanidins (Sarni-Manchado et al., 1999). When analyzing the perception of some flavan-3-ol monomers, Rossi and Singleton (1966) reported that (+)-catechins were only bitter and not astringent, however, Robichaud and Noble (1990), showed that (+)-catechin is both bitter and astringent.

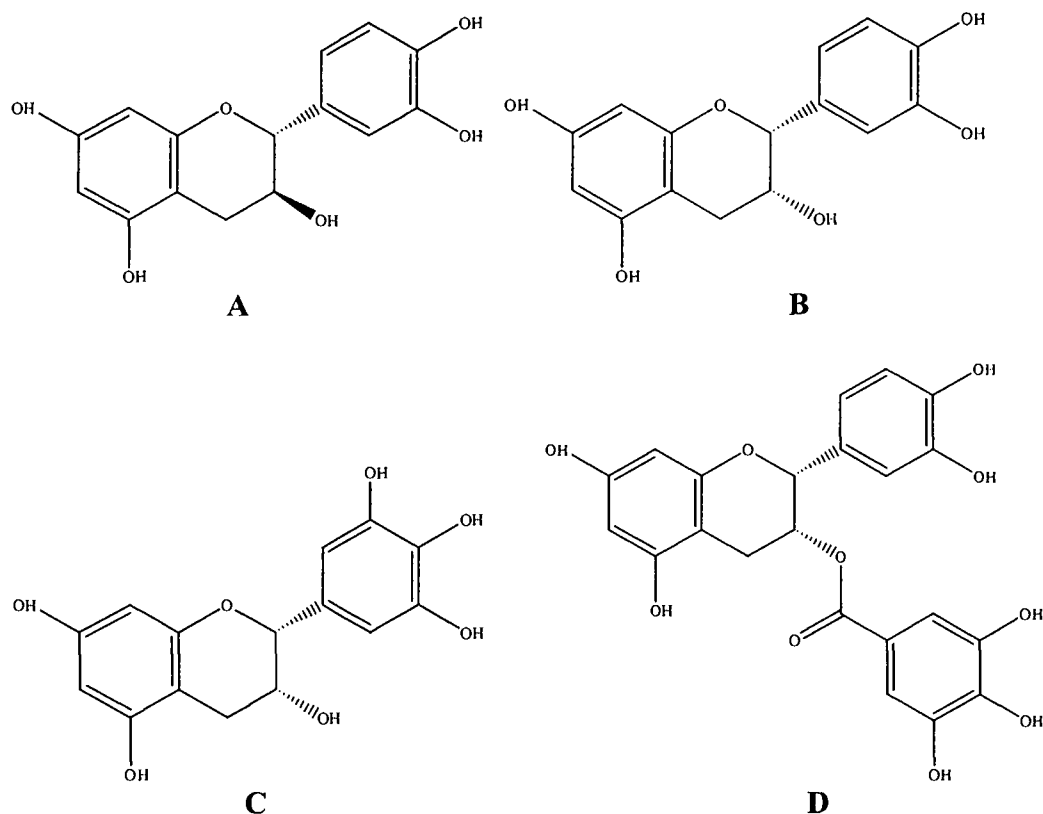
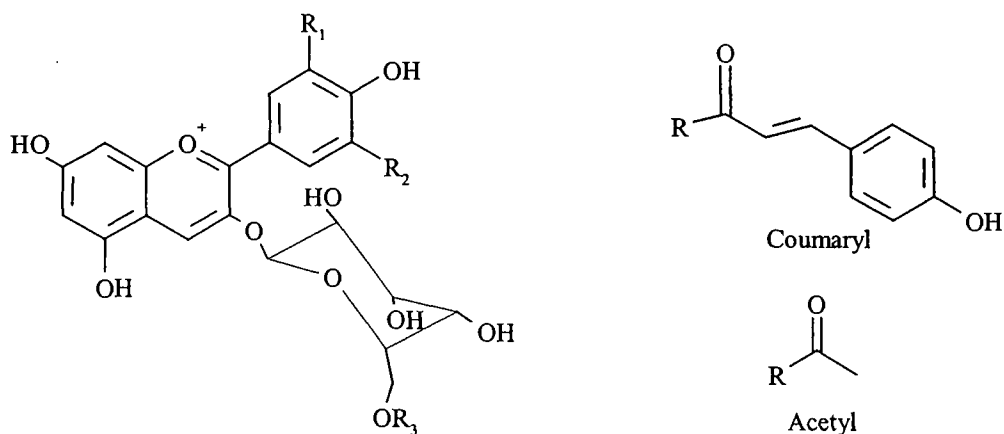


Figure 2.4. Flavan-3-ol monomers: **A.** (+)-catechin, **B.** (-)-epicatechin, **C.** (-)-epigallocatechin, **D.** (-)-epicatechin-3-O-gallate.

Proanthocyanidins are present in the seeds and skins of grapes. They are also present in wine due to extraction during the alcoholic fermentation.

Proanthocyanidins play an important role in wine flavor and mouthfeel, however, other compounds present in wine (sugar, acidity, alcohol, glycerol, etc) can affect their perception (Asquith, et al. 1987; Vidal et al., 2002; Vidal et al. 2004; Siebert and Chassy, 2003). In addition, the interactions between proteins and proanthocyanidins are affected by the solution conditions (solvent composition, ionic strength, pH, and temperature) (Asquith and Butler, 1986). Asquith et al. (1987) showed that carbohydrates could affect the binding ability of the proanthocyanidins because they influence the final protein-proanthocyanidin complex solubility.

Anthocyanins are another class of flavonoid compounds that are widely distributed among plants (Figure 2.5). It is generally thought that their function is to make fruit and flowers attractive to birds and insects for pollination and seed dispersal. They also play a role as a protectant against damaging radiation (Harborne and Williams, 2000). Anthocyanins are water soluble compounds, which can be acylated and glycosylated (Haslam, 1977).



ANTHOCYANIN	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
delphinidin-3-glucoside	OH	OH	H
cyanidin-3-glucoside	OH	H	H
peonidin-3-glucoside	OMe	H	H
petunidin-3-glucoside	OMe	OH	H
malvidin-3-glucoside	OMe	OMe	H
delphinidin-3-glucoside-6''-acetate	OH	OH	acetyl
cyanidin-3-glucoside-6''-acetate	OH	H	acetyl
peonidin-3-glucoside-6''-acetate	OMe	H	acetyl
petunidin-3-glucoside-6''-acetate	OMe	OH	acetyl
malvidin-3-glucoside-6''-acetate	OMe	OMe	acetyl
delphinidin-3-glucoside-6''-coumarate	OH	OH	coumaryl
cyanidin-3-glucoside-6''-coumarate	OH	H	coumaryl
peonidin-3-glucoside-6''-coumarate	OMe	H	coumaryl
petunidin-3-glucoside-6''-coumarate	OMe	OH	coumaryl
malvidin-3-glucoside-6''-coumarate	OMe	OMe	coumaryl

Figure 2.5. The anthocyanins of *Vitis vinifera*.

Anthocyanidins are the aglycones of the anthocyanins. In nature, there are six commonly occurring anthocyanidin structures. However, anthocyanidins themselves are rarely found in plants, rather they are almost always found as the more stable glycosylated derivatives. Sugars are present most commonly at the C-3 position, while a second site for glycosylation is the C-5 position and, more rarely, the C-7

position. The sugars that have been identified include glucose, rhamnose, xylose, galactose, arabinose and glucose (Francis, 1989). The sugars provide additional sites for modification as they may be acylated with acids. The acyl acids involved in order of occurrence, are *p*-coumaric, caffeic, ferulic, *p*-hydroxy benzoic, sinapic, malonic, acetic, succinic, oxalic and malic acids (Francis, 1989). Because of the diversity of glycosylation and acylation, there are at least 300 naturally occurring anthocyanins (Strack and Wray, 1993). They differ also in their hydroxylation pattern of the B ring, which is the major determinant of the color of these pigments (Harborne, 1963).

In most grape varieties, anthocyanins are restricted to the skin tissue. They are extracted during the fermentation of grape must and are responsible for the color of red wine. The anthocyanins found Pinot noir are malvidin, delphinidin, cyanidin, petunidin and peonidin-3-monoglucosides; malvidin-3-glucoside being the most abundant anthocyanin. Pinot noir does not contain acylated anthocyanins (Rankine, et al., 1958; Fong et al., 1971).

**Biosynthesis of phenolic compounds.** Flavonoid biosynthesis is the culmination of two metabolic pathways, the shikimate pathway and the acetate pathway (polyketide pathway). The acetate pathway provides the precursors for the flavonoid A ring from malonyl-CoA and the amino acid phenylalanine, which is produced in the shikimate pathway, provides the B ring (Swain and Williams, 1970; Dewick and Haslam, 1969).

Flavonoid biosynthesis starts with the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA. This reaction is carried out by the enzyme chalcone synthase (CHS). The chalcone is subsequently isomerised by the enzyme chalcone flavanone isomerase (CHI) to yield a flavanone (naringenin). From these central intermediates the pathway diverges into several side branches, each yielding a different class of flavonoids, as shown in Figure 2.6 (Koes et al., 1993).

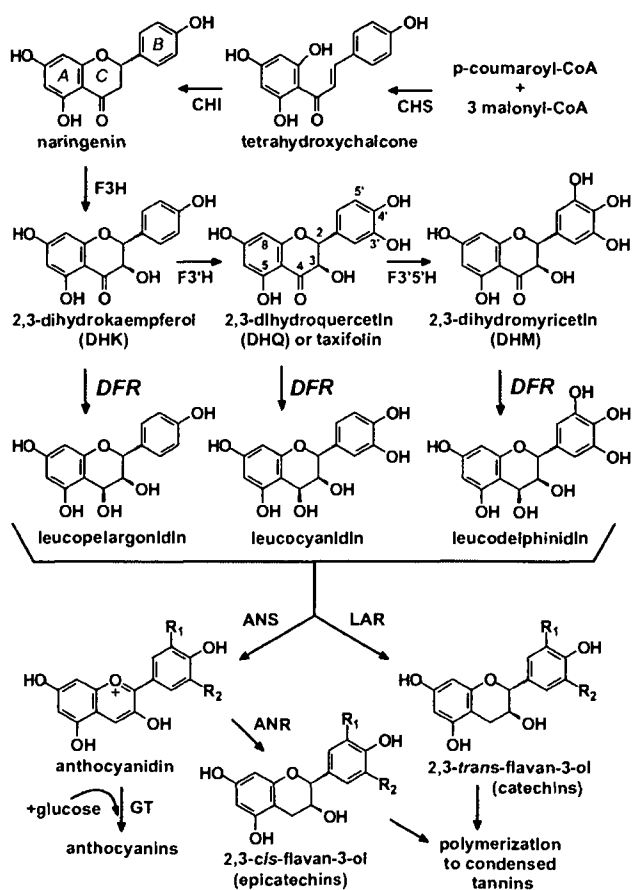


Figure 2.6. Simplified diagram of the flavonoid biosynthetic pathway. Enzymes: CHS chalcone synthase, CHI chalcone flavanone isomerase, LAR leucoanthocyanidin reductase, ANR anthocyanidin reductase, DFR dihydroflavonol reductase.

**Grape anatomy.** The grape cluster is a fleshy fruit containing berries organized into a cluster. Each berry is attached to the rachis by a small pedicel containing vessels, which supply the berry with water and nutritive substances (Ribéreau-Gayon et al., 2000B).

Each grape berry is composed of a group of tissues surrounding the seeds. According to Pratt (1971), the fruit wall from its outer surface to its inner surface adjacent to the seed is the pericarp and has five components: epidermis, hypodermis, outer wall, inner wall and inner epidermis. Viala and Péchoutre (1910) refer to the epidermis as epicarp, the middle of the wall as mesocarp and the inner epidermis as endocarp. However, Ribéreau-Gayon et al. (2000B), point out that the pericarp is divided into the exocarp (the skin), the mesocarp (the pulp) and the endocarp (the tissue that lines the seed receptacles containing the seeds but is not distinguishable from the rest of the pulp).

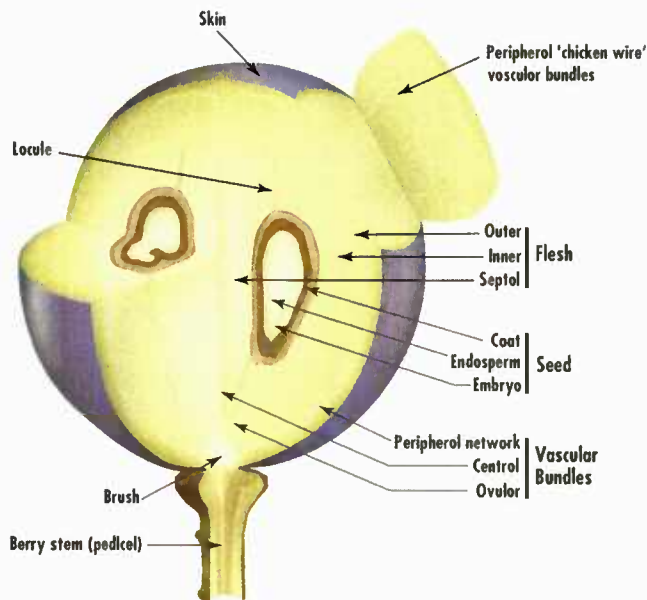


Figure 2.7. Anatomy of a grape berry (Illustration by Jordan Koutroumanidis and provided by Don Neel *Practical Winery and Vineyard*. Found in *Australian Viticulture* from text: “Ripening berries – a critical issue” by Dr. Bryan Coombe and Tony Clancy, 2001).

**Grape development.** The development of the grape berry has a double-sigmoidal growth curve (Terrier et al., 2001; Considine and Knox, 1979; Coombe, 1976; Harris et al., 1968; Winkler et al., 1974). There are three commonly described phases of growth: anthesis and first stage of rapid growth (cell division, enlargement, and pedicel growth), a period of no or slow growth (seed maturation and seed coat hardening), and a final rapid period of growth (fruit ripening, cell enlargement), which are commonly described as Stage I, Stage II and Stage III, respectively (Barnavon, 2000; Harris et al., 1968; Winkler et al., 1974).

The transition into fruit ripening occurs within a period of a few days where the berry goes from a stage of quiescence (lag phase = Stage II) to one of rapid growth, sugar accumulation, acid metabolism, and for a colored variety, anthocyanin

synthesis. Varieties without anthocyanins (e.g. Chardonnay) lose their green color through chlorophyll degradation and become translucent (Harris et al., 1968 cited by Creasy, 1991).

**Phenology.** In spring, after dormancy has been broken and temperatures increase, buds swell and shoots emerge. This process is generally called *bud break*. After buds burst, leaves appear and after 6 to 8 weeks, flowers open. Over the rather inconspicuous flower, a cap is positioned and when anthers are ready to be released the cap is shed and the pollen from the anthers falls on the stigma. This stage is referred as *capfall*, *anthesis* or *bloom*. Two to three days later, fertilization is complete and in another two to three days those flowers not fertilized begin to drop off – a stage known as *shatter*. *Set* refers to the flowers which have been pollinated and fertilized and which remain on the bunch (Jackson and Schuster, 1987). The term *fruit set* is used to describe the transformation of flowers into fruits. *Fruit set* may refer to the percentage of flowers in an inflorescence, which grow into fruits or to the physiological process involved in the early stages of fruit growth (Mullins et al., 1992).

The stage at which anthocyanin pigments first appear in a grape berry is known as *véraison*. At this stage, growth accelerates, berry begins to soften, glucose and fructose increase, acidity decreases, chlorophyll is lost and the color develops in red and black varieties. At *véraison*, acid levels are high, but after this the levels are reduced by dilution, caused by an inflow of water into the berries, and by the conversion of acids to salts (Jackson and Schuster, 1987; Mullins et al., 1992).

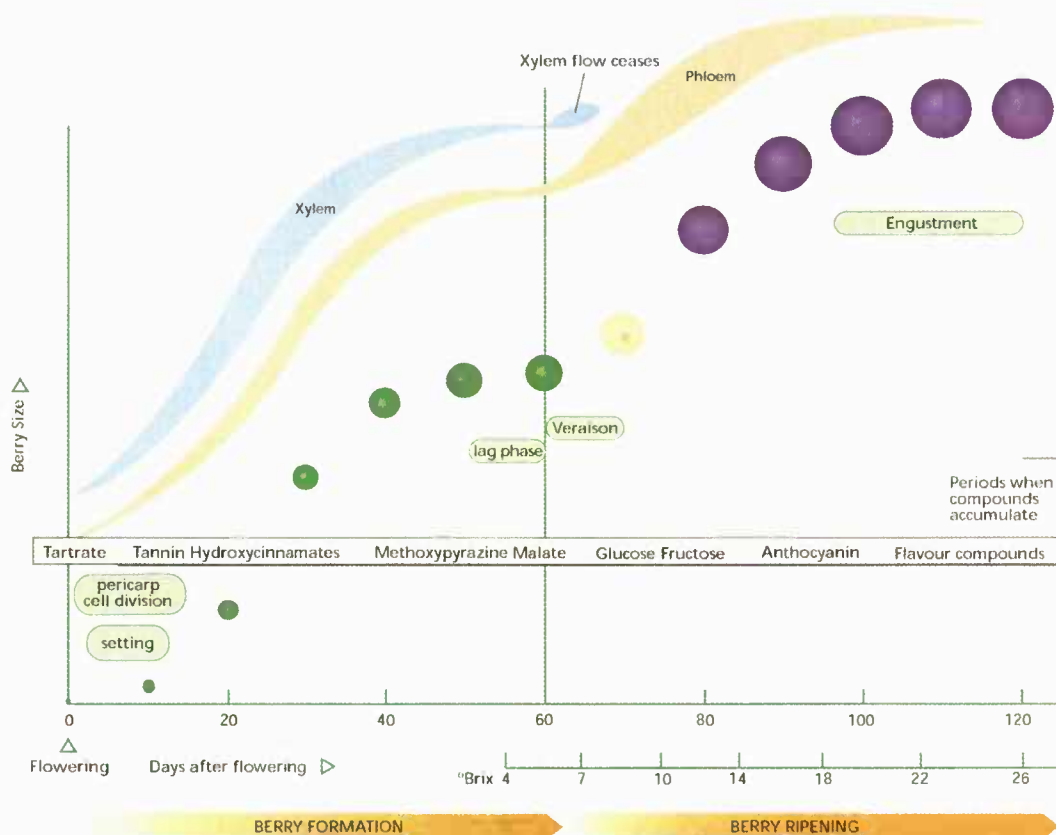


Figure 2.8. Stages of berry development showing the relative size and color of berries at ten day intervals after flowering. Also shown the periods when compounds accumulate, the levels of juice °Brix, and the rate of inflow of xylem and phloem vascular saps into the berry (Illustration by Jordan Koutroumanidis and provided by Don Neel *Practical Winery and Vineyard*).

**Chemical changes and solute accumulation.** According to Kennedy (2002) the grape berry is essentially an independent biochemical factory, in which the berry has the ability not only to transport the primary metabolites essential for plant survival (water, sugar, amino acids, minerals and micronutrients), but also to synthesize complex berry components (for example, flavor and aroma compounds) that define a particular wine.

The berry is supplied through the berry stem or pedicel by a vascular system composed of xylem and phloem elements. The xylem is the tissue that transports water, minerals, growth regulators, and nutrients from the root system to the aerial portions of the vine. The phloem is the vascular tissue involved in photosynthate transport from the canopy to the vine. The phloem also carries other organic compounds such as amino acids and organic acids (Winkler et al., 1974). According to Greenspan et al. (1994), the xylem has an almost exclusive role in water transport into the berry during the pre-véraison development. The phloem however, has a reduced function early in berry development, but becomes the primary source of ingress after véraison.

Sugars are the primary constituents of the grapes, essentially glucose and fructose. Fructose is always predominant (the glucose/fructose ratio is around 0.9). Sucrose, which is the migratory form of sugar in the plant (Swanson and El Shishiny, 1958), exists only in trace amounts in the grape.

Tartrate and malate are the most prevalent acids in grapes. These acids are distributed in the grape somewhat differently, with tartaric acid being highest

towards the outside of the developing berry, and malic acid being highest in the flesh. Tartaric acid accumulates during the initial stages of berry development while malic acid accumulates just prior to véraison (Ruffner et al., 1983; Possner et al., 1983; Hrazdina et al., 1984). These acids provide wine with acidity and are therefore critical to wine quality (Butzke and Boulton, 1997).

Approximate chemical indices for Oregon Pinot noir must are: 21.5-24.5 °Brix, 6-9g/L TA, 3.35-3.8 pH, 2-5 g/L malate and 2.5-8g/L tartrate; and wine: 11.5-13.5 % v/vethanol (reviewed in Miranda-Lopez, 1990).

The bulk of polyphenolic compounds occur in the skins, seeds and conductive tissues of grape berries at all stages of development (Hawker et al. 1972). The accumulation of proanthocyanidins occurs during the first period of berry growth. Proanthocyanidins mainly occur in the outer protective layers of both grape skin and seeds (Amrani-Joutei et al., 1994; Prieur et al., 1994; Souquet et al., 1996). They accumulate in the cell vacuoles and range from fine granules to more or less agglutinated masses (Fougere-Rifot et al., 1993). Amrani et al. 1994, localized three different tannins in grape skins: the first consisted of free tannins in solution in the vacuolar sap (these proanthocyanidins appear in the form of granules decreasing in size from the skin surface to the pulp). The two other groups were constituted by bound proanthocyanidins: those bound to the proteins of the internal face of the tonoplast, and those bound to the cell wall polysaccharides by osidic bonds. Anthocyanins develop at the beginning of the second stage. They also accumulate in the vacuole of the cells (Alfenito et al., 1998).

**Seed development.** Originating from the four ovules of the ovary, the grape berry contains a maximum of four seeds. However, the number of seeds is typically less than four because of the absence or abortion of one or several ovules (Winkler et al., 1974). According to Cawthon and Morris (1982), seed number per berry is related to accumulation of  $^{14}\text{C}$ -photosynthate, fresh berry weight and dry berry weight. In addition, percentage of soluble solids and intensity of juice color after véraison were inversely related to seed number. However, Hardie and Aggenbach (1996), found no relationship between number of seeds per berry and seed weight. Trellising, pruning level, canopy manipulation and regulation of bunch number per vine have had little effect on seed numbers or weight per berry. Ebadi et al. (1995) found a reduced number of seeds per berry when the vines were exposed to low temperatures just before and during flowering, and Hale and Buttrick (1974) reported that, three weeks after flowering, seed number and seed weight could no longer be influenced by varying temperature conditions. Scienza et al. (1978) observed that one-seeded berries contained a statistically higher amount of anthocyanin than two or three-seeded berries, in particular during the final phase of ripening in September. Moreover, seed number was positively correlated with the concentrations of gibberellin-like substances and abscisic acid (on a per-berry basis).

The seeds are an important source of phenolic compounds during red winemaking. Bourzeix et al. (1986), when analyzing the average content of proanthocyanidins in different grape varieties (Alicante-Bouschet, Aramon, Carignan, Cinsaut, Grenache noir, Mourvedre and Syrah), found that seeds

contained 38% of the total proanthocyanidins distributed between all parts of the grape (stalk, pulp, seed and skin).

Kennedy et al. (2000A) reported that seeds (cv. Shiraz) reached their maximum weight one week before véraison and thereafter, the fresh seed mass declined steadily. According to Kelly et al. (1992), the final phase of seed development involves the loss of water and cessation of reserve synthesis, followed by a metabolically inactive state. Dormancy is due to either the impermeability of the seed coat to water and/or gases, the mechanical prevention of radicle extension, or the seed coat preventing inhibitory substances from leaving the embryo. The hardening of the grape seed is due to lignification of the inner layers of the outer integument. The inner integument remains thin and adheres to the endosperm (Mullins et al., 1992).

**Effect of climate on berry development.** Climate is one of the major factors determining both where grapes can be grown and the quality of the wine produced from those grapes. If the mean temperatures are above 20 °C, the winters are mild and leaf fall and vine dormancy do not occur, or occurs only partially; temperate plants such as grape vines crop poorly under these conditions. In short summers, vines have insufficient time to ripen their fruit and cold winters may kill or seriously damage the plant (Jackson and Schuster, 1987). Grapes are native to the warm temperate zone and their culture is most successful between 34° and 49° north and south latitude. The time of ripening is determined primarily by variety and heat summation. Seasonal conditions, particularly temperature and heat summation,

markedly influence the rates of the development changes. *Vitis vinifera* requires long, warm-to-hot, dry summers and cool winters (Winkler, et al. 1974). Bonnardot (1997) reported that high spring temperatures, after a dry winter, lead to an early date of Pinot noir harvest, whereas low spring temperatures, after a rainy winter, lead to a late date of harvest.

Heat summation for the grapevine is generally defined as the average daily summation of temperatures above 50 °F (10 °C). The base line is set at 50 °F (10 °C) because there is almost no shoot growth below this temperature. The summation is expressed as degree-days (Winkler et al., 1974). However, this method does not take into account periods of fog, cloud cover, or wind; factors that change temperature during the period concerned (Williams, 1987).

Using mean temperatures, Prescott (1964) reported that the areas suitable for grape production should have the mean temperature of the warmest monthly period in excess of 66 °F (18.9 °C) and that of the coldest monthly period in excess of 30 °F (-1.1 °C). Grapes can be grown outside these limits but only in areas where there are compensating conditions.

According to Ribéreau-Gayon et al. (2000A), temperature affects photosynthetic activity and metabolism in the vine, and its action is not limited to the period of grape development. Its influence on bud burst and flowering dates also has important indirect consequences on grape quality. Ewart and Kliewer (1977) observed that ovule fertilization in several winegrape varieties was reduced at 15 °C day/10 °C night temperatures versus 25 °C/20 °C regimes. Grapevines are self

fertilized, but if the temperature is too cool, pollen may not grow quickly enough to fertilize the ovule and in rainy periods pollen may be washed from the clusters (Winkler et al., 1974).

The accumulation of some compounds in the grape is also affected by temperature conditions. Kliewer (1977) found that at 37/32 °C (day/night), soluble solids in grapes did not increase above 12.9 °Brix, whereas at mean daily temperatures of 20.3 °C, 21 °Brix was obtained. The synthesis and metabolism of organic acids in grapes have also been reported to be influenced by changes in temperature. Hence, increasing culture temperatures from 10 to 25 °C increased the synthesis of tartaric and citric acid; however, temperatures higher than this resulted in a reduction of these acids. (Kliewer, 1964).

Phenolic content in grapes seems also to be affected by weather conditions. Although few studies have been done to find the relationship between flavonoids and temperature, some studies regarding light exposure have been attempted. Hence, Price (1994) found higher accumulation of phenolics in epidermal tissues of grapes exposed to higher levels of solar irradiance. On the other hand, Downey (2003) did not observe an effect of shading on seed proanthocyanidins content and composition, but there were differences in the proanthocyanidin content of grape skins. Although these differences were pronounced around *véraison*, they were not significant at the end of the season.

Anthocyanin synthesis in grapes is related positively to light and negatively to heat. Hence, Spayd et al. (2002) found that when sun-exposed clusters in the vine

were cooled, they underwent an increase in anthocyanin content. However, heating shaded clusters decreased anthocyanin concentration in grapes. Similarly, Kliewer and Torres (1972) reported that grapes grown under cooler night temperatures had a higher accumulation of anthocyanins. Exposure to solar radiation increases concentration of flavonols, while temperature has little to no effect on their concentrations (Spayd et al., 2002). While heat may affect the biosynthesis of certain compounds in the grape, heat also seems to play a role in their degradation. Hence, significant oxidation reactions, especially those involving phenolics, occur at elevated temperatures (Mullins et al., 1992). Similarly, Kliewer (1964) reported a decrease in acidity in ripe grapes at high temperatures as a result of malic acid respiration.

**Phenolics in wine.** Phenolic compounds are responsible for the bitterness, astringency and color of wines. According to Haslam (1977), a wine without enough phenolic compounds would be perceived as flat and insipid, while a wine with too much phenolics would have a harsh and rough quality.

Wollan (1997) indicates that the quality of phenolics affecting the organoleptic quality in the wine depends on the state of ripeness of the fruit: Grapes that are harvested too early will produce wines light in color, thin in body and harsh “greenness” on the palate. On the other hand, high quality ripe fruit from good vineyards will give red wines that are dark and rich with soft acid, supple proanthocyanidins, balanced astringency, and just a hint of bitterness.

Although mainly due to the quality and concentration of proanthocyanidins, astringency and bitterness perception is also affected by the alcohol, polysaccharides, acidity, and sugar levels in wine (Noble and Shannon, 1987; Fischer and Noble, 1994; Asquith, et al. 1987; Vidal et al., 2002; Vidal et al., 2004)

An excessively long maceration period and elevated temperatures (above 35 °C) can result in a decrease in anthocyanins, both by absorption of these molecules on yeast and pomace, and by reactions of degradation and condensation with proanthocyanidins, respectively. A short maceration leads to a wine rich in anthocyanins but relatively poor in total phenolics (proanthocyanidins); if the maceration period is lengthened, anthocyanin content and color decrease, while total phenolics still increase but more slowly (Ribéreau-Gayon, 1982).

During wine aging, the chemical transformation of anthocyanins is accompanied by a modification of color hue, 'full red' in young wines, evolving gradually to 'orange-brown' in very old wines (Ribéreau-Gayon, 1982). The color change is due to the complex formation of flavonoids with anthocyanins, which produces an increase in both absorbance and bathochromic shift of the visible absorption maximum of the latter. This interaction is based mainly on hydrogen bond formation between a carbonyl group of the anthocyanin anhydrobase and aromatic hydroxyl groups of the complex-forming flavonoids. The larger the number of hydroxyl groups in the flavonoid molecule, the stronger the complex formation (Chen and Hrazdina, 1981). The chemical reactions which cause color change and

pigment stabilization, also cause a loss of astringency and the development of desirable sensory characters in an aged red wine (Williams, 1997).

Sulfur dioxide is important in the prevention of microbiological spoilage in wine but also has an antioxidant role in the prevention of the polymerization and condensation reactions described above. In this regard, the use of sulfur dioxide should be carefully moderated. While too much sulfur dioxide would block the processes necessary for the normal maturation of wine and too little would allow them to proceed too quickly (Wollan, 1997).

**Relationship between weather, grapes and wine.** The development of phenolic compounds during grape ripening has been described in previous research (Kennedy et al., 2000A; 2001). Different authors have studied the concentration of flavonoids in wine with particular emphasis on influencing factors such as different varieties, different regions, different winemaking processes, etc (Bourzeix et al. 1986; Carando, 1999; Waterhouse and Teissedre, 1997; Archier et al., 1993). Other studies involving wines made at different grape maturities have been done. Thus, Miranda-Lopez et al. (1992) reported that wines made with grapes at increasing levels of maturity had more odor-active peaks than wines from earlier harvested fruits. Similarly, Perez-Magariño and Gonzalez-San José (2004) studied the evolution of phenolics during aging of red wines made with grapes harvested at different maturities. However, there is no previous information about the relationship between weather conditions, grape maturity, wine quality and proanthocyanidin structure. Poorly understood is the link between climate and the accumulation of

anthocyanin and proanthocyanidins during berry development, as well as the other characteristics of grape phenolic composition that influence the ultimate properties of a red wine. Moreover, no research has been done that attempts to define the term “tannin ripeness”.

## **CHAPTER 3**

### **Evolution of Anthocyanins and Proanthocyanidins During Berry Development**

#### **Introduction**

Information about environmental factors affecting anthocyanin and proanthocyanidin biosynthesis in grapes is very limited. While some authors have reported that anthocyanin biosynthesis is affected by light (Kliewer, 1977), other studies have not found this relationship (Price et al., 1995). In addition, recent studies suggest that the combination of low temperatures and high sun exposure significantly enhance the accumulation of anthocyanins in grapes (Spayd et al., 2002). Information about factors affecting proanthocyanidin biosynthesis in grapes is more limited and inconclusive. Recent studies suggest the influence of light in skin proanthocyanidin biosynthesis (Downey, 2003). However, no studies have investigated the relationship between proanthocyanidin biosynthesis and temperature. The purpose of this chapter is to study the affect of vintage on anthocyanins and proanthocyanidins during grape berry development.

