Biosynthesis of Flavonoids in Grapevines (*Vitis vinifera* L.)

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Mark Downey

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University of Adelaide

Department of Horticulture, Viticulture and Oenology
Abstract

Flavonoids in grapes contribute to wine quality and to the health benefits derived from grapes and wine. This project sought to examine the spatial and temporal distribution, as well as the composition, of the flavan-3-ols, proanthocyanidins and flavonols in winegrapes throughout berry development. In addition, we sought to explore how modifying bunch exposure to light would impact upon flavonoid biosynthesis in the grape berry by comparing the flavonoid composition of shaded and exposed grapes. The expression of key genes in the flavonoid biosynthetic pathway was also investigated to understand how the pathway is regulated during berry development.

The flavan-3-ol and proanthocyanidin composition of both seeds and skin of grapes was determined by reversed-phase HPLC after acetone extraction and acid-catalysis in the presence of excess phloroglucinol. The main period of proanthocyanidin accumulation in grape seeds occurred immediately after fruit-set with maximum levels observed around veraison. Over two seasons there was variation in both the timing and content of proanthocyanidins in seeds. In skin, proanthocyanidin accumulation occurred from fruit set until 1-2 weeks after veraison. Proanthocyanidin subunit composition was different in seeds and skin and changed during berry development. The mean degree of polymerisation of the tannin polymers in skins was higher than in the seeds at all stages of berry development. Proanthocyanidin levels in both seeds and skin decreased between veraison and harvest. Additional proanthocyanidin subunits were released when the residues remaining after acetone extraction were subjected to direct acid-catalysis in the presence of phloroglucinol. In the seeds, these accounted for much of the post-veraison decrease, but not in grape skin. At harvest, 75% of extractable berry proanthocyanidin...
was in the seeds. Accumulation of proanthocyanidins in the seeds appears to be independent of that in the skins but in both tissues, synthesis occurs early in berry development and maximum levels are reached around veraison.

Flavonols were identified in the skin of developing berries, buds, tendrils, inflorescences, anthers and leaves of the grapevine. The dominant flavonols were quercetin glucosides with trace amounts of kaempferol glucosides detected in Shiraz flowers but not in developing berries. Flavonols were high in flowers and decreased toward berry-set then remained relatively constant through berry development. Total flavonols per berry were low until pre-veraison then increased during berry development, particularly before ripening, in Chardonnay and during ripening in Shiraz. Two genes encoding the enzyme flavonol synthase (FLS) were isolated from Shiraz flowers. The first of these, \( VvFLSI \), was expressed in leaves, tendrils, pedicels, buds and inflorescences as well as in developing grapes. Expression was highest between flowering and fruit set then declined, increasing again during ripening coincident with the increase in flavonols. Expression of \( VvFLS2 \) was much lower than for \( VvFLSI \) and did not change during berry development.

The results indicate that two distinct periods of flavonol synthesis occur in grapes, the first around flowering and the second during ripening of the developing berries.

To explore the influence of bunch exposure on grape, and thereby wine, quality a shading treatment comprising opaque boxes was applied to bunches of Shiraz grapes prior to flowering. The boxes were designed to maintain airflow while excluding light, to minimise changes in temperature and humidity. The effects of light exclusion on berry development, flavonoid accumulation and the expression of genes involved in flavonoid biosynthesis were assessed throughout berry development. There was no significant effect of shading on sugar accumulation and in two of the three seasons studied there was
no effect on berry weight. Chlorophyll concentration was much lower in the shaded fruit, which appeared pale yellow until veraison. The fruit coloured normally in the shaded bunches and in two of the three seasons there was no significant change in anthocyanin content. Expression of the gene encoding UDP-glucose flavonoid-3-O-glucosyl transferase (UFGT), a key gene in anthocyanin biosynthesis, increased after veraison and was similar in both shaded and exposed fruit. Anthocyanin composition was altered in the shaded fruit, which had a greater proportion of the dihydroxylated anthocyanins, the glycosides of cyanidin and peonidin. Shading had no significant effect on the levels of condensed tannins in the skin or seeds of ripe fruit. Shading significantly reduced the levels of flavonols in the grape skin. In the exposed fruit, flavonol concentration was highest around flowering then declined as the berries grew, but there was an increase in flavonols per berry during ripening. When the boxes were applied before flowering, shaded fruit had much lower levels of flavonols throughout berry development and at harvest the level of flavonols were less than 10% of that in exposed fruit. A gene encoding flavonol synthase (FLS) was expressed at flowering and during ripening in exposed grapes but its expression was greatly reduced in shaded fruit. The results indicate that shading had little effect on berry development and ripening, including accumulation of anthocyanins and tannins, but significantly decreased flavonol synthesis.

This research has contributed to our knowledge of grapevine biochemistry and physiology, particularly in relation to the biosynthesis of flavonoids. The pattern of accumulation of flavonoids has been determined in the seeds, skin and flesh of grapes during berry development. These investigations have included study of both the content and composition of the flavonoids, condensed tannins and anthocyanins during berry development. The results indicate that while anthocyanins, tannins and flavonols are all products of the flavonoid pathway, they are synthesised at different times during
development and in different tissues of the berry. These observations are consistent with
the pattern of flavonoid pathway gene expression reported here and elsewhere and
indicate that viticultural management to improve fruit quality needs to consider the
different timing of synthesis of these important compounds.

This research has also revealed much about the influence of bunch exposure on flavonoid
metabolism in grape berries. While there were significant yet subtle shifts in the
composition of anthocyanins and proanthocyanidins, only the synthesis of flavonols was
shown to be light dependent. Whether flavonoid accumulation or composition can be
effectively managed through viticultural practices, to what extent they can be modified or
manipulated, and how such changes might impact upon winemaking are all fields ripe for
future investigation.

Keywords: Grapevine, Vitis Vinifera L., Shiraz, Chardonnay, flavonoid, anthocyanin,
condensed tannin, proanthocyanidin, flavan-3-ol, flavan-3,4-diol, flavonol, gene
expression, flavonol synthase, BANYULS, LAR, chlorophyll, phloroglucinol, acid
catalysis, berry development, seeds, skin, leaves light, shading
Author’s note. Much of the research presented here, primarily Chapters 3, 4 and 5 has been published in the following papers:


Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge, no material presented here has been written or published by another person, other than where due reference has been made in the text.

I consent to the loan and/or photocopying of this thesis upon lodgement with the University of Adelaide Library.

SIGNED: .....                     DATE: 01 Feb 2004

Mark Downey
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Chapter 1. General Introduction

An ancient association exists between man and the grapevine and the wine produced from its fruit. This association has, at times, taken on religious and cultural significance, the wine variously worshipped and vilified, considered an elixir and a poison. As much today as ever, these views persist and while excess is indubitably incompatible with healthy living, there is accumulating evidence of nutritional and medicinal benefit associated with moderate consumption of wine. Much of this benefit, and a significant component of wine quality, is attributable to a class of plant secondary metabolites known as flavonoids. Although extensively studied in relation to their role as pigments in wine, the biosynthesis of flavonoids in the grapevine has, until recently, attracted little interest.

1.1. The Grapevine

The production of winegrapes, table grapes and raisins is based primarily on cultivars of the species *Vitis vinifera* of the family Vitaceae (Antcliff, 1988; Mullins *et al*., 1992). The Vitaceae are mainly woody shrubs, predominantly intertropical in distribution and characterised by pentamous flowers and the occurrence of tendrils and inflorescences opposite leaves (Mullins *et al*., 1992).

There are approximately 10,000 known cultivars of *Vitis vinifera* classified into three main groups, or proles: *Proles pontica*, *Proles orientalis* and *Proles occidentalis* (Negrul, 1936; Galet, 1979; Mullins *et al*., 1992; Bourquin *et al*., 1993). *Proles pontica* comprises the oldest cultivars, which arose around the Black Sea (Negrul, 1936; Antcliff, 1988; Mullins *et al*., 1992). *Proles orientalis* arose around the Caspian Sea and gave rise to Muscat wine grapes and many table grapes (Mullins *et al*., 1992). Neither *Proles pontica*
nor *orientalis* are suited to the cool climates of Northern Europe and the better-adapted *Proles occidentalis* were cultivated in these areas. Cultivars of *Proles occidentalis* now comprise most of the better-known wine grape varieties (Galet, 1979; Antcliff, 1988; Mullins *et al.*, 1992).

### 1.2. Grapegrowing and Winemaking

The domestication of the grapevine began around 5,000 BC along the shores of the Black Sea. Cultivation spread rapidly throughout the Middle East, Europe and North Africa, finding a prominent role in Greek, Roman and Egyptian civilisations (Hyams, 1965; Mullins *et al.*, 1992). The advent of winemaking was likely a serendipitous event resulting from the fermentation of stored grapes. The fruit of the grapevine being an ideal substrate for fermentation and the surface of the berry and bunch morphology provide a favourable habitat for yeasts (Hyams, 1965; Mullins *et al.*, 1992).

Briefly, the winemaking process consists of the extraction of juice from the fruit at various levels of maturity determined by the style of wine to be made and the fermentation of that juice with a cultured yeast (Berry, 1987; Ough, 1992; Boulton *et al.*, 1998; Rankine, 1998). Both the extraction and fermentation techniques are hugely variable, as are the varieties of grape and strains of yeast, all of which contribute to a diverse range of wine style and quality (Boulton *et al.*, 1996; Rankine, 1998). Wine quality is principally determined by the composition of the grapes in respect of colour, acid, sugar and phenolic content and the management of these parameters throughout vinification and maturation (Winkler *et al.*, 1974; Timberlake & Bridle, 1976; Haslam, 1980; Somers & Verette, 1988; Boulton *et al.*, 1996; Saint-Cricq de Gaulejac *et al.*, 1998).
1.2.1. The Wine Industry

International wine production is around 30 billion litres annually, 75% of which is produced in Europe and the former Soviet Union (Mullins et al., 1992; Australian Bureau of Statistics, 2001). Australia generates only 3% of world production, with almost half of Australian wine exported, a figure that has steadily grown since the mid-1980s when Australia was a net importer of wine (Australian Bureau of Statistics, 2001). The greatest export market has traditionally been the U.K. and this market still accounts for 50% of Australian wine exports (Australian Bureau of Statistics, 2001). The continued success of the Australian wine industry is dependent upon ongoing production of quality, rather than quantity wines (Osmond & Anderson, 1998).

1.2.2. The Health Benefits of Wine

Much of the recent literature on wine concerns the antioxidant properties of phenolic components and their role in reducing mortality from free radical associated diseases such as coronary heart disease, atherosclerosis, stroke and some cancers (St. Leger et al., 1979; Armand et al., 1988; Nagabhushan et al., 1988; Renaud & de Lorgeril, 1992; Artaud-Wild et al., 1993; Hertog & Hollman, 1996; Criqui & Ringel, 1994; McDonald et al., 1998). Numerous compounds of the broad general class of secondary metabolites known as flavonoids have been identified as antioxidants in wine. Prominent among these are the anthocyanins, flavonols and flavan-3-ols, as well as polymeric flavonoids (Bors & Saran, 1987; Pace-Asciak et al., 1995; Sato et al., 1996; Yamasaki et al., 1996; Baldi et al., 1997; Fauconneau et al., 1997; Plumb et al., 1998; Saint-Cricq de Gaulejac, 1999; Bors et al., 2001).
1.3. The Flavonoids

The flavonoids are a group of compounds based on the polyphenolic flavan skeleton (Figure 1.1), variously substituted with hydroxyl, methyl, galloyl, glucosyl and acyl moieties. In addition, flavonoids can form complexes with other flavonoids, metal ions and numerous other molecules (Asen et al., 1972; Brouillard et al., 1989; Strack & Wray, 1993; Haslam, 1998). Flavonoids also form oligomers and extensive polymers under a range of biological and chemical conditions (Harborne, 1967; Delcour et al., 1983; Haslam, 1998).

There are more than 4000 known flavonoids of diverse physiological function (Stafford, 1991; Koes et al., 1994; Shirley, 1996). Flavonoids occur in all vascular plants and in most mosses, but have not been detected in algae or bacteria and are only rarely found in fungi (Markham, 1982; Stafford, 1991; Weiss et al., 1999). Historically, flavonoids, particularly anthocyanins, have been extensively studied in their role as floral pigments and various colour mutants have aided the elucidation of the biosynthetic pathway (Harborne, 1958; Harborne, 1967; Stafford, 1990). Studies of these compounds in numerous species, including grapes, have been instrumental to our understanding of the genetic basis of pigmentation (Bateson, 1901; Lawrence, 1950; Barritt & Einset, 1969; Sparvoli et al., 1994; Holton & Cornish, 1995; Boss et al., 1996a; Boss et al. 1996b). The chromophoric nature of the flavonoid ring structure results in the absorption of light in
both the ultraviolet and visible spectra (Markham, 1982). Absorbing ultraviolet light protects plants from UV irradiation and this has been proposed as the archetypal role of flavonoid compounds (Koes et al., 1994; Shirley, 1996; Smith & Markham, 1998). Flavonoids that absorb both UV and visible light, including pigments such as aurones, chalcones and anthocyanins, also act as attractants for pollination and seed dispersal (Saito & Harborne, 1992; Koes et al., 1994; Shirley, 1996). Other physiological roles attributed to this diverse class of compounds include facilitating conditional male fertility in pollen (Ylstra et al., 1992; Taylor, 1995) and the establishment of symbioses with nitrogen fixing bacteria in the rhizosphere (Djordjevic et al., 1987; Clarke et al., 1992; Recourt et al., 1992; Koes et al., 1994; Shirley, 1996). Flavonoids also have demonstrated roles as deterrents to herbivory through the bitter and astringent nature of the flavan-3-ols and condensed tannins (Horowitz, 1964; Shaver & Lukefair, 1969; Feeny, 1976; Elliger et al., 1980; Dreyer et al., 1981; Nef, 1988; Wagner, 1988; Mole, 1989; Elmqvist et al., 1991; Harborne & Grayer, 1993; Lu & Bennick, 1998). In addition, many flavonoids and isoflavonoids have phytoalexin activity with roles in plant defence against fungal and bacterial pathogens (Skipp & Bailey, 1977; Gnanamanickam & Smith, 1980; Smith, 1982; Dixon & Lamb, 1990; Dakora et al., 1993).