## Materials and Methods

**Chemicals.** All chromatographic solvents were HPLC grade. Acetonitrile, methanol, ethanol, acetic acid, gallic acid, ascorbic acid and potassium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ). (+)-Catechin, (-)-epicatechin, quercetin, phloroglucinol and caffeic acid were purchased from Sigma (St. Louis, MO). Malvidin-3-glucoside was purchased from Polyphenols Laboratories (Sandness, Norway). Ammonium phosphate monobasic and orthophosphoric acid were purchased from Fisher Scientific (Santa Clara, CA). Hydrochloric acid, and sodium acetate anhydrous were purchased from E.M.Science (Gibbstown, NJ), and Mallinckrodt (Phillipsburg, NJ), respectively.

**Instrumentation.** A Hewlett-Packard, Model 1100 HPLC (Palo Alto, CA) with a vacuum degasser, autosampler, quaternary pump, DAD, column heater, Pentium III computer and chemstation software was used for chromatographic analysis. A pH-meter Thermo Orion model 370 was used for pH measurements. Soluble solids were measured using a hand-held refractometer (Amercian optical corporation). A platform shaker model Innova 2300 purchased from New Brunswick Scientific (Edison, NJ) was used for the extraction of phenolics. A freeze drier (Labconco, Kansas City, MO) and rotary evaporator #R-205 (Buchi, Kansas City, MO) were also used.

**Weather information.** Cumulative temperature information was taken from the Oregon State University's Hyslop monitoring station located in Corvallis, Oregon. The station is located just off U.S. Highway 20 at latitude 44.63 degrees N, 123.20 degrees W, and at an elevation of 225 feet above sea level. This data is available online: [www.ocs.orst.edu/pub\\_ftp/weather/hyslop](http://www.ocs.orst.edu/pub_ftp/weather/hyslop).

**Sampling of grapes.** Grapes (*Vitis vinifera* L. cv. Pinot noir, self-rooted Pommard clone) were collected from vines grown at the Oregon State University experimental vineyard located in Alpine, Oregon. Six rows containing approximately 50 vines each and two rows containing 20 vines were selected for study. Vineyard operations were consistent with commercially accepted practice. Over three consecutive vintages (2001-2003), five cluster samples (x5 replicates) were collected for analysis each week beginning approximately four weeks prior to véraison and continuing through commercial harvest. Berries were kept at 4 °C until processed.

To process, and for each replicate, grape berries were first removed from the cluster and then randomized. Berries were separated into two groups, the first for phenolic analysis and the second for pH, titratable acidity and sugar analysis. Berries selected for pH, titratable acidity and sugar analysis were kept at -20 °C prior to analysis.

**Extraction of phenolics.** A 150-berry subset of each replicate (x5 replicates) was used for the extraction and analysis of phenolics. Berries were counted, weighed and frozen at -20 °C until analysis. Seeds and skins were separated by

hand from frozen berries and rinsed with distilled-deionized water. Seeds were counted, weighed, freeze-dried and then reweighed; skins were also freeze-dried and weighed. Seeds and skins were then extracted separately in a 2:1 v/v acetone:water (1 ml/gm berry wt.) extraction solvent at room temperature for 24 hrs and at 100RPM on a platform shaker. After extraction, extracts were filtered through Whatman #1 filters and evaporated under reduced pressure at 38 °C to remove acetone. The remaining aqueous solution was adjusted to a volume of 100 ml with water. Extracts were frozen at -20 °C until phenolic analysis.

**Analysis of anthocyanins and flavan-3-ol monomers.** Total anthocyanin and flavan-3-ol monomer content were measured by reversed-phase HPLC. Separation of phenolics was achieved with a LiChrospher 100 RP-18 (4x250 mm, 5µm particle size) column, protected by a guard column containing the same material, and was purchased from Merck (Darmstadt, Germany). Solvent gradient conditions and mobile phases were prepared as described previously by Lamuela-Raventos and Waterhouse (1994). The aqueous solution containing the grape phenolics was thawed to room temperature, filtered using Teflon filters (0.45 µm, Acrodisc CR13) before injection. Eluting phenolics were monitored at 280 and 520 nm.

A standard was prepared by dissolving (+)-catechin (100 mg/L), (-)-epicatechin (100 mg/L) and malvidin-3-glucoside (30 mg/L) in a 20% v/v methanolic solution containing 3% v/v acetic acid.

**Analysis of proanthocyanidin composition.** Proanthocyanidin composition was determined by reversed-phase HPLC after acid-catalysis in the presence of excess

phloroglucinol (phloroglucinolysis). The column was Chromolith RP-18e (100-4.6mm) purchased from Merck (Darmstadt, Germany). The method utilized a binary gradient with mobile phases containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Solvent gradient conditions were the same as described previously by Kennedy and Taylor (2003).

Three milliliters of the aqueous solution containing the seed and skin phenolics were freeze dried to a dry powder. The resulting seed powder was dissolved in methanol and the volume was adjusted to 5 ml; skin powder was adjusted to 2 ml. A solution of 0.2 N HCl in MeOH, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid was prepared. The proanthocyanidin present in the methanolic solution was reacted with the same volume of this solution at 50 °C for 20 minutes and then combined with 5 volumes of 40mM aqueous sodium acetate to stop the reaction. This final solution was filtered through Teflon filters (0.45 µm PTFE acrodisc CR13) before injection. Proanthocyanidin cleavage products were estimated using their response factors relative to (+)-catechin (Kennedy and Jones, 2001), which was used as the quantitative standard. The standard was prepared by dissolving (+)-catechin (100 mg/L) and acetic acid (1 ml/L) in 12% v/v ethanol. To calculate the mean degree of polymerization (mDP), the sum of terminal and extension subunits (in moles) was divided by the sum of terminal subunits (in moles). The terminal subunits did not include flavan-3-ol monomers (they were subtracted using the data gathered from the flavan-3-ol monomer analysis).

**Analysis of titratable acidity, pH and sugar concentration.** The remaining berries were used to analyze titratable acidity, pH and sugar concentration. Approximately 70 gm of berries within each replicate (5 replicates) were randomly selected, thawed and crushed. The resulting juice was separated and used for the analysis of titratable acidity, soluble solids and pH. For titratable acidity analysis, 100 ml of boiling deionized water (pH previously adjusted to 8.2-8.4 with a few drops of 0.1 N KOH) were poured into a 250 ml beaker containing 5 ml of juice sample. After cooling to room temperature, the solution was titrated with 0.1 N KOH to a pH 8.2-8.4. The volume of KOH was used to calculate the titratable acidity expressed as tartaric acid. The soluble solids and pH were measured directly from the grape juice. The sugar content was estimated by refractometric measurement of total soluble solids in the grape juice.

**Statistical analysis.** Analysis of variance was conducted to compare data between these three vintages and Tukey analysis ( $\alpha=0.05$ ) was used to compare averages. SAS version 8.0 was used to perform all the statistical analysis.

## **Results**

**Phenological events.** Significant phenological events that occurred during the 2001-2003 vintages are shown in Table 3.1. Grapes were also picked after commercial maturity, so overripe grapes could be analyzed. The dates of

commercial ripeness were determined based upon the concentration of soluble solids. It is clear that 2003 reached ripeness at the earliest date.

**Table 3.1. Phenological events during the 2001-2003 growing seasons.**

Year	Bud break	Full bloom	Véraison	Commercial ripeness
2001	Apr-20	Jun-20	Aug-27	Sep-27
2002	Apr-16	Jun-23	Aug-25	Sep-26
2003	Apr-16	Jun-23	Aug-23	Sep-22

**Weather information.** Since vine development and metabolism is strongly affected by temperature (Mullins et al., 1992), information on temperature was collected for the three vintages. Other variables such as precipitation and humidity can play a role in vine temperature; however, given that the vintages under study were generally dry and warm without extreme temperatures (Corvallis Weather Station website), the weather data presented in this section focuses only on temperature and heat summation data.

The cumulative growing degree-days are shown in Figure 3.1. From fruit set through harvest, it is clear that 2003 accumulated considerably more heat than 2001 and 2002. During this period, 2002 accumulated more heat than 2001, however, before fruit set the pattern of heat accumulation was different. The cumulative heat summation in growing degree days at different dates during the growing season is shown in Table 3.2. As observed, 2001 accumulated more heat than 2002 and 2003 up to June 16<sup>th</sup>.

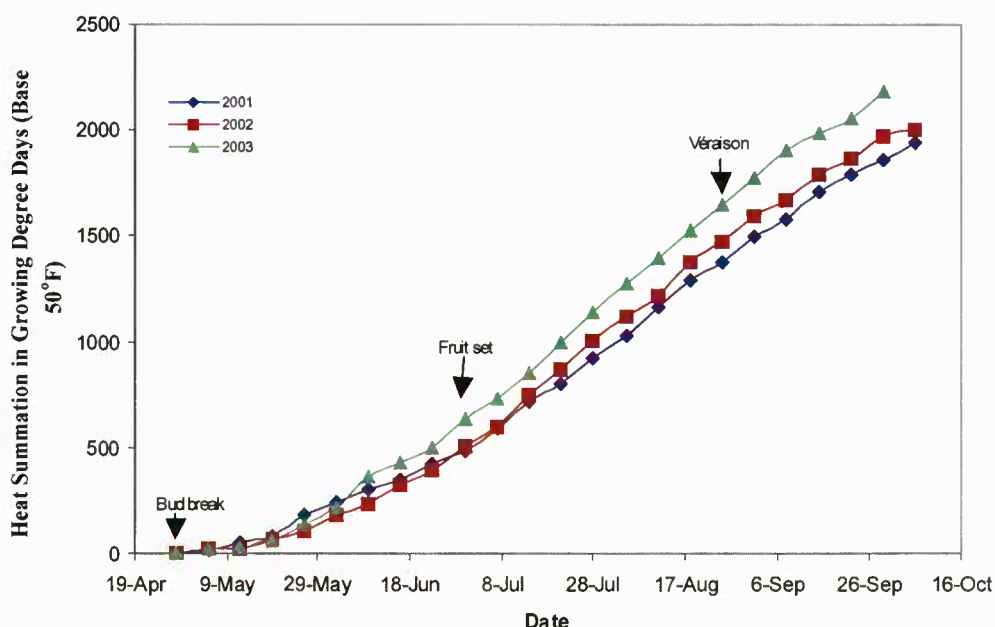


Figure 3.1. Heat summation expressed in cumulative growing degree-days (50 °F base) for the 2001-2003 growing seasons with approximate phenological events indicated.

Table 3.2. Growing degree days (50 °F base) at different dates during the three seasons.

Vintage	2-Jun	16-Jun	30-Jun	21-Jul	25-Aug
2001	244	345	485	803	1375
2002	180	325	503	867	1473
2003	220	429	632	999	1645

Growing degree-days provide information on the cumulative heat that the vine receives during a period of time and above a base temperature at which the plant begins to be active (50 °F or 10 °C). Most of the information in existing studies express the heat summation in growing degree days using a base line of 50 °F, however, the modern trend is focused on expressing the heat summation in SI units, with the corresponding change in the numeric values (10 °C baseline). In the present study, heat summation is expressed using 50 °F base in order to facilitate the

comparison to the existing information. However, to transform heat summation from 50 °C base units to 10 °C base units, we propose this formula:

$$\text{GDD (10 °C base)} = [\text{GDD (50 °F base)}]/1.8$$

This formula will provide the means to compare the heat summation in this study as expressed in 50 °F base line with modern studies and information available in the future.

Temperature is also relevant to vine metabolism and phenolic biosynthesis. Figures 3.2-3.3 summarize the average, maximum and minimum daily temperatures for the three vintages. Short- term variations in temperature can be observed in the three graphs.

The maximum daily temperature (Figure 3.2) for 2003 was consistently above 25 °C during the period between fruit set and véraison; 2002 was also above this temperature for much of the time, although temperatures were below 25 °C several times, for consecutive days in 2001. The three years had peak temperatures above 35 °C. 2002 exceeded 40 °C on one day. At fruit set, 2003 reached one of its maximum peaks (>35 °C) while 2002 also reached a high temperature at this time (32 °C), but never as high as 2003. About three weeks prior to véraison, 2001 and, to a lesser extent, 2002 experienced temperatures below 17 °C.

During the period between véraison and harvest, 2003 had peaks in temperature above 35 °C, while 2001 and 2002 remained below this.

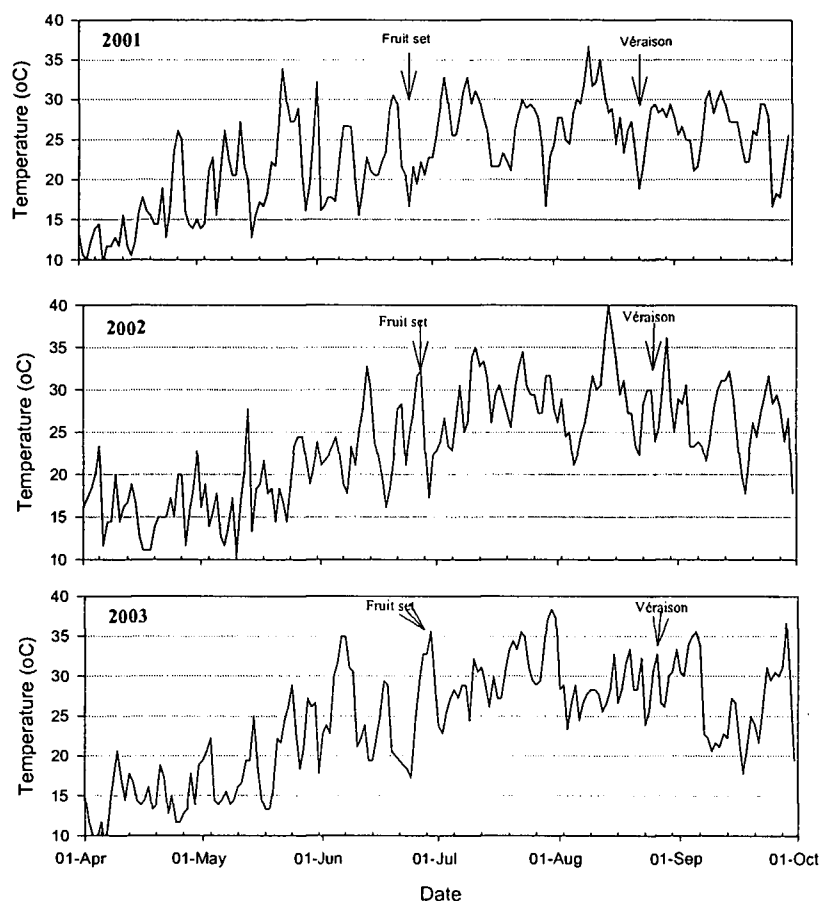


Figure 3.2. Maximum daily temperature over three seasons, at Corvallis Weather Station.

In analyzing the minimum daily temperature during the period between fruit set and véraison (Figure 3.3), it was observed that 2003 had several peaks exceeding 15 °C, while 2002 only reached one peak above this temperature (no temperatures above 15 °C were observed for 2001 during this period). The 2001 and 2002 experienced temperatures below 10 °C for several consecutive days, while 2003 reached temperatures below 10 °C fewer times and only once every few days. None of the three seasons experienced minimum temperatures below 5 °C. Before fruit set, and for several weeks, the 2003 season remained above 7 °C, while 2001 and 2002

experienced, several times and for consecutive days, temperatures below 4 °C. In a particular case in 2002, temperatures reached below 0 °C. At fruit set, the minimum daily temperature for 2003 was 15 °C, while 2001 and 2002 reached temperatures of 13 and 12 °C, respectively.

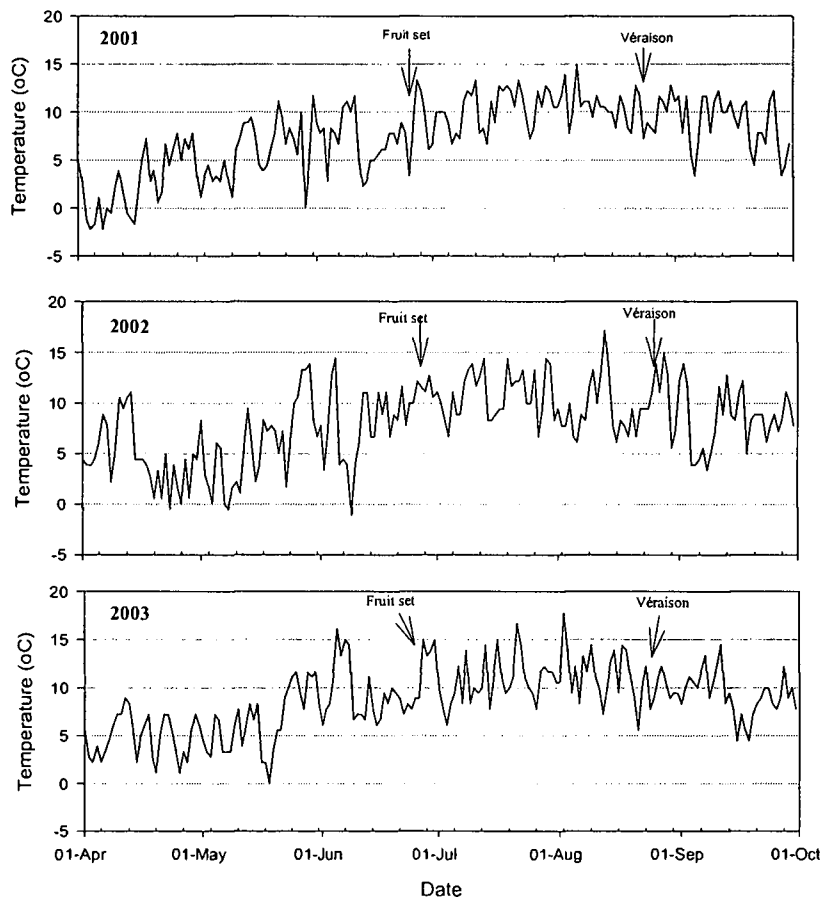


Figure 3.3. Minimum daily temperature over three seasons, at Corvallis Weather Station

**Berry development.** Berry weights were different for the three vintages, although they generally developed in parallel (Figure 3.4). Véraison (Figure 3.4 inset) was considered to be when approximately 50% of the berries had turned red. For 2003, this event occurred approximately three to four days earlier than for other years. A general pattern was observed for the three years: the berries increased in weight until two weeks post véraison, then they experienced a stabilization with a slight increase in weight followed by a decrease right after they reached commercial maturity. The berry weight for 2001 increased dramatically after véraison. At the time of harvest, the berry weight for the 2001 vintage (1.3 gm/berry) was significantly higher ( $p < 0.001$ ) than for the 2002 (1.1 gm/berry), and 2003 (1.1 gm/berry) vintages. The differences between 2002 and 2003 were not significant.

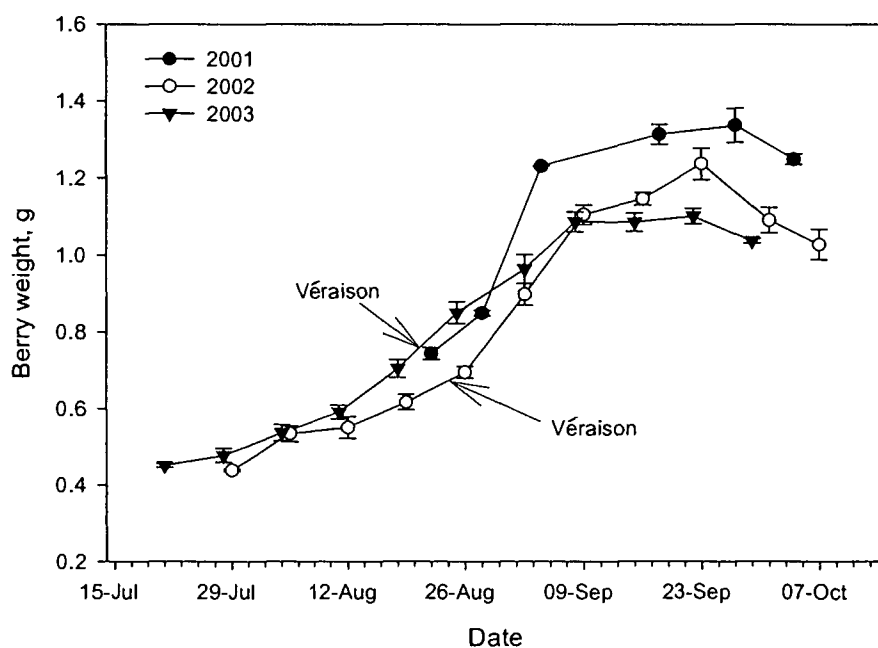


Figure 3.4. Berry weight change and véraison onset for the 2001-2003 growing seasons with error bars indicating  $\pm$  SEM (N=5).

In addition to information on berry weight, information on vineyard production were documented. Differences in overall fruit production varied between years (2001: 1.5 T/Ac; 2002: 2.4 T/Ac; 2003: 2.75 T/Ac). The lower production observed during the 2001 vintage was due to more cluster thinning at véraison.

The fresh and dry skin weights for 2002 and 2003 are shown in Figure 3.5. Although fresh skin weight developed in parallel for both years, for 2003, the fresh skin weight increase began one week earlier than 2002. At harvest, berries from both years had similar fresh skin weights ( $p=0.72$ ). The dry skin weight for 2002 and 2003 had the same pattern and weight across maturity and just at harvest, grapes from the 2002 growing season had an extra accumulation of skin weight, however, this did not lead to a significant difference in dry skin weight ( $p=0.0932$ ) at harvest.

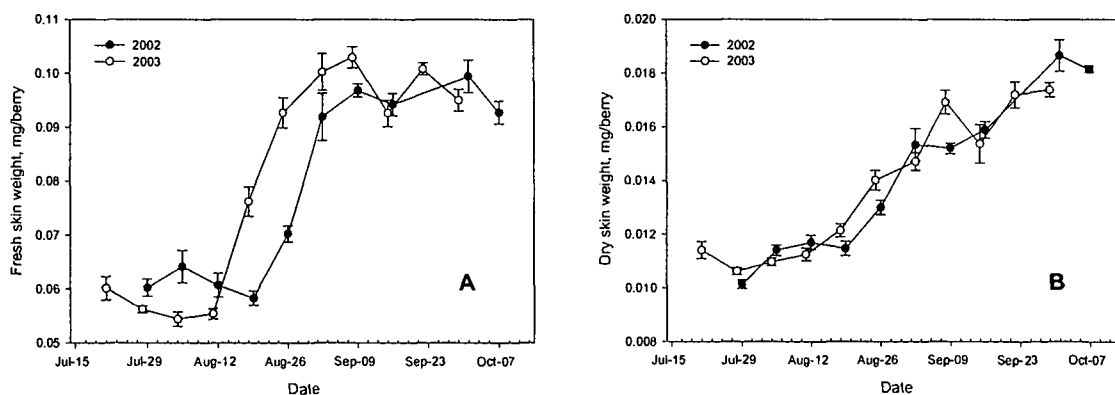


Figure 3.5 Skin weight change for the 2002 and 2003 growing seasons with error bars indicating  $\pm$  SEM (N=5). A) Fresh skin weight (mg/berry); B) Dry skin weight (mg/berry).

Very little difference in soluble solids was observed between the 2001-2003 vintages (Figure 3.6). Although there was an earlier sugar accumulation for 2003,

this difference was minimized two weeks after véraison. At harvest, the three vintages had similar sugar concentration. Fruit was considered ripe when the soluble solids were between 23.5 and 24.5 °Brix.

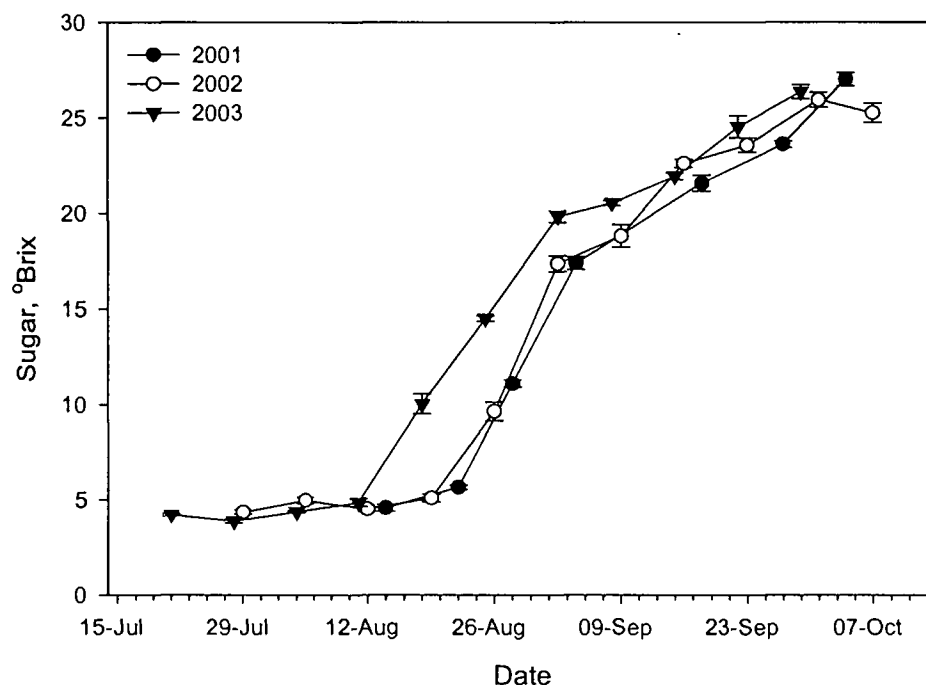


Figure 3.6. Soluble solids accumulation during the 2001-2003 growing seasons with error bars indicating  $\pm$  SEM (N=5).

The pH and titratable acidity (TA), summarized in Figures 3.7 and 3.8, followed expected trends, and large differences between years were found. The highest pH at harvest corresponded to the 2001-growing season. At harvest, the pH for 2001 was significantly higher ( $p < 0.001$ ) than the other two years. Differences in pH between 2002 and 2003 were also found; although they were not as high as those in 2001, the difference was still significant ( $p = 0.0046$ ).

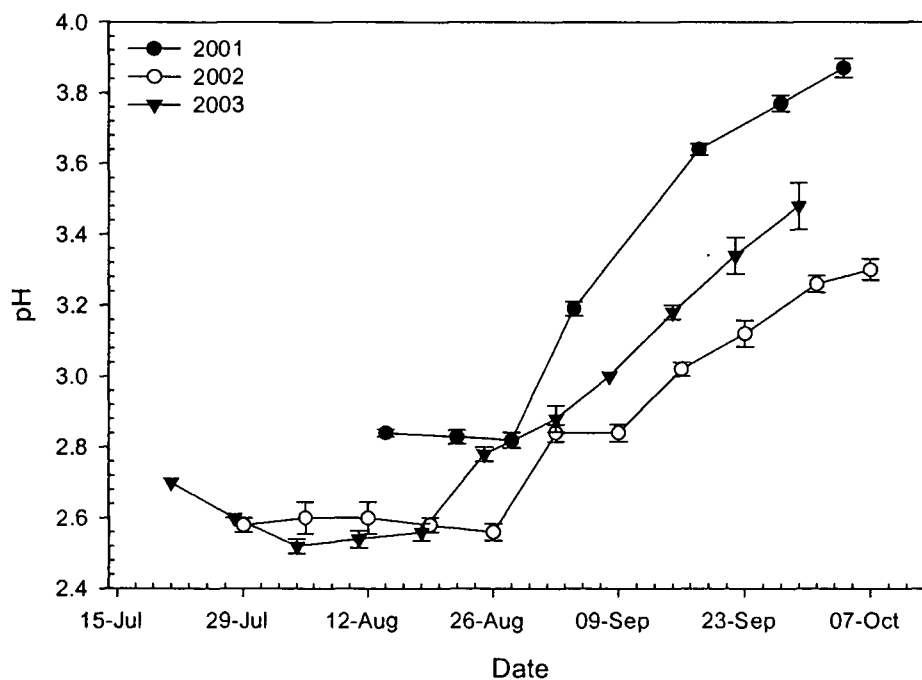


Figure 3.7. pH change during the 2001-2003 growing seasons with error bars indicating  $\pm$  SEM (N=5).

When analyzing the TA during berry development for the three years (Figure 3.8), the differences were not as dramatic as observed for pH analysis. The only year that was significantly different ( $p < 0.001$ ) in comparison with the other two was 2002 when analyzed at harvest time. The 2001 and 2003 growing seasons had similar TA at harvest time ( $p = 0.1812$ ).

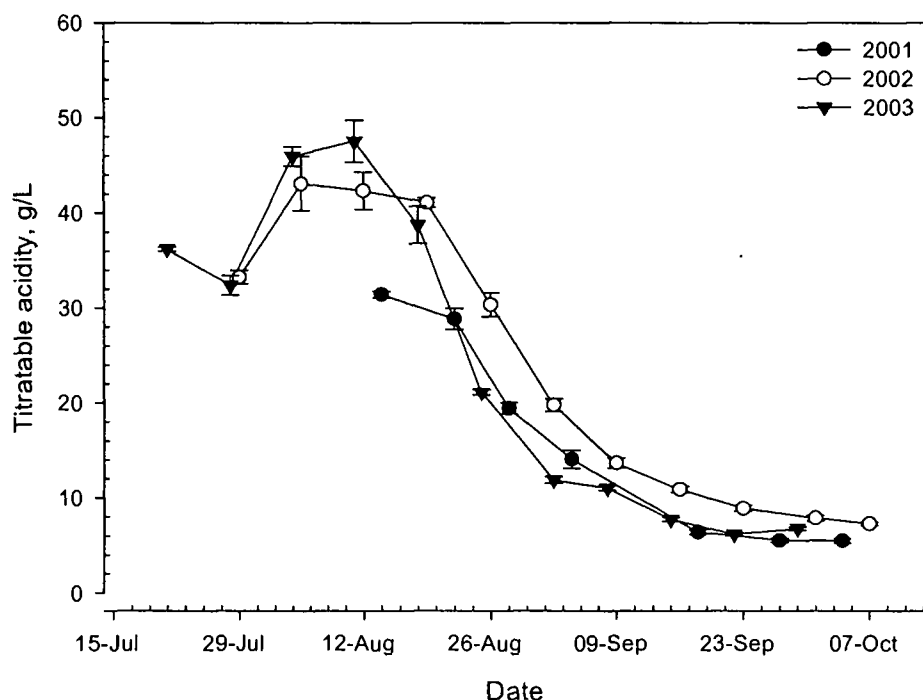


Figure 3.8. Titratable acidity (gm/L) change during the 2001-2003 growing seasons with error bars indicating  $\pm$  SEM (N=5).

**Seed development.** The seeds developed similarly for the three vintages.

Initially, prior to *véraison*, the seeds were green with a soft seed coat. Beginning at *véraison*, and continuing through harvest, the seeds lost their green color and became increasingly brown with time.

During the 2003-growing season, and based upon seed weight, the seeds developed earlier than in previous years (Figure 3.9), and also decreased in weight rapidly. At harvest time, 2003 had the lowest seed weight. The highest seed weight was reached within the two weeks of *véraison* for the 2001 and 2003 vintages, and one week before *véraison* for 2002 growing season.

No change in seed weight was observed through the final pick dates for the 2003 vintage. However, the 2001 and 2002 seed weights declined continuously through the end of the season. This suggests that the seeds for these two years were still developing at harvest time. The number of seeds per berry (Figure 3.9 inset) for 2003 growing season was significantly higher when compared with 2001 ( $p=0.0023$ ) and 2002 ( $p=0.0082$ ). No significant difference was found between 2001 and 2002 ( $p=0.6468$ ).