In contrast, the absence of flavonoids, particularly tannins, from pasture crops can result in the formation of protein foam during rumen fermentation leading to pasture bloat and death. The presence of dietary flavan-3-ols and proanthocyanidins precipitate the protein preventing foam formation and the onset of bloat (Tanner et al., 1995; Li et al., 1996; Bavage et al., 1997). The precipitation of protein by condensed tannin has been used to quantify the tannin content of tissues (Hagerman & Butler, 1980; Harbertson et al., 2002). Further, it is this ability to complex with proteins that led to their use in the
tanning of hides and gave this class of flavonoid compounds their generic name, tannins (Haslam, 1989). The mechanism by which tannins prevent pasture bloat and tan leather is not dissimilar to the interactions of tannins in wine with human salivary proteins and polysaccharides creating the mouthfeel that tannins contribute to red wine (Thorngate, 1997; Gawel, 1998; Lu & Bennick, 1998).

1.3.1. Flavonoids in Wine

1.3.1.1. Anthocyanins

Much of the visual character and taste of wine is due to the presence of flavonoid compounds derived from the seeds and skin of the grapes (Ribereau-Gayon, 1974; Glories, 1988). The classes of flavonoids that contribute significantly to wine are the anthocyanin pigments, the tannins and related monomeric flavan-3-ols, and to a lesser extent, the flavonols. Anthocyanins, extracted from the grape skin during crushing and fermentation, are fundamentally responsible for all of the colour observed in young red wines (Ribereau-Gayon & Glories, 1986; Mazza & Miniati, 1993; Mazza, 1995). Free anthocyanins can contribute as much as 99% of the colour of young red wines, with 30-50% of these anthocyanins held in copigment associations (Somers & Evans, 1977; Somers, 1978; Revilla et al., 1999; Boulton, 2001). However, much of this initial colour is lost during fermentation due to the inherent instability of the anthocyanin molecule (Somers & Evans, 1977; Somers & Evans, 1979; Somers & Verette, 1988). Around half of the anthocyanins originally present in the red wine are converted into polymeric pigments during the first year of storage (McCloskey & Yengoyan, 1981; Somers & Verette, 1988; Cheynier et al., 1997). As red wines age, pigment composition becomes progressively more complex, with the colour of old wines being predominantly due to
polymeric pigments formed through complex associations between anthocyanins and tannins (Somers, 1971; Timberlake & Bridle, 1976; Nagel & Wulf, 1979; McCloskey & Yengoyan, 1981; Bakker et al., 1986b; Somers & Verette, 1988; Brouillard et al., 1997).

1.3.1.2. Tannins

Tannins are an important component of gustatory impact of wine with flavan-3-ols and proanthocyanidin oligomers and polymers contributing to the body and mouthfeel of the wine (Glories, 1988; Thorngate, 1997; Gawel et al., 2000). Astringency is a feature of young wines and is usually correlated with flavan-3-ol content (Goldstein & Swain, 1963; Fulcrand et al., 1996; Czochanska et al., 1979). Many polyphenolic compounds, including the flavan-3-ols and related dimers and oligomers, have a harsh astringent taste and produce in the palate a feeling of roughness, dryness and constriction caused by the precipitation of proteins and mucopolysaccharides in the saliva (Somers, 1971; Glories, 1988; Jackson & Lombard, 1993; Thorngate, 1997; Haslam, 1998). The flavan-3-ols also have a bitter sensory character and a significant difference in perception exists between different monomers, with epicatechin perceived as more bitter for a longer duration than catechin (Noble, 1994; Thorngate, 1997). In addition, the presence of ethanol enhances bitterness intensity and duration (Noble, 1994). The bitterness and astringency of flavonoids decreases as the molecular weight increases with polymerisation (Noble, 1994; Brossaud et al., 2001; Vidal et al., 2002). The loss of astringency occurs with the formation of large polymeric compounds through the oxidative cross-linking of proanthocyanidin oligomers (Wildenradt & Singleton, 1974; Timberlake & Bridle, 1976; Somers & Wescombe, 1987; Fulcrand et al., 1996; Thorngate, 1997; Haslam, 1998). These large molecules are insoluble and tend to precipitate from the wine. However, the formation of tannin complexes with anthocyanins increases the solubility of large
molecular weight polymers (Cheynier et al., 1997). The tannins readily identified in red wines include the flavan-3-ols catechin, epicatechin and epicatechin-gallate, as well a variety of dimers, oligomers and extensive heterogeneous polymers (Salagoity-Auguste & Bertrand, 1984; Singleton, 1988; Fulcrand et al., 1999).

1.3.1.3. Flavonols

In addition to anthocyanins and tannins, glycosides of the flavonols myricetin, quercetin and kaempferol are commonly found in wines. While tannins and anthocyanins in wines tend to form large polymeric compounds during vinification and maturation, flavonol glycosides are hydrolysed by acidic conditions and consequently, the flavonol aglycones are also commonly found in wines (Somers & Verette, 1988; Blanco et al., 1998; Goldberg et al., 1998; McDonald et al., 1998).

The phenolic profile of white wines is dominated by gallic acid and the hydroxycinnamates, caftaric acid and coumaric acid (Ong & Nagel, 1978a; Singleton et al., 1978). There are virtually no flavan-3-ols or tannins in white wine due largely to the method of winemaking in which skins and seeds are removed after crushing (Singleton & Trousdale, 1983; Romeyer et al., 1985; Somers et al., 1987; Somers & Verette, 1988). Flavonols in white wines may be derived from the skin during pressing, or the result of contamination with leaf material. Flavonol glycosides and their breakdown products produced during winemaking have low solubility in aqueous solution and can cause a hazing effect in white wine (Somers & Ziemelis, 1985).

While the conditions of winemaking and aging significantly effect the final concentration of flavonoids in wine, it is the amount and composition of flavonoids in the fruit at harvest that will largely determine the flavonoid profile of the mature wine. Around 65%
of the phenolics in wine come from the skins and the remaining 35% from the seeds. However, much higher levels of phenolic compounds are reported in grape seeds reflecting the lower extractability of phenolic compounds from seeds, which is thought to be due to lignification of the seed (Singleton & Draper, 1964; Meyer & Hernandez, 1970; Ribereau-Gayon & Glories, 1986; Singleton, 1988; Thorngate & Singleton, 1994; Revilla et al., 1997).

1.3.2. Flavonoids in Grapevines

1.3.2.1. Anthocyanins

Anthocyanins are probably the most studied phenolic component of grapes because of their contribution to the colour of red wines (Mazza & Miniati, 1993; Mazza, 1995). Anthocyanins occur in the vacuoles of dedicated cells in the epidermal layers of the skin of red grapes and in the pulp of red flesched varieties (Mueller & Greenwood, 1978; Pecket & Small, 1980; Moskowitz & Hrazdina, 1981; Sparvoli et al., 1994). The anthocyanins in grapes are the \(-3-O\text{-glucosides}, -3-O\text{-acetylglucosides and }-3-O-p\text{-coumaroylglucosides of cyanidin, petunidin, peonidin, delphinidin and malvidin (Figure 1.2). Malvidin is the major anthocyanin in red grapes, with malvidin glucosides accounting for around 60% of total pigment (Crippen & Morrison, 1986b; Mazza & Miniati, 1993b; Mazza, 1995). Anthocyanin pigments are also observed in the young vine leaves of several varieties but not generally in mature leaves (Pirie & Mullins, 1976; Darne, 1993; Darne, 1996; Ezzili et al., 1999). White grapes do not produce anthocyanins because of mutations that result in their failure to express the final steps in anthocyanin synthesis (Boss et al., 1996b).
Figure 1.2. Anthocyanins in *Vitis vinifera* Shiraz are based on the anthocyanidins cyanidin, peonidin, delphinidin, petunidin and malvidin occurring in three glycosidic forms, the glucoside, acetylglucoside and coumaroylglucoside, shown here as the glycosides of malvidin.

1.3.2.2. Flavonols

Several studies have been made of flavonols in grapes focussing on the induction of flavonols by UV light and exploring the apparent correlation between this phenomenon and the quality of grapes and subsequent wines (Morrison & Noble, 1990; Price et al., 1995). The glucosides of quercetin (Figure 1.3) are the dominant flavonols in grapes, although glycosides of myricetin and kaempferol have also been reported, albeit at significantly lower levels (Cheynier & Rigaud, 1986; Spanos & Wrolstad, 1990; Price et al., 1995). Flavonols occur in the upper epidermis of the grape berry, consistent with a role in UV screening and have not been reported in the seeds or flesh of the berries (Flint...
Flavonols found in Shiraz are based on the aglycones kaempferol, quercetin and myricetin. The most prevalent flavonols being quercetin glucoside and quercetin glucuronide.

et al., 1985; Beggs et al., 1987; Somers & Verette, 1988; Stafford, 1990; Haselgrove, 1997). Glucosides of kaempferol, quercetin and myricetin also occur in the leaves of *Vitis* spp., at levels considerably greater than those in the fruit (Somers & Ziemelis, 1985; Darne, 1996; Hmamouchi et al., 1996). Stems of grapevines have also been reported to contain flavonols, primarily glucosides of quercetin (Souquet et al., 2000).

1.3.2.3. Tannins

The flavan-3-ol content of grapes has also been extensively studied due to the contribution of the related tannins to wine quality and more recently because of their nutraceutical potential. In the seeds and skin of grapes, the flavan-3-ols catechin, epicatechin and epicatechin-gallate are the common monomers (see Figure 1.4) with over twenty dimeric and trimeric proanthocyanidins also identified (Lea et al., 1979; Romeyer et al., 1986; Escribano-Bailon et al., 1992; Prieur et al., 1994; Santos-Buelga et al., 1995;
Of the oligomers, only the B-type proanthocyanidins are present in grapes (Figure 1.5). These mainly have 4β-8 linkages with small amounts of dimers and trimers containing 4β-6 linkages (Escribano-Bailon et al., 1992; Cheynier et al., 1997; Fuleki & Ricardo da Silva, 1997).

In the grape seeds, flavan-3-ols and proanthocyanidins are found in the seedcoat. The levels of catechin and epicatechin are reputedly similar with lesser amounts of epicatechin-gallate. In addition, all of the B-type dimers (Figure 1.5), and the C2 trimer have been reported in grape seeds at varying levels (Lea et al., 1979; Romeyer et al., 1986; Prieur et al., 1994; Thorngate & Singleton, 1994; Santos-Buelga et al., 1995; Cheynier et al., 1997; Katalinic & Males, 1997; Saint-Cricq de Gaulejac et al., 1997; de Freitas & Glories, 1999).

The dominant stereochemistry of grape seed oligomers and polymers is epicatechin with the ratio epicatechin:catechin increasing from 2:1 to 12:1 with increasing polymerisation with catechin relatively more abundant as the terminal subunit unit (Prieur et al., 1994; Souquet et al., 1996a; Cheynier et al., 1997).

The difference between seed and skin tannins is primarily the presence of the trihydroxylated prodelphinidins in the skin and a higher degree of polymerisation (Foo & Porter, 1980; Souquet et al., 1996a; Cheynier et al., 1997). The dominant flavan-3-ol monomer in grape skin is catechin (Escribano-Bailon et al., 1995) with the trihydroxylated products, gallatechin, epigallocatechin and epigallocatechin-gallate.
detected in addition to catechin, epicatechin and epicatechin-gallate (Foo & Porter, 1980; Foo & Porter, 1983; Souquet et al., 1996a; Haslam, 1998). In skins, only the dimers B1 to B4 are commonly reported, although trace amounts of the B5 dimer, which is one of the most prevalent in the seed, have also been reported (Santos-Buelga et al., 1995; Souquet et al., 1996a; Saint-Cricq de Gaulejac et al., 1997; de Freitas & Glories, 1999). In addition, proanthocyanidins in grape skin are reported to form complex associations with other cellular components, including cell wall polysaccharides, lignins and proteins (Amrani-Joutei et al., 1994; Escribano-Bailon et al., 1995; Cheynier et al., 1997; Saint-Cricq de Gaulejac et al., 1997).

Flavan-3-ols are not detected in the pulp of either red or white grape berries, although they have been reported in the vascular traces of the fruit (Lea et al., 1979; Lee & Jaworski, 1989; Ricardo da Silva et al., 1991a; Souquet et al., 1996a). Flavan-3-ols have also been detected in the stems of the grape bunch, with the composition of stems similar

![Diagram of proanthocyanidin dimers](image)

Figure 1.5. Proanthocyanidin dimers occurring in *Vitis vinifera* Shiraz. All dimers shown have been detected in grape seeds, while only dimers B1-B4 with traces of B5 are reported in grape skin.
to that of the fruit (Ricardo da Silva et al., 1991b). Tannins have also been observed in the leaves of *Vitis* spp. (Darne, 1993). The tannin composition of the seeds and skin of white grapes is similar to that of red grapes, although there is considerable variation in the relative proportions between varieties (Lea et al., 1979; Romeyer et al., 1986; Ricardo da Silva et al., 1991b; Amrani-Joutei et al., 1994; Cheynier et al., 1997).

### 1.4. Biosynthesis of Flavonoids

Flavonoid biosynthesis is the culmination of two metabolic pathways, the shikimate pathway and the phenylpropanoid pathway. The phenylpropanoid pathway synthesises flavonoids from carboxylated acetyl-CoA (malonyl-CoA) and the amino acid phenylalanine, which is produced in the shikimate pathway (Dewick & Haslam, 1969; Heller & Forkmann, 1993). Under normal growth conditions, about 20% of the carbon fixed by plants flows through the shikimate pathway and about 2% of all carbon flows from the shikimate pathway into phenylpropanoid metabolism (Markham, 1982; Herrmann, 1995).

The phenylpropanoid pathway is generally considered to culminate in anthocyanin synthesis (Figure 1.6). Along the way precursors for a number of other products, are formed including lignin, lignan, aurones, flavones, isoflavonoids, flavonols and flavan-3-ols (Harborne, 1967; Gerats & Martin, 1992; Haslam, 1998). Much of the biosynthetic pathway of the flavonoids has been elucidated through characterisation of colour mutants and genes encoding many enzymes in the pathway have been cloned (Forkmann, 1993; Helariutta et al., 1993; Holton et al., 1993a; Diallinas & Kanellis, 1994; Sparvoli et al., 1994; Charrier et al., 1995; Tanaka et al., 1996; Gong et al., 1997; Tanner et al., 2003; Xie et al., 2003).
Figure 1.6. The general phenylpropanoid pathway involved in the biosynthesis of flavonoids. PAL – phenylalanine ammonia lyase; C4H – cinnamate-4-hydroxylase; C3H – p-coumarate-3-hydroxylase; 4CL – 4-coumarate CoA ligase; CCoA3H – p-coumaroyl-CoA-3-hydroxylase; SiSy – stilbene synthase; CHS – chalcone synthase; CHR – chalcone reductase (in concert with CHS); CHI – chalcone isomerase; F3H – flavanone-3β-hydroxylase; F3′H – flavonoid-3′-hydroxylase; F3′5′H – flavonoid-3′,5′-hydroxylase; FLS – flavonol synthase; DFR – dihydroflavonol reductase; LAR – leucoanthocyanidin reductase; LDOX – leucoanthocyanidin dioxygenase (a.k.a. ANS – anthocyanidin synthase); ANR – anthocyanidin reductase; UFGT – UDP glucose: flavonoid-3-O-glucosyltransferase; IFS – isoflavone synthase; IOMT – isoflavone-4′-O-methyltransferase; IFOH – isoflavone-2′-hydroxylase; IFR – isoflavone reductase; PTS – pterocarpan synthase.
1.4.1. Flavonoid Biosynthesis in Grapevines

1.4.1.1. Anthocyanins

Flavonoid biosynthesis in grapevines has to date only been studied in the skin of the berry and has primarily focused on anthocyanin biosynthesis (Boss et al., 1996a, Ford et al., 1998). In red grapes, anthocyanin accumulation commences with veraison, the onset of ripening (Somers, 1976; Mullins et al., 1992; Boss et al., 1996a). Initially, only glucosides of the dihydroxylated anthocyanins, cyanidin and peonidin accumulate, followed by the trihydroxylated anthocyanins delphinidin, petunidin and malvidin (Mazza & Miniati, 1993b; Boss et al., 1996b; Katalinic & Males, 1997; Keller & Hrazdina, 1998). The accumulation of anthocyanins in the skin of red grapes coincides with expression of the gene encoding the final step in anthocyanin biosynthesis, UFGT (Boss et al., 1996a). In white grapes, there are no anthocyanins present in the skin and the gene encoding UFGT is not expressed (Boss et al., 1996b).

While genes encoding many flavonoid biosynthetic enzymes have been cloned, it is relatively rare for all of them to be cloned from the same species (Helariutta et al., 1993; Charrier et al., 1995; Liew et al., 1995; Pelletier & Shirley, 1996; Fukada-Tanaka et al., 1997; Gong et al., 1997; Rosati et al., 1999; Saito et al., 1999). Fortunately, Vitis vinifera is one such species with phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3β-hydroxylase (F3H), dihydroflavonol reductase (DFR), leucoanthocyanidin dioxygenase (LDOX a.k.a. ANS – anthocyanidin synthase) and UDP glucose: flavonoid-3- O-glucosyltransferase (UFGT) having been cloned (Sparvoli et al., 1994). The expression studies during berry development showed all of these genes except UFGT to be expressed from flowering until just prior to veraison.
when transcript levels decreased. At veraison, expression of these genes plus UFGT was observed (Boss et al., 1996a). The enigma of these results is that the pre-veraison expression of most of the flavonoid pathway genes occurs in the absence of anthocyanin synthesis. The accumulation of other flavonoids such as the tannins and flavonols could explain the early expression of genes in this pathway.

**1.4.1.2. Tannins**

Tannins appear to accumulate early in berry development reaching a peak around veraison and then decreasing towards harvest, with similar patterns of accumulation reported in both the seeds and skin of the grape berry (Somers, 1976; Czochanska et al., 1979; Darne, 1982; Romeyer et al., 1986; Lee & Jaworski, 1989; Katalinic & Males, 1997; de Freitas & Glories, 1999; Kennedy et al., 2001). Recent work in this area suggests the peak in seed tannin accumulation may actually occur several weeks prior to veraison (Kennedy et al., 2000b). Grape tannins are primarily polymers comprised of flavan-3-ol terminal subunits and flavan-3,4-diol extension subunits (Figure 1.7). Prior to veraison the composition of these polymers is primarily the dihydroxylated subunits catechin and epicatechin, whereas post-veraison the trihydroxylated epigallocatechin is also detected in the grape skin (Czochanska et al., 1979; Katalinic & Males, 1997; Foo & Porter, 1980).

The enzymatic step involved in the biosynthesis of flavan-3-ols appears to be catalysed by two enzymes accounting for the 2,3-trans and 2,3-cis stereochemistry of the products catechin and epicatechin (Figure 1.4). The first of the two enzymes involved in flavan-3-ol biosynthesis is leucoanthocyanidin reductase (LAR, see Figure 1.6), which converts the leucoanthocyanidins to the corresponding flavan-3-ol with 2,3-trans stereochemistry, for example leucocyanidin to catechin (Stafford & Lester, 1985; Kristiansen, 1986;
The second enzyme, anthocyanidin reductase (ANR, see Figure 1.6), converts the anthocyanidins to their respective 2,3-cis flavan-3-ols, for example cyanidin to epicatechin (Albert et al., 1997; Devic et al., 1999; Xie et al., 2003).

The mechanism of tannin polymer formation is uncertain. While several enzymatic mechanisms have been postulated there is little convincing evidence to support them (Haslam, 1998). In contrast, extensive investigations in wine and model solutions have

![Diagram of flavan-3-ol stereochemistry](image)

<table>
<thead>
<tr>
<th>Flavan-3-ol</th>
<th>2-3 Stereochemistry</th>
<th>R₁</th>
<th>R₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>trans</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>cis</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Epicatechin-gallate</td>
<td>cis</td>
<td>H</td>
<td>Gallic Acid</td>
</tr>
<tr>
<td>Epigallocatechin</td>
<td>cis</td>
<td>OH</td>
<td>H</td>
</tr>
</tbody>
</table>

Figure 1.7. General proanthocyanidin structure showing terminal flavan-3-ol subunits, flavan-3,4-diol extension subunits and interflavan bond linking polymeric subunits, as well as carbon numbering of the flavan skeleton and nomenclature of monomers.
demonstrated several non-enzyme catalysed mechanisms for polymer formation (lurd, 1972; Delcour et al., 1983; Bakker & Timberlake, 1997; Saucier et al., 1997; Mateus et al., 2002b; Vidal et al., 2002).

1.4.1.3. Flavonols

The pattern of flavonol accumulation in grape berries has been little studied to date (Haselgrove, 1997). Flavonol accumulation appears to occur prior to veraison as the level reported at veraison was the maximum reported between that time and harvest (Haselgrove et al., 2000). Biosynthesis of flavonols from dihydroflavonols (see Figure 1.6) is catalysed by flavonol synthase (FLS) (Forkmann et al., 1986). While the FLS gene has not been cloned in Vitis vinifera, it has been cloned in Petunia, Arabidopsis, Solanum and Citrus (Holton et al., 1993b; Pelletier et al., 1997; van Eldik et al., 1997; Moriguchi et al., 2002).

1.4.2. Factors Affecting Flavonoid Biosynthesis

The factors reputedly affecting flavonoid biosynthesis in plants are myriad and include light, temperature, altitude, soil type, water and nutritional status, microbial interactions, pathogenesis, wounding and defoliation, plant growth regulators and developmental processes. These factors are also closely intertwined, for example in forest clearings where light intensity is greater than inside the forest, plants have higher levels of tannins. Initially, this appeared as an effect of light on tannin accumulation. However, it was also observed that in these clearings, the incidence of defoliating insects was significantly greater due to the presence of a high proportion of juvenile plant material (Mole et al., 1988; Mole & Waterman, 1988). In this case, tannin accumulation was induced by
herbivorous attack rather than light, a widely reported phenomenon (Feeny, 1976; Walters & Stafford, 1984; Wagner, 1988; Elmqvist et al., 1991).

The effect of incident radiation upon flavonoid accumulation in the plant is also intertwined with changes in photosynthesis and a number of investigations into the effects of sun exposure and shading on both development and flavonoid metabolism have been conducted on grapevines. Where whole canopies have been shaded, the flavonoid content of grapes was observed to decrease significantly (Buttrose et al., 1971; Kliwer, 1977; Crippen & Morrison, 1986b; Smart et al., 1988). However, there was also a general decrease in carbon fixation in these grapevines, which resulted in lower levels of other metabolites such as sugars and organic acids (Kliwer et al., 1967; Kliwer, 1977; Smart et al., 1988; Rojas-Lara & Morrison, 1989). Although, these differences have also been attributed to greater water loss from exposed berries than from shaded fruit (Crippen & Morrison, 1986a).

Furthermore, sun-exposure results in an increase in temperature in the plant, either through direct heating of the plant from incident radiation or increases in the ambient air temperature (Jones, 1992). As the temperature of a biological system increases, so too does metabolism with an associated increase in development (Hawker, 1982; Jones, 1992; Ebadi et al., 1995a; Dokoozlian & Kliwer, 1996). However, at higher temperatures metabolism ceases (Jones, 1992), in grapevines this temperature is around 30°C (Coombe, 1987b). Similarly, decreasing temperature slows metabolism and results in reduced carbon fixation as well as reduced production of secondary metabolites (Jones, 1992). In grapevines, temperatures below 9-10°C are generally considered to slow metabolism to the point where little or no growth occurs (Winkler et al., 1974; Jackson & Spurling, 1988; Jackson & Lombard, 1993; Boulton et al., 1998). Below a certain
temperature metabolism ceases and at some point chilling injury will occur (Jones, 1992). In grapevines, freezing injury to the wood or dormant buds occurs at around -15°C, although this is rarely a problem in Australia (Jackson & Spurling, 1988). Of greater concern is the effect of low temperature around the time of flowering, which has been shown to significantly impact upon fruit-set (Buttrose & Hale, 1973; Ebadi et al., 1995a; Ebadi et al., 1995b). Both high and low temperatures have also been shown to effect the anthocyanin and tannin content and composition of many plants including grapevines (Kliewer, 1970; Buttrose, 1970; Lees et al., 1994; Bel-Tal & King, 1997; Morris & Robbins, 1997; Leng et al., 2000). Unfortunately, the application of shading treatments has also been shown to substantially alter the temperature and humidity within grapevine canopies (Kliewer et al., 1967; Rojas-Lara & Morrison, 1989; Haselgrove et al., 2000). Increased humidity in the canopy has a two-fold effect, by lowering the vapour pressure deficit, transpiration and photosynthesis are decreased, reducing growth, and possibly also flavonoid accumulation (Jones, 1992). In addition, high humidity increases the risk of pathogenesis through fungal or bacterial infection (Emmett et al., 1992), which may cause a wound response inducing flavonoid accumulation (Mehdy & Lamb, 1987; Vogt et al., 1994).

Chilling injury can also effect flavonoid accumulation (Leyva et al., 1995), but is likely part of a general wound-response that has also been documented to increase flavonoid biosynthesis (Chalmers & Faragher, 1977; Vogt et al., 1994). In some instances this is related to defoliation as described above, while in other cases, pathogenic inducers effect flavonoid accumulation (Langcake & Pryce, 1976; Langcake & Pryce, 1977b; Harrison & Stickland, 1980; Pool et al., 1981; Marschner, 1995; Hipskind et al., 1996; Lo & Nicholson, 1998). In addition to pathogens, soil microorganisms, both mycorrhizal
(Chabot et al., 1992; Harrison & Dixon, 1994) and rhizobial (Clarke et al., 1992), have been shown to affect flavonoid accumulation. The induction of flavonols in the formation of symbioses with nitrogen-fixing bacterium occurs in response to low soil nitrogen (Zaat et al., 1989; Recourt et al., 1992). In the presence of high soil nitrogen, a different group of flavonoids are exuded to inhibit nodulation (Firmin et al., 1986; Djordjevic et al., 1987; Dakora et al., 1993). Thus, what appears to be microbial induction of flavonoid accumulation is in fact a response to nutrient status. Nevertheless, the impact of wounding and microbial association upon phenylpropanoid biosynthesis in grapevines has been extensively investigated in relation to the accumulation of the stilbene phytoalexins resveratrol and viniferin (Langcake & Pryce, 1976; Langcake & Pryce, 1977a; Langcake & Pryce, 1977b; Langcake & McCarthy, 1979; Pool et al., 1981; Creasy & Coffee, 1988; Dercks & Creasy, 1989; Jeandet et al., 1995). However, this work has yet to be extended to flavonoids such as flavonols, tannins and anthocyanins.

Nutrient availability has a fundamental influence upon plant growth (Russell, 1961; Marschner, 1995; Keller et al., 1998). Nutrient status has been shown to effect the flavonoid composition of plant tissues. Both low and excessively high levels of nitrogen fertiliser have been shown to decrease colour in grape berries (Kliwer, 1977; Keller & Hrazdina, 1998) and high potassium has been reported to decrease colour in grapes (Jackson & Lombard, 1993). While in forage legumes, high phosphate levels inhibited tannin accumulation (Morris & Robbins, 1997), the effect on grape tannins has not been examined. Large increases in N, P and K have been shown to decrease phenolic content in plants. However, while increasing fertilisation generally had a positive effect on flavonoid content, it can lead to excessive vigour resulting in decreased flavonoid accumulation.
In addition to the chemical characteristics of a soil, amended or otherwise, physical characters can also effect flavonoid accumulation (Jackson & Lombard, 1993; McDonald et al., 1998). Such characters as the parent material and the age of the soil that largely determine the micronutrient pool, structure and texture have a significant effect on plant growth (Russell, 1961; Northcote, 1992; Marshner, 1995). However, the major consequence of soil type is the capacity of the soil to hold water while remaining sufficiently well-drained to avoid waterlogging (Russell, 1961; Northcote, 1992). Irrigation alleviates reduced development and growth through water stress, although some reports suggest water deficit increases tannin and anthocyanin content in grapevines (Nadal & Arola, 1995; Dry et al., 1998). However, other workers suggest excessive water decreases tannin content (Kennedy et al., 2000a), and that water deficit has no effect on tannin or anthocyanin accumulation (Kennedy et al., 2000a; Stoll, 2000). In contrast to the vines, anthocyanin biosynthesis in grape cell cultures is extremely sensitive to osmotic stress (Do & Cormier, 1991; Suzuki, 1995).

The difficulty with interpreting water deficit treatments is the range of other effects that occur throughout the plant, that are regulated by plant hormones in response to stress. Stomatal closing in response to water deficit reduces photosynthesis, thereby reducing all metabolite accumulation (Jones, 1992). Furthermore, plant growth regulators such as, abscisic acid, ethylene, cytokinins, gibberellins and auxins have all been reported to influence the flavonoid content of plants, including grapevines (Matsushima et al., 1989; Woltering & Somhorst, 1990; Deikman & Hammer, 1995; Moalem-Beno et al., 1997; Morris & Robbins, 1997; Stoll, 2000).

Generally, a large number of variables have been shown to impact upon flavonoid composition in many plants, including grapevines. In addition, many of these factors are
closely related and difficult to isolate experimentally. Despite the broad variation observed with many of these parameters, the greatest affectors of flavonoid content in grapevines are site and season (Bakker et al., 1986a; Gonzalez-San Jose et al., 1990; Revilla et al., 1997; McDonald et al., 1998; de Freitas & Glories, 1999; Guidoni et al., 2002).