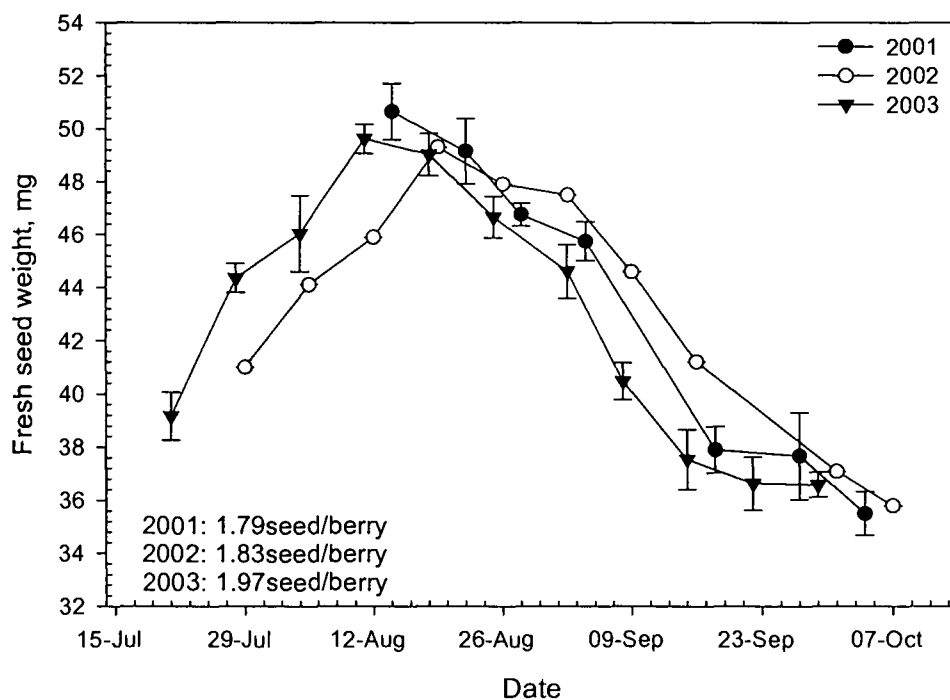


Figure 3.9. Fresh seed weights for the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM ( $N=5$ ), and the corresponding number of seeds per berry for each year.

Dry seed weights (Figure 3.10) from 2002 and 2003 berries increased markedly from the beginning of the season. Right after véraison, the dry seed weight remained constant up to harvest. The 2001 growing season also experienced the same pattern,

but the increase before *véraison* was not so marked. Dry seed weight did not experience a large change after *véraison* for the three vintages. At harvest, the three vintages had similar dry seed weights ( $p=0.2050$ ).

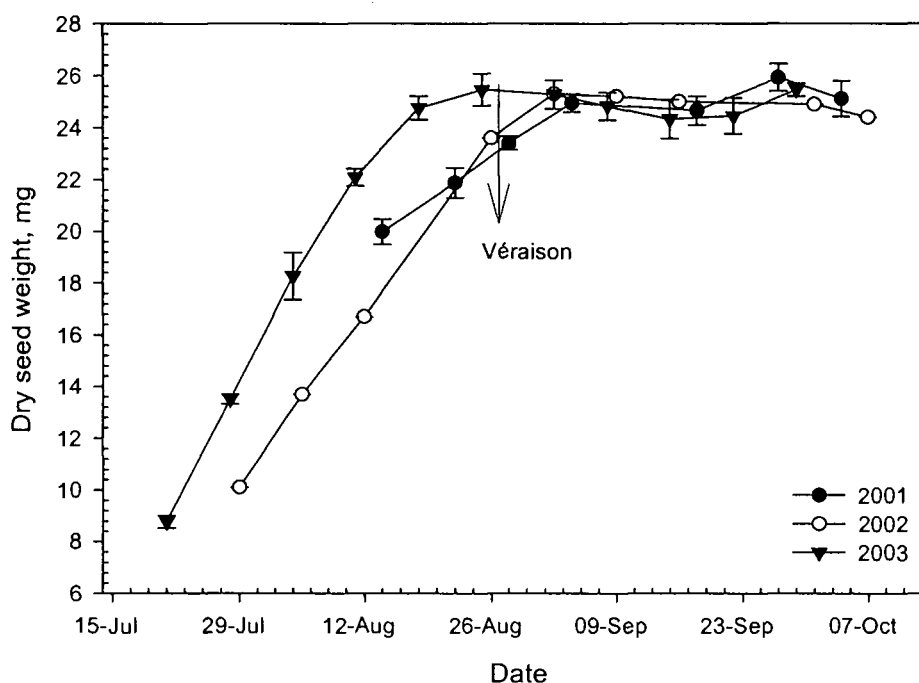


Figure 3.10. Dry seed weights for the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM ( $N=5$ ), and the corresponding number of seeds per berry for each year.

**Identification of phenolics.** After extraction of phenolics from seeds and skins, the resulting aqueous extract containing the phenolics were analyzed for free monomers and proanthocyanidin cleavage products. Phenolics were identified by comparison of their elution time and absorbance spectra with those of commercial standards. In addition, the elution times were compared with those reported in literature (Lamuela-Raventos and Waterhouse, 1994; Kennedy and Jones, 2001).

Figure 3.11 shows the separation of an aqueous extract from grape seeds. The elution times for the free monomers (+)-catechin and (-)-epicatechin were consistent with those of commercial standards. The analysis of seed monomers (Figure 3.11) shows the presence of (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate flavan-3-ol monomers, while skin analysis (Figure 3.12) only shows the presence of (+)-catechin. Similarly, in Figure 3.13, the separation and identification of anthocyanins one week after véraison is also shown. Malvidin-3-glucoside was the primary anthocyanin; however, delphinidin, cyanidin, petunidin and peonidin were also observed.

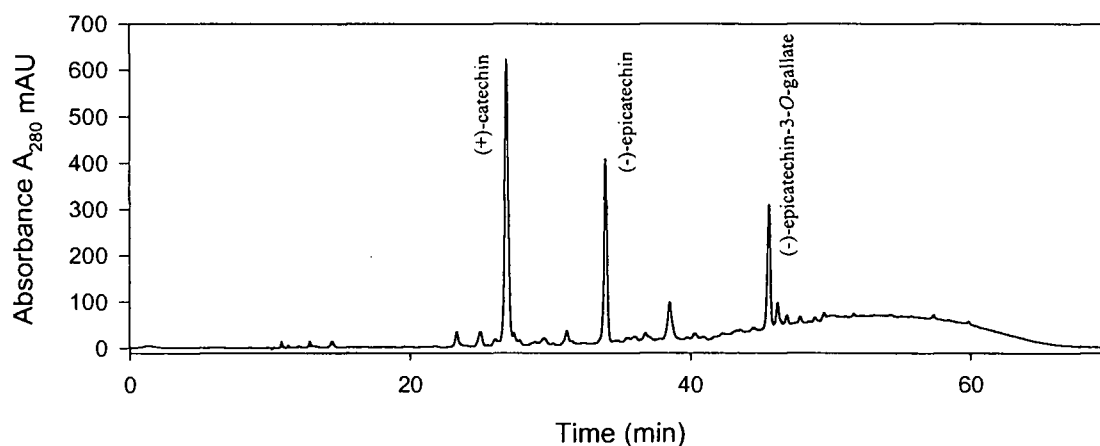


Figure 3.11. HPLC separation of free flavan-3-ol monomers from Pinot noir seeds.

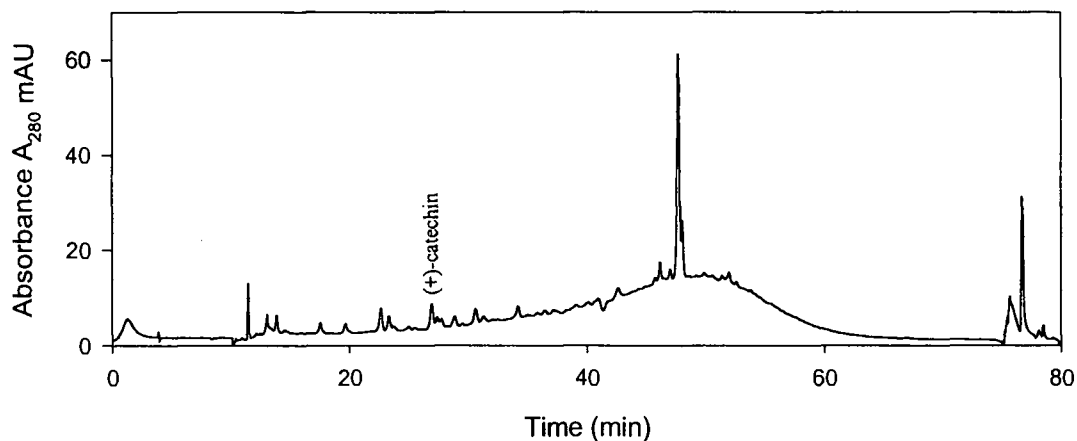


Figure 3.12. HPLC separation of free flavan-3-ol monomers from Pinot noir skins.

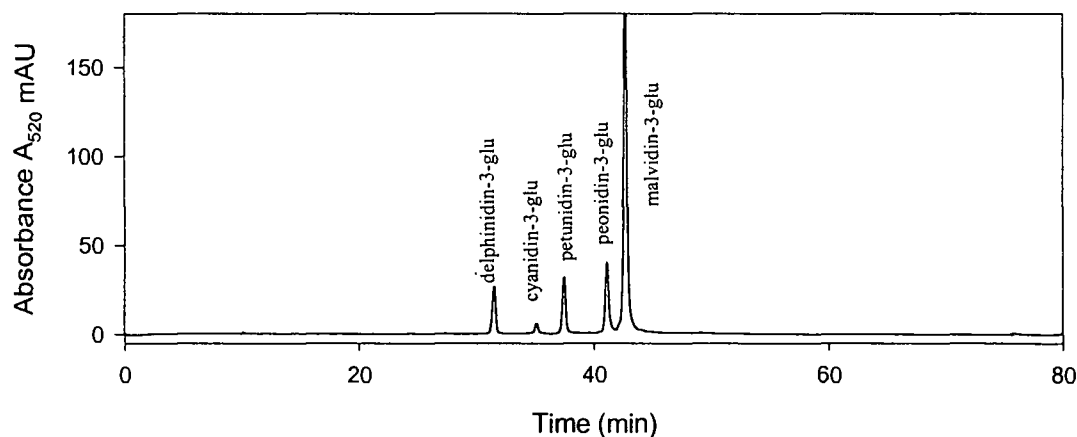


Figure 3.13. HPLC separation of Pinot noir anthocyanins.

Following acid catalyzed cleavage of the proanthocyanidins, phloroglucinol adducts of the proanthocyanidin extension subunits were observed. In the seed extract (Figure 3.14) (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate monomers and their corresponding phloroglucinol adducts were observed, (+)-catechin-phloroglucinol, (-)-epicatechin-phloroglucinol and (-)-epicatechin-gallate-

phloroglucinol. For skin extract after acid catalysis (Figure 3.15), (+)-catechin, (+)-catechin-phloroglucinol, (-)-epicatechin-phloroglucinol, (-)-epicatechin-3-*O*-gallate-phloroglucinol and (-)-epigallocatechin-phloroglucinol were found.

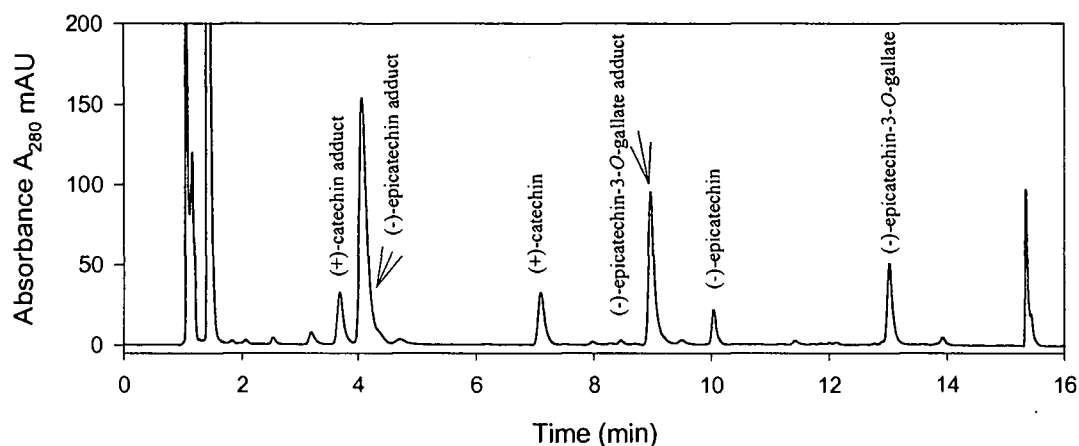


Figure 3.14. HPLC separation of Pinot noir seed proanthocyanidin subunits after acid catalysis in the presence of phloroglucinol.

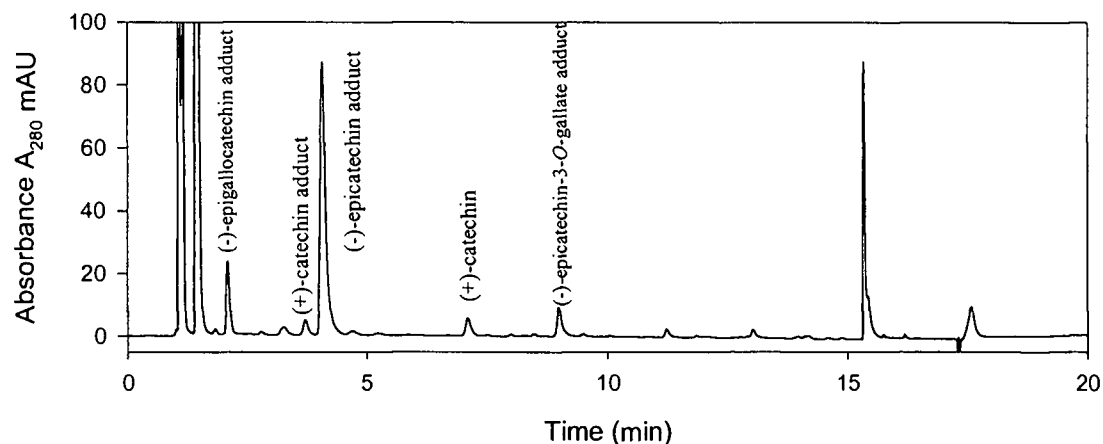


Figure 3.15. HPLC separation of Pinot noir skin proanthocyanidin subunits after acid catalysis in the presence of phloroglucinol

**Proanthocyanidin development in seeds.** Seed proanthocyanidins developed in a manner consistent with other varieties (Kennedy et al., 2000A; Kennedy et al.,

2000B). Figure 3.16 shows the development of flavan-3-ol monomers, extension and terminal subunits in berries collected during the 2001-2003 growing seasons.

On a per seed basis, the flavan-3-ol monomers appeared earlier in the 2003-growing season, reaching their maximum amount per seed near *véraison* and then decreasing. For 2001 and 2002, the maximum peak was observed approximately one week after *véraison*, with the amount per seed being higher for the 2001 growing season. When expressed on a per gram fresh berry weight basis, grapes from 2003 reached their maximum concentration of flavan-3-ol monomers around one week prior to *véraison*, and their concentration was considerably higher when compared to the two previous years. Although differences were obvious through the season, no significant difference in monomer concentration per gram of berry was found at harvest ( $p=0.4280$ ) for the three years.

The amount of total extension subunits in seeds was similar for the 2001 and 2002 growing seasons. On a per seed basis, these two years experienced a decrease in extension subunits after reaching a maximum concentration near *véraison*. 2003 reached highest concentration approximately 2 weeks before *véraison*, and remained nearly constant for the following 4 weeks. The pattern was different when expressed on a per gram of berry basis: For the three vintages, the maximum concentration was observed at the beginning of the season, decreasing continuously through the growing season. The three years followed a somewhat parallel behavior across the season. At harvest, the extension subunit content was significantly lower ( $p<0.001$ )

for 2001 in comparison with the other years. No significant difference was found between 2002 and 2003.

Analyzing the seed terminal subunits on a per seed basis, it is clear that 2003 had a different pattern when compared with the two previous years. Grapes from this particular year experienced a continuous increase until one week after *véraison*. Then a dramatic decrease occurred followed by a constant concentration (and slight increase) through the end of the season. Grapes from 2001 and 2002 growing seasons had the lowest concentration of seed terminal subunits near *véraison*, reaching the maximum peak three weeks after that. It is interesting to note that the three years had a peculiarly high accumulation of terminal subunits three weeks after *véraison*, followed by a slight decrease (less pronounced for 2002) and then a slight increase until harvest. On a per gram of berry basis, the story was different: the three years reached their maximum peak several weeks prior to *véraison*. Although the concentrations were very different, especially for the 2003-growing season, the pattern of accumulation was similar. The three years decreased continuously until approximately one week after *véraison*, then the concentration remained almost constant and an increase was observed just at the end of the season (one week after commercial harvest, due in part to berry shriveling). On a per gram berry basis, the terminal subunit content in seeds was significantly higher for 2003 in comparison with 2001 ( $p=0.0020$ ) and 2002 ( $p=0.0029$ ). No significant difference was found between 2001 and 2002 ( $p=0.9786$ ).

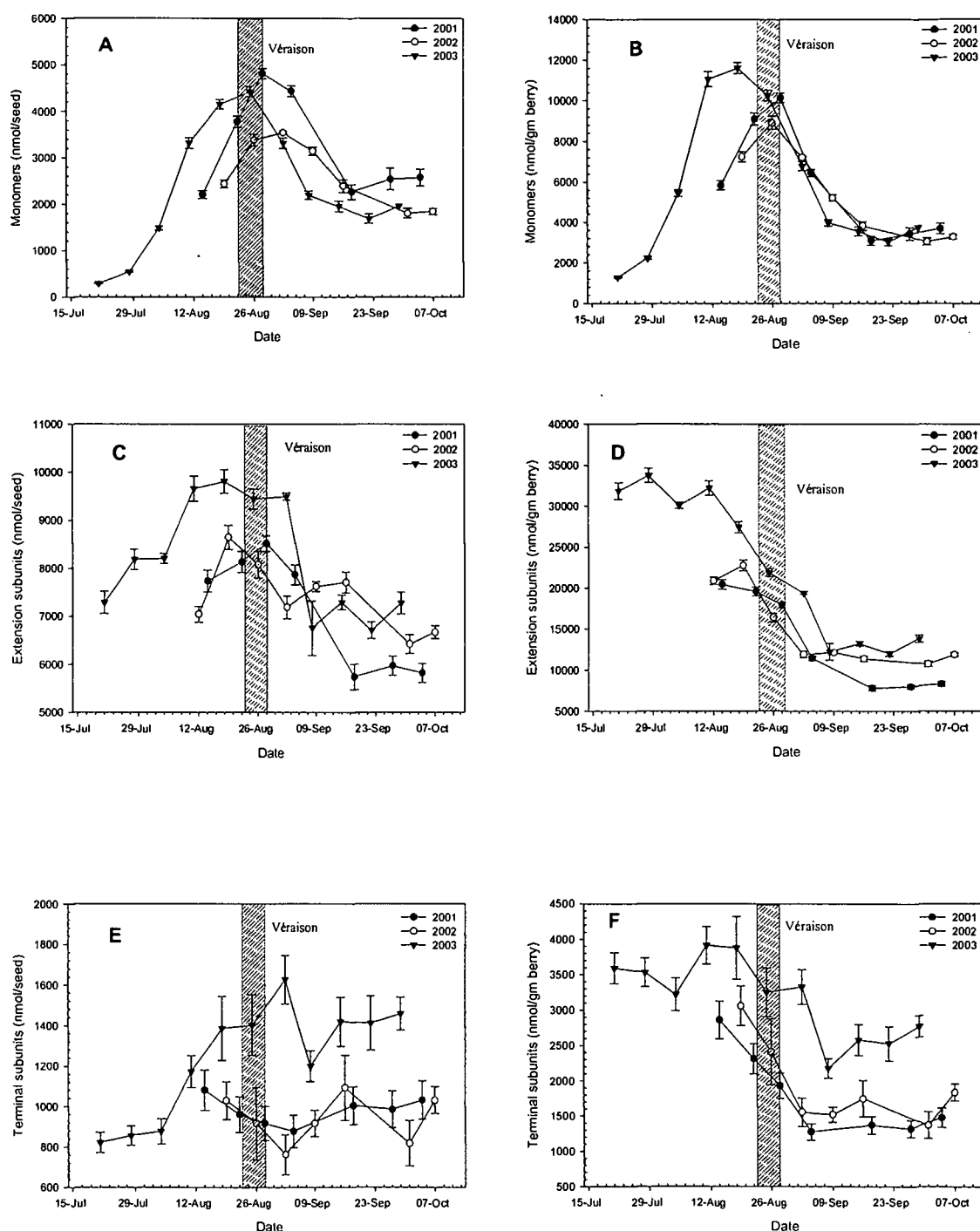


Figure 3.16. Development of flavan-3-ol components in grape seeds during the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM (N=5). **A)** Monomers (nmol/seed); **B)** Monomers (nmol/gm berry); **C)** Extension subunits (nmol/seed); **D)**

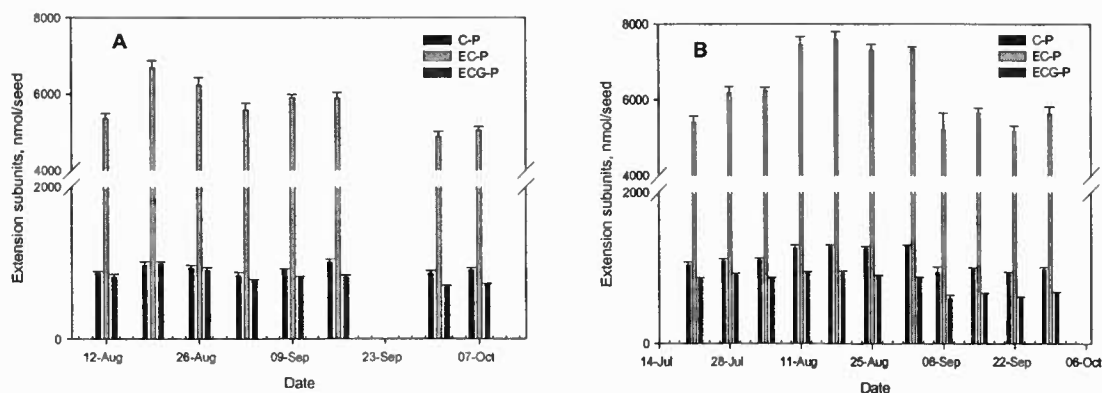
Extension subunits (nmol/gm berry); **E**) Terminal subunits (nmol/seed); **F**) Terminal subunits (nmol/gm berry).

**Seed proanthocyanidin composition.** Extension subunit composition in Pinot noir seeds throughout berry development was predominantly (-)-epicatechin. Near véraison, 77% (by mol) of total extension subunits were (-)-epicatechin, while (+)-catechin accounted for 13% and (-)-epicatechin-3-*O*-gallate made up the remaining 10% of total extension subunits. When comparing extension subunit composition between 2002 and 2003 (Figure 3.17), 2003 experienced a slight increase in the proportion of catechin extension subunits; however, this increase was only around 2% in comparison with 2002. In general, both years kept roughly the same proportion of these subunits. At véraison, the total extension subunit in 2002 was around 88% of that in 2003. For both years, the maximum amount of extension subunits was reached around véraison, and then a decrease in concentration was observed. After véraison, seed subunits during the 2003 growing season had a greater decrease in comparison with 2002. At harvest, 2002 represented 95% of the total extension subunits observed in 2003.

The compositional changes of flavan-3-ol monomers showed a different pattern. At véraison, approximately 56% of the flavan-3-ols were (+)-catechin while the proportions of (-)-epicatechin and (-)-epicatechin-3-*O*-gallate were 36 and 8%, respectively. Similarly, seeds from 2003 showed a higher concentration of flavan-3-ols in comparison with 2002. At véraison, total flavan-3-ol monomers in 2002 represented only 89% of the flavan-3-ols present in the 2003 seeds.

Larger differences were observed when analyzing the terminal subunit composition between 2002 and 2003. At *véraison*, 2002 had a higher proportion of (+)-catechin (65.7%) and lower proportion of (-)-epicatechin (10.5%) in comparison with 2003, whose concentrations of (+)-catechin and (-)-epicatechin were 56 and 27.3%, respectively. The 2003 vintage had also higher concentration of terminal subunits. At *véraison*, 2002 represented 37% of the terminal subunits present in the 2003 seeds.

Although there were differences in terminal subunits between vintages, when summing terminal and extension subunits (Figure 3.17 G-H), the proportions were similar. At *véraison*, for both vintages, the proportion of flavan-3-ol subunits (monomers and terminal subunits) was similar. Approximately 57% of the flavan-3-ols were (+)-catechin while the proportions of (-)-epicatechin and (-)-epicatechin-3-*O*-gallate were 32 and 11%, respectively. In addition, this graph also shows that after *véraison*, the proportion of subunits remain fairly constant for both years.



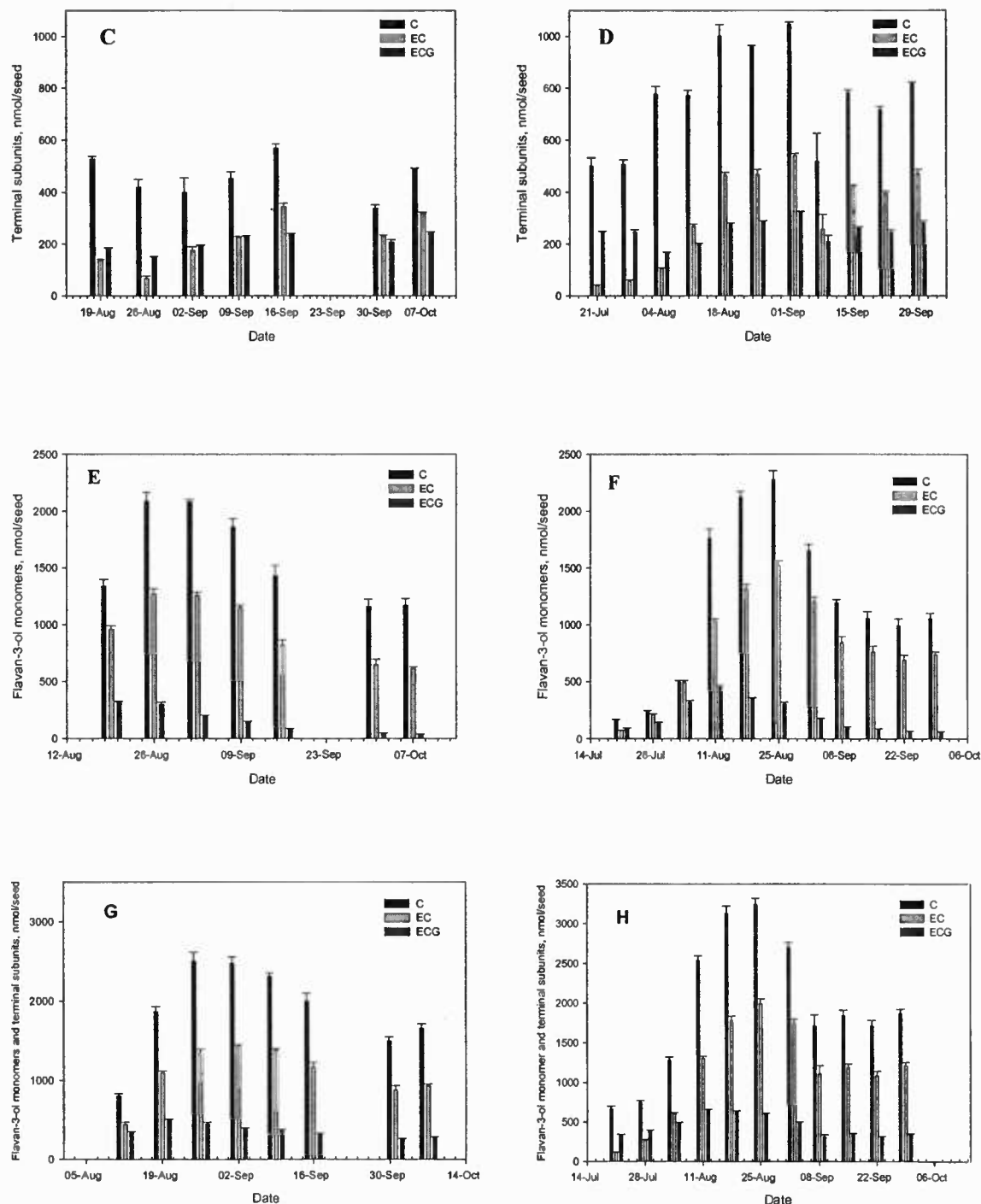


Figure 3.17. Subunit composition of seeds after acid-catalyzed cleavage of proanthocyanidins. A) Extension subunit development for the 2002 growing season (nmol/seed); B) Extension subunit development for the 2003 growing season

(nmol/seed); **C**) Terminal subunit composition for the 2002 growing season (nmol/seed); **D**) Terminal subunit composition for the 2003 growing season (nmol/seed); **E**) Flavan-3-ol monomer composition for the 2002 growing season; **F**) Flavan-3-ol monomer composition for the 2003 growing season; **G**) Flavan-3-ol monomer and terminal subunit composition for the 2002 growing season (nmol/seed); **H**) Flavan-3-ol monomer and terminal subunit composition for the 2003 growing season (nmol/seed). Abbreviations: **C** (+)-catechin; **EC** (-)-epicatechin; **ECG** (-)-epicatechin-3-*O*-gallate; **C-P** (+)-catechin-phloroglucinol; **EC-P** (-)-epicatechin-phloroglucinol; **ECG-P** (-)-epicatechin-3-*O*-gallate-phloroglucinol.

**Proanthocyanidin development in skins.** The development of total skin proanthocyanidins is shown in Figure 3.18. Skin extension subunits accounted for much of the skin tannin content. Monomers and terminal subunits in skins were responsible for 0.5 and 3.0% of the total skin tannin amount, respectively, and this proportion remained nearly constant across maturity and vintage.

On a per berry basis, the amount of proanthocyanidins in skin tissue produced over the 2001 and 2002 vintages were similar in both pattern of accumulation and in amount. Skin proanthocyanidins from 2001 had the highest concentration around véraison while 2002 skins reached the highest concentration two weeks after véraison. The skins from these two years experienced a decrease in tannins two weeks after véraison. At harvest, no significant difference ( $p=0.6351$ ) was found between 2001 and 2002 skin proanthocyanidins. In contrast, skin tannin during the 2003 growing season had an earlier and greater accumulation reaching a maximum concentration near véraison. This pattern was similar to that observed in seed proanthocyanidin development for the same year. Although proanthocyanidin accumulation at véraison was higher when compared to the two previous years, differences were reduced towards the end of the season. At harvest, 2003 was

significantly different from 2001 ( $p=0.0214$ ) and 2002 ( $p=0.0041$ ) in terms of skin proanthocyanidin content. For the three years, the skin proanthocyanidin content at harvest declined to 65% of the maximum amount observed around véraison.

On a berry weight basis, the three vintages developed in similar manner. They reached a maximum concentration of skin proanthocyanidin several weeks prior to véraison and then a constant decrease was observed throughout maturity. At harvest, 2001 had the lowest concentration of skin proanthocyanidin, however, when compared with 2002, this difference was not significant ( $p=0.1907$ ). The skin proanthocyanidin content at harvest was significantly higher for the 2003 vintage when compared to 2001 ( $p=0.0002$ ) and 2002 ( $p=0.0052$ ).

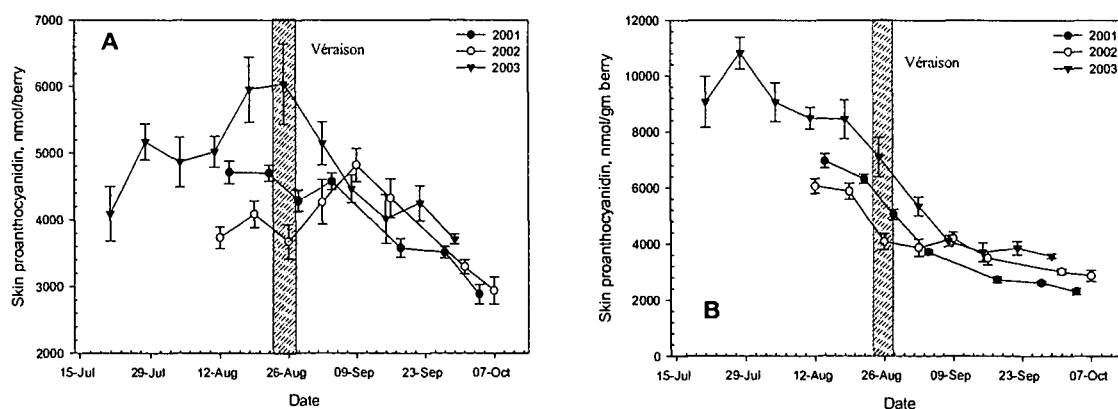


Figure 3.18. Development of total proanthocyanidin in grape skin during the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM ( $N=5$ ). **A)** nmol/berry; **B)** nmol/gm berry

**Skin proanthocyanidin composition.** In Pinot noir grape skin, (-)-epigallocatechin extension subunits were found in addition to (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate. Extension subunit composition in skins

was primarily (-)-epicatechin and (-)-epigallocatechin. From the total skin proanthocyanidin pool, approximately 60% (by mol) corresponded to (-)-epicatechin and 33% to (-)-epigallocatechin. (+)-Catechin was the only flavan-3-ol monomer found in this analysis and was present in low concentration (3% of the total pool).

As observed in Figure 3.19, there was a greater accumulation of extension subunits during the 2003-growing season. At harvest, the total subunit content in skins from 2002 was 77.5% of the content present in 2003 skins.

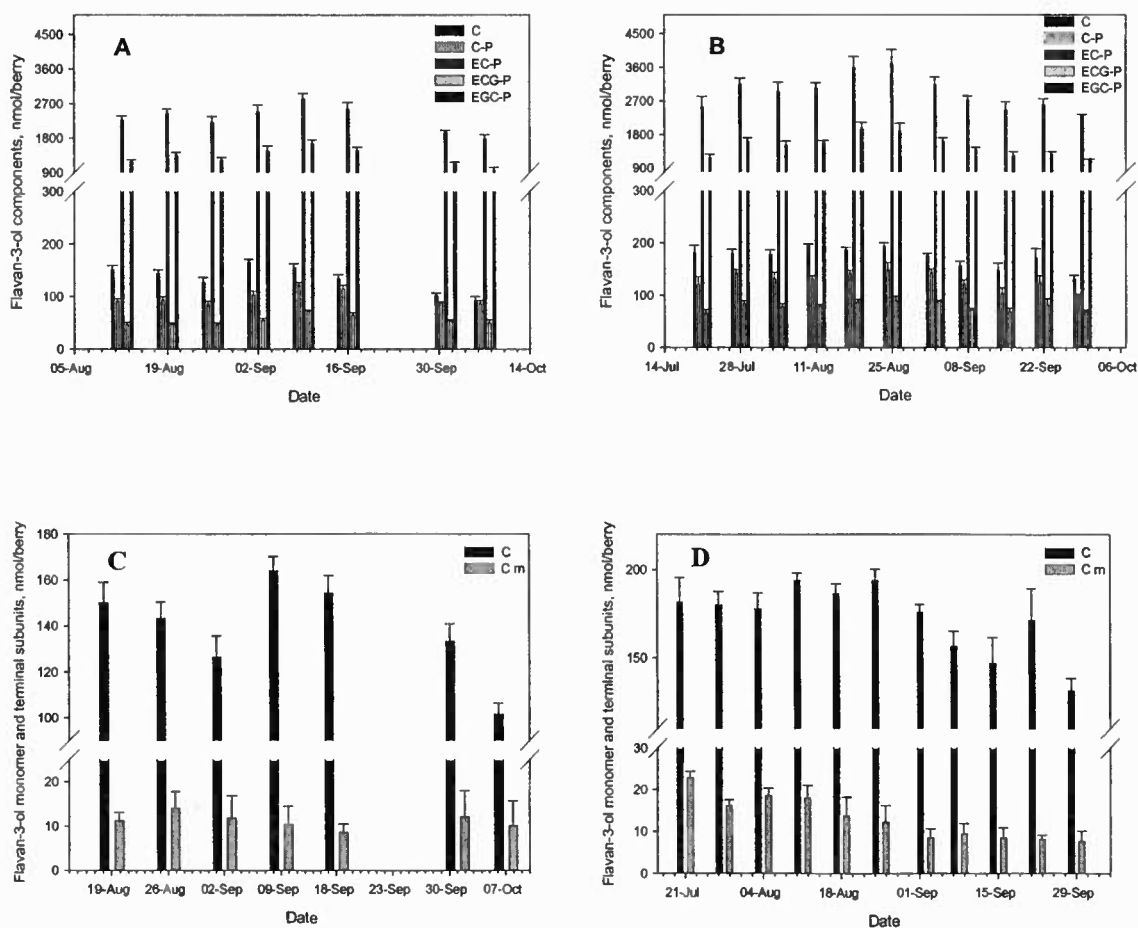


Figure 3.19. Subunit composition in skin after acid-catalysis cleavage of proanthocyanidins, with error bars indicating  $\pm$  SEM (N=5). **A)** Subunit development during the 2002 growing season ((+)-catechin includes terminal and flavan-3-ol monomers) (nmol/berry); **B)** subunit development during the 2003 growing season ((+)-catechin includes terminal and flavan-3-ol monomers) (nmol/berry); **C)** flavan-3-ol monomers during the 2002 growing season (nmol/berry); **D)** flavan-3-ol monomers and terminal subunits during the 2003 growing season (nmol/berry). Abbreviations: **C** (+)-catechin (including monomers and terminal subunits); **C m** (+)-catechin (only monomers); **C-P** (+)-catechin-phloroglucinol; **EC-P** (-)-epicatechin-phloroglucinol; **ECG-P** (-)-epicatechin-3-O-gallate-phloroglucinol; **EGC-P** (-)-epigallocatechin-phloroglucinol .

**Mean degree of polymerization of seed and skin proanthocyanidins.** The apparent mean degree of polymerization (mDP) of seed proanthocyanidins (Figure 3.20) followed similar trends over the 2001 and 2002 vintages, however, these values differed greatly from the 2003-growing season. While the 2001 and 2002 growing seasons experienced a decrease in mDP after *véraison*, the 2003 mDP decreased constantly from several weeks prior to *véraison* (10 units) until the end of the season (6 units). At harvest time, 2003 had the lowest mDP in seeds. In contrast, seed proanthocyanidin from 2002 had the highest mDP across the season, reaching the maximum peak at *véraison* (11.5 units), followed by a decrease throughout harvest (9.5 units). The mDP at harvest was significantly different ( $p=0.010$ ) between 2002 and 2003. No significant difference was found between 2001 and the other two years.

The mean degree of polymerization (mDP) of skin proanthocyanidins (Figure 3.21) was higher than those observed in seed proanthocyanidin. The three vintages showed different patterns. While 2001 and 2002 seemed to increase throughout maturity, 2003 remained fairly constant with a slightly higher value around *véraison*.

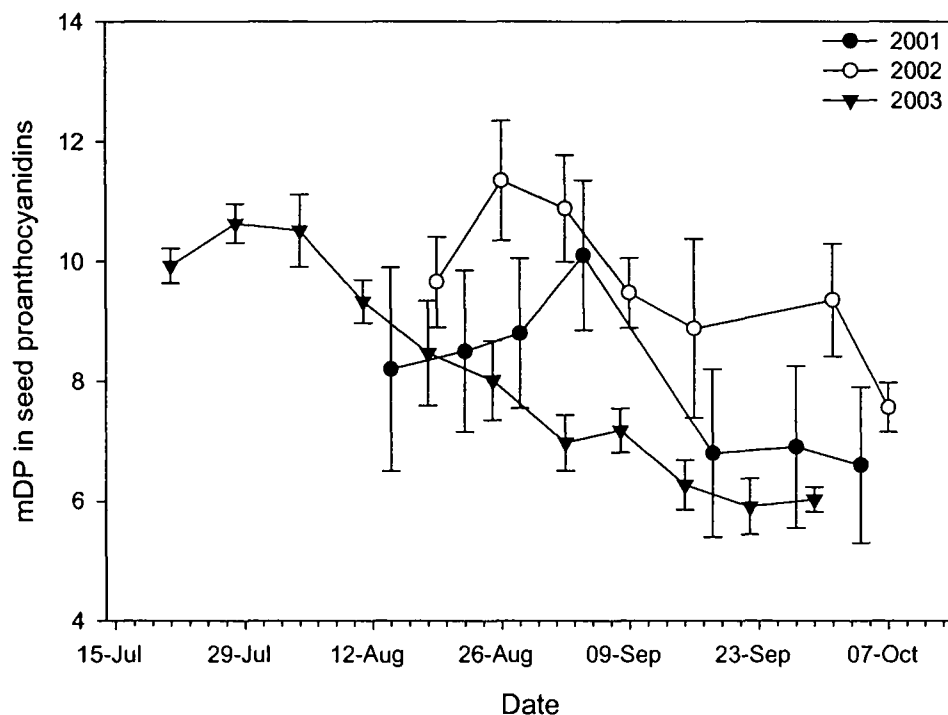


Figure 3.20. Mean degree of polymerization of grape seed proanthocyanidins for cv. Pinot noir during berry development for the 2001-2003 growing season with error bars indicating  $\pm$  SEM (N=5).

At harvest, 2002 had the highest skin mDP, which was significantly higher ( $p < 0.001$ ) when compared with other vintages. The mDP from grape skins grown during this year experienced the highest rate of increase from 30 units before véraison to 42 units at harvest.

The 2001 growing season had the lowest mDP values, starting with 20 units before véraison, and finishing with 26 units at harvest. Grape skin proanthocyanidins from the 2003-growing season did not experience a dramatic increase in mDP. Although grapes from 2003 had a higher mDP than 2002, these differences were not significant ( $p = 0.5107$ ).

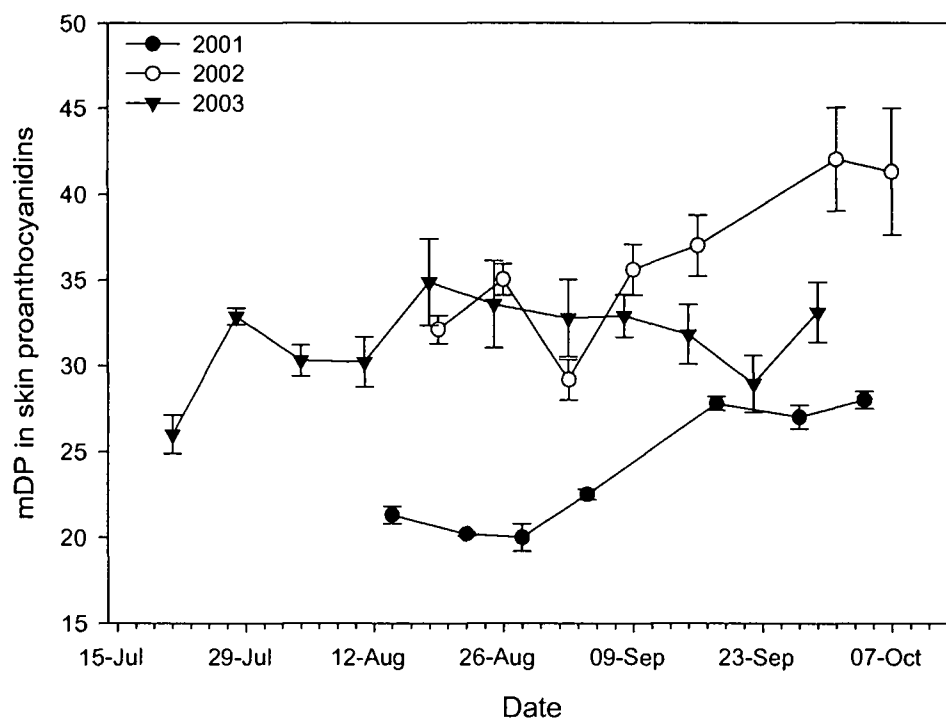


Figure 3.21. Mean degree of polymerization of skin proanthocyanidins for cv. Pinot noir during berry development for the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM (N=5).

**Relationship between seed monomers and seed weight.** Figure 3.22 shows the relationship between flavan-3-ol monomers present in seeds and their corresponding fresh seed weight for the 2002 and 2003 growing seasons. As observed in the previous section (Figure 3.9), the fresh seed weight experienced two clear phases of development: an increase in weight until véraison and a decrease in weight thereafter. The data considered in this relationship corresponds to the second phase of seed development (after véraison) in which only a decrease in weight is observed. These results indicate that the loss of seed weight was accompanied by a loss of flavan-3-ol monomers with a good correlation.

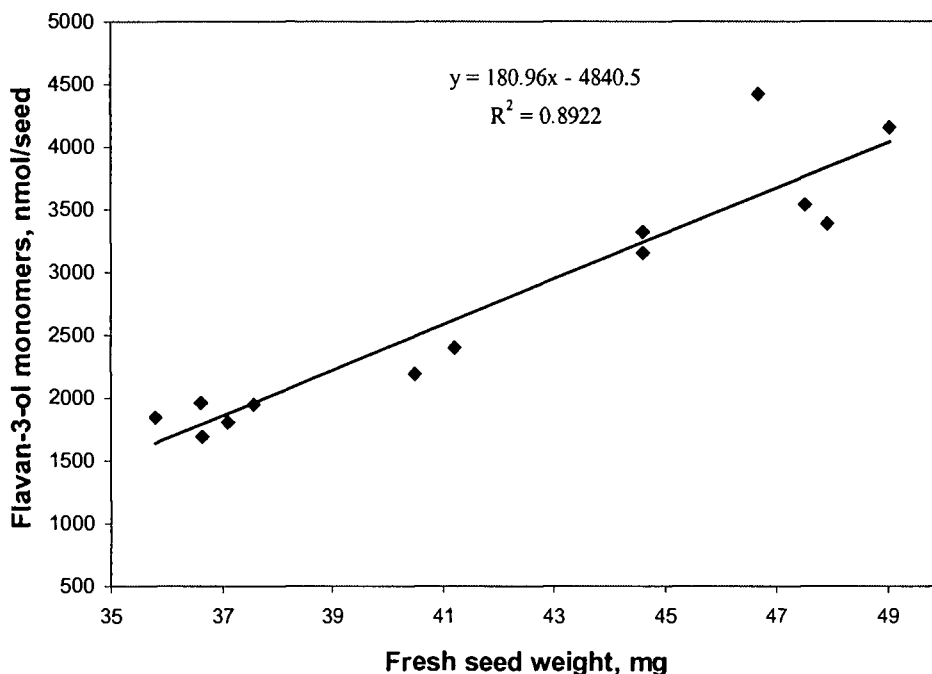


Figure 3.22. Relationship between flavan-3-ol monomers present in seeds and the fresh seed weight (mg/seed) from the 2002 and 2003 growing seasons

**Anthocyanin development.** Figure 3.23 shows the pattern of anthocyanin accumulation in Pinot noir berries during the 2001-2003 growing seasons. The total anthocyanin content was calculated as the sum of the individual compounds determined by HPLC and expressed as malvidin-3-glucoside equivalents. The anthocyanin development was similar for the three vintages in both pattern of accumulation and concentration. Only the first vintage had a higher concentration of anthocyanins especially at harvest times, however, while the anthocyanin amount per berry was generally higher during the 2001 growing season, the lower harvest berry weight observed during the 2002 and 2003 growing seasons minimized these differences at the winemaking level (Figure 3.23-B). While fruit from 2001 and

2003 experienced a decrease in anthocyanin accumulation one week post harvest, 2002 remained constant until the end of the season.

At harvest, and on a per berry basis, the anthocyanin concentration for 2001 was significantly higher than for 2002 ( $p=0.0019$ ). No significant differences were found between 2003 and the other two years. On a per gram of berry basis, although the differences were minimized, there were still significant differences between 2001 and 2002 ( $p=0.0327$ ).

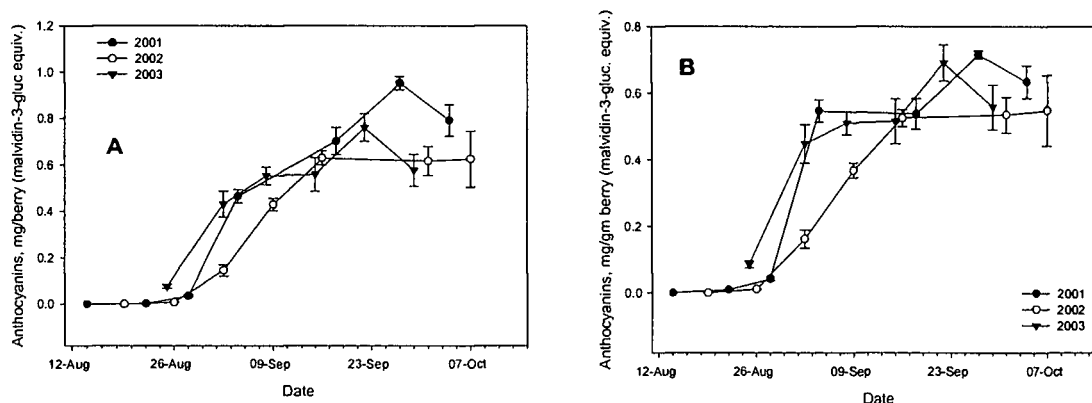


Figure 3.23. Development of anthocyanins during the 2001-2003 growing seasons, with error bars indicating  $\pm$  SEM ( $N=5$ ). A) mg/berry; B) mg/gm berry.

**Anthocyanin composition.** Anthocyanin composition in grapes from the 2002 and 2003 growing seasons are shown in Figure 3.24. Anthocyanin composition in Pinot noir berries was predominantly malvidin-3-glucoside. At harvest, 62.5% (by weight) of the total anthocyanins were malvidin-3-glucoside, with delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside and peonidin-3-glucoside accounting for the 8, 2, 10 and 17.5%, respectively.

As observed, grapes from the 2003 vintage accumulated slightly more anthocyanin in comparison with 2002. While malvidin-3-glucoside content

remained somewhat constant during the last weeks of the 2002 vintage, peonidin-3-glucoside increased slightly through the end of the season.

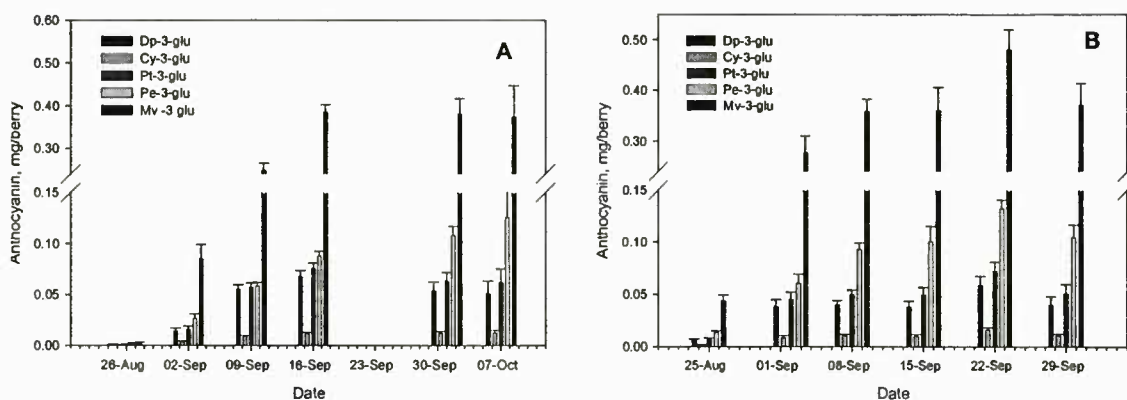


Figure 3.24. Anthocyanin composition in grape skin during the 2001-2002 growing seasons, with error bars indicating  $\pm$  SEM (N=5). **A)** Anthocyanin components during the 2002 growing season; **B)** Anthocyanin components during the 2003 growing season. Abbreviations: **Dp** Delphinidin; **Cy** Cyanidin; **Pt** Petunidin; **Pe** Peonidin; **Mv** Malvidin.

## Discussion

The overall objective of this chapter was to understand the relationship between climate and phenolic development in grapes.

Differences in temperature (and therefore heat accumulation) were observed between the three vintages. Vine growth is responsive to temperature (Mullins *et al.*, 1992). Some substances such as sugar, acids, and pigments are dependent upon temperature and heat accumulation (Winkler *et al.*, 1974). Hence, Kliewer (1964) reported that increasing temperatures from 10 to 25 °C increased the synthesis of tartaric and citric acid; however, temperatures higher than this resulted in a

reduction of these acids. Similarly, Kliewer and Torres (1972) reported that grapes grown under cooler night temperatures had higher accumulation of anthocyanins. Since 2003 was a warmer year, it would be expected to have an earlier development and a different pattern of accumulation for some components.

When analyzing the berry weight development during the three growing seasons (Figure 3.4), it is clear that berries grown during the 2003-growing season experienced an earlier increase in berry size in comparison with the other years. However, this growth stopped about 3 weeks before the end of the season. At harvest time, 2003 had the lowest berry weight and 2001 the highest. These differences in berry weight could be due to differences in vineyard management practices, which was considerably different for 2001 in comparison with the other vintages (i.e.: significantly more crop was thinned in 2001). The reduction in crop load is associated with berries of bigger size. Here, a more vigorous cluster thinning is compensated by the vine by producing larger berries. Similar results have been reported previously (Bravdo et al., 1985).

Heat summation could have also played a role in the smaller berry size observed for the 2003 vintage. McCarthy (1999) reported smaller berries in years with higher growing degree-days. He proposed two mechanisms by which berry weight decreased during warmer years. The first suggests a decrease in the rate of cell division in pericarp tissue. Thus, Kliewer (1977) reported an adverse influence of high temperatures on cell division during the first stage of berry development. Similarly, Hale and Buttrose found that high day/night temperatures during the first

stage of berry development irreversible reduced final berry size. However, high temperatures during the second rapid berry growth phase had no effect on final berry size. Since our results showed that 2003 had berries with bigger sizes during the early stage of berry development (pre-véraison), it would not be likely that the small berry weight at harvest observed for this year is due to changes during the early phase of berry development. The second possible mechanism is related to shrinkage of berries during advanced stages of ripening. The berry growth for the 2003 growing season stopped three weeks before commercial harvest while for the other vintages the berry weight kept increasing. Warmer years could promote a higher water loss in berries (McCarthy, 1999).

Berry weight at harvest time has important implications from a winemaking perspective. Smaller berries would be expected to produce a more concentrated wine assuming the same per berry quantity of components like phenolics, polysaccharides and aroma compounds.

The commencement in berry weight reduction for the three years occurred on similar dates and soluble solids concentration. 2002 experienced the most dramatic decline in berry weight, which occurred when the soluble solids were 23.6 °Brix. The 2003-growing season on the other hand experienced a slight decrease when the fruit had reached 24.5 °Brix. Similar results were reported by Uhlig (1993) and McCarthy (1999); both found a decline in berry weight for several years when the berries had reached 22-24 °Brix.

Similar to the pattern observed for the whole berry analysis, skin weight increased earlier for the 2003 growing season (Figure 3.5A). However, when the grape skins were dried (Figure 3.5B), the resulting weights were very similar for all years. These results suggest that even when the warmer year had an earlier increase in skin and berry weight, this increased weight was likely the result of larger cell volume as a result of water accumulation. The dry skin weight increased throughout the ripening period, and reached a constant weight for the last two harvest dates. Similar results have been reported (Pirie and Mullins, 1977).

The sugar accumulation for the 2003 growing season occurred earlier when compared with the other vintages. Similar to patterns observed for berry and skin weight, the differences between soluble solids for 2003 and the other vintages were minimal during the harvest window. At commercial harvest, the 2003 growing season had higher soluble solids (24.5°Brix) in comparison to the other vintages.

Although the berry weight loss was pronounced (17% berry weight loss) at the end of the 2002 growing season, the sugar content did not experience a dramatic increase as a result of concentration for the last three pick dates (Figure 3.6). Moreover, the soluble solids decreased 11% during the last three pick dates. Since the optimum temperature for photosynthesis is between 25 and 30 °C (Kriedemann, 1968), the decline in sugar accumulation would not be due to photosynthesis decline as a result of warm conditions. Partial breakdown of glucose by the way of pentose phosphate cycle, as reported by Kliwer (1965) in glucose-decreasing grapes at the end of the berry maturation, could be the reason why sugar content decreased in the

concentrated juice. He observed a decrease in glucose from 10 to 6.5% (percent of fresh weight), which corresponded to a decrease in 16% (by weight) of glucose on a per berry basis during the last four weeks of ripening. In our study, the decline in sugar per berry basis was observed only during 2002. For the other two vintages, the sugar accumulation kept increasing (only slightly for 2003) until the end of the season.

Titrateable acidity (TA) and pH during the three vintages (Figure 3.7-3.8) had unexpected behaviors. Although the patterns across maturity were comparable to previous studies (increase in pH and decrease in TA) (Harris et al., 1968), there was no relationship between pH and TA between vintages. Only 2002 had the highest TA and the lowest pH in comparison with the other years. The pH was significantly higher for the 2001 growing season, however, the TA was very similar to that observed during the 2003 vintage. Since tartaric acid is respired at temperatures above 30°C (Winkler et al., 1974) and malic acid is quickly respired at temperatures above 25°C (Lakso and Kliever, 1978), it is likely that the low TA observed during the 2003 growing season was the result of acid degradation due to the warmer conditions for this particular year. In addition, the low TA for 2001 might have been due to the dilution of acids as a result of the larger berry size for this year (Ruffner et al., 1983).

Although soil management practices were constant during the three vintages, it could be possible that higher intake levels of potassium in 2001 berries played a role in the higher pH observed in this year, in comparison with 2003. Zelleke and

Kliewer (1979) reported that potassium intake was significantly higher when root temperatures were higher. Since potassium ions are interchangeable with hydrogen ions within the grape (Boulton, 1980; Butzke and Boulton, 1997), higher potassium content could produce a decrease in hydrogen ions with a corresponding increase in pH.

The similar values of TA between vintages in contrast with the dramatic differences in pH indicate that even when the free hydrogen ion concentration in grapes was different, the total acidity (dissociated and undissociated) remained constant.

The grape seed weight (Figure 3.9-3.10) followed patterns similar to those reported by Kennedy et al. (2000A). From véraison through harvest, the seeds did not gain dry weight, while the fresh seed weight decreased constantly from véraison to harvest. No change in fresh seed weight was observed during the last pick dates for the 2003 vintage. 2001 and 2002 seed weights declined continuously throughout fruit ripening. This suggests that the seeds for these two years were still developing at harvest time. These differences could affect the extractability of seed proanthocyanidins. The early weight gain observed in 2003 seeds might have been due to the higher heat accumulation experienced during this year.

Similarly, temperature is also associated with the seed number per berry. A significantly higher number of seeds per berry were found during the 2003 growing season. This particular year experienced higher maximum and minimum daily temperatures (Figure 3.2-3.3) at fruit set and a few weeks before that. These results

are consistent with those found by Ebadi et al. (1995) who reported a reduced number of seeds per berry when the vines were exposed to low temperatures just before and during flowering. Similarly, Ewart and Kliewer (1977) found that the number of seeds per berry in vines grown (from one week before fruit set through véraison) at 25/10° and 25/20° (day/night) were approximately double than that of vines held at 15/10 °C. Low temperatures are also reported to reduce the ovule fertilization (Buttrose and Hale, 1973; Kobayashi et al., 1965). According to Cawthon and Morris (1982), the seed number per berry is directly related to accumulation of <sup>14</sup>C-photosynthate, fresh weight and dry weight, and little relationship with berry content of abscisic acid. The knowledge of the number of seeds per berry has important implications from a winemaking standpoint. Grapes with a higher number of seeds would be expected to produce a more tannic wine assuming the same quantity of proanthocyanidins on a per seed basis given a similar berry weight.

Two well defined stages were observed in the seed proanthocyanidin biosynthesis (on a per seed basis) during the three growing seasons (Figure 3.16): i) a stage of accumulation that took place since several weeks before véraison, and ii) a stage of decline from véraison through harvest. The synthesis and accumulation of proanthocyanidins in seeds coincides with seed development. The peak of maximum seed weight and seed proanthocyanidin was reached at similar dates. During fruit ripening, the proanthocyanidin content decreased, apparently due to oxidation reactions (Kennedy, 2000B). In addition, this decrease could also be

attributed to a reduction in extractability resulting from the conjugation of proanthocyanidins with other cellular components. Thus, Downey (2003) reported that, after acetone extraction, substantial amounts of terminal and extension subunit residues were retained in post-véraison seeds.

Seed monomers developed just before véraison similar to other varieties (Romeyer et al., 1986; Kennedy et al., 2000A). The proanthocyanidins (extension subunits) on the other hand were already present and were maximal around véraison, consistent with other studies (Kennedy et al., 2000A; Kennedy et al., 2000B). When comparing the three vintages in terms of proanthocyanidin accumulation, it is clear that grapes from 2003 accumulated higher amounts of flavan-3-ol monomers and proanthocyanidins earlier. The warmer conditions observed during this year might have enhanced the activity of some enzymes responsible for the biosynthesis of phenolics. Separating the effects of temperature and sunlight on grape berry composition is difficult because many of the biochemical pathways are both light and temperature sensitive. Although high heat accumulation and temperature peaks were observed during the 2003 growing season, light intensity may also be involved in the greater tannin content observed for this year. Swain and Williams (1970) reported that light greatly enhanced the synthesis of phenolic compounds in plant tissues. Similarly, Crippen and Morrison (1986) found that soluble phenols were significantly higher in sun exposed berries when expressed on a per berry basis. Even when the accumulation of monomers and extension subunits was higher at the beginning of the 2003 season (per seed basis), at harvest, the concentration was

similar to the other years. Terminal subunits did not experience the decline observed in extension subunits and monomers after *véraison*. The concentration for these years remained fairly constant after *véraison* and for 2003, the terminal subunit concentration increased beginning approximately three weeks before *véraison* (on a per seed basis).

The patterns observed in proanthocyanidin biosynthesis and accumulation on a per gram of berry basis are consistent with those reported in literature (Kennedy, 2000A; Downey, 2003). However, since the berry sizes were very different for these vintages, the analysis and discussion of proanthocyanidin accumulation are focused on the per seed or per berry basis.

The seed flavan-3-ol monomers observed in this study (Figure 3.17 C-D), included (+)-catechin, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate. (+)-Catechin was the major component of the flavan-3-ol monomers and terminal subunits followed by (-)-epicatechin. Similar results have been reported in Pinot noir (Santos-Buelga et al., 1994) and in other varieties (Prieur et al., 1994; Kennedy et al., 2000B; Su and Singleton, 1969). In contrast, when analyzing the extension subunits (Figure 3.17 A-B), it was found that (-)-epicatechin was the major subunit, followed by (+)-catechin and (-)-epicatechin-3-*O*-gallate. Although research done on other varieties support (-)-epicatechin as the major extension subunit present in seeds, the next subunit found in largest amount was reported to be (-)-epicatechin-3-*O*-gallate (Kennedy et al., 2000A; Kennedy et al., 2000B), which is not consistent with our results. Even when the concentration of (+)-catechin and (-)-epicatechin-3-

*O*-gallate were very similar, (+)-catechin was higher than (-)-epicatechin-3-*O*-gallate.