1.5. Conclusions and Project Aims

This project grew out of the original work of Paul Boss (1998), who examined the expression of flavonoid pathway genes in *Vitis vinifera* L. cv. Shiraz during berry development. While examining the biosynthesis of anthocyanins in grape berries, he observed that the genes encoding the whole biosynthetic pathway with the exception UFGT, the gene encoding the final step in anthocyanin biosynthesis, were expressed throughout most of berry development. UFGT was only expressed post-veraison when anthocyanin synthesis commenced in the berry skin. What purpose then did expression of the pathway prior to veraison serve? The most likely explanation is that the pathway is producing other flavonoid compounds early in berry development, likely candidates being the flavonols and flavan-3-ols, two classes of compounds that diverge from the pathway before anthocyanin synthesis (Figure 1.6).

It is rare that the whole or even a substantial part of the flavonoid biosynthetic pathway has been examined in a single plant and these generally at a single point in development. *Vitis vinifera* is an ideal system to examine flavonoid biosynthesis as it produces all of the major classes of flavonoids formed by branchpoints in the pathway. An examination of the spatial and temporal pattern of accumulation of these compounds, and the expression
of genes involved in their synthesis would substantially increase our understanding of flavonoid biosynthesis.

While many studies have been conducted on grape flavonoids these have also tended to focus on flavonoid composition at harvest or changes during ripening. In addition, the bulk of this work has been conducted on important European or North American cultivars grown under the prevailing conditions at those sites. As wine quality reflects that of the fruit, and flavonoids are a significant component of wine and grape quality, defining the pattern of flavonoid synthesis in winegrapes would also be a useful contribution to the production of high quality wine.

This project sought to make a comprehensive examination of tannin, anthocyanin and flavonol accumulation in tissues of the major Australian winegrape varieties, Shiraz and Chardonnay, throughout the growing season and in seeds and skin during berry development over successive seasons. In addition, it was intended to clone the genes encoding the determinant enzymes in the biosynthesis of flavonols (FLS) and flavan-3-ols (LAR). Moreover, the prevailing zeitgeist suggests light has a strong influence on all aspects of flavonoid metabolism, although much of this work has been complicated by microclimate affects such as temperature and humidity. Therefore, flavonoid composition and gene expression studies would be extended to a shading treatment of the fruit that sought to eliminate these effects.

Thus, in summation, the aims of this project were:

- To determine when and where flavonols are synthesised during berry development.

- To determine when and where flavan-3-ols and proanthocyanidins (tannins) are synthesised during berry development.
To determine the influence of light on flavonoid biosynthesis in grapes.

To clone and characterise key genes in flavonol and tannin biosynthesis in grapes and to determine their expression pattern and correlate it with biosynthesis.

To investigate the basis of pre-veraison expression of most of the flavonoid biosynthetic pathway as observed by Boss et al. (1996a).
Chapter 2. Grape Berry Development

2.1. Introduction

The fruit of the Vitaceae are berries, the archetypal grape being a black berry with anthocyanins in both the flesh and skin (Mullins et al., 1992). White and pink skinned grapes are widely cultivated, as are several pink-fleshed varieties, although the flesh of most extant grape cultivars is not pigmented (Somers, 1976; Mullins et al., 1992). Berry development is bimodal, the initial growth phase being mainly cell division followed by a lag period before the second phase of growth, which is predominantly cell expansion (Cawthorn & Morris, 1982; Coombe, 1992; Mullins et al., 1992; Coombe & McCarthy, 2000). Ripening occurs during the second phase of development (Figure 2.1) and is characterised by softening of the berry, accumulation of sugars and metabolism of organic acids (Winkler et al., 1974; Pirie & Mullins, 1980; Coombe, 1988; Nunan et al., 1998). In addition, pigments, flavour and aroma compounds accumulate during ripening (Pirie & Mullins, 1980; Coombe, 1988; Coombe, 1992; Mullins et al., 1992; Coombe & McCarthy, 1997). The relative contribution of each of these components to the grape and wine is influenced considerably by site, season, viticultural practice and the timing of harvest.

Figure 2.1. Schematic representation of grape berry development.
For a given site in an irrigated vineyard, it is assumed that characteristics such as soil will remain invariant, nutrition will be adequate and viticultural practices will not vary greatly from year to year. Thus, the primary seasonal difference at such a site will be climatic, predominantly temperature and light, which have demonstrated impacts on berry development and flavonoid metabolism.

As this project sought to compare flavonoid composition in both exposed and shaded fruit, between cultivars and across seasons, it was necessary to quantify fruit development in each treatment to enable valid comparisons. Failure to do this might attribute differences in flavonoid content directly to treatment effects where in fact the cause may have been differences in fruit development.

### 2.1.1. Shading Treatments

A large body of historical data exists on the effects of sun-exposure on grape berry composition. Some of these investigations involved the application of physical shade treatments such as plastic sheeting (Kliwerer et al., 1967), shade-cloth (Smart et al., 1988), bags (Kliwer & Antcliff, 1970) and foil-clad cages (Haselgrove et al., 2000). Others have tried binding canes under the canopy (Archer & Strauss, 1989), sampling different parts of the canopy where the light regimen was perceived to be different (Price et al., 1995; Haselgrove et al., 2000; Dergvist et al., 2001) and defoliation (Kliwerer & Antcliff, 1970; Hunter et al., 1995). In addition, treatments have been applied at different developmental stages from fruit-set to veraison, with samples taken primarily during ripening, or at harvest. In many of these studies, the treatments have altered other aspects of vine physiology, such as cane length, leaf number and foliage exposure as well as bunch exposure. These approaches have also produced considerable variation in
light/dark levels between studies, as well as a range of microclimate effects, notably changes in temperature with concomitant impacts on photosynthesis and metabolism. Thus, it has sometimes been difficult to discriminate between the effects of treatments and other microclimate influences. To address this, it was proposed that a shading treatment be applied that would eliminate other microclimate effects while achieving light exclusion.

2.2. Materials and Methods

2.2.1. Design of Light-Proof Boxes

Of the previous strategies (Section 2.1.1) the application of bags to developing bunches seemed an economic and straightforward approach. A preliminary investigation into the temperature difference between the ambient air and the inside of an aluminised paper bag was conducted. Temperature was logged over several days using a Tinytag Plus™ logger (Hastings Data Loggers, Australia) with an external temperature probe inserted into the bag. A significant difference in temperature was recorded, particularly in full sunlight, with the inside of the bag significantly warmer (data not shown). In addition, the inside of the bag became extremely moist from condensation indicating high humidity within the bag. These observations indicated an opportunity for a radical new approach to shading.

To shade bunches of grapes, the method of shading needed to fulfil several requirements. It must be light-proof while maximising airflow to maintain the ambient vineyard atmosphere within the box. In addition, the design had to enclose the bunch without impacting upon grapevine physiology and, being in a commercial vineyard, needed to be sufficiently unobtrusive yet robust, that normal viticultural operations such as spraying
and trimming could continue. The chosen material was polypropylene sheet (0.6 mm) to make a light-weight box that could hang in the canopy without damaging the vines, which would produce a wound response (Mehdy & Lamb, 1987; Vogt et al., 1994; Dixon & Paiva, 1995). The box was designed such that it could be assembled easily, but pack flat for storage and transportation. Application of the box enclosed a bunch upon the cane as the box was closed, then the box was attached to a neighbouring cane to support some of the weight and prevent excessive movement in high wind (Figure 2.2). The hanging points also acted as a closing mechanism for the box. The box design employed a darkroom-door type arrangement on each side with the inside of the box painted black to prevent reflected light entering the box (Figure 2.2). The outside of the box was white to maximise reflection and prevent black-body heating (Smart & Sinclair, 1976; Jones, 1992).

Initially two prototype boxes were made from polypropylene sheet, one black, the other white. A trial to assess the temperature inside both boxes was set up in the Waite

![Figure 2.2. Design and assembly of polypropylene boxes used to shade grape berry bunches. A) Opened box as for application to the vine showing foam padding around slots to prevent cane damage. B) Closed box as applied to the vine with tabs for securing the box to the cordon or trellis visible at top.]
vineyard. Temperature inside the boxes was logged as was the external air temperature and the temperature inside a Stevenson Screen. The Stevenson Screen was considered to represent an acceptable level of microclimate amelioration with respect to the ambient atmosphere. The temperature was logged over several days (01-08 September 1999), using a temperature logger (DataTaker 50™, Australia).

The results of these trials showed very little difference between the temperature regime in the Stevenson Screen and the outside air temperature (Figure 2.3A), although it was slightly cooler inside the Screen at low temperatures. A similar result was observed

Figure 2.3. Temperature trials on prototype shading boxes. A. Comparison between ambient field temperature and Stevenson Screen, used as box control; B. Comparison of temperature inside Stevenson Screen and inside white box; C. Comparison between white box and ambient field temperature; D. Comparison between box made from white polypropylene and black polypropylene.
between the Stevenson Screen and the white box (Figure 2.3B), with the temperatures virtually the same, except for a slightly lower temperature in the box at night. In addition, the fourth and fifth days showed a slightly higher temperature inside the box. Meteorological data for these days (04 September and 05 September 1999) showed a front passing through with associated high winds (Bureau of Meteorology data, not presented). This indicates that there was better airflow through the Stevenson Screen than through the light-proof box, although it is important to remember that the Stevenson Screen is not light-proof.

Nevertheless, perfect airflow was not achieved as the box afforded some protection from the wind resulting slightly higher temperature inside the box. Between the box and the outside air temperature, the box was cooler at night and warmer in high wind (Figure 2.3C). There was a marked difference in temperature between the box made from black polypropylene and that made from white polypropylene with the inside painted black (Figure 2.3D). The results of these trials, indicated that the white box would be suitable for the purpose of this investigation as the temperature regime was similar to that of the ambient air temperature and performed similarly to a Stevenson Screen.

Airflow through the box is important not only for rapid temperature equilibration between the interior of the box and the ambient atmosphere, but also for maintaining the same relative humidity inside and outside of the box. Thus, relative humidity was also logged with two TinyTag Plus™ relative humidity/temperature loggers (Hastings Data Loggers, Australia) to determine whether the shading treatment would impact upon this aspect of bunch microclimate. Readings were made at 5 minute intervals and the hourly average was calculated. Relative humidity readings in and out of the box for a month were compared using Students T-test. While there were differences in relative humidity,
in some instances of up to 20% for individual readings, there was no significant
difference in the relative humidity (Figure 2.4) on an hourly, daily or monthly basis
between the interior of the box and the outside atmosphere. The average hourly
difference in relative humidity between the ambient environment and the inside of the
boxes used as a shading treatment in this project over a one month period was ±2.9%
(p=0.6975).

Figure 2.4. Relative humidity inside and outside of light-proof boxes over a five day period in April,
showing relatively small differences in relative humidity between the ambient environment and the
inside of the polypropylene boxes used as a shading treatment in this project.

The extent to which box design achieved light exclusion was also investigated. The light
environment inside the box was measured using a quantum sensor, LI-COR™ (LI 6400,
USA). This measured the level of photosynthetically active radiation (PAR, 400-700 nm,)
inside the box and this was compared to levels outside of the box. PAR inside the box
was measured at four orientations of the box about the vertical axis (north, south, east and
west) with the quantum sensor facing north (towards the sun). Recordings were also
made at four orientations of the box about the horizontal axis (up, down, east and west)
with the quantum sensor facing upwards. In all cases the level of PAR inside the box was less than 0.5% of that outside the box.

In addition, transmission of light through the box material was determined using a Shimadzu™ (Japan) spectrophotometer. Transmission through the white polypropylene sheet with one side painted black was less than 0.1% between wavelengths of 220 and 800 nm, less than 0.01% below 400 nm.

Furthermore, as a physiological measure of the light environment inside the boxes, chlorophyll in both shaded and exposed fruit was measured. Chlorophyll synthesis is light induced and in the absence of light no synthesis occurs, thus shaded fruit would have less chlorophyll (Zucker, 1972; Raven et al., 1992). Chlorophyll was measured by the method of Harborne (1998). Briefly, chlorophyll was extracted in 80% acetone from developing inflorescences and grape berry and skin throughout the collection series (1999-2000 and 2000-2001), from exposed and shaded fruit. The absorbance of the extract was recorded at 646 and 663 nm and total chlorophyll was determined according to the function:

$$\text{Total chlorophyll (mg/L)} = 17.3A_{646} + 7.18A_{663}$$

### 2.1.2. Timing and Application of Treatments

In the first season (1999-2000), two shading treatments were trialed. The first shading experiment applied 56 boxes to inflorescences at the onset of cap-fall to the north and south sides of two rows. The second experiment applied 21 boxes after fruit-set. Boxes applied to canes can be seen in Figure 2.5.

Based on data collected during the 1999-2000 season, two shading experiments were conducted during the second experimental season. The first shading treatment in 2000-
Figure 2.5. Light-proof boxes used to shade bunches of *Vitis vinifera* L. cv. Shiraz berries in the field at the time of application one week post-flowering during the 1999-2000 season in Willunga, S.A.

2001 applied 45 boxes to buds in two rows just prior to bud-burst so that fruit would develop from inflorescences that had not been exposed to light. When the developing canes were beginning to fill the boxes, and adjacent exposed shoots were clearly showing inflorescences, the boxes were opened at night and repositioned to enclose only the inflorescence. The remainder of the cane developed in light. The second shading treatment during the 2000-2001 season repeated that of the first season on a larger-scale with 72 boxes applied across two rows as inflorescences became apparent.

During the 2001-2002 season, a single large-scale shading experiment was conducted to enable winemaking from the shaded and exposed fruit. Based on the results of the trials
conducted in 1999-2000 and 2000-2001, shading was applied to 425 inflorescences prior to flowering, in the same block as previously. A two-panel and two-row buffer was employed for all experiments to avoid end effects. Boxes were applied to both the north and south sides of rows. Boxes were examined regularly and where cane growth resulted in bunches emerging from boxes, these were discarded. The winemaking component of this research comprises a separate research project (R. Ristic, University of Adelaide) and will not be considered here.

2.1.3. Sampling of Grape Berries

Tissues of *Vitis vinifera* L. cultivars were collected from Shiraz and Chardonnay vines grown on modified Scott-Henry trellises in a commercial vineyard in Willunga, South Australia (34° 46' south, 138° 32' east). The region has a maritime climate with a mean daily temperature of 23°C during berry development (Bureau of Meteorology data, not presented). Sampling dates for Chardonnay and Shiraz over three seasons are presented in Table 2.1. Shiraz samples were collected at weekly intervals from flowering until harvest during the 1999-2000 and 2001-2002 seasons and from bud-burst until harvest in 2000-2001. Chardonnay fruit was collected from flowering to harvest during the 1999-2000 season. Flowering was defined as the time when approximately 50% of the opercula had fallen from the flowers (50% cap-fall).

Bunches were collected randomly from 180 Shiraz vines spread across six rows and ten panels of a single block and from 72 Chardonnay vines across four rows and six panels. A minimum of twenty bunches were collected on each date and the berries removed and bulked. A sub-sample of approximately 100 berries was taken for measures of grape
berry development. The remaining berries were immediately frozen in liquid nitrogen then stored at -80°C for later analysis of metabolites and RNA extraction.

During the first season (1999-2000), seeds and whole berries of both Shiraz and Chardonnay were collected. In the following season (2000-2001), only Shiraz tissues were collected and berries were dissected into seeds and skin in the field, on several occasions during berry development, grape flesh was also collected. In the third and final season (2001-2002), only Shiraz skin was collected during berry development with a sample of seeds made at harvest.

Sampling of grape tissues from shading experiments was similar to that of the exposed fruit. At each sampling date during the 1999-2000 and 2000-2001 seasons (Table 2.2), 5-6 boxes were opened and bunches removed for analysis. At harvest all remaining boxes were opened and their contents removed for analysis. In the final season (2001-2002), to retain the maximum number of shaded bunches for winemaking, sampling was limited to removing single berries from shaded and exposed bunches. Exposed bunches sampled on these occasions had been tagged at the time of applying the shading treatment. Boxes applied to bunches in the canopy and tagged control bunches can be clearly seen in Figure 2.2. Between 50 and 100 berries were collected on each sampling date. Seeds and flesh were separated from the skin of the berry by rolling between thumb and forefinger to disrupt cell wall integrity in the flesh of the berry and the contents expelled by squeezing. At harvest, the fruit was distributed into five replicates for winemaking and each replicate was sampled for later analysis of metabolites and RNA extraction.
Table 2.1 Grape berry sampling of Chardonnay 1999-2000 and Shiraz over three seasons 1999-2000, 2000-2001 and 2001-2002

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\(^t\) Flowering; \(^t\) Veraison; \(^o\) Harvest

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* Shading treatment applied; † Flowering; ‡ Veraison; * Harvest

Note: In 1999-2000, veraison in Experiment 1 (shading treatment applied one week post-flowering, -9V) was delayed by one week in the shaded fruit.
2.1.4. Analysis of Berry Development

During the 1999-2000 and 2000-2001 seasons, measurements of berry deformability and total soluble solids (°Brix) were made in the field. Deformability was determined using skin-fold callipers applied to berries prior to their removal from the bunch (Coombe, 1992). Each berry thus measured was then removed and total soluble solids (°Brix) for that berry measured by hand-held refractometer. A sub-sample of 100 berries was taken back to the lab for dissection and measurement of seed and skin weight, seed number and bulk weight of berries. The weight of each component was determined by dividing the bulk weight of seeds or skin by berry number and for seeds also by seed number.

At the end of the first season (1999-2000), pH and titratable acidity were also determined as the fruit approached harvest. This was considered particularly important during that season as a pronounced occurrence of “hen and chicken” (Stannard et al., 1974; Cholet et al., 1998) resulted in many small, under-ripe berries at harvest. This phenomenon was not observed during the two successive seasons. Titratable acidity and pH were determined by the methods outlined in Iland et al. (1993).

During the 2001-2002 season, Shiraz skin was collected in the field and frozen immediately in liquid nitrogen. The flesh and seed portion was retained and this was used to determine total soluble solids (°Brix). Because of this approach, no standard error for °Brix was available for the 2001-2002 data, except for the harvest sample. Bulk berry weight and skin weight were determined in the field. Seeds were only collected at harvest during the 2001-2002 season and at that time seed weight and seed number were determined. Berry deformability was not measured during the 2001-2002 season.
Immediate dissection and freezing of samples was important to limit changes due to sampling and transportation, particularly for subsequent RNA extractions. Snap freezing minimises RNAse activity (Sambrook et al., 1989) in the excised berry. Furthermore, immediate freezing prevents expression artefacts caused from sampling, such as gene expression related to wound response (Mehdy & Lamb, 1987; Vogt et al., 1994; Dixon & Paiva, 1995).

Berry weight, skin weight and seed weight were determined by measuring bulk weight of 50–100 berries divided by berry or seed number and thus no statistical analysis was possible. Total soluble solids ('Brix) and deformability we determined for 50–100 individual berries and thus statistical comparisons were possible. Figures representing these data are shown with standard error bars, often these are smaller than the symbols for each data point.

The tendency of many workers towards analysis of freeze-dried samples has been resisted here. It was considered that the main beneficiaries of this research would be grapegrowers and winemakers, who deal with fresh fruit. Trying to relate data from dry weight back to fresh weight can at best be irksome, otherwise, prone to error. All measurements are expressed here as mg/g fresh weight of tissue or mg per whole fresh berry.

In addition, much research on grape berry development and the changes in metabolites during grape berry development has, in the past, been presented with reference to the date each sample was taken. The reason for this is uncertain given that the dates for flowering, veraison and harvest are highly variable between sites and between seasons at the same site (as shown below, Section 2.3.11). This makes sample dates an arbitrary scale of little
practical value, an observation that becomes painfully obvious when referring to studies conducted in the Northern Hemisphere. A reference point in berry development that is meaningful to researcher, grapegrower and winemaker alike is veraison, thus all samples analysed here have been referred to as weeks pre-veraison or post-veraison. Abbreviations such as +6V or -3V have been used to denote six weeks post-veraison or three weeks pre-veraison respectively. Actual sampling dates are presented in Table 2.1.

Veraison is the term used by viticulturalists to denote the onset of ripening. Historically, this has referred only to colour change, although it is now commonly used to refer to berry softening, resumption of growth, sugar accumulation, decreases in titratable acidity, increasing pH and loss of chlorophyll (Coombe, 1973; Mullins et al., 1992). There appears no clear pattern to the order in which these process occur, although berry softening, deformability, has been suggested as a useful objective measure (Coombe, 1973; Coombe & Hale, 1973). While sugar accumulation, softening, colouration and increasing size collectively constitute veraison, it is sugar accumulation in the flesh of the berry that increases most dramatically and is most readily measured, which is the best indication of the onset of ripening (Coombe, 1989; Coombe & McCarthy, 2000). In comparison, colour development can spread across several weeks (Pirie, 1977; Pirie & Mullins, 1977), with small changes in anthocyanin concentration not readily detected by the naked eye, particularly when masked by chlorophyll pigments in the berry. Here we have used sugar accumulation in the berry to determine veraison. This determination was made based on total soluble solids (Brix) measured in 50 individual berries and the average Total Soluble Solids (Brix) calculated. Veraison (0V) was then defined as the sample prior to an increase in Total Soluble Solids (Brix), having established base level prior to veraison.
2.1.5. Environmental Differences between Seasons

In order to estimate the environmental differences between experimental seasons, temperature in the vineyard was logged from before flowering until harvest over the three experimental seasons, 1999-2000, 2000-2001 and 2001-2002 using a TinyTag™ data-logger (Hastings Data-Loggers, Australia). The temperature logger was suspended in the canopy and upon application of shading treatments, a temperature probe was attached to the data-logger, which was inserted into a box adjacent to the logger. Temperature recordings were made at 15 minute intervals throughout the season and these data were used to calculate the mean daily temperature as well as the daily minimum and maximum, both in the canopy and inside the box.

2.2. Results

2.2.1. Chardonnay Berry Development 1999-2000

Sampling of Chardonnay in 1999-2000 commenced prior to flowering, which occurred around 21 October 1999 (-9V), and continued at weekly intervals until harvest. Berry weight increased in a double sigmoidal pattern considered typical (Cawthorn & Morris, 1982) of grape berry development (Figure 2.6). Berry weight increased slowly from flowering through fruit-set (-8V) and then more rapidly until one week pre-veraison. A brief lag phase was observed...
for a week, whereafter berry growth recommenced. During this phase of growth, berry weight increased for three weeks from veraison, then remained static for the following two weeks followed by a decline in the final week until harvest.

The pattern of sugar accumulation (°Brix) in the berry followed a similar pattern to berry weight in the period post-veraison (Figure 2.7). Veraison, defined as the onset of ripening, is usually determined by an increase in sugar in the berry. Here we have designated veraison as the week prior to an increase in sugar concentration. This phase of sugar accumulation closely matched the second phase of berry growth from veraison to three weeks post-veraison. As with berry weight, sugar accumulation then remained stable and as berry weight decreased in the last week total soluble solids increased, suggesting water loss from the berry.

As the berry begins to ripen, numerous processes within the berry are responsible for the breaking down of the cell walls in the flesh (Coombe, 1976; Nunan et al., 1998). Thus, the berry begins to soften and this
can be measured as the deformability of the berry (Coombe & Bishop, 1980). In Chardonnay berries during 1999-2000, berry deformability was seen to increase post-veraison towards harvest (Figure 2.8). While deformability is a strong indication of ripening, it is highly susceptible to irrigation and rainfall events increasing berry turgor, shown as a decrease in deformability four weeks post-veraison (Figure 2.8).

Chardonnay seeds were collected from the time they were easily separated from the remainder of the berry under field conditions, during the 1999-2000 season this was possible at three weeks pre-veraison. From this time, seed weight per berry increased over the following two weeks, thereafter seed weight per berry remained relatively stable (Figure 2.9). At four weeks post-veraison an increase was observed in seed weight followed by a marked decline in the final week before harvest. The increase observed late in berry development when the seed had hardened off is difficult to rationalise other than as sampling variability. The most likely explanation being that slightly larger bunches or berries were inadvertently sampled at this point. Larger berries having been shown to have more, and/or larger seeds (Cawthorn & Morris, 1982; Boulton et al., 1998). However, seed number in Chardonnay was stable (1.493±0.031 seeds per berry, n=9 weeks), and berry size remained constant during this late increase in seed weight (Figure 2.6). These data indicate that the main period of seed growth in Chardonnay occurred prior to the first sampling at three weeks pre-veraison.

Figure 2.9. Seed weight per berry (g) for Chardonnay grapes during the 1999-2000 season, Willunga S.A.
2.2.2. Shiraz Berry Development 1999-2000

Changes in Shiraz berry weight during the 1999-2000 season (Figure 2.10) showed the classic double sigmoidal pattern of development (Cawthorn & Morris, 1982; Coombe, 1992; Mullins et al., 1992). Berry weight increased from flowering (-9V) until two weeks pre-veraison, followed by a lag until one week pre-veraison then berry weight increased towards harvest. A decline in berry weight was observed from four weeks post-veraison as the berry began to shrivel, however a rainfall event immediately prior to harvest resulted in an increase in berry weight at this time.

These changes in berry weight were reflected in sugar accumulation (Figure 2.11). Total soluble solids increased steadily from veraison towards harvest. At the time when berry weight began to decrease (Figure 2.10) °Brix continued to increase, suggesting an increase in concentration due to water loss. Further, at harvest when rain resulted in an increase in berry weight, °Brix declined.
Shiraz berry deformability increased with ripening from veraison towards harvest (Figure 2.12). The initial period of increasing deformability was slow, but from two until four weeks post-veraison there was a sharp increase in deformability. Following this, deformability remained stable for two weeks, then decreased at harvest consistent with an increase in turgor due to rainfall.

Shiraz seed weight per berry in 1999-2000 increased sharply from the first sample at five weeks pre-veraison for two weeks, reaching 80% of the observed maximum at three weeks pre-veraison (Figure 2.13). Seed weight per berry increased slowly from that point reaching its maximum (0.093 g/berry) one week post-veraison, whereafter seed weight per berry began to decline towards harvest. The decline in seed weight per berry was likely the result of decreased weight of individual seeds as the seedcoat lignified and the seed dehydrated (Raven et al., 1992; Kennedy et al., 2000b). Seed number per berry in Shiraz during the 1999-2000 season was 1.538±0.033, calculated from samples of 50-100 berries per week over 13 weeks.
During the 1999-2000 season, there was a large proportion of very small berries in bunches of Shiraz. These small berries, upon dissection, contained only seed traces. Berry development was retarded with no sign of colouration in these small berries as normal fruit passed through veraison. However, at around four weeks post-veraison in the normal berries, the small berries began to colour. At five weeks post-veraison a sample of these small berries was taken and total soluble solids (°Brix), titratable acidity (TA) and pH was compared with that of the normally developing fruit (Table 2.3). The smaller fruit had lower sugar (°Brix) and higher levels of acidity, both as lower pH and a higher level of titratable acidity expressed as tartaric acid equivalents (Table 2.3).

Table 2.3. Comparison of berry weight (g), total soluble solids (°Brix), pH and titratable acidity (TA; g/L tartaric acid equivalents) in normally developing berries and smaller underdeveloped berries of Vitis vinifera cv. Shiraz during 1999-2000 at five weeks post-veraison.

<table>
<thead>
<tr>
<th></th>
<th>Normal Berries</th>
<th>Small Berries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td>1.41</td>
<td>0.26</td>
</tr>
<tr>
<td>°Brix</td>
<td>22.1</td>
<td>20.7</td>
</tr>
<tr>
<td>pH</td>
<td>3.24</td>
<td>2.98</td>
</tr>
<tr>
<td>TA (g/L)</td>
<td>9.98</td>
<td>14.10</td>
</tr>
</tbody>
</table>

2.2.3. Shiraz Berries Shaded One Week Post-Flowering 1999-2000

Shading of Shiraz bunches one week post-flowering delayed veraison by one week compared to the exposed fruit (Table 2.2). Shading also had a noticeable effect on berry weight.
weight (Figure 2.14). At veraison, shaded berries weighed less than the exposed fruit as a result of reduced berry growth post-veraison. At harvest, shaded berries weighed 35% less than exposed berries.

Despite the difference in berry weight, shaded berries ripened at a similar rate to the exposed fruit and reached the same level of total soluble solids at harvest, around 23.5°Brix (Figure 2.15).

2.2.4. Shiraz Berries Shaded Five Weeks Post-Flowering 1999-2000

Application of the shading treatment five weeks post-flowering in the 1999-2000 season also resulted in reduced berry weight (Figure 2.16). However, the impact of shading on berry weight was less in berries shaded five weeks post-flowering than observed in berries shaded one week post-flowering (Section 2.3.4). Berries shaded five weeks post-flowering were only around 20% lighter at harvest than the exposed fruit.
There was no significant difference observed in the pattern of accumulation of total soluble solids (°Brix) in fruit shaded from five weeks post-flowering and the exposed fruit (Figure 2.17). Although at harvest, the level of total soluble solids (°Brix) was slightly higher in the shaded fruit.