When analyzing the composition of subunits in grape seeds, it is noteworthy that even when there were differences in concentration and proportion between monomers and terminal subunits, the sum of these two compounds yielded constant concentrations during the post-véraison stage. Similarly, 2002 and 2003 experienced different proportions of seed terminal subunits at véraison, however, when flavan-3-ol monomers and terminal subunits were considered together (Figure 3.17 G-H), the proportions were similar across vintages. For 2003, on September 8<sup>th</sup> the concentration of all the subunits of flavan-3-ol monomers was slightly higher in comparison with the next sampling dates (Figure 3.17 E-F). The composition of terminal subunits for the same date, was lower than the next sampling dates and increased throughout harvest (Figure 3.17 C-D). Similarly, the proportion of (-)-epicatechin within the terminal subunits for this date was lower in comparison with the next sampling dates. However, when considering flavan-3-ol monomers and terminal subunits together (Figure 3.17 G-H), the proportion of subunits remained constant after véraison. In addition, it can be observed that (-)-epicatechin is the second major component within the flavan-3-ol monomers during the entire season for 2002 and 2003. Within the terminal subunits, (-)-epicatechin represents the lowest proportion in the pre-véraison stage, however, it becomes the second largest subunit at post-harvest. These results along with the decrease in mean degree of polymerization could indicate the incorporation of monomers into terminal subunits.

Through acid-catalyzed degradation, terminal subunits may experience transformation to monomers and vice versa.

In grape skins, the extension subunits accounted for almost all the subunits present in skins (96.5%). These results are consistent to those reported in cv. Merlot in which the monomers represented 2% of the total flavanols extracted from skins (Souquet et al., 1996). As observed, 2003 had the highest accumulation of proanthocyanidins at the beginning of the season. Although the differences were minimized after *véraison*, 2003 still had the highest concentration of skin proanthocyanidins at harvest. This behavior is similar to that observed in seed proanthocyanidins: warmer years had higher proanthocyanidin accumulation. Price et al. (1995) found that exposure to sunlight increased total phenol concentration in Pinot noir skin disks without affecting anthocyanin concentrations. Wicks and Kliewer (1983) reported that total phenolic levels were significantly affected by light in the skin of Emperor grapes and, in contrast with what was reported by Price (1995), the anthocyanin content was also affected. Although in our experiment it was not possible to separate light and heat, the correlations observed between heat accumulation and proanthocyanidin content provide good indication of the factors that can influence the proanthocyanidin biosynthesis.

Although the nature of the decrease still remains unclear, the decline in grape skin proanthocyanidins between *véraison* and harvest (per berry basis) could be attributed to proanthocyanidins in the skins forming increasingly stable associations with other cellular components. Thus, previous studies reported proanthocyanidins

forming associations with other cellular components such as cell wall polysaccharides or proteins (Kennedy et al., 2001; Escribano-Bailón et al., 1995; Haslam, 1980).

The proanthocyanidin subunits in skins were composed primarily of (-)-epicatechin extension subunits. Skin also contained significant proportion of (-)-epigallocatechin extensions subunits. The only flavan-3-ol monomer observed in skins was (+)-catechin. Although (-)-epicatechin has been reported to be present in skins in other grape varieties (Katalinic, 1999), Escribano-Bailón et al. (1995) did not find monomers of (-)-epicatechin in Pinot noir skins. Similarly, Kennedy et al. (2001) did not report (-)-epicatechin or (-)-epicatechin-3-*O*-gallate as terminal subunits in skins from cv. Shiraz. The degradation products released after the acid catalysis of the grape skins yielded (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate and (-)-epigallocatechin. The same extension subunits with comparable proportions (60% (-)-epicatechin) were reported in skins from cv. Merlot grapes (Souquet et al., 1996). In the present study, the flavan-3-ol components released after acid catalysis decreased after véraison but their proportion remained fairly constant throughout the end of the season. For 2003, the monomers of (+)-catechin remained constant during post-véraison while the terminal subunits decreased and experienced an increase only on the date that corresponded to commercial ripeness (Figure 3.19 C-D). Since monomers remained constant despite the changes in terminal subunits, the transformation of monomers into terminal subunits, as hypothesized in seeds, does not appear to occur in skin proanthocyanidins.

The mean degree of polymerization (mDP) in seeds from 2003 decreased prior to *véraison*. Proanthocyanidins for this vintage formed earlier than in the other 2 years, and experienced a decrease in molecular size throughout harvest. On the other hand, grapes from 2001 and 2002 growing seasons reached the maximum chain length around *véraison* and declined after that. The mDP for skin proanthocyanidins had a different pattern. The molecular weight of skin proanthocyanidins kept increasing (especially for 2001 and 2002) until the end of the season. Similar results were reported in literature (Kennedy et al., 2001; Souquet et al., 1996; Kennedy et al., 2002). As expected these results indicate that proanthocyanidin from skins have a larger molecular size (5 times) than seed proanthocyanidins.

When grape seed proanthocyanidins are analyzed by high performance gel permeation chromatography (Kennedy and Taylor, 2003), a reduction in the molecular weight distribution was observed with maturity, suggesting that the mDP observations are indicative of actual degree of polymerization.

The decrease in seed weight after *véraison* is proportional to the decrease in seed flavan-3-ol monomers. Since the dry seed weight remains fairly constant after *véraison*, it is clear that the decline in fresh seed weight is due mainly to water loss. Speculating, the loss of water that seeds experience during the second phase of berry growth would produce reduction in cell size with a corresponding reduction in permeability. Hence, the extractability of compounds like flavan-3-ol monomers would be reduced in more mature seeds. Further research will necessary in order to

confirm this relationship and to find other possible explanations for this phenomenon.

Five anthocyanin monoglucosides (cyanidin, delphinidin, petunidin, peonidin and malvidin) were found after reversed-phase analysis, consistent with previous studies (Rankine, et al., 1958; Fong et al., 1971). The amount of anthocyanin extracted per gram of berry in Pinot noir was comparable to those reported in literature (Puissant and Leon, 1967). Since grapes from the 2001 growing season were bigger, it would be expected to have less anthocyanin content on a per berry weight basis. However, anthocyanin accumulation in grapes are enhanced by decreasing the crop load (Kliewer and Weaver, 1971), so that the low yield observed in the 2001 vintage could have accounted for the higher anthocyanin content present in grapes at harvest.

Light intensity and temperature are generally two interrelated factors. In most cases, an increase in temperature is observed as a result of more sun exposure. These two factors produce contrasting effects in the anthocyanin synthesis. Hence, Kliewer (1977) showed that low light intensity greatly reduced the coloration of Emperor grapes compared to fruit ripened at high light intensity. In contrast, Downey (2003) did not find differences between anthocyanin levels in light exposed and shaded grapes in two of the three seasons studied. However, Kliewer and Torres (1972), using controlled growth rooms, reported that 68 °F day temperature (night temperature constant at 59 °F), greatly increased the level of pigments in skins of Pinot noir grapes compared to fruit ripened at 86 °F day temperature. Assuming

similar light intensities for the three years, the higher anthocyanin content observed in the 2001 vintage could be attributed to cooler weather conditions.

Grapes grown during the 2001 and 2003 growing seasons experienced a decrease in anthocyanin content for the last pick date (over-ripeness). Similar results were reported by Hrazdina et al. (1984), who explained that the decline in anthocyanin content was related to the activity of an enzyme responsible for the anthocyanin biosynthesis (phenylalanine ammonia lyase) present during the development of berries, which declined toward the end of the developing period.

When comparing the anthocyanin components between vintages, it was found that even when there were slight differences in concentration, the proportion of the five anthocyanins remained nearly constant across vintages and across maturity. Only peonidin was observed to increase slightly for the last two pick dates in the 2002 growing season. Although 2003 was the warmest year, no significant difference in anthocyanin content was found between 2003 and the other years. Downey (2003) suggested an association between vines grown at higher temperatures and a change in the proportions of anthocyanin components with a particular increase of trihydroxylated anthocyanins. He also observed that grapes grown under shaded conditions were consistently lower in anthocyanins with three oxygen substituents on the ring B and higher in anthocyanins with two oxygen substituents on the flavonoid ring B. The results observed by Downey (2003) are not consistent with those reported in the present study. The lack of dramatic change in anthocyanin composition between 2002 and 2003 vintages, in addition to the lack of

dramatic variation in total anthocyanin concentration, indicates that the differences in temperature between these years did not produce an effect in the anthocyanin behavior.

. In summary, the relationship between anthocyanin content and weather conditions remains poorly understood. It is possible that the lack of logic between these factors is due to the lack of well defined dates and stages of ripeness. The extractability of the anthocyanins may be governed by the states of maturity that controls the breakdown of skin cells (Ribéreau-Gayon et al., 2000B).

### **Conclusion**

Heat accumulation and temperature changes during grape development were associated with differences in the accumulation of phenolic compounds. Based upon this study, warmer years were associated with higher rates of proanthocyanidin accumulation and higher proanthocyanidin content in grape seeds and skin. Proanthocyanidin composition did not vary dramatically during ripening or across vintages. Anthocyanin accumulation did not vary with vintage.

## **CHAPTER 4**

### **The Effect of Grape Maturity on Wine Phenolics**

#### **Introduction**

Grape berries experience many changes during the last stages of ripening. Some of these changes include berry shriveling, cell wall permeability (Nunan et al., 1998) and oxidation reactions (Kennedy, 2002), and could affect the extraction and perception of phenolics in wines. Hence, wines made with more mature grapes could have different sensory properties. With regard to the subject of this thesis, a common explanation for the observed differences in wine astringency perception as a function of berry ripeness is that there is a change in the composition of proanthocyanidins either through an increase in skin proanthocyanidin proportion or possibly through direct modification as a result of berry maturity. The term that is often applied to this phenomenon is “tannin ripeness”. The increase in “tannin ripeness” is considered to be an improvement in bitterness and astringency perception. Although this observation is generally accepted in the wine industry, the chemical basis for this observation has yet to be elucidated. The overall objective of this chapter was to gain an improved understanding of how phenolics are extracted during wine production as a function of grape maturity. With this information it

was hoped that an improvement in our understanding of “tannin ripeness” could be made.

## **Materials and Methods**

**Chemicals.** The chromatographic solvents were HPLC grade. Acetonitrile, methanol, ethanol, acetic acid, gallic acid, ascorbic acid and potassium metabisulfite were purchased from J.T. Baker (Phillipsburg, NJ). (+)-catechin, (-)-epicatechin, quercetin, phloroglucinol and caffeic acid were purchased from Sigma (St. Louis, MO). Malvidin-3-glucoside was purchased from Polyphenols Labs (Sandness, Norway). Ammonium phosphate monobasic and orthophosphoric acid were purchased from Fisher Scientific (Santa Clara, CA). Hydrochloric acid, ethanol and anhydrous sodium acetate were purchased from E.M.Science (Gibbstown, NJ), Aaper (Shelbyville, KY) and Mallinckrodt (Phillipsburg, NJ), respectively.

**Instrumentation.** A Hewlett-Packard, Model 1100 HPLC (Palo Alto, CA) with a vacuum degasser, autosampler, quaternary pump, DAD, column heater, Pentium III computer and chemstation software was used for chromatographic analysis. A pH-meter Thermo Orion model 370 was used for pH measurements. Soluble solids were measured using a hand-held refractometer (Amercian optical corporation). A platform shaker model Innova 2300 purchased from New Brunswick Scientific (Edison, NJ) was used for the extraction of phenolics. A freeze drier (Labconco, Kansas City, MO) and rotary evaporator #R-205 (Buchi, Kansas City, MO) were also used.

**Wine production.** Wines were made using conditions according to commercial winemaking practices. For all wines, attention was directed to making wines in a consistent manner so that differences in wine composition could be attributed to grape maturity. Harvested grapes were divided into lots, destemmed and crushed in a destemmer-crusher (Coccaglio, USA), and 50 mg/L SO<sub>2</sub> was added. Musts were kept at ~5 °C overnight. Following cold storage, musts were allowed to warm to room temperature (~8 hours) and were then inoculated with 1 g/L Lalvin RC 212 Bourgorouge yeast (Lallemend). Once fermentation commenced, fermenting wines were punched down twice a day. Fermentations were monitored with a hydrometer and temperatures were maintained below 31 °C. Upon dryness (~7-8 days), wines were pressed (up to 2.5 bars) with a balloon-type press (Willmes, Germany). Approximately one month after pressing, wines were racked and inoculated with 0.025 gm/gal *Oenococcus oenos* OSU strain, malolactic bacteria (Lallemend). Four months later, after completion of malolactic fermentation, wines were racked and 25 mg/L SO<sub>2</sub> was added. Wines were stored at ~5 °C until bottling. Before bottling, SO<sub>2</sub> was added to the wines in order to achieve 30 mg/L free SO<sub>2</sub>.

For the 2001 vintage, grapes were harvested at two different maturities, on September 27<sup>th</sup> and October 4<sup>th</sup> with corresponding soluble solids of 23.7 and 27.0 °Brix, respectively. For each date, grapes were divided into two replicate lots, although one lot was pressed after one week (end of fermentation) and the second lot was pressed after 2 weeks.

For the 2002 vintage, grapes were harvested at three different maturities, on September 16<sup>th</sup>, September 26<sup>th</sup> and October 7<sup>th</sup> with corresponding soluble solids of 20.1, 23.4 and 25.5 °Brix, respectively. Approximately 115 kg of grapes were harvested on each date. They were randomly divided into three lots (replicates). An additional 115 kg of grapes were harvested at the intermediate level of ripeness (23.4 °Brix). For these grapes the fermenting must was pressed on the third day of fermentation to minimize proanthocyanidin extraction. For this wine, additional purified proanthocyanidins were added for astringency assessment.

For the 2003 vintage, grapes were harvested at three different maturities, on September 15<sup>th</sup>, 22<sup>nd</sup> and 29<sup>th</sup> with corresponding soluble solids of 21.9, 24.5 and 26.4 °Brix. Approximately 115 kg of grapes were harvested on each date. Harvested fruit was divided into three replicate lots. Although fermentation conditions were similar, wines were pressed after 8 days as a result of a longer fermentation for this year.

**Analysis of anthocyanins and flavan-3-ol monomers.** Total anthocyanin and flavan-3-ol monomer content were measured by reversed-phase HPLC. Separation of phenolics was achieved with a LiChrospher 100 RP-18 (4x250 mm, 5µm particle size) column, protected by a guard column containing the same material, and was purchased from Merck (Darmstadt, Germany). Solvent gradient conditions and mobile phases were prepared as described previously by Lamuela-Raventos and Waterhouse (1994). The aqueous solution containing the grape phenolics was

thawed to room temperature, filtered using Teflon filters (0.45  $\mu$ m, Acrodisc CR13) before injection. Eluting phenolics were monitored at 280 and 520 nm.

A standard was prepared by dissolving (+)-catechin (100 mg/L), (-)-epicatechin (100 mg/L) and malvidin-3-glucoside (30 mg/L) in a 20% v/v methanolic solution containing 3% v/v acetic acid.

**Analysis of proanthocyanidin composition.** Proanthocyanidin composition was determined by reversed-phase HPLC after acid-catalysis in the presence of excess phloroglucinol (phloroglucinolysis). The column was Chromolith RP-18e (100-4.6mm) purchased from Merck (Darmstadt, Germany). The method utilized a binary gradient with mobile phases containing 1% v/v aqueous acetic acid (mobile phase A) and acetonitrile containing 1% v/v acetic acid (mobile phase B). Solvent gradient conditions were the same as described previously by Kennedy and Taylor (2003).

For the analysis of proanthocyanidin composition in wine, 10 ml of wine was freeze-dried and the resulting powder was dissolved in 6 ml of water. The aqueous solution was applied to a C18-SPE column (1 g Alltech), previously activated with 10 ml of methanol followed by 15 ml of water. The applied sample was washed with 15 ml of water and eluted with 12 ml of methanol. The methanolic solution was freeze-dried and then reconstituted to 2 ml of methanol solution. A solution of 0.2 N HCl in MeOH, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid was prepared. The methanolic wine extract was reacted with the same volume of this solution at 50 °C for 20 minutes and then combined with 5 volumes of 40 mM aqueous sodium acetate to stop the reaction. This final solution was filtered using

Teflon filters (0.45  $\mu$ m PTFE acrodisc CR13) prior to injection. Proanthocyanidin cleavage products were quantified using their response factors relative to (+)-catechin, which was used as the quantitative standard. The standard was prepared by dissolving (+)-catechin (100 mg/L), (-)-epicatechin (20 mg/L) and acetic acid (1 ml/L) in 12% ethyl alcohol. To calculate the mean degree of polymerization (mDP), the sum of flavan-3-ol monomers, terminal and extension subunits (in moles) was divided by the sum of flavan-3-ol monomers and terminal subunits (in moles) and expressed as percentage (Kennedy and Jones, 2001). In addition, proportion of seed and skin proanthocyanidin was calculated using a previously described method (Peyrot des Gachons and Kennedy, 2003).

**Wine extract.** Ten milliliters of each wine was evaporated to eliminate water and ethanol. The final weight was measured, divided by its initial volume (10 ml) and expressed as percentage (w/v) of wine extract.

**Astringency assessment.** Wines produced from the 2002 vintage and pressed on the third day of fermentation were manipulated using isolated and purified seed and skin proanthocyanidin. Grapes and methods used to isolate and purify seed and skin proanthocyanidins were the same as described by Jorgensen et al. (2004). Wines pressed on the third day of fermentation had 60% of the proanthocyanidins present in wines made under normal winemaking conditions.

Three wines were made by adding different proportions of skin and seed proanthocyanidins: i) 100% seed proanthocyanidin addition, ii) 100% skin proanthocyanidin addition and iii) 50:50% seed and skin proanthocyanidin addition.

Proanthocyanidins were added to the wines approximately ten days before sensory evaluation. After proanthocyanidin addition, the adjusted proportions of seed-skin proanthocyanidins were 31-69, 94-6 and 63-37%, respectively. The total proanthocyanidin concentration was kept constant for all the wines. Manipulated wines were informally evaluated by a panel consisting of four experienced Oregon winemakers with a considerable amount of experience in proanthocyanidin assessment. Additionally, four Oregon State University faculty and students were involved in the study. The purpose of this panel was to evaluate the wines for gross differences in astringency (while keeping the quantity constant) and to provide direction for future sensory studies on astringency quality.

A second informal sensory study was conducted using 2003 wines produced at three different maturities. Approximately 40 Oregon winemakers tasted these wines in an informal sensory evaluation. Their preferences and observations on the astringency of these wines were made.

## **Results**

During the 2001-2003 vintages, wines were made from grapes harvested at three levels of grape maturity (two for the 2001 vintage) (Table 4.1). The three harvest dates were designed to investigate extremes of what industry might observe. Here, the earliest harvest date was clearly an underripe crop whereas the latest harvest date was tending towards an overripe crop.

Table 4.1. Grape harvest dates for the 2001-2003 wines.

Wine	2001	2002	2003
Early	----	16 Sept	15 Sept
Mid	27 Sept	26 Sept	22 Sept
Late	4 Oct	7 Oct	29 Sept

Although the three years had similar soluble solids between the three grape maturities (Table 4.2), the dates of harvest varied. In order to get the desired soluble solids, grapes were harvested earlier for the 2003-growing season. In addition, for this particular year, grapes experienced a faster sugar accumulation at the end of the season in comparison with the other two years. While the difference between early and mid-maturity during the 2002 vintage was 10 days, the difference for 2003 was only 7 days.

Table 4.2. Soluble solids (°Brix) of harvested grapes.

Wine	2001	2002	2003
Early	----	20.1	21.9
Mid	23.6	23.4	24.5
Late	27.0	25.5	26.4

Titrateable acidity (TA) was measured for the wines made at three different maturities during the 2003 vintage (Table 4.3). The analysis was carried out five weeks before astringency assessment. The wine made from late maturity grapes had a slightly higher TA in comparison with the two previous years.

Table 4.3 Titrateable acidity for the 2003 wines.

Wine	T. A. (g/L)
Early	6.05±0.05
Mid	5.90±0.05
Late	6.55±0.05

**Temperature profile during fermentation.** Wines were made under similar conditions for the different vintages. None of the wines experienced temperatures above 32.5 °C, however, different temperature profiles with different fermentation lengths were observed for the 2002 and 2003 vintages.

Wines made from grapes harvested at the early maturity experienced clear differences in temperature profile across vintages (Figure 4.1). The maximum temperature (32.5 °C) was reached earlier (2<sup>nd</sup> day) for the 2002 vintage, while 2003 musts had their maximum temperature around the 3<sup>rd</sup> day, and never exceeded 30 °C. The highest recorded temperature was in 2002 and was followed by a rapid decrease. In contrast, 2003 had a lower temperature profile; however, the temperatures remained more constant during fermentation. Wines made from grapes harvested earlier during the 2002-growing season completed fermentation on the fourth day. Musts from the 2003 vintage took almost two days longer to complete fermentation.

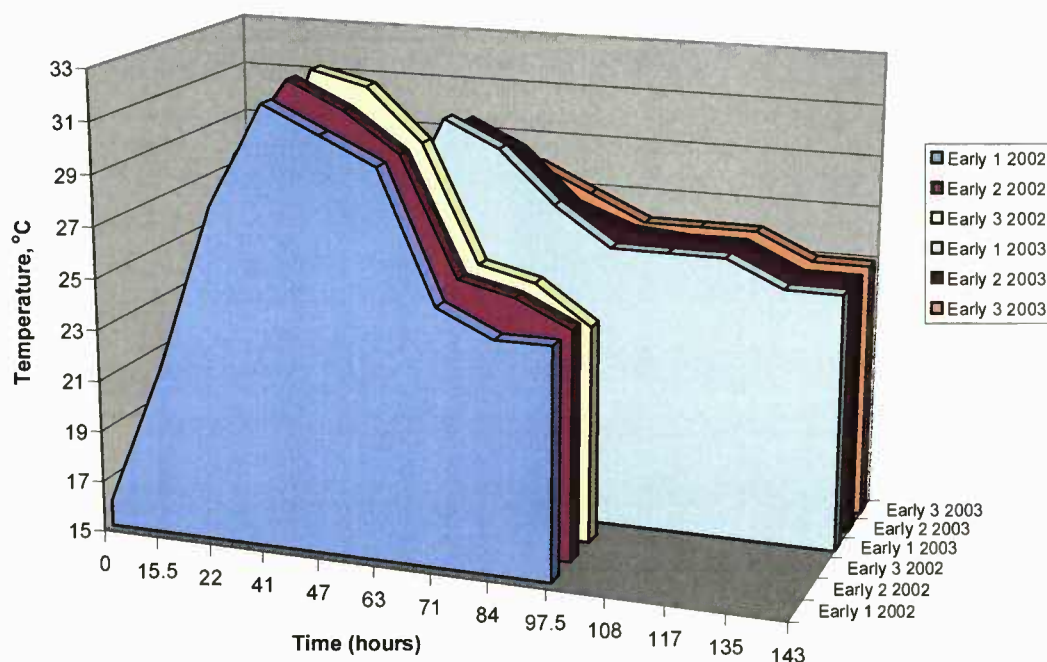


Figure 4.1. Temperature profile during fermentation of musts produced from early maturity grapes and from the 2002 and 2003 vintages.

Wines made with mid maturity grapes had temperature profiles different from those observed for the early wines (Figure 4.2). The differences in fermentation lengths were less than the early set. Musts from 2003 completed their fermentation at the beginning of the 6<sup>th</sup> day, while 2002 musts completed their fermentation approximately nine hours earlier. Mid-maturity wines produced in the 2002 vintage experienced a peak temperature during the 2<sup>nd</sup> day followed by a decrease; however, the highest temperatures (32.5 °C) were observed closer to the end of the fermentation, around the 5<sup>th</sup> and 6<sup>th</sup> day. Musts from 2003 mid-maturity grapes (as observed also in the early maturity musts) experienced a more constant temperature

during fermentation. For this year, the highest temperature was 30 °C and was reached on the 2<sup>nd</sup> day of fermentation. The duration of the fermentation for the 2003 musts was 146 hours, approximately 3 hours longer than the corresponding early musts. The largest difference was observed when the 2002 mid and early fermentations were compared. Musts from 2002 experienced fermentations 40 hours longer than the corresponding wines made of early maturity grapes.

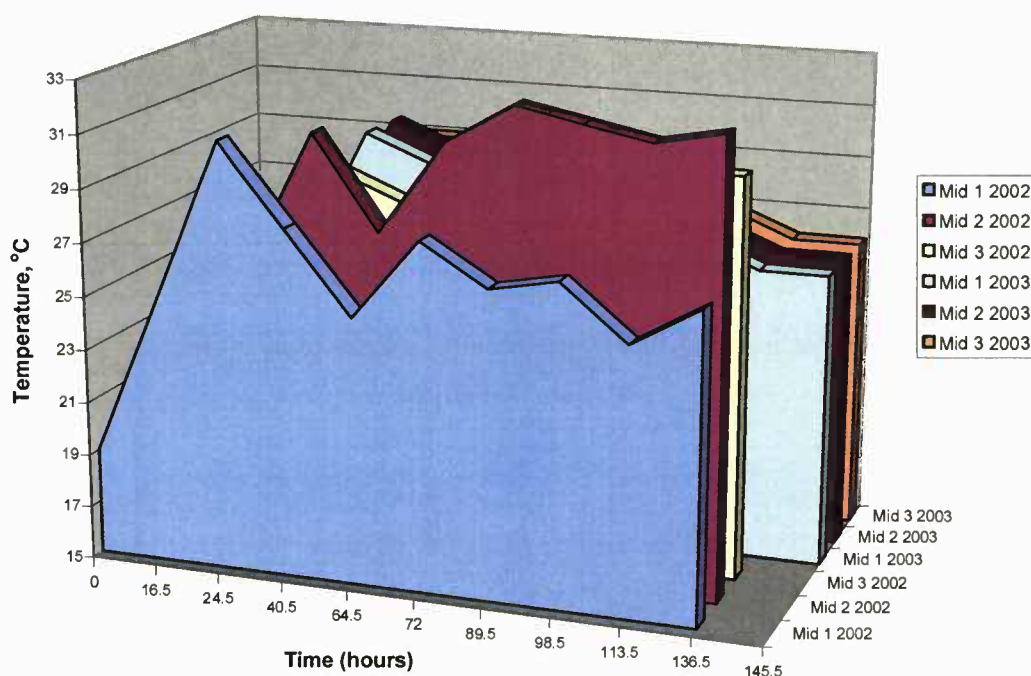


Figure 4.2. Temperature profile during fermentation of musts produced from mid maturity grapes and for the 2002 and 2003 vintages.

Fermentation temperature profiles for the late musts (Figure 4.3) were more similar between 2002 and 2003. For both years, the highest temperatures were 30°C and were reached around the 2<sup>nd</sup> day of fermentation, although 2002 also had high temperatures during the 5<sup>th</sup> and 6<sup>th</sup> days. In contrast with temperature profiles

observed during the early and mid must fermentations (particularly for 2002), late maturity musts for both years experienced more constant temperatures. The fermentation for 2003 lasted approximately 189 hours (7.9 days) while 2002 musts finished their fermentation in approximately 184 hours (7.7 days).

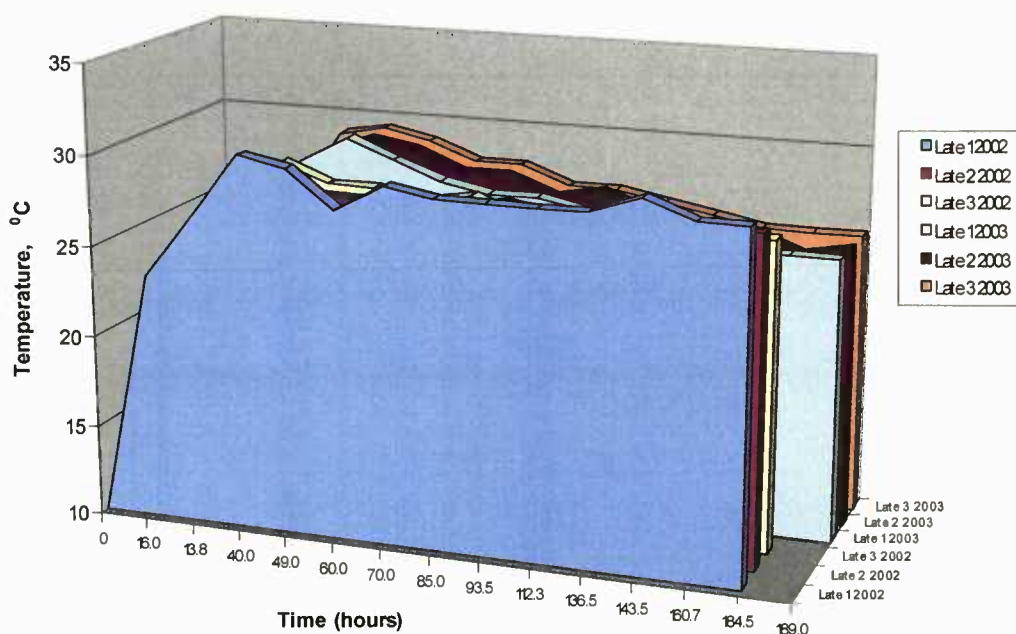


Figure 4.3. Temperature profile during fermentation of musts produced from late maturity grapes and for the 2002 and 2003 vintages.

**Proanthocyanidin content in wine.** The proanthocyanidin concentration of wines made during the 2002 -2003 vintages and at the three different maturities is summarized in Figure 4.4. The 2003 vintage had a higher concentration of

proanthocyanidins in comparison with 2002. For the late maturity wines, there was a large difference in proanthocyanidin concentration between these two vintages.

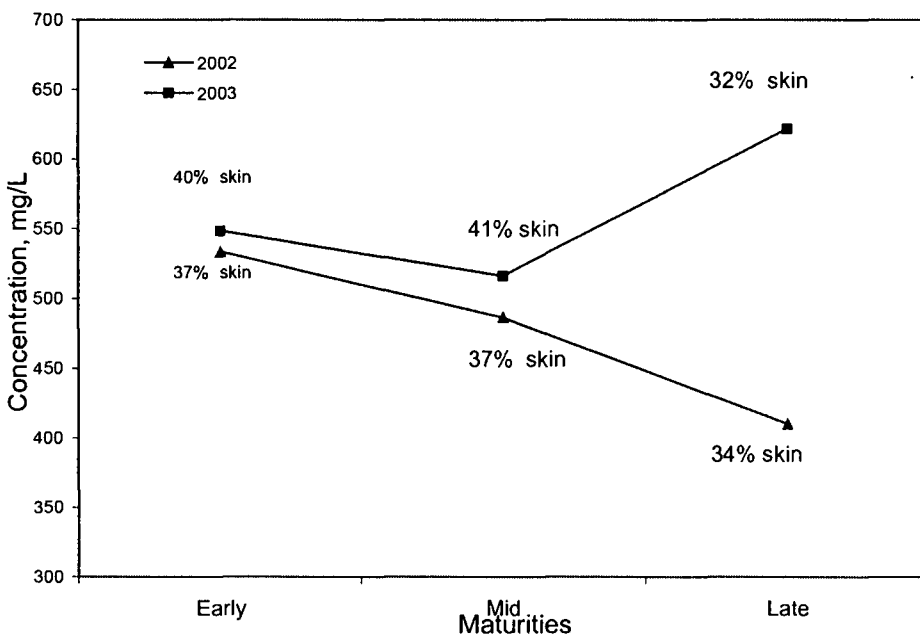


Figure 4.4. Total proanthocyanidin concentration in wine produced at three different grape maturities for 2002 and 2003 growing seasons, with the corresponding proportion of skin proanthocyanidin.

Although the proanthocyanidin concentration for the late maturity increased for the 2003 growing season, the proportion of skin proanthocyanidins decreased. Wines produced during the 2003 vintage had a higher proportion of skin proanthocyanidins in comparison with 2002. For both years, the maximum skin proanthocyanidin extraction was observed in mid maturity wines. The amount of seed proanthocyanidins (Table 4.4) decreased across maturity for the wines produced in the 2002 vintage (336-271 mg/L). However, in the 2003 vintage, the wines experienced an increase in seed proanthocyanidin content, on the last pick

date (from 330-423 mg/L). For both years, the skin proanthocyanidin content in wine decreased with berry maturity. The rate of decrease was greater for wines made during the 2002 vintage.

Table 4.4. Concentration of seed and skin proanthocyanidin in wine produced at three different grape maturities for the 2002 and 2003 growing seasons.

Wine	2002		2003	
	*Seed proanthocyan idin	*Skin proanthocyan idin	*Seed proanthocyan idin	*Skin proanthocyan idin
Early	336	197	330	219
Mid	306	180	304	212
Late	271	139	423	199

\* in mg/L

**Proanthocyanidin composition in wine.** In wine, the extension subunit composition (Figure 4.5) was primarily (-)- epicatechin (70%) as seen for the grapes . The second highest extension subunit concentration observed was (-)- epigallocatechin (15%).

Wines produced during the 2002 vintage had the highest extension subunit concentration during the early maturity date followed by a slight decrease across maturity (Figure 4.5 A). Wines produced with mid maturity grapes and pressed earlier (3<sup>rd</sup> day of fermentation) experienced a lower extraction of extension subunits which represented 60% of its corresponding mid-maturity-7-day-pressed wine, as expected. The proportion of (-)-epigallocatechin present in this low-tannin wine was higher (27%) than the one observed in the 7-day-pressed wine (15%). In contrast with what was observed for the 2002 wines, the extension subunits present in wines made during the 2003 vintage experienced a slight increase across maturity

(Figure 4.5 B), especially for the last two pick dates. In addition, the total concentration was slightly higher for the 2003 wines at all maturities.

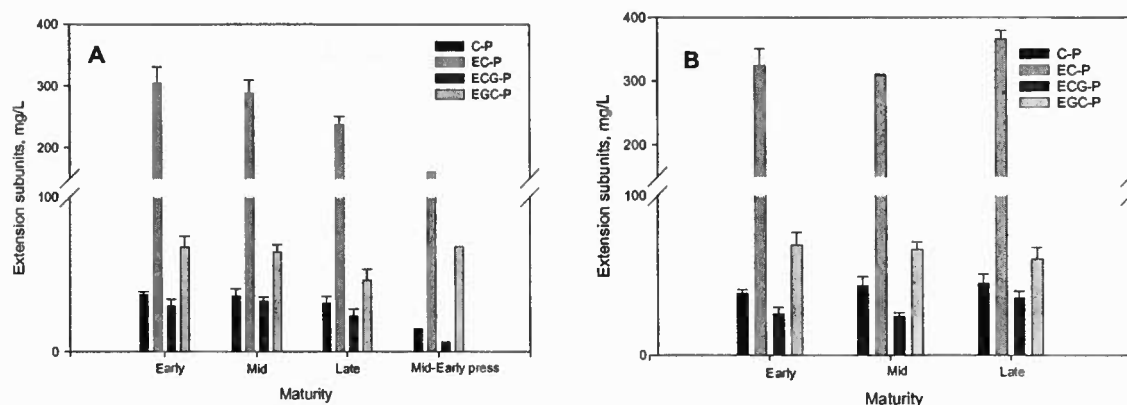


Figure 4.5. Extension subunit composition in wines made from grapes harvested at different maturities. **A)** Wines made during 2002 vintage; **B)** Wines made during 2003 vintage. Abbreviations: **C-P** (+)-catechin-phloroglucinol; **EC-P** (-)-epicatechin-phloroglucinol; **ECG-P** (-)-epicatechin-3-*O*-gallate-phloroglucinol; **EGC-P** (-)-epigallocatechin-phloroglucinol.

The concentration of flavan-3-ols (monomers and terminal subunits) showed a pattern similar to that observed in the extension subunits (Figure 4.6). The 2002 wines experienced a slight decrease across maturity while wines produced during the 2003 vintage increased with ripeness. (+)-Catechin was the principal subunit. For both years, (+)-catechin was 2/3 and (-)-epicatechin 1/3 of the total monomer and terminal subunit concentration. The low-proanthocyanidin wine had 35% of the total flavan-3-ols observed in the corresponding 7-day-pressed wine. In contrast with the differences in subunit proportions observed in the extension subunit analysis, the early pressed wine had a constant (+)-catechin/(-)-epicatechin ratio (67/33) when compared with other 7-day-pressed wines.

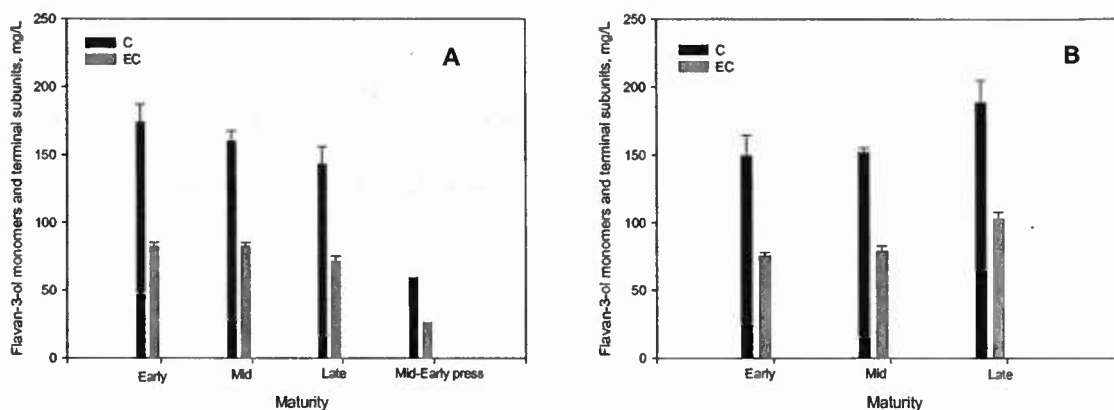


Figure 4.6. Flavan-3-ol monomer and terminal subunit composition in wines made from grapes harvested at different maturities. **A)** Wines made during 2002 vintage; **B)** Wines made during 2003 vintage. Abbreviations: **C** (+)-catechin; **EC** (-)-epicatechin.

**Mean Degree of Polymerization of wine proanthocyanidins.** Across grape maturity, the mean degree of polymerization (mDP, including flavan-3-ol monomers) in wine was relatively constant and low (Table 4.5). Both years experienced a slight decrease across maturity, particularly subtle for the 2002 wines.

Table 4.5. Mean Degree of Polymerization for the wines produced during the 2002 and 2003 vintages.

Wine	mDP	
	2002	2003
Early	2.54	2.98
Mid	2.52	2.86
Late	2.42	2.69

**Wine extract.** Wine extract experienced an increase across maturity (Table 4.6), suggesting that some compounds are more readily extracted as the fruit ripens.

Table 4.6. Wine extract (w/v) for the wines produced during the 2002 and 2003 vintages.

Wine	Wine extract % (w/v)	
	2002	2003
Early	2.24±0.01	2.39±0.01
Mid	2.75±0.14	2.71±0.02
Late	2.86±0.04	3.24±0.02

**Anthocyanin content in wine.** Anthocyanin concentration in wines made from grapes harvested at different maturities (Figure 4.7) followed the same pattern of accumulation for 2002 and 2003. There was an increase until mid-maturity and then a decrease. Anthocyanin content was higher for the wine produced in the 2002 vintage. The highest concentration was reached at mid-maturity for both vintages.

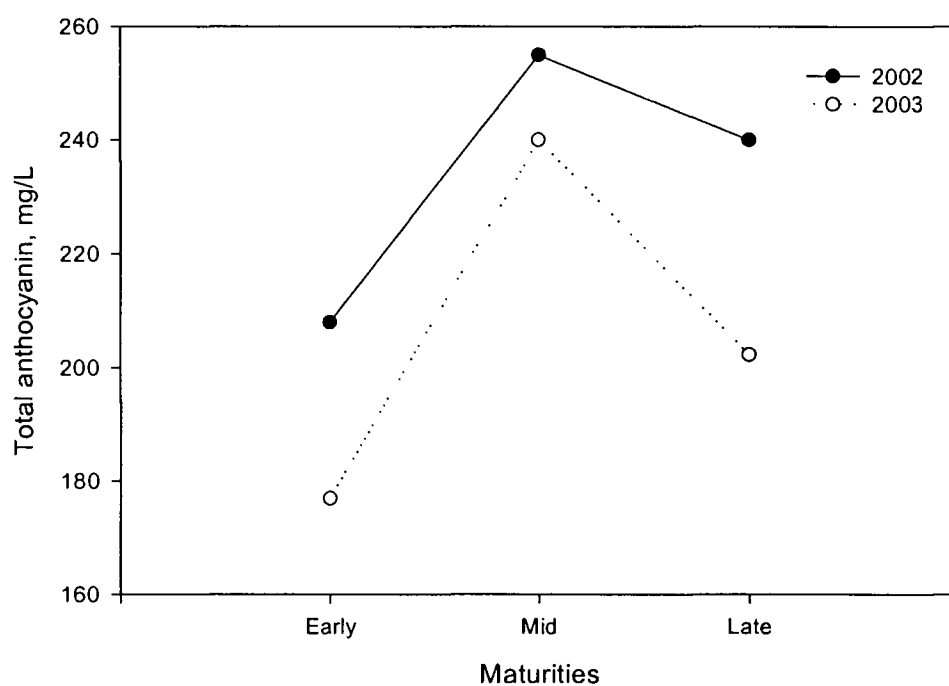


Figure 4.7. Total anthocyanin concentration in wine made from grapes harvested at three different grape maturities for 2002 and 2003 growing seasons.

**Astringency assessment.** Wines produced during the 2002 vintage were manipulated using isolated seed and skin proanthocyanidins. These proanthocyanidins were added to a wine that was pressed early (low proanthocyanidin content). In Table 4.7, the proportion of seed and skin proanthocyanidins in the manipulated wines are shown. The initial proanthocyanidin concentration in these wines was 279 mg/L, while the monomer concentration was 64 mg/L. After proanthocyanidin addition, the total proanthocyanidin content in the wines was around 910 mg/L, however, the proportion of seed and skin proanthocyanidin was considerably different.

Table 4.7. Wines manipulated with different proportions of seed and skin tannins.

Wine	Added proanthocyanidins (mg/L)	Total proanthocyanidins (mg/L)	% Skin proanthocyanidin	% Seed proanthocyanidin
1	575	915	31	69
2	560	903	94	6
3	577	921	63	37

The purpose of this study was to evaluate wines for gross differences in astringency quality (while keeping the quantity constant) and to provide direction for future sensory studies on astringency quality.

Considering the extreme variation in skin proanthocyanidin proportion (30-90%), the qualitative differences in astringency were subtle and inconclusive. The panelists were not able to describe clear differences. The results of this preliminary sensory evaluation seem to be inconsistent with the perceived relationship between proanthocyanidin origin (skin or seed) and “tannin ripeness”.

In another study, wines from the 2003 vintage, produced from grapes harvested at three maturities, were tasted in an informal sensory evaluation. Here, most of the panelists agreed that wines improved in astringency quality with grape maturity (70% of whom preferred the late maturity wine).

**Phenolics in wine for the three vintages.** In Table 4.8, a summary of the individual phenolic components for the mid-maturity wines is shown. The proanthocyanidin concentration increased with vintage, while the anthocyanin content in wine decreased slightly. The mean degree of polymerization also increased. Despite the differences observed in proanthocyanidin concentration, flavan-3-ol monomers were similar between wines produced across vintages. From grape analysis, the 2003 growing season had the lowest concentration of flavan-3-ol monomers and 2001 the highest. However, 2003 had the highest number of seeds per berry and 2001 had the lowest. These contrasting effects probably minimized the differences observed in wine.

Table 4.8. Flavonoid composition in mid-maturity wines.

Wine	Tannin <sup>a</sup>	Monomer <sup>a</sup>	Antho <sup>a</sup>	mDP <sup>b</sup>
2001	444	149	284	2.3
2002	486	169	255	2.5
2003	516	160	240	2.9

Notes) <sup>a</sup> mg/L

<sup>b</sup> Average mean degree of polymerization including flavan-3-ol monomers

## Discussion

The purpose of this chapter was to improve our understanding of how fruit maturity influenced phenolic extraction during winemaking, with particular attention being focused on proanthocyanidins.

Temperature profiles during fermentation for the 2002 and 2003 wines are shown in Figures 4.1-4.3. Early maturity wines produced during the 2002 and 2003 vintages experienced a greater difference in fermentation profiles (fermentation took 40 hours longer for the 2002 early wines). Fermentation profiles for the late maturity wines were similar. The difference in temperature for these wines during fermentation was 0.5 °C and there was little difference in the length of fermentation. Overall, differences in proanthocyanidin and anthocyanin concentration were greater in comparison with the early and mid maturity wine fermentations, despite having similar fermentation profiles (temperature and time). Previous studies have shown that anthocyanin extraction occurs therein the early days of fermentation (Scudamore et al. 1990; Boulton et al., 1998). Anthocyanin content generally decreases afterwards (Scudamore et al. 1990; Boulton et al., 1998). Given that the fermentation lengths were similar, it is likely that the differences in anthocyanin content observed between 2002 and 2003 (Figure 4.6) are due to differences in the fruit.

Previous research has shown that the concentration of proanthocyanidins increases with maceration with the highest rates of extraction occurring over the

first ten days (Boulton et al., 1998; Meyer and Hernandez, 1970). Hence, longer fermentation lengths (i.e.: 2003 early maturity wines) would be more likely to produce an effect on overall proanthocyanidin extraction in comparison with the late maturity wine fermentations in which the difference in fermentation length was much less. However, despite the small differences in maceration time (and temperature) in the late maturity wines, the differences in proanthocyanidin concentration were greater than the early and mid-maturity wines, suggesting again that fruit maturity played a role in the differences observed.

During the 2003 growing season, wines made from increasingly mature grapes (mid versus late maturity) resulted in an increase in proanthocyanidins. Perez-Magariño and Gonzalez-San José (2004) reported that dimer and trimer flavan-3-ols tended to reach higher levels in wines made from increasingly ripe grapes. This pattern was also observed in the berry analysis (previous chapter) in which terminal and extension subunits increased on the last two pick dates.

An opposite pattern was observed in wines made during the 2002-growing season. Although grapes experienced a slight increase in terminal and extension subunits for the last two pick dates, the proanthocyanidin content in wines decreased with grape maturity, particularly with the late maturity grape-wine comparison.

There is a tendency in the wine industry to associate riper grapes with better astringency perception. The term that is often applied to this is “tannin ripeness”. A common explanation for improved astringency as a function of berry ripeness is that there is an increase in skin proanthocyanidin proportion as a result of berry maturity.

In stark contrast to this, the results from this study show that wines made with riper grapes have a higher proportion of seed proanthocyanidin (Table 4.4).

In Figure 4.5 and for 2002 and 2003, it was observed that the maximum anthocyanin accumulation corresponded to the wines made from mid maturity grapes. Similar results were observed by Hrazdina et al. (1984) when analyzing Chaunac grapes and by Perez-Magariño and Gonzales-San José (2004) when analyzing Cabernet Sauvignon wines. Data gathered from anthocyanin analysis in grapes (Figure 3.24) showed that anthocyanin content (mg/gm berry) for the 2002-growing season remained constant during the final two pick dates. Since these results do not correlate with those observed in the wine, suggests (at least for the 2002 data) that the anthocyanin peak observed in mid maturity wines was not solely influenced by grape concentration.

Data gathered during the 2003-growing season experienced similar patterns between grape and wine anthocyanin content for the different maturities. The decrease in anthocyanin content observed in late wines from the 2003-vintage could be due to berry senescence and a loss of cellular redox potential (Thompson et al., 1987).

Both vintages had low anthocyanin concentrations in wines made from early maturity grapes in comparison with mid-maturity. Although the anthocyanins were present in the berries, they did not appear to be extracted into the wine to the extent that they were in the mid-maturity wines. The increasing permeability of the cell

wall as the berry ripens (Nunan et al. 1998; Nunan et al. 2001) could have contributed to the increased extractability of anthocyanins observed.

It is noteworthy that the 2003 growing season had a higher anthocyanin content in grapes than 2002; however, this difference was not the case when their corresponding wines were analyzed. Specifically, wines made during the 2002 vintage had a higher anthocyanin content at all grape maturities than those made during the 2003 vintage. These results could suggest that anthocyanin content in grapes is not a good indicator of anthocyanin content in the corresponding wine.

As observed in Table 4.6, wine extract increased with grape maturity. Since wines made with riper grapes had longer fermentation times (Figure 4.1-4.3), it could be hypothesized that mid and late maturity wines have higher extracts due to the longer pomace contact. However, previous studies have shown that fermentations carried out with different maceration times produced similar extracts with the same maturity grapes (Yokotsuka et al., 2000).

Two informal sensory studies were conducted in order to improve our understanding of the concept of “tannin ripeness”. The first study was conducted on a modified wine and was intended to provide information about the sensory differences (from a descriptive standpoint) in wines containing different proportions of seed and skin proanthocyanidins. Proanthocyanidins isolated from pre-véraison seeds and skins were used to manipulate the wines. According to Jorgensen et al. (2004), these proanthocyanidins contained little if any residual flavan-3-ol monomers. Consequently, the mean degree of polymerization (mDP) of seed and

skins proanthocyanidins were 8.1 and 22.3 (including monomers), respectively, while the mDP for the wines made in this project were far less (<3.0 including monomers, Table 4.9). Hence, it is important to note that following proanthocyanidin addition, these wines contained distinctly different proanthocyanidins (skin versus seed) yet, the molecular weight distribution of the proanthocyanidins in the final wines were much different than would likely be expected under normal winemaking conditions. Despite this, this study provided initial and preliminary information on the sensory differences between skin and seed proanthocyanidins.

Manipulated wines had differences in seed and skin proanthocyanidin proportions larger than those normally expected in wines. As observed in Figure 4.4, wines made according to commercial winemaking practices experienced smaller variations in the proportions of seed and skin proanthocyanidins across maturity. Hence, the decrease in skin proanthocyanidin proportion between mid and late maturity was from 37 to 34% for 2002 and 41 to 32% for 2003. Although the differences in proanthocyanidin proportion in the manipulated wines were much different than those observed in the wine as a result of grape maturity, the differences in astringency perception were subtle. Since all the variables were held constant (acidity, sugar, alcohol, flavors, polysaccharides, harvest date, etc) with the exception of proanthocyanidins, these results would suggest that differences in astringency perception would not be due to proanthocyanidin structure, and the notion of “tannin ripeness”. It must be stressed however that this study needs to be

repeated with proanthocyanidin fractions (seed and skin) and molecular weight distribution that are more relevant. In addition, this study should be conducted under more controlled conditions.

A second informal sensory study was conducted with the 2003 wines, and was intended to explore “tannin ripeness” from a preference standpoint. The results of the sensory study concluded that wines made with more mature grapes improved in astringency quality. The changes in phenolic composition during this time (i.e.: skin versus seed, concentration, mDP) does not support the idea of tannin composition as being involved in this improvement.

Haslam (1977) reported similar results when analyzing blackberries. He found that even when there was an increase in palatability of blackberry, the change during the ripening process in the concentration of ellagitannins was of little significance, which indicated that other factors, besides a reduction in quantity of complex polyphenols, are important in determining the loss of astringency in ripening fruit.

The dramatic increase in wine extract with berry ripening is consistent with other compounds being present. These compounds may play a role in astringency perception. Potential compounds would appear to include: acidity, sugar, polysaccharides, alcohol and flavor compounds (Asquith, et al. 1987; Vidal et al., 2002; Vidal et al. 2004; Siebert and Chassy, 2003). Recent studies suggest that polysaccharides in wine affect astringency perception (Vidal et al., 2004A, 2004B). As described before, during fruit ripening the cell wall softens and some polysaccharides (e.g. galacturonan) become more soluble (Nunan et al., 1998;

Silacci and Morrison, 1990). Consequently, riper grapes would be expected to contain higher concentration of soluble polysaccharides. Taira and Ono (1997) reported that the interaction of proanthocyanidins with soluble pectins released during ripening impedes their binding to salivary proteins.

### **Conclusion**

Higher anthocyanin extractions were observed in wines made from commercially ripe grapes (23.4-24.5 °Brix). Wines made with more mature grapes experienced a reduction in the proportion of skin proanthocyanidins. While tasters considered that astringency and bitterness perception improved in wines made with riper grapes, the change in phenolic composition during this time does not support the idea of proanthocyanidin structure as being related to “tannin ripeness”. Compounds other than phenolics are likely to play a role in the perception of astringency quality. Based on previous studies, the changes in astringency and bitterness perception observed in our study could be due to the changes of polysaccharides across grape maturity. Future research should be focused on the mechanism and effect of polysaccharides on tannin perception.

## CHAPTER 5

### **Wine Tannin Prediction Based upon Grape Analysis: A Few Observations**

#### **Introduction**

Given the results of the previous chapters, the purpose of this chapter is to explore how the nature of the vintage relates to wine phenolic composition, with particular attention being directed to tannin concentration.

#### **Results and Discussion**

**Grape and wine tannin.** In Figure 5.1, the amount of tannin in grapes and their corresponding wines over three vintages is shown. There were differences in grape tannin content for all years. As predicted from grape analysis, the wines made during the 2003 vintage had the highest concentration of tannins, but only 9% of total grape tannins were extracted into wine. Wine produced during the 2001 vintage had the highest tannin extraction (12.8%). Grapes with higher tannin content experienced a lower rate of tannin extraction. In Figure 5.2, the relationship between the extraction rate of tannin in the wine and the original tannin content in the corresponding grape is shown. As observed, the higher tannin content present in grapes correlates to a lower rate of tannin extraction in the wine. Considering

theoretical concepts, diffusion is the net movement of a substance from an area of high concentration towards an area of lower concentration (Devlin and Witham, 1983). Based upon this theory, tannins will move out of the plant cell and into the developing wine.

Diffusion of non-electrolyte molecules across a semi-permeable membrane (i.e.: the plant cell) depends upon the *diffusion coefficient* as well as the *permeability coefficient* and thickness of the membrane. The *diffusion coefficient* is the ability of a substance to diffuse under defined conditions of temperature and pressure. It depends upon the mass and tendency of diffusing substances to “flow”. The *permeability coefficient* is the measure of the permeability of the membrane. Factors affecting diffusion are: i) density of the diffusing particles, ii) resistance of the medium, iii) temperature of the system, and iv) diffusion pressure gradient (Sinha, 2004). Since diffusion pressure is proportional to the concentration of diffusing particles (Sinha, 2004); among all of the variables affecting diffusion, gradient pressure could be considered the only one, which experienced substantial difference between vintages. 2003 had the highest tannin concentration in grapes, and hence the highest concentration gradient. However, even when tannin concentration gradient was the highest for this year, the resulting wine had only a slightly higher tannin concentration in comparison with the other vintages (Figure 5.1). Assuming that the permeability coefficient was the same for the three vintages, a possible explanation for this phenomenon is that only certain amount of tannin was able to pass through the membrane, yielding similar tannin concentration in wine for the

three vintages. Correspondingly, high concentrations of tannin in grape do not necessarily favor a high extraction.

Weimberg et al. (1983) proposed two phases for the mechanism of solute diffusion through the plant cell membrane. The first phase represents solute leakage from the cytoplasm, and the second phase is the release of solutes from the vacuole, suggesting that vacuole leakage does not begin until the first phase has ended, and the beginning of the second phase is controlled by the decrease in the concentration of solute in the cytoplasm.

It could be hypothesized that the likely mechanism by which phenolics are extracted into the wine is as follows: During crushing, some cells are disrupted and their cell walls lose integrity. For those cells, the diffusion of solutes occurs during the first stages of maceration when alcohol is not yet present. Since ethanol can penetrate plant cell membranes (Sinha, 2004), for those cells whose cell wall remains intact, the mechanism of tannin extraction will be dependant on the presence of ethanol. Hence, alcohol would not only induce permeability of the cell membrane with the corresponding leakage of solutes, it would also produce permeability in the tonoplast with the corresponding release of tannins. Accordingly, Lerner et al. (1978) induced pores in plant cell membranes using toluene-ethanol solutions. They reported that the pores were large enough to allow the diffusion of sugars, amino acids and short chains of organic acids. In addition, they also found a rapid leakage of solutes from the vacuole as compared with the slow release of solutes from the cytoplasmic compartment, suggesting that pores induced in the

tonoplast were larger than those formed in the multimembranes separating the subcompartments of the cytoplasm. In our study, the winemaking conditions were similar for the three vintages, so that the level of berry crushing and alcohol was similar. Consequently, diffusion of tannins for the three vintages would be limited by the same amount and size of pores induced by alcohol, yielding similar tannin concentration in wine for the three vintages.

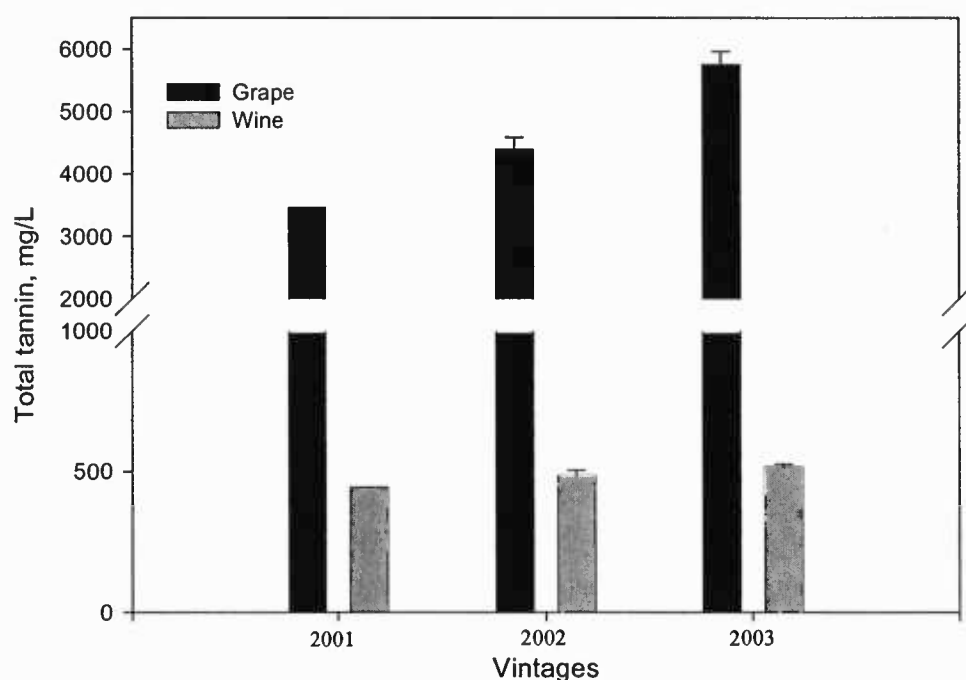


Figure 5.1 Amount of tannins in grapes and the equivalent amounts in the corresponding wine for the 2001-2003 growing seasons. Error bars indicating  $\pm$  SEM (N=5, grape data; N=3, wine data).

Membrane permeability may also increase due to the action of pectolytic enzymes, which hydrolyze wall pectins, and increase in activity during fruit ripening (Lecas and Brillouet, 1994). Although the soluble solids were similar

between vintages, the state of maturity that controls the breakdown of cells could have been different (Ribereau-Gayon, 2000B), with the consequent difference in tannin extractability.

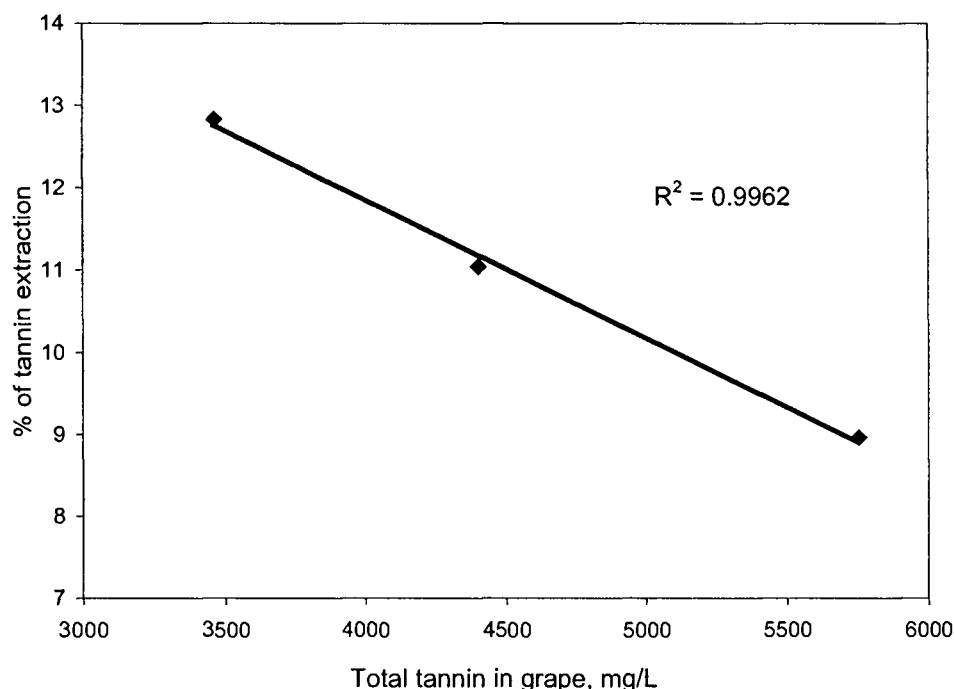


Figure 5.2 Relationship between the extraction rate of tannin into wine and the original tannin content in the corresponding grape for the 2001-2003 growing seasons.

**Grape and wine anthocyanins.** The total anthocyanin content in grapes and wine for the three vintages is shown in Figure 5.3. It appears that, there is no correlation between anthocyanin content in grapes and their amount in wines. While there were large differences in grape anthocyanins content between years (especially for 2002), these differences were not observed in the wines. In addition, the differences observed in the wines did not follow the same pattern observed in grapes.

Ribéreau-Gayon et al., (2000B) explains that under comparable winemaking conditions, grapes with higher anthocyanin content do not always produce wines with more color. Grapes have a variable extraction potential or extractability apparently to differences in maturity. This notion of anthocyanin extractability apparently depends on the breakdown of skin cells. In addition, similar mechanisms to those described above regarding the tannin extraction could also play a role in the differences observed in anthocyanin extraction between vintages.

Wines produced during the 2002 vintage experienced the highest extraction of grape anthocyanins into wine (47.8%). The 2003 wines, on the other hand, had the lowest rate of anthocyanin extraction (34.8%). These differences in extraction rate helped to minimize the differences in anthocyanin content observed in the wines.

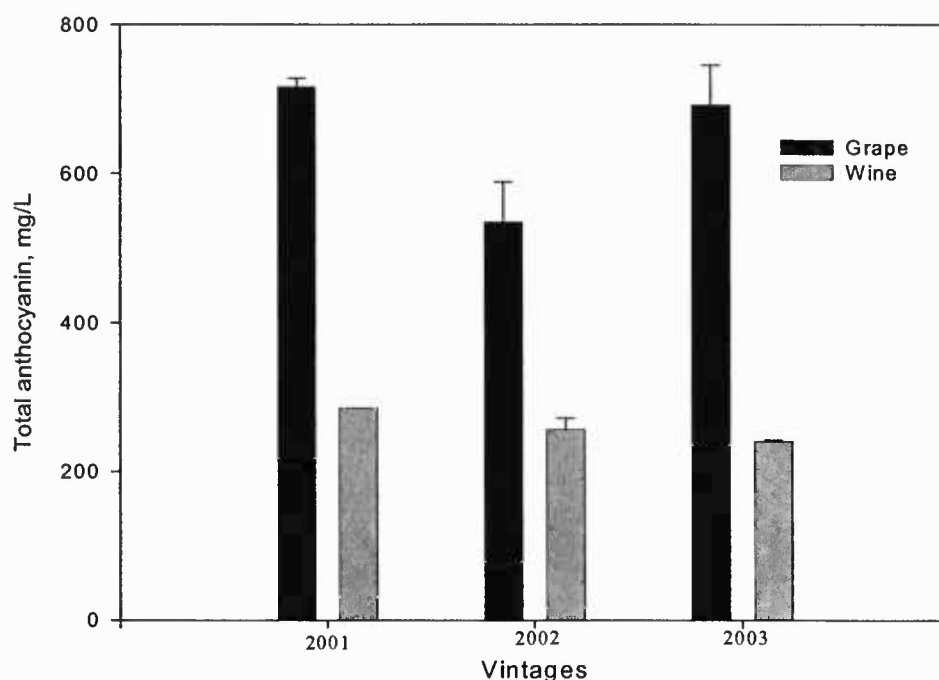


Figure 5.3. Amount of anthocyanins in grapes and the equivalent amounts in the corresponding wine for the 2001-2003 growing seasons. Error bars indicating  $\pm$  SEM (N=5, grape data; N=3, wine data).