2.2.5. Shiraz Berry Development 2000-2001

During the 2000-2001 season, the pattern of Shiraz berry growth, as described by changes in berry weight, did not exhibit the typical sigmoidal pattern of development (Figure 2.18) so apparent in the previous season (Figure 2.10). During the 2000-2001 season, berry weight increased steadily from fruit-set (-7V) towards veraison, when a lag phase at two weeks pre-veraison was observed for one week. Berry weight then increased steadily until five
weeks post-veraison, after which time berry weight began to decrease. As seen in Figure 2.19, total soluble solids (°Brix) increased steadily during this time. Sugar accumulation from five weeks post-veraison until harvest appears to be the result of water loss from the ripe berry as berry weight decreased over this period (Figure 2.18).

Berry deformability, being so susceptible to changes in berry turgor, as well as large diurnal fluctuations (Coombe & Bishop, 1980), its value beyond determining the onset of ripening is of little value. While this data was collected, it will not be presented here.

Seed weight per berry in the 2000-2001 season increased steadily from a week after fruit-set (-6V) for two weeks (Figure 2.20). From four weeks pre-veraison there was a two week lag followed by further increase in seed weight per berry. This level (0.13 g/berry) began to decline steadily from one week post-veraison towards harvest, losing around 35% of the maximum weight recorded. Seed number during the 2000-2001 season was 2.93±0.04 seeds per berry (n=16 weeks).

2.2.6. Shiraz Berries Shaded Five Weeks Pre-Flowering 2000-2001

In the second season (2000-2001), the difference in berry weight between shaded and exposed fruit (Figure 2.21) was less noticeable than that observed during the 1999-2000 season (Figure 2.14). The shading treatment was applied four weeks before flowering (-13V) and samples taken seven, three and one week pre-veraison (Table 2.2). Before
veraison, berry weight in the shaded fruit was lower than that in the exposed (Figure 2.21). However, post-veraison increases in the berry weight of shaded fruit resulted in similar weights by five weeks post-veraison. At harvest, berry weights were similar between shaded and exposed fruit (approximately 0.96 g/berry).

There was also virtually no difference in total soluble solids between shaded and exposed fruit throughout ripening during the 2000-2001 season (Figure 2.22). This was similar to the trend observed the previous year (Figure 2.15).

During the 2000-2001 season, grape skin was collected from both shaded and exposed fruit (Figure 2.23). Skin weight in the shaded fruit was lower than that recorded for the exposed fruit.
Skin weight was lower prior to veraison, but increased to levels comparable with those in the exposed fruit at around three to four weeks post-veraison. After that time, skin weight of the shaded fruit began to decline towards harvest. A similar decline was observed in the skin weight of exposed fruit, but this decrease occurred later (+5V) and was lesser in magnitude. Shaded berry skin decreased around 25% between the peak three weeks post-veraison and harvest, while exposed skin decreased around 15% from seven weeks post-veraison until harvest.

During the 2000-2001 season, seeds were collected from shaded as well as the exposed fruit. Seed weight per berry in shaded fruit was slightly lower than in the exposed fruit before veraison, but there was no difference in seed weight per berry at harvest (Figure 2.24). Around veraison, seed weight per berry was slightly higher in the exposed fruit. Immediately post-veraison, the weight of seeds per berry in exposed fruit began to decrease and reached a similar level to the slowly increasing seed weight per berry in the shaded fruit. Seed weight per berry continued to decrease until around three weeks post-veraison whereafter seed weight per berry in exposed fruit was more or less constant. From around one week post-veraison, seed weight per berry in the shaded fruit also began a slow decline towards veraison, mirroring the values of the exposed fruit in the last few weeks to veraison (+7V, +9V). Differences in seed weight per berry between treatments could be due to differences in seed number per berry. While seed number in the shaded fruit (2.70±0.08 seeds per berry, n=7 weeks) was slightly lower than that in the exposed

![Figure 2.24. Effect of shading treatment on Shiraz seed weight (g) per berry during 2000-2001](image-url)
fruit (2.93±0.04 seeds per berry, n=17 weeks), this difference was not significant in a
Student’s T-test (p=0.1117, α=0.05).

2.2.7. Shiraz Berries Shaded at Bud-burst 2000-2001 and Comparison of
Shading Treatments

The second shading experiment conducted during the 2000-2001 season arose from
observations of berry development and flavonoid accumulation made during the 1999-
2000 season. These observations (Chapter 5) prompted the question of what effect would
complete light exclusion throughout shoot, inflorescence and berry development have on
flavonoid composition. Complete light exclusion of the whole cane in the field was
impractical at this time given the constraints of the shade box. Nevertheless, buds were
boxed before bud-burst and later the boxes were rearranged in darkness to enclose only
the inflorescence. Initially, 45 boxes were applied however, practical difficulties greatly
reduced this number. While it was possible to take several small samples during the
growing season (Table 2.2), obtaining a representative sample at harvest rather than a
developmental series was considered more valuable. Nonetheless, at harvest only five
boxes from the original experiment had survived.

At harvest, the weight per berry of the exposed fruit was 0.96 g/berry based on a sample
of 100 berries. A similar sample of berries from the shading treatment applied before
flowering showed berry weight to be 0.87 g/ berry (n=90), while those from the shading
treatment applied prior to bud-burst weighed 0.95 g/berry (n=100).

The levels of total soluble solids in the exposed fruit and fruit from each of the shading
experiments conducted in 2000-2001 are presented in Table 2.4. At harvest, there was no
significant difference in total soluble solids (°Brix) between the exposed fruit and the fruit from the shading treatment applied prior to bud-burst (Experiment 1) using Student’s T-test (p=0.3672, α=0.05). However, there was a significant difference in total soluble solids between the exposed fruit and that from the shading treatment applied prior to flowering (Experiment 2) (p=0.0001, α=0.05). No significant difference was found in the level of total soluble solids (°Brix) between shading treatments (p=0.6243, α=0.05).

Table 2.4. Difference in total soluble solids (°Brix) at harvest between exposed fruit and fruit from two shading experiments, showing standard deviation (SD) and number of berries sampled (n). **Experiment 1**: Shading treatment applied to canes prior to bud-burst; **Experiment 2**: Shading treatment applied to inflorescences prior to flowering (Table 2.2).

<table>
<thead>
<tr>
<th></th>
<th>Exposed</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>°Brix</td>
<td>29.30</td>
<td>27.83</td>
<td>27.35</td>
</tr>
<tr>
<td>SD</td>
<td>2.29</td>
<td>2.39</td>
<td>1.52</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>

2.2.8. Shiraz Berry Development 2001-2002

The change in Shiraz berry weight during the 2001-2002 season is shown in Figure 2.24. In the third experimental season at the Willunga site (2001-2002), the classic pattern of berry growth was much less pronounced than in either of the previous two seasons (Figures 2.10 & 2.18). Berry weight increased slowly from the first collection two weeks after fruit-set (-8V), which had occurred two weeks after flowering...
(-10V). After veraison, berry weight began to increase more steeply reaching a maximum (1.37 g/berry) four weeks post-veraison. Berry weight then declined slightly towards harvest (1.16 g/berry), appearing at first to drop then rise again. This may have been related to an irrigation event as no rainfall was recorded at this time. This final sample represented a five-fold increase in sample size with five replicates of 50 berries rather than a single sample of 50 berries.

Total soluble solids (°Brix) were measured from three weeks after fruit-set during the 2001-2002 season (Figure 2.25). The level remained virtually unchanged before veraison, but increased sharply from veraison towards harvest. As seen in both of the previous seasons (1999-2000, 2000-2001), sugar accumulation in the weeks immediately prior to harvest was coincident with decreases in berry weight due to water loss.

Apart from berry weight and °Brix, only skin weight was monitored throughout berry development in the 2001-2002 season (Figure 2.26). Skin weight increased slowly from the initial sample towards veraison, whereafter skin...
weight increased sharply until three weeks post-veraison. Skin weight then remained stable, although an increase was observed in the final week to harvest.

Seeds were collected at harvest from 450 berries for each treatment. Total seed weight per berry at harvest was 0.046±0.002, with 1.78±0.034 seeds per berry, each seed weighing 0.026±0.001 grams.

2.2.9. Shiraz Berries Shaded Pre-Flowering 2001-2002

The effect of shading on berry weight during the third experimental season (2001-2002) is shown in Figure 2.27. There was virtually no difference in berry weight throughout development during the 2001-2002 season with berry weight increasing steadily from fruit-set towards harvest in both the exposed and shaded fruit. The increase in the weight of the exposed berries between three and four weeks post-veraison was not observed in the shaded fruit and suggests a sampling error in the exposed sample.

Similarly, there was no discernible difference in the level of total soluble solids.
(°Brix) between the exposed control and shaded berries (Figure 2.28) at any time during berry development in the 2001-2002 season. At harvest, there was no significant difference in total soluble solids between the shaded and exposed fruit (Table 2.5).

Skin weight throughout berry development was monitored for both the exposed and shaded fruit (Figure 2.29). Towards harvest in the third season (2001-2002) there were slight differences in skin weight between the exposed and shaded fruit. The weight of the grape skin from the shaded fruit increased slightly more than that of the exposed fruit, but then declined to a level lower than the exposed fruit. The practice of measuring bulk skin weight rather than individual weights during development, while expedient, precluded statistically testing differences in skin weight. At harvest, the replicates for winemaking purposes were sampled and skin weight, seed weight and total soluble solids determined for both the shaded and exposed fruit. This allowed statistical analysis of the differences observed. At harvest, skin weight in the shaded fruit, while slightly lower, was not significantly different from that of the exposed fruit (Table 2.5).

At harvest, there was a significant difference in seed weight between the shaded and exposed fruit (Table 2.5). Seed weight per berry was significantly higher (p<0.007) in the shaded fruit than it was in the control bunches, which had been exposed to normal light levels. The difference in seed weight per berry was due partly to the significantly greater
weight of each seed (p=0.048), but also a higher seed number per berry in the shaded fruit (p=0.024).

Table 2.5. Harvest data for exposed and shaded fruit 2001-2002 showing average, standard deviation and standard errors with p-value for Student’s T-test for difference between means (α=0.05). All weights in grams (g); number of samples (n) represents number of 50 berry sub-samples from each treatment.

<table>
<thead>
<tr>
<th>Berry Weight</th>
<th>°Brix</th>
<th>Skin Weight</th>
<th>Seed Weight per Berry</th>
<th>Seed Weight</th>
<th>Seed Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exposed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>1.161</td>
<td>26.356</td>
<td>0.443</td>
<td>0.046</td>
<td>0.026</td>
</tr>
<tr>
<td>SD</td>
<td>0.069</td>
<td>1.109</td>
<td>0.078</td>
<td>0.007</td>
<td>0.003</td>
</tr>
<tr>
<td>SE</td>
<td>0.018</td>
<td>0.370</td>
<td>0.026</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td><strong>Shaded</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>1.101</td>
<td>26.489</td>
<td>0.379</td>
<td>0.066</td>
<td>0.034</td>
</tr>
<tr>
<td>SD</td>
<td>0.129</td>
<td>0.824</td>
<td>0.091</td>
<td>0.013</td>
<td>0.009</td>
</tr>
<tr>
<td>SE</td>
<td>0.033</td>
<td>0.275</td>
<td>0.030</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>T-test</td>
<td>0.122</td>
<td>0.786</td>
<td>0.185</td>
<td>0.007</td>
<td>0.048</td>
</tr>
</tbody>
</table>

2.2.10. Seasonal Differences in Shiraz Berry Development

Table 2.6 shows the flowering, veraison and harvest dates for Shiraz in a commercial vineyard in Willunga, SA. Considerable variation was observed in the timing of flowering over the three seasons examined here (1999-2000, 2000-2001 & 2001-2002). Data collected from this site during the past decade indicates that flowering usually occurred in the second or third week of November (Table 2.6) and the period from flowering to veraison was generally nine weeks. Harvest commonly occurred around 16 weeks post-flowering, although this varied with season and other unrelated factors such as winery capacity. Thus, 16 weeks post-flowering has been used here to compare ripeness, as measured by sugar accumulation (°Brix), between seasons.
In the first season (1999-2000), flowering occurred earlier than previously reported, the time from flowering to veraison was nine weeks, thus veraison was also earlier. However, by 16 weeks post-flowering, °Brix indicates that these fruit had reached a similar level of ripeness to fruit in other seasons at this site (Table 2.6). In the third season (2001-2002), flowering occurred relatively late and the time to veraison was longer than in previous years. However, by 16 weeks post flowering, °Brix was the highest reported at that time.

The highest sugar level recorded was 29.3°Brix at harvest in 2000-2001.

<table>
<thead>
<tr>
<th>Season</th>
<th>Flowering</th>
<th>Weeks to Veraison</th>
<th>Veraison</th>
<th>°Brix at 16 Weeks Post-Flowering</th>
<th>Weeks to Harvest</th>
<th>Harvest</th>
<th>°Brix at Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996-1997</td>
<td>21-Nov-96</td>
<td>9</td>
<td>31-Jan-97</td>
<td>20</td>
<td>29-Mar-97</td>
<td>23.8</td>
<td></td>
</tr>
<tr>
<td>1997-1998</td>
<td>18-Nov-97</td>
<td>9</td>
<td>23-Jan-98</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1998-1999</td>
<td>28-Jan-99</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999-2000</td>
<td>04-Nov-99</td>
<td>9</td>
<td>06-Jan-00</td>
<td>23.5</td>
<td>7</td>
<td>24-Feb-00</td>
<td>23.5</td>
</tr>
<tr>
<td>2000-2001</td>
<td>16-Nov-00</td>
<td>8</td>
<td>11-Jan-01</td>
<td>25.0</td>
<td>9</td>
<td>13-Mar-01</td>
<td>29.3</td>
</tr>
<tr>
<td>2001-2002</td>
<td>23-Nov-01</td>
<td>10</td>
<td>31-Jan-02</td>
<td>26.4</td>
<td>6</td>
<td>15-Mar-02</td>
<td>26.4</td>
</tr>
</tbody>
</table>

Data collected 1993 to 1998 courtesy of Dr. Chris Davies, CSIRO Plant Industry, Adelaide Laboratory

Shiraz berry development as measured by berry weight for the three seasons 1999-2000, 2000-2001 and 2001-2002 is shown in Figure 2.30. Berry weight in each season followed a similar pattern, although in the third season (2001-2002) berry weight was lower than in the two previous seasons.
Despite berry weight being lower in 2001-2002 than in previous seasons, the pattern and level of sugar accumulation (°Brix) was virtually identical (Figure 2.31). The final harvest level of total soluble solids (°Brix) in the second season was higher than in other seasons. However, at six weeks post veraison, the harvest date for the third season, the levels were similar across the three seasons. Nevertheless, a Student’s T-test of the mean values for total soluble solids at 16 weeks post-flowering showed a significant difference between each season (1999-2000 23.47±0.24°Brix; 2000-2001 24.96±0.23°Brix; 2001-2002 26.39±0.37°Brix).
Shiraz skin, collected during two seasons (2000-2001 and 2001-2002), showed a similar pattern of increasing skin weight. Prior to veraison, both berry weight and skin weight were higher in the 2000-2001 season. Towards harvest, berry weight was slightly higher in 2000-2001, while skin weight was lower.