**Factors determining tannin extraction into wine.** It is widely accepted that the tannins in wines are derived from seed and skin tissue. The proportions of seed and skin tannin found in the wine however, differ from those found in the grape (Table 5.1), indicating that during fermentation, skin tannins are more easily extracted. This observation is summarized in Figure 5.4. Thus, although more tannins are present in the seeds, a higher proportion of skin tannin extraction is typically achieved during maceration.

Table 5.1. Proportion of seed and skin tannin present in grape and wine.

Wine	Grape		Wine	
	% seed tannin	% skin tannin	% seed tannin	% skin tannin
2002	80	20	63	37
2003	79	21	59	41

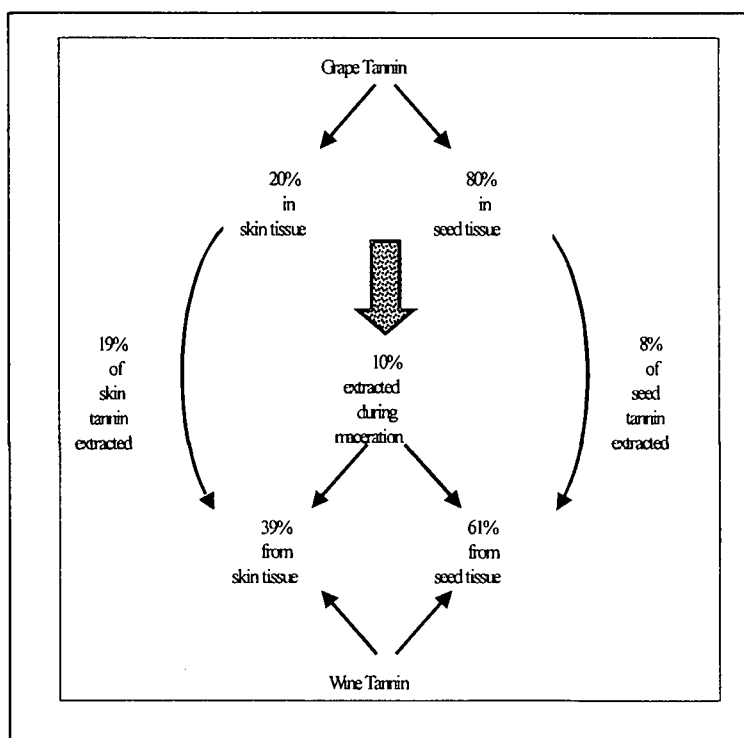


Figure 5.4. Extraction of seed and skin tannins during winemaking.

In order to predict tannin content in wine based upon grape analysis, it is important to correct the deficiencies of assuming that the rates of seed and skin tannin diffusion during maceration are the same. In Table 5.2, the concentration of seed and skin tannin in grapes and wine from six wines produced during the 2002 and 2003 growing season is shown. The corresponding percentages of individual seed and skin tannin extraction from the grapes are also calculated. Although the differences in seed tannin are large (as those observed between 2002 mid and 2003 late), the rate of seed tannin extraction from grape to wine remains relatively constant (8.7 and 8.8%, respectively). Skin tannin diffusion was also relatively constant for the different wines.

On average and for these vintages, the seed tannin content present in wine is 7.8% of the seed tannins from grape. The amount of skin tannin in wine represents 19% of the skin tannins found in grapes.

Table 5.2. Seed and skin tannin content in grapes and wine for the 2002 and 2003 vintages.

Wine	* Grape tannin		Wine tannin		% of individual extraction from seed and skin	
	Seed mg/kg	Skin mg/kg	Seed mg/L	Skin mg/L	% Seed	% Skin
2002 Early	4075.8	1095.2	335.8	197.2	8.2	18.0
2002 Mid	3524.2	877.1	306.2	179.8	8.7	20.5
2002 Late	3979.1	830.9	270.5	139.4	6.8	16.8
2003 Early	4587.4	1073.5	329.2	219.4	7.2	20.4
2003 Mid	4212.5	1119.3	304.4	211.6	7.2	18.9
2003 Late	4817.0	1039.1	422.9	199.0	8.8	19.2
<i>Average</i>					<b>7.8</b>	<b>19.0</b>

\* Grapes were analyzed at the corresponding maturity

From this information and with the help of chromatographic methods to get the seed and skin tannin content in grapes at harvest, the following formula was developed to predict wine tannins.

The tannin content in Pinot noir wine in mg/L and in this study is:

$$\frac{7.8\%(\text{tannin per seed}) \times (\# \text{ seeds per berry}) \text{ mg/berry} + 19\%(\text{skin tannin in berry}), \text{ mg/berry}}{\text{Berry weight gm}}$$

It is noteworthy that not only seed and skin tannin concentration is important in this prediction. The number of seeds per berry and berry weight play also a role in the final tannin concentration. In this study, 2003 happened to have both, higher tannin content in seeds and higher number of seeds per berry. However, assuming similar seed tannin content for both vintages, 2003 would still have a higher final tannin concentration as a result of a higher number of seeds per berry. Similarly, the berry weight is also important in the calculations of tannin content. Smaller berry weight would favor a more concentrated wine assuming similar amounts of tannins in a per berry basis. During vintages with smaller berry weights, winemakers would be forced to use higher number of berries with the resulting higher tannin content in the wine. Hence, the number of seeds per berry, berry weight, skin tannin and seed tannin concentration would be the four variables to consider in order to predict the tannin content in wine.

The comparison between the total tannin concentration predicted with this formula and the actual concentration found in the wines is shown in Table 5.3.

Although there were differences between these two values, the formula predicted efficiently the higher tannin content in wine observed during the 2003 vintage.

**Table 5.3.** Comparison between actual and predicted tannin content in wine based on grape analysis at harvest.

Wine	Tannin content, mg/L	
	Predicted	Actual
2002 Early	526.3	533
2002 Mid	441.8	486
2002 Late	468.6	410
2003 Early	562.2	549
2003 Mid	541.6	516
2003 Late	573.6	622

The process developed so far resulted in a formula for predicting wine tannin content from grape data gathered at harvest. However, winemakers require this information with many weeks in advance in order to plan the adjustments of tannin extraction. Facing this problem, another formula was developed following the same procedure described above, but taking into consideration the tannin content in grapes at véraison. The tannin proportion in grapes at véraison differs greatly from that observed at harvest (Table 5.4). However, since there is a strong correlation between tannin content in grapes at harvest (mid maturity) and at véraison (Figure 5.5), the formula can accurately predict the tannin content in wine. In this case, the mid maturities for the three vintages were considered.

Table 5.4. Seed and skin tannin content in grapes (at véraison) and wine for the 2001-2003 vintages.

Wine	Grape tannin		Wine tannin		% of individual extraction from seed and skin	
	Seed mg/kg	Skin mg/kg	Seed mg/L	Skin mg/L	% Seed	% Skin
2001	6352.1	1834.7	239.8	204.2	3.8	11.1
2002	8338.1	1923.5	306.2	179.8	3.7	9.3
2003	9082.6	2452.4	304.4	211.6	3.4	8.6
			<i>Average</i>		<i>3.6</i>	<i>9.7</i>

Based on grape analysis at véraison, the tannin content in Pinot noir wine in mg/L is:

$$\frac{3.6\%(\text{tannin per seed}) \times (\# \text{ seeds per berry}), \text{mg/berry} + 9.7\%(\text{skin tannin in berry}), \text{mg/berry}}{\text{Berryweight, gm}}$$

The predicted and actual tannin content in wine is shown in Table 5.5 and the correlation for these two values across vintages is observed in Figure 5.5.

Table 5.5. Comparison between actual and predicted tannin content in wine.

Wine	Tannin content, mg/L	
	Predicted	Actual
2001	406.7	444
2002	486.8	486
2003	564.9	516

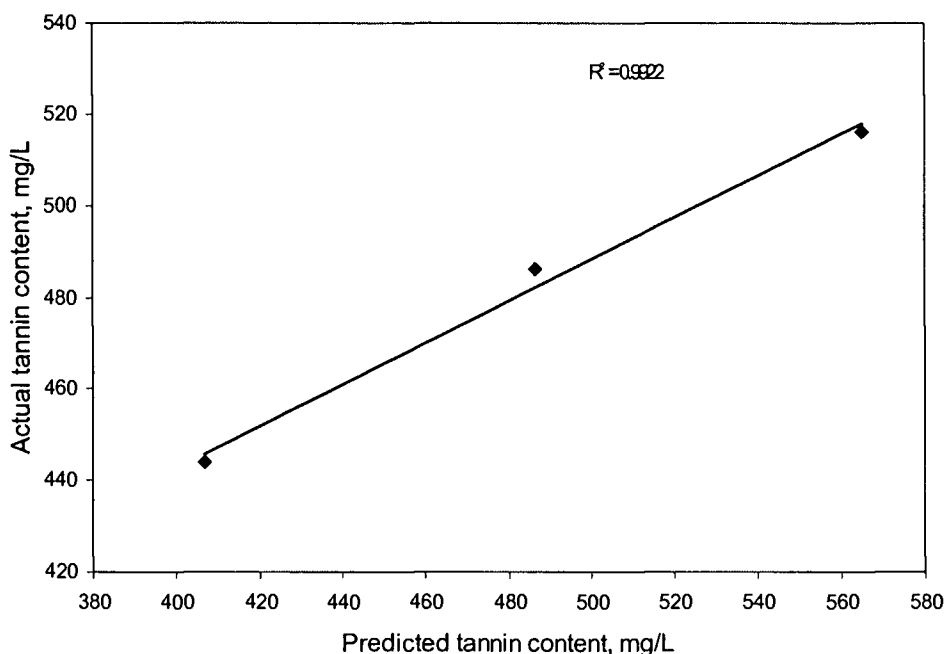


Figure 5.5. Comparison between actual and predicted tannin content in wine based on grape analysis at véraison.

Building these formulas is an important tool for the prediction of tannins. This formula will help in the prediction of tannin content in wine with enough time ahead to plan the actions to control the excess or deficiency of tannins expected in the wine. Winemakers experiencing vintages with considerably high tannin content predicted in grapes at véraison will have plenty of time to plan the modifications and corrective actions in the winemaking process so that the tannin extraction could be minimized. In contrast, winemaking practices facing vintages with low tannin content in grapes would be focused on maximizing tannin extraction or addition of tannic material.

In addition, these formulas consider were made considering four variables: seed tannin concentration, skin tannin concentration, number of seeds per berry and the

berry weight. The knowledge of these four variables will give the winemakers the tools for the prediction of tannin content in wine.

It is important to highlight that these formulas were built with a limited number of data points, since only three vintages have been studied. For better predictions and considering future studies, it is advisable to include as many years as possible to get the most representative values. Moreover, winemaking conditions should be kept constant for a more accurate prediction.

**Early prediction of tannin.** Although a formula for the prediction of tannin content in wine was developed based on grape information, it still remains the question whether or not there is correlation between the tannin content in grapes and tannin content in wines. The relationship between tannin content in wines produced for the three vintages and tannin content in their corresponding grapes is shown in Figure 5.6. As observed, the higher the tannin content in grapes, the higher the tannin content in the corresponding wine.

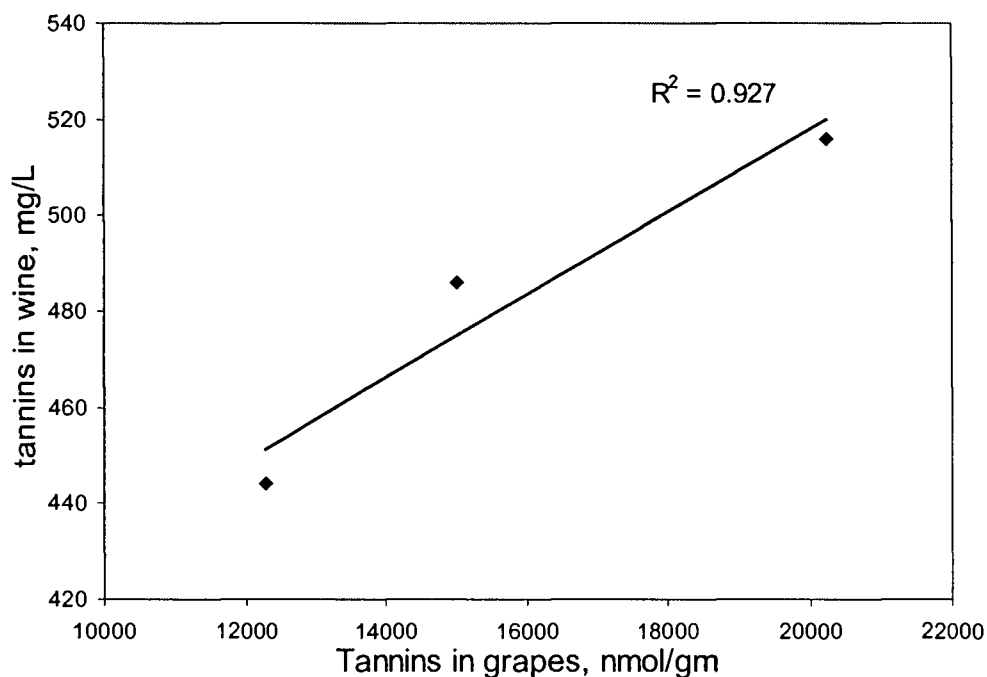


Figure 5.6. Relationship between total tannins in wine and total tannins in grapes at harvest time, for the 2001-2003 growing seasons.

The grapes considered in this analysis were included only mid-maturity grapes, which were selected because these grapes were considered to be “ideal” for commercial purposes. While the number of data points is limited, the good correlation suggests that it is possible to know the relative tannin amount in wine based upon grape analysis. However, to be of value to the wine industry, the prediction of tannin potential in wine should be known prior to harvest.

When analyzing the tannin accumulation in grapes for the three vintages (expressed on a “per gram of berry” basis), the three vintages developed in parallel (Figure 5.7). This suggests that it is possible to relate tannin concentration in grapes at different stages of maturity. Hence, Figure 5.8 shows the relationship between tannin content in grapes at véraison and tannin content in grapes at harvest. The

relationship found supports the idea of predicting wine tannin from grape analysis at véraison.

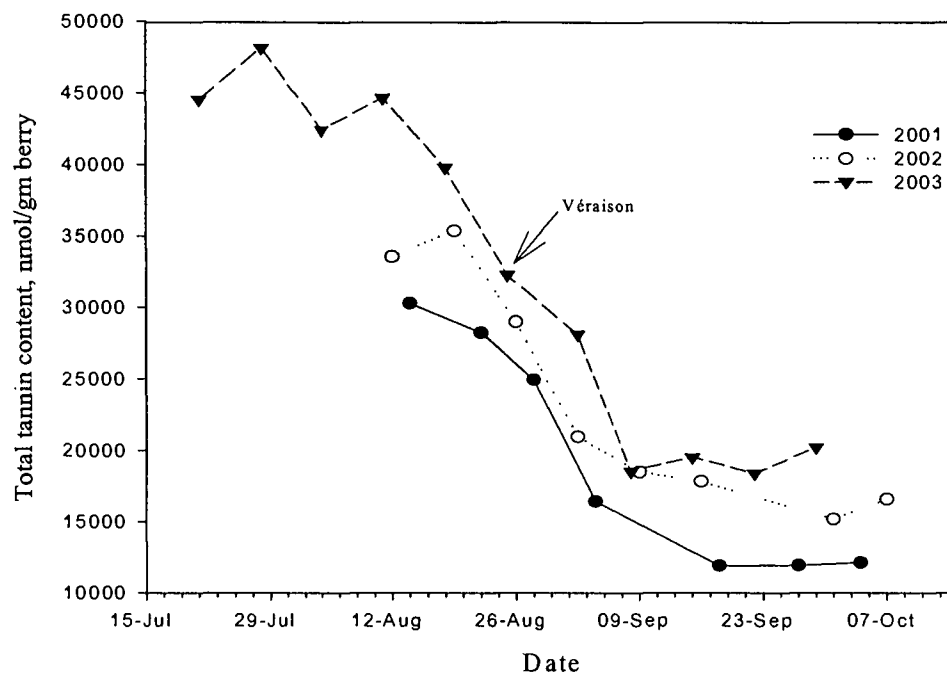


Figure 5.7. Development of total tannins in grapes during the 2001-2003 growing seasons

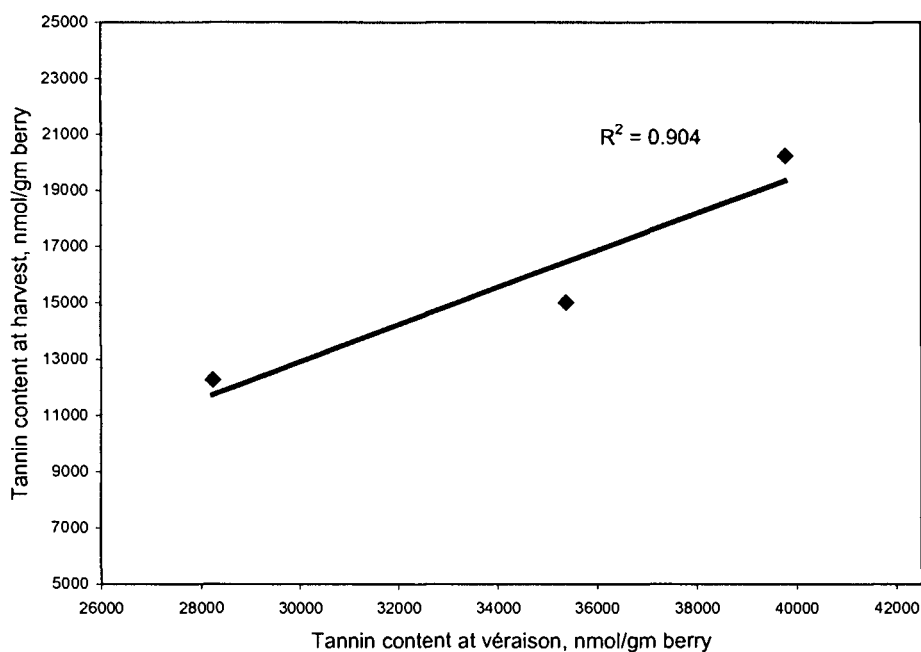


Figure 5.8. Relationship between total tannin at véraison and total tannin at harvest in grapes for the 2001-2003 growing seasons

When connecting these two pieces of information, the relationship between tannin content in grapes at véraison and the tannin content in wine for the three vintages is shown in Figure 5.9. The correlation suggests that, at véraison, it could be possible to predict relative vintage tannin content in wine.

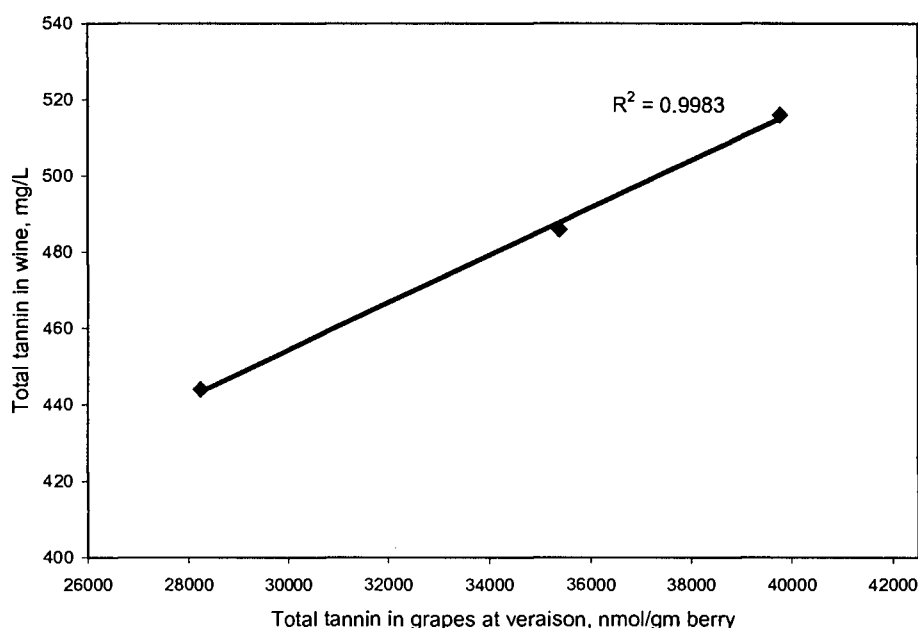


Figure 5.9. Relationship between total tannins in wine and total tannins in grapes at véraison, for the 2001-2003 growing seasons.

The prediction of tannin content in wine, based upon grape analysis at véraison was successful. However, the use of chromatographic methods was necessary to get this information. Since HPLC analysis is not an affordable option for the wine industry, it is necessary to consider other ways to predict tannin content. In Table 5.6, information on grape and wine tannins, and growing degree-days at véraison are shown. This data shows that years with higher heat summation had grapes and wine with higher tannin content.

Considering that tannin biosynthesis in grapes begins after fruit set and continues through véraison, the heat accumulated during this period can be considered as an indicator of the potential tannin synthesis. The relationship between tannin content in grapes at véraison and the cumulative growing degree-days is shown in Figure

5.10. Although the tannin content increases with heat summation, the correlation is poor ( $R^2=0.707$ ).

Table 5.6. Grape information relevant for the prediction of tannin.

Year	Grape tannin <sup>a</sup>	Wine tannin <sup>b</sup>	Heat summation <sup>c</sup>	Heat summation <sup>d</sup>	Bud break	Full bloom
2001	20975	444	1030	1443	Apr-20	Jun-20
2002	21786	486	1148	1547	Apr-16	Jun-23
2003	28009	516	1216	1750	Apr-16	Jun-23

Notes: <sup>a</sup> at véraison in nmol/berry

<sup>b</sup> in mg/L

<sup>c</sup> growing degree days from fruit set to véraison

<sup>d</sup> growing degree days from January to véraison

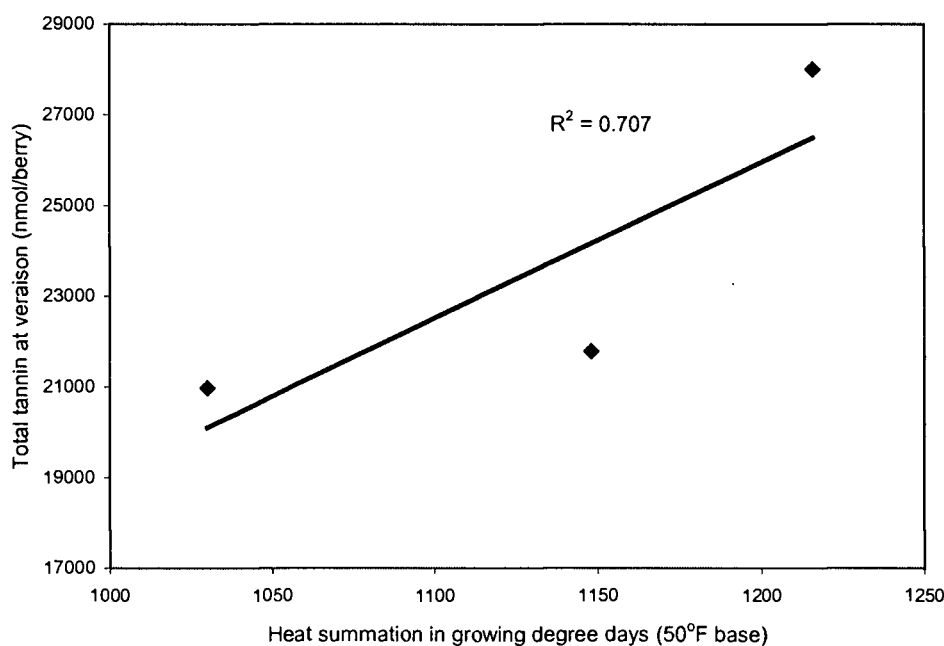


Figure 5.10. Relationship between total tannin content in grapes at véraison and the cumulative growing degree-days during the period fruit set – véraison, for the 2001-2003 growing seasons.

When heat summation is considered from January through véraison, the correlation improves considerably ( $R^2=0.946$ ) as shown in Figure 5.11. The inclusion of heat summation data for the period when vines are otherwise dormant

would seem to be counterintuitive; yet, certain growth regulators are affected by temperature during plant dormancy. Auxins for example, are growth regulators that cause cell elongation in plants. They also promote cell division in tissue and organ cultures. (Sinha, 2004). Cytokinins have been reported to participate in the regulation of many plant processes like cell division, morphogenesis of shoots and roots, chloroplast maturation, cell enlargement and senescence (Taiz and Zeiger, 1991). The combined effect of auxin and cytokinin has also been observed: High levels of auxin relative to cytokinin favors the root formation while high levels of cytokinin relative to auxin favors the shoot formation. In addition, balanced ratios of auxin and cytokinin stimulates both shoot and root formation (Sinha, 2004).

Sorce et al. (2000) studied the effect of certain auxins (indole-3-acetic acid) in potatoes. They monitored the time course of free and conjugated indole-3-acetic acid (IAA) during the dormancy of potatoes (*Solanum tuberosum*) at two different temperatures (3 and 23 °C). They found differences in the distribution of IAA between three distinct parts of the plant (eyes, subeye tissues and pith): the concentration of conjugated IAA was higher than free IAA in eyes and subeye tissues at 23 °C, while at 3 °C conjugated IAA prevailed only in eyes. Similarly, Jako (1981) found a correlation between cytokinin content of grape flower buds during winter and variations of rudimentary inflorescences per dormant bud. She also observed a good correlation of these variables with temperature. Thus, the number of inflorescences per dormant bud decreased when exposed to low temperatures.

Other plant regulators have also been reported to be associated to changes in temperature during dormancy. Li et al. (2004) reported that silver birch (*Betula pendula* Roth) experienced a change in abscisic acid during dormancy when exposed to low temperatures. The abscisic acid experienced a net increase when temperatures were lowered from 20 to 4 °C, however, below 4 °C, the abscisic acid decreased reaching the lowest levels at 0 °C. They explained that abscisic acid could play a role in freezing tolerance in woody plants. Similar changes in growth regulators could occur in vines during dormancy as a response to changes in temperature. The effect of abscisic acid in flavonoid biosynthesis and L-phenylalanine ammonia-lyase (PAL) activity has also been reported in literature. Kataoka et al. (1984) found that abscisic acid treatment to 'Kyoho' clusters at 1000 ppm in the beginning of ripening enhanced both anthocyanin accumulation and PAL activity even at high temperature or in the dark. Consequently, the inclusion of heat summation data in dormant vines may be partially justified. Based upon this information, it could be hypothesized that warmer conditions as those observed during the 2003 vintage could have promoted the synthesis of growth regulators like abscisic acid, auxins or cytokinins, which being present in greater quantities even before fruit set could have enhanced the biosynthesis of flavonoids during the early stage of berry ripening or promote a rapid growth of the vines with the corresponding greater leaf area and greater ability to synthesize flavonoids.

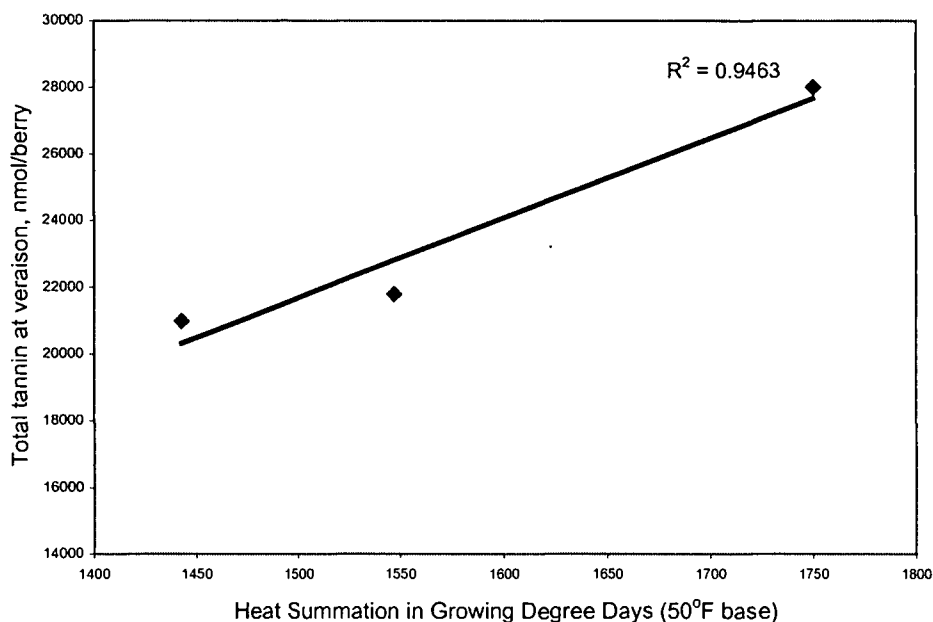


Figure 5.11. Relationship between total tannin content in grapes at véraison and heat summation from January through véraison, for the 2001-2003 growing seasons.

When comparing the tannin content in berries and growing degree days from fruit set, a poor correlation was observed; however, the correlation improved when growing degree days for the full calendar year were included. In grapes, it has been found that certain compounds like organic acids are responsive to temperature. Hence, the synthesis, accumulation and degradation of malic acid in grapes have been reported extensively in literature (Possner et al., 1983; Hrazdina et al., 1984) and coincide with the tannin synthesis and decline observed in our results. Organic acids appear to behave similarly to tannins in terms of accumulation and degradation/modification. Kliewer (1964) reported that concentrations of tartaric and citric acids increased in green berries when temperatures increased from 10 to 25 °C. Malic acid also increased in green berries when temperatures increased from

10 to 20 °C, however, as the berry ripened, malic acid content decreased with increasing temperatures. In addition, Lakso and Kliewer (1975) studied the enzymes responsible for the synthesis (PEP carboxylase) and degradation (malic enzyme) of malic acid. They found that PEP carboxylase activity increased until 38 °C; above this temperature there was a sharp decrease in activity. The malic enzyme on the other hand, showed a constant increase in activity between 10 and 46 °C.

Similar enzymatic behavior may govern the synthesis and degradation of tannins. During the first stage of berry development (green berries), the activity of tannin producing enzymes (i.e. phenylalanine ammonia lyase) may be enhanced by high temperatures. In the second stage (after véraison), this activity would be reduced and high temperatures could enhance the tannin degradation.

Swain and Williams (1970) reported a good correspondence between increases in the activity of phenylalanine ammonia lyase (PAL) and the rate of synthesis of soluble phenolic compounds in the tissues on exposure to light. However, the authors recognized that these two phenomena could not be directly related. In our study, we propose that temperature is associated with the changes in proanthocyanidins observed in the grapes. The basis of this mechanism may be based on the optimal temperature for enzyme activity or a response of this enzyme to thermal stress. Rivero et al. (2001) reported that PAL activity and phenolic content in tomatoes were affected by heat. They grew tomatoes at three different temperatures (15, 25 and 35 °C). The lowest biomass production occurred in tomatoes at 35 °C, representing a 44% reduction with respect to the highest values at

25 °C. They found a twofold increase in PAL activity and almost threefold increase in phenolic concentration in tomatoes grown under heat stress. However, when watermelons were grown under the same conditions, the highest PAL activity and phenolic concentration was observed under cold stress conditions (15 °C) for this particular fruit. They concluded that the mechanism in both plants would consist of the accumulation of phenolic compounds as a possible form of adapting to the stress (cold and heat). Since this experiment measured total phenolics, it is not possible to know accurately if the effect of heat favored only tannins with a different effect in other phenolics like anthocyanins (as observed in our results). Nozzolillo et al. (1989) found increasing anthocyanin concentration in jack pine seedlings (*Pinus banksiana*) grown under cold temperatures. They hypothesized that the reason why pigment biosynthesis increased in response to cold was that cold-induced permeability changes in the membranes permit enzymes to gain access to anthocyanin precursors previously inaccessible. The results of this project, though very limited, suggest that it may be possible to predict relative tannin potential in wine based upon climatological data. These results are clearly preliminary and require a much longer period of study to draw definitive conclusions.

## Conclusion

Changes in temperature and heat summation between vintages are strongly associated with changes in tannin content in grapes and wine. Warmer years were associated with higher tannin content in grapes and wine. There is also a strong relationship between tannin content in grapes and tannin content in wine. It is possible to predict tannins in wine based on early grape analysis. No relationship was found between anthocyanin content in grapes and anthocyanin content in wine, and between anthocyanin content and weather conditions. There exist differences in tannin extractability and diffusion for seeds and skin during winemaking. Based on this study, Pinot noir wine had 7.8% of the seed and 19% of the skin tannin present in grapes analyzed at harvest, and 3.6% of the seed and 9.7% of the skin tannin present in grapes analyzed at *véraison*. Number of seeds per berry and berry weight are important variables to be considered in the prediction of total tannin content in wine. Future research should consider more years of study in order to predict more accurately the relationship between tannin biosynthesis and temperature. In addition, complementary analysis on growth regulators during vine dormancy and phenylalanine ammonia lyase activity during the first stage of berry development would be required to fully understand the mechanism of tannin biosynthesis in response to different temperatures. Finally, the monitoring of heat stress conditions

in the vine will be required in order to know whether or not the increase in tannin biosynthesis is due to certain levels of high temperature or to heat stress.

## Bibliography

- Abass, M. and C.B. Rajashekar. 1993. Absciscic acid accumulation in leaves and cultured cells during heat acclimation in grapes. Div. Hortic., Kansas State Univ., Manhattan, KS, USA. Horticultural Science. 28:50-52.
- Albert, S., M. Delseny and M. Devic. 1997. BANYULS, a novel negative regulator of flavonoid biosynthesis in the Arabidopsis seed coat. The Plant Journal. 11:289-299.
- Alfenito, M.R., E. Souer, C.D. Goodman, R. Buell, J. Mol, R. Koes and V. Walbot. 1998. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. The Plant Cell. 10:1135-1149
- Amrani Joutei, K. and Y. Glories. 1994. Etude En Conditions Modeles De L'Extractibilite Des Composes Phenoliques Des Pellicules Et Des Pepins De Raisins Rouges. Journal Internationals des Sciences de la Vigne et du Vin. 28:303-317
- Amrani Joutei, K. and Y. Glories. 1995 Tanins et anthocyanes: localisation dans la baie de raisin et mode d'extraction, Rente Française d'Enologie. 153:28-31.
- Archier, P., S. Coen and J.P. Roggero. 1993. Changes in the phenolic content of single-variety wines after the first nine months of storage. Sciences Des Aliments 13:483-490.
- Asquith, T. N., J. Uhlig, H. Mehansho, L. Putman, D.M. Carlson and L. Butler. 1987. Binding of condensed tannins to salivary proline-rich glycoproteins: The role of carbohydrate. J. Agric Food Chem. 35:331-334.
- Asquith, T. N. and L.G. Butler. 1986. Interactions of condensed tannins with selected proteins. Phytochem. 25:1591-1593.
- Barnavon, L., T. Doco, N. Terrier, A. Ageorges, C. Romieu, and P. Pellerin. 2000. Analysis of cell wall neutral sugar composition, Beta-galactosidase activity and a related cDNA clone throughout the development of Vitis vinifera grape berries. Plant Physiol. Biochem. 38:289-300
- Barz, W. and U. Mackenbrock. 1994. Constitutive and elicitation induced metabolism of isoflavones and pterocarpan in chickpea (*Cicer arietinum*) cell suspension cultures. Plant Cell Tissue Organ. Cult. 38:199-211.
- Bharti, A.K. and J.P. Khurana. 1997. Mutants of Arabidopsis as tools to understand the regulation of phenylpropanoid pathway and UVB protection mechanisms. Photochem. and Photobiol. 65:765-776.

- Bonnardot, V. 1997. Some climatic indices for Pinot noir maturation at a meteorological station in Burgundy. *S. Afr. J. Enol. Vitic.* 18:19-23
- Bourzeix, M., D. Weyland, N. Heredia and C. Desfeux. 1986. A study of catechin and procyanidins of grape clusters, the wine and other by-products of the vine. *Bull. de l'O.I.V.*: 1171-1254.
- Boulton, R.B., V.L. Singleton, L.F. Bisson and R.E. Kunkee. 1998. Principles and practices of winemaking. Aspen publishers. Gaithersburg, Maryland.
- Boulton, R. 1980. The relationships between total acidity, titratable acidity and pH in wine. *Am. J. Enol. Vitic.* 31:76-80.
- Bravdo, B., Y. Hpnar, C. Loinger, S. Cohen and H. Tabacman. 1985. Effect of crop level and crop load on growth, yield, must and wine composition and quality of Cabernet Sauvignon. *Am. J. Enol. Vitic.* 36:125-131.
- Butzke, C.E. and R.B. Boulton. 1997. Acidity, pH, and potassium for grape growers. *Grapegrowing*. September/October. 10-16.
- Buttrose, M.S. and C.R. Hale. 1973. Effect of temperature on development of the grapevine inflorescence after bud burst. *Am. J. Enol. Vitic.* 24:14-16.
- Camm, E.L., and G.H.N. Towers. 1973. Phenylalanine ammonia lyase. *Phytochem.* 12:961-973.
- Cawthon, D. L. and J. R. Morris. 1982. Relationship of seed number and maturity to berry development, fruit maturation, hormonal changes, and uneven ripening of 'Concord' (*Vitis labrusca* L.) grapes. *J. Amer. Soc. Hort. Sci.* 6:1097-1104
- Chen, L.J. and G. Hrazdina. 1981. Structural aspects of anthocyanin-flavonoid complex formation and its role in plant color. *Phytochem.* 20:297-303.
- Cheynier, V., H. Fulcrand, P. Sarni and M. Moutounet. 1997. Progress in phenolic chemistry in the last ten years. Australian Society of Viticulture and Oenology Seminar: *Phenolics and Extraction*. Proceedings of a seminar held on October 1997 in Adelaide
- Coombe, B., and T. Clancy. 2001. Ripening berries – a critical issue. *Australian Viticulture*. March/April. 5-11.

- Crippen, D.D. Jr., and J.C. Morrison. 1986. The effects of sun exposure on the phenolic content of Cabernet Sauvignon berries during development. *Am. J. Enol. Vitic.* 37:243-247.
- Das, D. K. 1994. Naturally occurring flavonoids: structure, chemistry, and high-performance liquid chromatography methods for separation and characterization. *Methods in Enzymology* 234:410-453.
- De Freitas, V. and N. Mateus. 2001. Structural features of procyanidin interactions with salivary proteins. *J. Agric Food Chem.* 49:940-945
- Devlin, R.M. and F.H. Witham. 1983. *Plant physiology*. Fourth edition. Willard Grant Press. Boston.
- Dewick, P.M. and E. Haslam. 1969. Phenol biosynthesis in higher plants: gallic acid. *Biochemical journal.* 113:537-542
- Dixon, R.A. and N.L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. *The Plant Cell.* 7:1085-1097.
- Downey, M. 2003. Biosynthesis of flavonoids in grapevines (*Vitis vinifera* L.). Thesis completed for Doctor of Philosophy. Department of Horticulture, Viticulture and Oenology. University of Adelaide. Australia.
- Ebadi, A., B.G. Coombe and P. May. 1995. Fruit-set on small Chardonnay and Shiraz vines grown under varying temperature regimes between budburst and flowering. *Aust. J. Grape Wine Research.* 1:3-10.
- Escribano-Bailon, M.T., M.T. Guerra, J.C. Rivas-Gonzalo and C. Santos-Buelga. 1995. Proanthocyanidins in skins from different grape varieties. *Z. Lebensm Unters Forsch.* 200:221-224.
- Ewart, A. and W.M. Kliewer. 1977. Effects of controlled day and night temperatures and nitrogen on fruit-set, ovule fertility, and fruit composition of several wine grape cultivars. *Am. J. Enol. Vitic.* 28:88-95.
- Fisher, U. and A.C. Noble. 1994. The effect of ethanol, catechin concentration, and pH on sourness and bitterness of wine. *Am. J. Enol. Vitic.* 45:6-10
- Fong, R.A., R.E. Kepner and A.D. Webb. 1971. Acetic acid acylated anthocyanin pigments in the grape skins of a number of varieties of *Vitis vinifera*. *Am. J. Enol. Vitic.* 22:150-154

- Fougere-Rifot, M., J.J. Delavaux, N. Benharbit el Alami, J. Bouard and O. Brun. 1993. Importance des tanins vacuolaires dans le developpement des ovaires et des ovules de la vigne. *Vitis*. 32:127-136
- Francis, I.L., S. Kassara, A.C. Noble and P.J. Williams. 1999. The contribution of glycoside precursors to Cabernet Sauvignon and Merlot aroma In: Chemistry of wine flavor. A.L. Waterhouse and S.E. Ebeler Eds. American Chemical Society Washington.
- Francis, F.J. 1989. Food colorants: Anthocyanins. *Crit. Rev. Food Sci. Nutr.* 28:273-314.
- Gawel, R. 1998. Red wine astringency: A review. *Aust. J. Grape and Wine Research* 4:74-95.
- Greenspan, M.D., K.A. Shackel and M.A. Mathews. 1994. Developmental changes in the diurnal water budget of the grape berry exposed to water deficits. *Plant Cell & Environment*. 17:811-820.
- Hale, C.R. and M.S. Buttrick. 1974. Effect of temperature on ontogeny of berries of *Vitis vinifera* L. cv. Cabernet Sauvignon. *J. American Soc. Hort. Sci.* 99:390-394
- Hale, C.R., and R.J. Weaver. 1962. The effect of developmental stage on direction of translocation of photosynthate in *Vitis vinifera*. *Hilgardia*. 33:89:131.
- Harborne, J. B. and C. A. Williams. 2000. Advances in flavonoid research since 1992. *Phytochem.* 55:481-504.
- Harborne, J.B. 1963. Ultraviolet spectroscopy of polyphenols. In: Methods in polyphenol chemistry. Proceedings of the plant phenolics Group Symposium Oxford. April. The Macmillan Company. New York.
- Harborne, J.B. 1994. The flavonoids, advances in research since 1986. Chapman and Hall, London.
- Harris, J. M., P. E. Kriedemann and J. V. Possingham. 1968. Anatomical aspects of grape berry development. *Vitis* 7:106-119
- Haslam, E. 1977. Review: Symmetry and promiscuity in procyanidin biochemistry. *Phytochem.* 16:1625-1640.
- Haslam, E. 1980. In *Vino Veritas*: Oligomeric procyanidins and the ageing of red wines. *Phytochem.* 19:2577-2582.

Hawker, J.S., M.S. Buttrose, A. Soeffky and J.V. Possingham. 1972. A simple method for demonstrating macroscopically the location of polyphenolic compounds in grape berries. *Vitis*. 11:189-192

Heldt, H.W. 1997. Plant biochemistry and molecular biology. Institute of Plant Biochemistry, Göttingen. Oxford University press. New York.

Hemingway, R.W. and J.J. Karchesy. 1985. Chemistry and significance of condensed tannins. Plenum Press. New York

Ho, C.T. 1992. Phenolic compounds in food – An overview. *In: Phenolic compounds in food and their effects on health*, Vol 1: Ho, C.T., C.Y. Lee and M.T. Huang. ACS Symposium Series.

Hrazdina, G., G.F. Parsons and L.R. Mattick. 1984. Physiological and biochemical events during development and maturation of grape berries. *Am. J. Enol. Vitic.* 35:220-227.

Jackson, D.I. and D. Schuster. 1987. The productions of grapes and wine in cool climates. Butterworths horticultural books. New Zealand.

Jackson, D.I. and P.B. Lombard. 1993. Environmental and management practices affecting grape composition and wine quality – A review. *Am. J. Enol. Vitic.* 44:409-430

Jako, Nora. 1981. Changes in fertility and cytokinin content in buds of the variety Welschriesling from the beginning of the dormant season until bud burst. *Mitteilungen Klosterneuburg*. 31:98-102.

Jorgensen, E.M., A.B. Marin and J.A. Kennedy. 2004. Analysis of the oxidative degradation of proanthocyanidins under basic conditions. *J. Agric. Food Chem.* 52:2292-2296.

Joutei Amrani, K., Y. Glories and M. Mercier. 1994. Localization of tannins in grape berry skins. *Vitis*. 33:133-138

Kataoka, I., Y. Kubo, A. Sugiura and T. Tomana. 1984. Effects of temperature, cluster shading and some growth regulators on L-phenylalanine ammonia-lyase activity and anthocyanin accumulation in black grapes. *Fac. Agric., Kyoto Univ., Kyoto, Japan. Memories of the College of Agriculture, Kyoto University.* 124:35-44.

Katalinic, V. 1999. Grape catechins- Natural antioxidants. *J. Wine Research*. 10:15-23.

Kelly, K. M., J. Van Staden and W. E. Bell. 1992. Seed coat structure and dormancy. *Plant Growth Regulation*. 11:201-209

Kennedy, J.A. 2002. Understanding grape berry development. July/August. [www.practicalwinery.com/JulyAugust02](http://www.practicalwinery.com/JulyAugust02).

Kennedy, J. A., G. J. Troup, J.R. Pilbrow, D.R. Hutton, D. Hewitt, C.R. Hunter, R. Ristic, P.G. Iland and G.P. Jones. 2000A. Development of seed polyphenols in berries from *Vitis vinifera* L. cv. Shiraz. *Aust. J. Grape Wine Research* 6:244-254.

Kennedy, J.A., M.A. Matthews and A.L. Waterhouse. 2000B. Changes in grape seed polyphenols during fruit ripening. *Phytochem*. 55:77-85.

Kennedy, J.A., M.A. Matthews and A.L. Waterhouse. 2002. Effect of maturity and vine water status on grape skin and wine flavonoids. *Am. J. Enol. Vitic*. 53:268-274

Kennedy, J.A., Y. Hayasaka, S. Vidal, E.J. Waters and G.P. Jones. 2001. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric Food Chem*. 49:5348-5355

Kennedy, J.A. and A.W. Taylor. 2003. Analysis of proanthocyanidins by high-performance gel permeation chromatography. *J. Chromatography A*. 995:99-107.

Kennedy, J.A., Y. Hayasaka, S. Vidal, E.J. Waters and G.P. Jones. 2004. Composition of grape skin proanthocyanidins at different stages of berry development. *J. Agric Food Chem*. 49:5348-5355.

Kliwer, W.M. 1964. Influence of environment on metabolism of organic acids and carbohydrates in *Vitis vinifera*. I. Temperature. *Plant physiology*. 39:869-880

Kliwer, W.M. 1977. Influence of temperature, solar radiation and nitrogen on coloration and composition of emperor grapes. *Am. J. Enol. Vitic*. 28:96-103

Kliwer, W.M. and R.E. Torres. 1972. Effect of controlled day and night temperatures on grape coloration. *Am. J. Enol. Vitic*. 23:71-77

Kliwer, W.M. 1977. Effects of high temperatures during the bloom-set period on fruit-set, ovule fertility, and berry growth of several grape cultivars. *Am. J. Enol. Vitic*. 28:215-222.

Kliwer, W.M. and R.J. Weaver. 1971. Effect of crop level and leaf-area on growth, composition and coloration of Tokay grapes. *Am. J. Enol. Vitic*. 22:172-177.

- Kliewer, W.M. 1965. Changes in concentration of glucose, fructose, and total soluble solids in flowers and berries of *Vitis vinifera*. Am. J. Enol. Vitic. 16:101-110.
- Kobayashi, A., H. Yukinaga and E. Matsunaga. 1965. Studies on the thermal conditions of grapes. V. Berry growth, yields and quality of Muscat of Alexandria as affected by night temperature. J. Jpn. Soc. Hortic. Sci. 34:152-158.
- Koes, R. E., F. Quattrocchio and M. Mol. 1994. The flavonoid biosynthetic pathway in plants: function and evolution. BioEssays 16:123-132.
- Kriedemann, P.E. 1968. Photosynthesis in vine leaves as a function of light intensity, temperature and leaf age. Vitis. 7:213-220.
- Lakso, A.N. and W.M. Kliewer. 1975. The influence of temperature on malic acid metabolism in grape berries. Plant Physiol. 56:370-372.
- Lakso, A.N. and W.M. Kliewer. 1978. The influence of temperature on malic acid metabolism in grape berries. II. Temperature responses of net dark CO<sub>2</sub> fixation and malic acid pools. Am. J. Enol. Vitic. 29:145-149.
- Lecas, M. and J.M. Brillouet. 1994. Cell wall composition of grape berry skins. Phytochem. 35:1241-1243.
- Lerner, H.R., D. Ben Bassat, L. Reinhold and A. Poljakoff Mayber. 1978. Induction of pore formation in plant cell membranes by toluene. Plant Physiol. 61:213-217.
- Li, C., O. Junttila, P. Heino and E.T. Palva. 2004. Low temperature sensing in silver birch (*Betula pendula* Roth) ecotypes. Plant Science. 167:165-171.
- Loveys, B.R. and P.E. Kriedemann. 1973. Rapid changes in abscisic-like inhibitors following alterations in vine leaf water potential. Plant Physiol. 28:476-479.
- Mann, J., R.S. Davidson, J.B. Hobbs, D.V. Banthorpe and J.B. Harborne. 1996. Natural products: their chemistry and biological significance. Longman. England.
- McCarthy, M.G. 1999. Weight loss from ripening berries of Shiraz grapevines (*Vitis vinifera* L. cv. Shiraz). Aust. J. Grape Wine Research 5:10-16.
- Meyer, B. and J.R. Hernandez. 1970. Seed tannin extraction in Cabernet Sauvignon. Am. J. Enol. Vitic. 21:184-188.
- Miranda-Lopez, R. 1991. A maturity trial study of Pinot noir wines: aroma profile by sniffing gas chromatographic effluent. Thesis for the degree of Master of Science in Food Science and Technology. Oregon State University.

Miranda-Lopez, R., L.M. Libbey, B.T. Watson and M.R. McDaniel. 1992. Odor analysis of Pinot noir wines from grapes at different maturities by a gas chromatography-olfactometry technique (OSME). *J. Food Sci.* 57:985-1019

Mullins, M.G., A. Bouquet and L.E. Williams. 1992. *Biology of the grapevine* Cambridge University Press. Great Britain.

Ndung'u, C.K., S. Masumi, G. Okamoto and K. Hirano. 1997. Absciscic acid, carbohydrates and nitrogen content of Kyoho grapevines in relation to bud break induction by water stress. *Am. J. Enol. Vitic.* 48:116-122.

Noble, A.C. and M. Shannon. 1987. Profiling Zinfandel wines by sensory and chemical analyses. *Am. J. Enol. Vitic.* 39:1-5

Nozzolillo, C., P. Isabelle and G. Das. 1990. Seasonal changes in the phenolic constituents of jack pine seedlings (*Pinus banksiana*) in relation to the purpling phenomenon. *Canadian J. Botany.* 68:2010-2017.

Nunan, K.J., I.M. Sims, A. Bacic, S.P. Robinson and G.B. Fincher. 1997. Isolation and characterization of cell walls from the mesocarp of mature grape berries (*Vitis vinifera*). *Planta.* 203:93-100.

Nunan, K. J., I.M. Sims, A. Bacic, S.P. Robinson and G.B. Fincher. 1998. Changes in cell wall composition during ripening of grape berries. *Plant Physiol.* 118:783-792.

Nunan, K.J., S.P. Robinson and G.B. Fincher. 2001. Expression patterns of cell wall-modifying enzymes during grape berry development. *Planta* 214: 257-264.

Peleg, H., Bodine, K.K. and Noble A.C. 1998. The influence of acid on astringency of alum and phenolic compounds. *Chemical senses.* 23:371-378

Perez-Magariño, S. and M.L. Gonzalez-San José. 2004. Evolution of flavanols, anthocyanins, and their derivatives during the aging of red wines elaborated from grapes harvested at different stages of ripening. *J. Agric Food Chem.* 52:1181-1189

Peyrote des Gachons C. and J.A. Kennedy. 2003. Direct method for determining seed and skin proanthocyanidin extraction into red wine. *J. Agric Food Chem.* 51:5877-5881.

Pickering, G. J., D.A Heatherbell, L.P. Vanhanen and M.F. Barnes. 1998. The effect of ethanol concentration and the temporal perception of viscosity and density in white wine. *Am. J. Enol. Vitic.* 49:306-318

- Pirie, A. and M.G. Mullins. 1977. Interrelationships of sugars, anthocyanins, total phenols and dry weight in the skin of grape berries during ripening. *Am. J. Enol. Vitic.* 28:204-209.
- Poni, S., C. Intrieri and O. Silvestroni. 1994. Interactions of leaf age, fruiting, and exogenous cytokinins in Sangiovese grapevines under non-irrigated conditions. I. Gas exchange. *Am. J. Enol. Vitic.* 45:71-78.
- Possner, D., H.P. Ruffner and D.M. Rast. 1983. Regulation of malic acid metabolism in berries of *Vitis vinifera*. *Acta Hort.* 139:117-121.
- Pratt, C. 1971. Reproductive anatomy in cultivated grapes - A review. *Am. J. Enol. Vitic.* 22:92-109
- Prescot, J.A. 1965. The climatology of the vine (*Vitis vinifera* L.) the cool limits of cultivation. *Trans. Roy. Soc. S. Aust.* 89:5-23
- Price, S.F. 1994. Sun exposure and flavonols in grapes. Thesis for the degree of Doctor of Philosophy in Plant Physiology. Oregon State University.
- Price, S.F., P.J. Breen, M. Valladao and B.T. Watson. 1995. Cluster sun exposure and quercetin in Pinot noir grapes and wine. *Am. J. Enol. Vitic.* 46:187-194.
- Prieur, C., J. Rigaud, V. Cheynier and M. Moutounet. 1994. Oligomeric and polymeric procyanidins from grape seeds. *Phytochem.* 36:781-784.
- Puissant, A. and H. Leon. 1967. La matiere colorante des grains de raisins de certains cépages cultivés en Anjou en 1965. *Ann. Technol. Agr. Paris.* 16:217-225.
- Ribéreau-Gayon, P. 1982. The anthocyanins of grapes and wines. *In: Anthocyanins as food colors.* Markakis, P. Ed. Academic Press, Inc. New York.
- Ribéreau-Gayon, P., Y. Glories, A. Maujean and D. Dubourdieu. 2000A. Handbook of enology – Volume 1. The microbiology of wine and vinifications. Wiley LTD. New York
- Ribéreau-Gayon, P., Y. Glories, A. Maujean and D. Dubourdieu. 2000B. Handbook of enology – Volume 2. The chemistry of wine stabilization and treatments. Wiley LTD. New York
- Robichaud, J. L. and A. C. Noble. 1990. Astringency and bitterness of selected phenolics in wine. *J. Sci Food Agric.* 53:343-353.

- Romeyer, F. M., J.J. Macheix and J.C. Sapis. 1986. Changes and importance of oligomeric procyanidins during maturation of grape seeds. *Phytochem.* 25:219-221.
- Rossi, J.A. and V.L. Singleton. 1966. Flavor effects and adsorptive properties of purified fractions of grape seed phenols. *Am. J. Enol. Vitic.* 17:240-246
- Ruffner, H.P., S. Brem and U. Malipiero. 1983. The physiology of acid metabolism in grape berry ripening. *Acta Hort.* 139:123-128
- Santos-Buelga, C., E. M. Francia-Aricha and M.T. Escribano-Bailon. 1995. Comparative flavan-3-ol composition of seeds from different grape varieties. *Food Chem.* 53:197-201.
- Sarni-Manchado, P., V. Cheynier and M. Moutounet. 1999. Interactions of grape seed tannins with salivary proteins. *J. Agric. Food Chem.* 47:42-47
- Scienza, A., R., Miravalle, C. Visai and M. Fregoni. 1978. Relationships between seed number, gibberellin and abscisic acid levels and ripening in Cabernet Sauvignon grape berries. *Vitis.* 17:361-368.
- Scudamore-Smith, P.D., R.L. Hooper and E.D. McLaran. 1990. Color and phenolic changes of Cabernet Sauvignon wine made by simultaneous yeast/bacteria fermentation and extended pomace contact. *Am. J. Enol. Vitic.* 41:57-67
- Serafini, M., G. Maiani. A. Ferro-Luzzi. 1997. Effect of ethanol on red wine tannin-protein (BSA) Interactions. *J. Agric Food Chem.* 45: 3148-3151.
- Seymour, G.B. and K.C. Gross. 1996. Cell wall disassembly and fruit softening. *Postharv. News Inf.* 7:45-52
- Siebert, K.J. and A.W. Chassy. 2003. An alternate mechanism for the astringent sensation of acids. *Food Qual. Pref.* 15:12-18
- Silacci, M.W. and J.C. Morrison. 1990. Changes in pectin content of Cabernet Sauvignon grape berries during maturation. *Am. J. Enol. Vitic.* 41:111-115.
- Singleton, V.L., and P. Esau. 1969. Phenolic substances in grapes and wine, and their significance. Academic press. New York.
- Sinha, R.K. 2004. Modern plant physiology. Alpha Science International Ltd. England.
- Smith A.K., June H. Noble A.C. 1996. Effects of viscosity on the bitterness and astringency of seed grape tannins. *Food Qual. Pref.* 7:161-166

- Smith, A.K. and A.C. Noble. 1998. Effects of increased viscosity on the sourness and astringency of aluminium sulfate and citric acid. *Food Qual. Pref.* 9:139-144.
- Sorce, C., R. Lorenzi, N. Ceccarelli and P. Ranalli. 2000. Changes in free and conjugated IAA during dormancy and sprouting of potato tubers. *Aust. J. Plant Physiology.* 27:371-377.
- Souquet, J.M., V. Cheynier, F. Brossaud and M. Moutounet. 1996. Polymeric proanthocyanidins from grape skins. *Phytochem.* 43:509-512.
- Spayd, S.E., J.M. Tarara, D.L. Mee and J.C. Ferguson. 2002. Separation of sunlight and temperature effects on the composition of *Vitis vinifera* L. cv. Merlot berries. *Am. J. Enol. Vitic.* 53:171-182
- Stafford, H. A. and H.H. Lester. 1984. Flavan-3-ol Biosynthesis. *Plant Physiol.* 76:184-186.
- Strack, D. and V. Wray. 1993. The anthocyanins. *In: Advances in flavonoids since 1986.* Edited by J.B. Harborne. Chapman and Hall. London.
- Su, C.T. and V.L. Singleton. 1969. Identification of three flavan-3-ols from grapes. *Phytochem.* 8:1553-1558.
- Swain, T. and C.A. Williams. 1970. The role of phenylalanine in flavonoid biosynthesis. *Phytochem.* 9:2115-2122.
- Swanson, C.A. and E.D.H. El Shishiny. 1958. Translocation of sugars in the Concord grape. *Plant Physiology.* 33:33-37
- Taira, S. and M. Ono. 1997. Reduction of astringency in permixon caused by adhesion of tannins to cell wall fragments. *Acta Hort.* 436:235-241.
- Taiz, L. and E. Zeiger. 1991. *Plant physiology.* The Benjamin/Cummings Publishing Company, Inc. California.
- Terrier, N., F.X. Sauvage, A. Ageorges and C. Romieu. 2001. Changes in acidity and in proton transport at the tonoplast of grape berries during development. *Planta.* 213:20-28
- Thompson, J.E., R.L. Legge and R.F. Barber. 1987. The role of free radicals in senescence and wounding. *New Phytol.* 105:317-344

- Van Staden, J., J.C. Manning and K.M. Kelly. 1989. Legume seeds – the structure: function equation. In: CH Stirton and J.L. Zarucchi, eds, *Advances in Legume Biology. Monogr. Syst. Bot. Missouri Bot. Garden* 29:417-450
- Vidal, S., L. Francis, S. Guyot, N. Marnet, R. Gawel, V. Cheynier and E.J. Waters. 2002. The mouth-feel properties of grape and apple proanthocyanidins in a wine like medium. *J. Sci. Food Agric.* 83:564-573.
- Vidal, S., P. Courcoux, L. Francis, P. Williams, M. Kwiatkowski, R. Gawel, V. Cheynier, E. Waters. 2004A. The mouth-feel properties of polysaccharides and anthocyanins in a wine like medium. *Food Chem.* 85:519-525.
- Vidal, S., L. Francis, P. Williams, M. Ktiawkoski, R. Gawel, V. Cheynier and E. Waters. 2004B. Use of an experimental design approach for evaluation of key wine components on mouth-feel perception. *Food Qual. Pref.* 15:209-217.
- Weimberg, R., H.R. Lerner and A. Poljakoff-Mayber. 1983. Induction of solute release from *Nicotiana tabacum* tissue cell suspensions by polymyxin and EDTA. *J. Exp. Botany.* 34:1333-1346.
- Wicks, A.S. and W.M. Kliever. 1983. Further investigations into the relationship between anthocyanins, phenolics and soluble carbohydrates in grape berry skins. *Am. J. Enol. Vitic.* 34:114-116.
- Williams, P.J. 1997. Structures, rates of formation and sensory properties of red wine pigmented tannins and the influences of viticultural practices on these tannins. Australian Society of Viticulture and Oenology Seminar: *Phenolics and extraction*. Proceeding of a seminar held on 9 October 1997 in Adelaide
- Williams, V. M., L.J. Porter and R.W. Hemingway. 1983. Molecular weight profiles of proanthocyanidin polymers. *Phytochem.* 22:569-572.
- Winkel-Shirley, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology and biotechnology. *Plant Physiol.* 126:485-493.
- Winkler, A. J., J.A. Cook, W. M. Kliever, and L.A. Lider. 1974. General viticulture. University of California Press, Berkeley, California.
- Wollan, D. 1997. Tannin extraction and manipulation –Managing the options. Australian Society of Viticulture and Oenology Seminar: *Phenolics and extraction*. Proceeding of a seminar held on 9 October 1997 in Adelaide.
- [www.chem.qmul.ac.uk/iubmb/enzyme/reaction/phenol/flavonoid.html](http://www.chem.qmul.ac.uk/iubmb/enzyme/reaction/phenol/flavonoid.html)

[www.honeybee.helsinki.fi/MMSBL/Gerberalab/anthocyanin\\_path.html](http://www.honeybee.helsinki.fi/MMSBL/Gerberalab/anthocyanin_path.html)

Yokotsuka, K., M. Sato, N. Ueno and V. L. Singleton. 1999. Colour and sensory characteristics of Merlot red wines caused by prolonged pomace contact. *J. Wine Research*. 11:7-18

Zelleke, A. and M.W. Kliewer. 1979. Influence of root temperature and rootstock on bud break, shoot growth, and fruit composition of Cabernet sauvignon grapevines grown under controlled conditions. *Am. J. Enol. Vitic.* 30:312-317.

Zitzlaff, M. 1989. The effect of pomace contact on wine composition and quality of Cabernet Sauvignon. *The Australian Grapegrower & Winemaker*. April. 38-43.

Zoecklein, B. 2002. Red wine phenolic management. In *Enology notes number 61*, [www.fst.vt.edu/extension/enology/contentextenologynotes61.htm](http://www.fst.vt.edu/extension/enology/contentextenologynotes61.htm).