Shiraz seeds were collected throughout berry development during the 1999-2000 and 2000-2001 seasons and at harvest in 2001-2002. Seed weight per berry was lower during the 1999-2000 season than it was during 2000-2001, however at harvest seed weight per berry was lowest in 2001-2002. Despite lower seed weight per berry during 1999-2000, individual seeds weighed more than in 2000-2001, thus the lower seed weight per berry in 2001-2002 was due to smaller rather than fewer seeds. Based on samples of 50 berries per week, average seed number in 2000-2001 (2.93±0.043, n=17 weeks) was higher than during the previous season, 1999-2000 (1.54±0.033, n=13 weeks), or the following season, 2001-2002 (1.78±0.043, n=9 replicates of 50 berries at harvest).

2.2.11. Seasonal Differences in Temperature

Temperature in the vineyard was recorded throughout each of the three experimental seasons. The data logger (TinyTag™) was attached to the vine trellis on the first visit to the site each season. The temperature was logged at 15 minute intervals and the readings for each 24 hour period from midnight were used to determine the mean daily temperature and the daily maximum and minimum. Figure 2.32 shows these data for the 1999-2000 season in Willunga, SA with flowering and veraison indicated by red data points.
It is interesting to note that there were several days during the 1999-2000 season when the daily mean approaches the minimum temperature at which grapes metabolise, 10°C (Winkler et al., 1974; Jackson & Spurling, 1988; Jackson & Lombard, 1993; Boulton et al., 1998). The lowest daily mean was 9.8°C on 08 November 1999, just four days after flowering. In addition, during the early part of the season, daily minima were often recorded below 9°C with several of these occurring in the period immediately post-flowering (Figure 2.32). On a large number of days, the daily maximum exceeded 30°C above which grape metabolism is also reduced (Coombe, 1987b), with some daily maxima exceeding 40°C. Post-veraison, there were several days when the mean daily temperature was above 30°C.

In the second season, 2000-2001, sampling commenced with bud-burst in mid-August (Figure 2.33). At the time of flowering, 16 November 2000, the mean daily temperature was around 23°C with daily maxima at that time frequently exceeding 30°C. This was in.
contrast to the previous season when temperatures were low around flowering. While daily minima below 9°C were recorded during berry development, the first of these did not occur until two weeks after flowering, 30 November 2000. During the 2000-2001 season, numerous days had mean temperatures above 30°C with daily maxima frequently exceeding 40°C.

![Figure 2.33. Main daily temperature in Willunga, SA during the 2000-2001 season, vertical bars show daily maximum and minimum. Red data points at 16 November and 11 January represent flowering (-8V) and veraison (V) respectively.](image)

In the third experimental season, 2001-2002, the temperature at the time of flowering, 23 November 2001, was around 15°C (Figure 2.34). This was lower than in 2000-2001, but not as low as during 1999-2000 (Figure 2.32). Daily minima were frequently less than 9°C and maxima greater than 30°C, but at no point during the season did the mean daily temperature fall below 9°C, rarely in fact did it fall below 15°C. Furthermore, while the daily maxima exceeded 40°C on several occasions, the daily mean never exceeded 30°C and often did not exceed 25°C.
That 2001-2002 was a milder season than the two previous seasons is clear in Figure 2.35, where the mean daily temperature for each of the three seasons has been overlaid. Throughout the 2001-2002 season (black line, Figure 2.35), daily means were lower than in the previous seasons. Of the three seasons, the second, 2000-2001 (yellow line), was
the warmest with more hot days both during early berry development and post-veraison, with even the cooler days not recording temperatures as low as those experienced during 1999-2000 and 2000-2001. The first season, 1999-2000 (blue line), recorded more cool days and generally colder days during berry development than either of the two successive seasons.

2.2.12. Treatment Differences in Temperature

Temperature was also recorded in the boxes applied for the shading treatments during the three seasons, 1999-2000, 2000-2001 and 2001-2002. In the first season, 1999-2000 (Figure 2.36), box temperature was measured early in the season to test the box under field conditions. The data logger and a box were placed in the canopy and the temperatures recorded. The box was removed and the data downloaded approximately one week pre-flowering and then reapplied at flowering (04 November 1999).

During berry development, the temperature difference between the shaded box and in the canopy was approximately 0.5°C, with the box being slightly cooler. Unfortunately, temperature recording inside the box did not commence immediately after the application of boxes at flowering. The fault was detected during the first data transfer two weeks later and rectified.

In the following seasons, 2000-2001 and 2001-2002, the temperature inside the boxes was also lower than the ambient canopy temperature. Given the daily temperature range, the difference in mean daily temperature between the canopy and the box was not significant, however the difference in mean temperature was tested over three hour periods (n=12) during the coolest and warmest periods of the 1999-2000 season. On the morning of 23 October 1999 from 3:30 AM there was a significant difference (p<0.0001,
Figure 2.36. The effect of shading treatment on temperature in the field during the 1999-2000 season at Willunga, SA. Note: A preliminary trial commenced 20 September 1999 removed a week prior to flowering (04 November 1999), shading treatments were applied to berries at flowering, but temperature logging in the shade box experienced technical difficulties.

\( \alpha=0.05 \) in the temperature between the ambient canopy temperature (5.90±0.22°C) and the temperature inside the box (5.10±0.27°C). Later in the season, on 04 February 2000 between noon and 3:00 PM, the temperature difference between the canopy air temperature (38.7±0.26°C) and the box (37.7±0.27°C) was also significantly different (\( p<0.0001, \alpha=0.05 \)) using Student’s T-test.

During both the 2000-2001 and 2001-2002 seasons, the maximum temperature difference between the inside of the box and the ambient temperature in the canopy was less than 1.0°C. This difference was observed consistently throughout the season. Furthermore, this difference was consistent throughout each day despite large daily temperature ranges as seen during the 2000-2001 season or during extended hot periods (1999-2000) or cool periods (2001-2002).
These observations prompted a trial of the data logger under a range of temperatures. These included refrigeration (3.25±0.034°C), ambient indoor temperature (17.91±0.19°C) and ambient outdoor temperature (19.06±0.61°C) each run over a 48 hour period. In each case there was a significant difference (p<0.00001) between the logger and probe temperatures, which ranged from 0.5°C indoors, to 0.8°C under refrigeration. However, the greatest differences were recorded at high temperatures outdoors when during part of the day the logger and probe were exposed to direct sunlight. The difference between the mean temperatures over two days was around 3°C, while the difference for a given recording ranged from 15°C at the hottest part of the day to virtually zero when the ambient temperature was around 11-13°C and conditions were overcast. Thus, a significant warming effect was observed when the logger was in full sun. Except for those times, the differences between the logger and the thermocouple in the probe rarely exceeded 1.0°C throughout the trial. This trial of the logger and thermocouple probe also showed that the logger was less sensitive to temperature changes than the thermocouple, heating and cooling more slowly than the probe. Consequently, the greatest differences occurred when the temperature was highly variable. Nevertheless a statistically significant difference was observed in the field between the temperature recorded by the data logger and that recorded by the probe located inside the boxes.

2.2.13. Treatment Differences in Chlorophyll Accumulation

Chlorophyll levels of shaded fruit and fruit exposed to normal levels of light were determined for grape berries sampled throughout the 1999-2000 and 2000-2001 seasons. Figure 2.37A shows the concentration of total chlorophyll in developing Shiraz berries from the expanding inflorescence (-12V) until harvest (+6V). During the 1999-2000
season, chlorophyll concentration in whole berries was seen to decrease as the berry expanded. Figure 2.37B shows total chlorophyll per berry increasing with berry development. The main period of chlorophyll synthesis during berry development occurred from six to three weeks pre-veraison, which was coincident with the main period of berry growth (Figure 2.18). As the berry began to increase in size, chlorophyll per berry increased substantially in the exposed fruit, but only slightly in the shaded fruit. Field observations reflected these results with berries noticeably reduced in chlorophyll pigmentation (Figure 2.38).

During the second experimental season, chlorophyll levels were again determined in Shiraz berries from Willunga, SA over the main

![Figure 2.37. Total chlorophyll levels in shaded fruit (light green line) and fruit exposed to normal light (dark green line) in Shiraz berries during the 1999-2000 season in Willunga, SA. A) mg per berry; B) mg per gram fresh weight of berry (±SEM; n=3).](image)

![Figure 2.38. Shiraz berries pre-veraison showing differences in chlorophyll accumulation between bunches developing inside boxes (left) and those in the canopy exposed to normal levels of light (right) from the 1999-2000 season in Willunga, SA.](image)
period of berry development (Figure 2.39). In the exposed fruit the total chlorophyll per berry increased sharply with the initial period of berry growth, up until three weeks pre-veraison (Figure 2.39A). From one week pre-veraison until two weeks post-veraison, chlorophyll per berry in the exposed fruit began to decline as the berries increased in size. From three weeks post-veraison towards harvest chlorophyll again appeared to increase. In the shaded fruit, the total chlorophyll per berry was very low pre-veraison, but increased steadily from three weeks post-veraison until harvest. In both the shaded and exposed fruit, the concentration of chlorophyll per gram fresh weight of berry decreased with increasing berry size (Figure 2.39B). In the shaded fruit the minimum was reached three weeks pre-veraison, while in the exposed fruit this decline continued until around three weeks post-veraison. From three weeks post-veraison the chlorophyll concentration increased towards harvest in both shaded and exposed fruit.

![Figure 2.39](image_url)  
Figure 2.39. Chlorophyll accumulation in shaded (light green line) and exposed (dark green line) Shiraz berries in Willunga, SA during the 2000-2001 season, shown as A) mg per berry; and B) mg per gram fresh weight of berry (+SEM; n=3).

The apparent increase in chlorophyll post-veraison was unexpected. Subsequently, an opportunity arose to repeat this analysis on similarly treated fruit during the 2002-2003 season. Fruit of the cultivars Chardonnay and Shiraz at a field site in Sunraysia, Victoria (35° 74’ south, 142° 77’ east) were shaded using the same methods described above (Section 2.2.2). The shading treatment was applied around flowering and samples were
collected at weekly intervals throughout berry development from the exposed (control) fruit. Samples were collected from the shaded fruit less frequently pre-veraison to preserve the number of boxes available for sampling later in berry development. In Shiraz, the shading treatment was applied one week before flowering (-7V). Boxes were applied to Chardonnay bunches on the same date as Shiraz (29 November 2002), which was the time of flowering for Chardonnay (-6V).

In Shiraz berries from the 2002-2003 season (Figure 2.40A), chlorophyll was lower in the shaded fruit than in the exposed pre-veraison. The concentration in the exposed fruit approached a level similar level to that in the shaded fruit at veraison and remained the same in the period immediately post-veraison when the berries began to expand (data for berry weight not presented). Post-veraison there was again an apparent increase in chlorophyll concentration in both the shaded and exposed Shiraz fruit commencing from around three weeks post-veraison. In Chardonnay, the level of chlorophyll was also lower.

![Figure 2.40. A). Chlorophyll concentration in shaded and exposed Shiraz berry skins during the 2002-2003 season in Sunraysia, Vic. B). Chlorophyll concentration in shaded and exposed Chardonnay berry skins during the 2002-2003 season in Sunraysia, Vic. (+SEM; n=3).](image)
in the shaded fruit pre-veraison. The level of chlorophyll in the exposed fruit decreased towards veraison to reach a level similar to that in the shaded fruit by one week post-veraison (Figure 2.40B). Post-veraison, the chlorophyll concentration in both the shaded and exposed fruit of Chardonnay continued to decrease towards harvest.

2.3. Discussion

The work described here defines developmental differences in the fruit of *Vitis vinifera* between the cultivars Shiraz and Chardonnay as well differences in Shiraz berries between successive seasons. In addition, differences in berry development between Shiraz fruit exposed to normal levels of light within the canopy and artificially shaded fruit over three seasons have been examined and documented.

To achieve the shading treatment for these experiments a polypropylene box was designed to maximise airflow for constant temperature and humidity while excluding light. Measurements of the temperature between the shaded and exposed treatments showed that there was as much as 1.0°C difference in mean daily temperature between the boxes and the ambient temperature in the canopy. For a single reading, differences could be as great as 5.0°C, but rarely exceeded an average of 1-2°C over a three hour period at any time during the season, although these differences were significant. However, the temperature differences between the data logger and its external thermocouple under controlled conditions (Section 2.3.14) were commensurate with differences in field observations between the box and canopy. Thus, it was concluded that there was not a significant temperature effect created by the shading treatment.
Similarly, relative humidity was measured in and outside of the boxes to determine if the shading treatment affected humidity. While differences were observed between individual readings, there was no significant difference in relative humidity with shading when these readings were calculated as hourly averages. Thus, it was concluded that the boxes designed for the shading treatment had no significant impact on relative humidity.

While application of the shading treatment did not substantially alter temperature or humidity, the light regime was significantly changed. The box material transmitted less than 0.1% of light and there was not a significant level of PAR recorded inside the box. As a physiological test of light exclusion, chlorophyll was measured during berry growth over several seasons, 1999-2000, 2000-2001 and 2002-2003. In both shaded and exposed Shiraz berries chlorophyll was seen to decrease towards veraison, with the initial level of chlorophyll lower in the shaded fruit. This decline in chlorophyll pre-veraison was consistent with increases in berry size during that time and with the conclusions of Pandey and Farmahan (1977), that berry photosynthate makes a negligible contribution to berry growth. Post-veraison, chlorophyll appeared to increase in both shaded and exposed berries. This increase was unexpected as previous research has shown chlorophyll biosynthesis to be light induced (Zuker, 1972) and that the chlorophyll content of grape berries declines during ripening (Fougère-Rifot, et al., 1995b). This suggests that a process other than de novo chlorophyll biosynthesis was occurring post-veraison. It was considered possible that this observation could be attributed to chlorophyll degradation products that also absorb in the wavelengths measured for total chlorophyll. However, the molar absorptivities of these compounds are lower than those of the chlorophylls (Joslyn & Mackinney, 1938; Vernon, 1960; White et al., 1963). Thus, while the full extent of
chlorophyll degradation might be masked by the presence of these compounds, their presence would not account for the apparent increase in chlorophyll post-veraison.

Another possibility that might account for the apparent increase in chlorophyll post-veraison is the presence of anthocyanins in the skins of Shiraz berries during ripening. Observation of absorbance spectra of the 80% acetone extract from grape skins show a broad $A_{520}$ peak in absorbance that extends into the range at which chlorophylls absorb, vis 646 and 663 nm. In addition, a commercial standard of malvidin-3-glucoside (Extrasynthese, France) also showed absorbance at 646 and 663 nm. Furthermore, when the chlorophyll level was determined in the skin of shaded and exposed berries from the Chardonnay cultivar, there was no increase in chlorophyll concentration post-veraison. In fact, the chlorophyll concentration in both shaded and exposed fruit continued to decrease post-veraison, even after berry expansion ceased. This confirms chlorophyll degradation in grape skins post-veraison, and is consistent with recent research on chlorophyll fluorescence in grapes (During & Davtyan, 2002). Furthermore, the decrease in chlorophyll in Chardonnay grape skins post-veraison supports the supposition that chlorophyll degradation products are unlikely to account for the increased absorbance at 646 and 663 nm in Shiraz skin post-veraison. Comparison of absorbance spectra from Chardonnay and Shiraz extracts post-veraison and the absorbance spectrum of anthocyanins are convincing indications that the apparent increase in chlorophyll in Shiraz post-veraison is an artefact of anthocyanin accumulation in the ripening fruit. Thus, the lower level of chlorophyll in shaded fruit is further evidence that the light environment inside the boxes was below photosynthetically active levels.

The development of both Chardonnay (1999-2000) and Shiraz berries (1999-2000, 2000-2001 and 2001-2002), was seen to follow the classic pattern of berry development
(Winkler et al., 1974; Coome, 1992; Mullins et al., 1992; Coome & McCarthy, 2000). Excluding light from developing grape berries had little impact on sugar accumulation. The exception was a delay in ripening observed with shading at flowering during the first season. Dokoozlian and Kliewer (1996) reported such a delay, however in successive seasons no such delay was observed with sugar accumulation the same between treatments.

The effect of shading on berry development was less than initially anticipated with the greatest difference observed in berry weight during the first season (1999-2000). This result suggested that substantial differences would be observed in successive seasons, however this was not the case. During the first season, berry weight in both the fruit shaded at flowering and at fruit-set was lower than the exposed fruit, also the fruit shaded at flowering was lower in weight than that shaded at fruit-set. Furthermore, in the first season, veraison in the fruit shaded at flowering was delayed by approximately one week with respect to the exposed fruit and the fruit shaded at fruit-set. While shading has previously been reported to delay fruit ripening (Bureau et al., 2000), the delay in sugar accumulation, or ripening, and reduced berry size observed here was complicated by the incidence of “hen and chicken”, a phenomenon that also exhibits these characteristics (Cholet et al., 1998). During the first season, 1999-2000, the phenomenon known variously as millerandage (Fougère-Rifot et al., 1996) in France, “hen and chicken” (Stannard et al., 1974) in Australia or as shot berries elsewhere (Scienza, 1976; Kharjuria & Bakhshi, 1988), resulting in a high proportion of small under-developed berries, was observed in Shiraz. The cause of this condition is uncertain, although it has been shown that low temperature around flowering is a significant contributor to reduced fruit-set and abnormal ovule development (Ebadi et al., 1995a; Ebadi et al., 1995b). At the time of
flowering in the 1999-2000 season, the temperature in the field was low with several minima below 5.0°C recorded in the weeks either side of flowering (Figure 2.32) and this may have contributed to the phenomenon. The suggestion is that a proportionally greater number of the smaller berries may have been sampled from within the boxes during the 1999-2000 season contributing to the apparent difference in development, particularly in the absence of such an effect in subsequent seasons.

In addition, the observation that shading had no significant effect on berry development is in agreement with that of Haselgrove (1997), which reported no significant difference in sugar between exposed and shaded fruit. These results and previous reports indicate that sugar accumulation in the berry is largely independent of other process in the berry and the vine in general. Sugar levels across cultivars were similar throughout ripening with both Chardonnay and Shiraz berries reaching the same level of ripeness at the time of harvest (around 23.5°Brix, Figure 2.14). This level was consistent with reported values for the production of table wines (Boulton et al., 1998). Berries of the Chardonnay cultivar weighed less than those of Shiraz and seed weight per berry was also lower in Chardonnay. This was partly due to smaller seeds (by weight) and to lower seed number per berry. This relationship between seed number and berry weight has previously been documented in grapes (Cawthorn & Morris, 1982; Boselli et al., 1995; Boulton et al., 1998). Further, Cawthorn and Morris (1982) reported that while increasing seed number resulted in larger fruit, it did not substantially impact upon the accumulation of sugar in the fruit. This observation is consistent with the results presented here and suggests that sugar accumulation is independent of berry size and seed number and that the sink strength of the ripening fruit is considerable. In fact, it has been previously reported that, once begun, sugar accumulation is “massive and undeviating” unaffected even by
substantial defoliation, presumably due to mobilisation of carbohydrate reserves from roots or wood (Coombe, 1989).

Differences in berry weight between Shiraz and Chardonnay berries showed that Chardonnay berries developed slowly at first then more quickly towards veraison. In Shiraz, the more rapid period of growth occurred earlier in development and slowed towards veraison. This pattern of berry growth may be characteristic of the respective cultivars, or may reflect cooler temperatures earlier in the season (Figure 2.32), Chardonnay having flowered two weeks before Shiraz. That Shiraz berry growth is initially rapid and slows toward veraison is apparent from a comparison of berry weights over several seasons (Figure 2.30), suggesting that this is indeed the pattern for this cultivar. Increases in berry weight post-veraison exhibited the same pattern in both cultivars. This indicates that although Chardonnay berries were expanding more slowly, they accumulated sugar more quickly resulting in the same increase in weight as Shiraz.

Over the three seasons of sampling of Shiraz berries, while the patterns of berry development were similar, differences in development were observed. By weight, berries were larger in the first season (1999-2000) than either of the two following seasons with berry weight lowest in the third season (2001-2002). Berry weight increased most rapidly in the second seasons (2000-2001) and most slowly in the third season (2001-2002). As postulated with respect to the differences between Shiraz and Chardonnay, this more rapid increase in berry weight in 2000-2001 was possibly due to warmer temperatures in the second season (Figure 2.35). The generally lower temperatures during the third season (2001-2002) coincided with slower increases in berry weight, which further supports this notion.
Where deformability was measured during berry development in Shiraz and Chardonnay berries it was observed that the initial increase in deformability coincided with veraison, as defined by changes in sugar accumulation. In this regard, deformability is a valuable indicator of ripening, where the timing of changes in other parameters, such as berry weight, are not always synchronised with veraison. Apart from indicating veraison, measures of deformability during ripening are of little value as berry deformability is extremely sensitive to the water status of the vine. In addition, large diurnal fluctuations in grape berry deformability have been reported (Coombe & Bishop, 1980). Thus, berry deformability post-veraison seems to be a better indicator of turgor pressure than the progress of ripening.

As previously observed by other authors (Cawthorn & Morris, 1982; Boselli et al., 1995; Boulton et al., 1998) and as described above, there was a strong correlation between berry weight and seed weight and number. In the third experimental season examined here (2001-2002), berry weight was lowest and this corresponded to the lowest individual seed weight and lowest seed weight per berry. Of the first two seasons (1999-2000 and 2000-2001), seed weight was higher in the first year, but seed weight per berry lower as a result of lower seed numbers, nevertheless berry weight was greater. In the second year (2000-2001) the seeds were smaller than in the first year, but seed number was higher resulting in a higher seed weight per berry, yet berry weights were lower. These data indicate that individual seed size has a greater influence on berry weight than seed number. A few large seeds impact more on berry growth than many small seeds.

Seeds increased rapidly in weight from fruit-set towards veraison with as much as 80% of the final seed weight achieved three to four weeks pre-veraison. Seed weight then increased slightly towards veraison and decreased from veraison towards harvest. In the
first season (1999-2000), seeds did not reach their maximum weight until one to two weeks later than was observed in the following season (2000-2001) suggesting that season can effect seed development. The decrease towards harvest was consistent with the lignification and dehydration of seeds generally observed with ripening in many species and previously reported in grapevines (Raven et al., 1992; Kennedy et al., 2000b).

Despite significant differences in berry weight between seasons, the pattern and level of sugar accumulation (°Brix) in each season was similar. This observation lends support to the notion that sugar accumulation in the fruit is independent of other processes occurring in the berry during ripening, particularly other factors affecting berry growth. This is further reinforced by the observation that the timing of the second phase of berry development does not always coincide with the onset of sugar accumulation as seen with Shiraz berry weight during 1999-2000 (Figures 2.10 and 2.11).

Interestingly, despite berries being smaller in the third season (2001-2002) skin weight per berry was greater than in the previous season. It has previously been suggested that high light and temperature contribute to thicker skin in grapes (McDonald et al., 1998). In 2000-2001 the skin represented 28.3% of berry weight, while in 2001-2002 the skin accounted for 38.2% of total berry weight, these levels were relatively constant throughout the season from fruit-set until harvest. Such a calculation is sensitive to variations in grape water potential affecting berry weight, thus comparisons were also made of fruit at similar weights (=1.15 g) at around 24°Brix. Percent skin weight, as well as absolute skin weight, was higher in the 2001-2002 season than in the previous season (2000-2001). This is inconsistent with the suggestion that high light and temperature contribute to thicker skins because monthly average temperature and solar radiation
levels were both lower in 2001-2002 (Bureau of Meteorology data, not presented). In the second season (2000-2001), shading applied pre-flowering resulted in lower berry weights early in development, but little difference in berry weight or seed weight at harvest. Neither the timing of veraison, nor the accumulation of sugar appeared to be effected by shading during the second season. The only parameter that was noticeably and consistently effected by shading was skin weight, which was lower in the shaded fruit in both the 2000-2001 and 2001-2002 seasons. In the 2000-2001 season, skin from the shaded fruit represented 24.3% of berry weight, while skin from the exposed fruit accounted for 28.3% of berry weight. In the following season, shaded berry skin was 33.9% of berry weight and exposed skin 38.2%. These observations are consistent with the report of McDonald et al. (1998) that the skin of exposed fruit was thicker, as well as those of Haselgrove (1997) that skin weight was greater in exposed compared to shaded fruit. While this was not reflected in the meteorological data for 2000-2001 and 2001-2002 as described above, the empirical data presents a convincing argument for a strong positive relationship between light exposure and skin weight or thickness.

Here we have shown that grape berry development was largely unaffected by the light environment in the vicinity of the fruit with growth and ripening occurring normally in the absence of light. The main factors affecting berry development were cultivar and season, however shading did result in reduced skin weight. The significance of these observations will be discussed further in relation to flavonoid biosynthesis in *Vitis vinifera* fruit in the absence of light (Chapter 5).
Chapter 3. Analysis of Grapevine Tannins

3.1. Introduction

The term tannin, in grapes and wine, describes a range of polyphenolic compounds that includes flavan-3-ol monomers and proanthocyanidins. Proanthocyanidins are polymers comprised of flavan-3-ol terminal subunits and flavan-3,4-diol extension subunits as shown in Figure 1.7 (Goldstein & Swain, 1963; Geissman & Dittmar, 1965; Czochanska et al., 1979; Botha et al., 1982; Delcour et al., 1983; Haslam, 1998). These compounds have been reported in the seedcoat and skin of the grape berry (Fougère-Rifot et al., 1993; Amrani-Joutei et al., 1994; Thorngate & Singleton, 1994) and their structures and composition in grapes and wines has been studied extensively. However, most of these studies have focused on the flavan-3-ol and proanthocyanidin composition at harvest (Kantz & Singleton, 1990; Prieur et al., 1994; Escribano-Bailon et al., 1995; Souquet et al., 1996a, 1996b; Fuleki & Ricardo da Silva, 1997; de Freites et al., 1998), or changes during grape ripening (Czochanska et al., 1979; Romeyer et al., 1986; Katalinic & Males, 1997; Saint-Cricq de Gaulejac et al., 1997; de Freitas & Glories, 1999; de Freitas et al., 2000; Kennedy et al., 2000a).

The flavan-3-ols, catechin, epicatechin and epicatechin-gallate (see Figure 1.4) are readily detected in grapes and occur as both free monomers and terminal subunits in the seeds and skin of the grape berry. In grape seeds, catechin, epicatechin and epicatechin-gallate all commonly occur as terminal subunits, while in the skin the terminal subunit is primarily catechin (Romeyer et al., 1986; Prieur et al., 1994; Escribano-Bailon et al., 1995; Souquet et al., 1996a). The extension subunits catechin, epicatechin, epicatechin-
gallate and epigallocatechin are all detected in grapes, although epigallocatechin extension subunits have only been reported in the grape skin and not in the seedcoat (Tsai-Su & Singleton, 1969; Santos-Buelga et al., 1995; Escribano-Bailon et al., 1995; Moutounet et al., 1996; Souquet et al., 1996a; Cheynier et al., 1997). Total tannin content is reported to be significantly higher in seeds than skins, although polymer length is generally several-fold lower in the seeds (Prieur et al., 1994; Escribano-Bailon et al., 1995; Moutounet et al., 1996; Cheynier et al., 1997).

Previous studies of grape berry tannins have observed that substantial amounts of flavan-3-ols and proanthocyanidins are present in both the seeds and skins of the berry at veraison (Katalinic & Males, 1997; de Freitas & Glories, 1999; Kennedy et al., 2000b; Kennedy et al., 2001). In many such studies, determination of monomers, dimers and trimers has been made but the polymeric fraction was not analysed (Katalinic & Males, 1997; Jordão et al., 2001a; Mateus et al., 2001). In addition, molecular studies of anthocyanin biosynthesis have shown that genes encoding enzymes required for both anthocyanin and tannin biosynthesis are expressed prior to veraison, when anthocyanins are not synthesised (Boss et al., 1996a). This suggests that the expression of these genes might be related to the accumulation of other flavonoid products, such as flavan-3-ols and proanthocyanidins in the developing fruit.

Tannins have also been reported in other grapevine tissues. Tannins in the internodes of Vitis vinifera L. cv. Ugni blanc grapevine canes are reportedly highly condensed (Darne, 1982). Phenolic content was examined at flowering, veraison and leaf-fall with the highest levels observed at veraison. The lower phenolic content in the mature and lignified stems was likely a result of decreased extractability. In grapevine shoots of the cultivar Carignan, mainly monomers and oligomeric proanthocyanidins have been
reported (Boukharta et al., 1988; Boukharta et al., 1994). The dimers observed included both 4-6 and 4-8 linkages in the dimers B1 to B4 and B7 as well as two trimeric proanthocyanidins. Upon hydrolysis, both catechin and epicatechin appeared as terminal subunits with the extension subunits primarily epicatechin and epicatechin-gallate (Boukharta et al., 1994). However, Bourziex et al. (1986) had earlier reported the terminal subunits in stems to be around 94% catechin in the cultivar Alicante-Bouschet, considering the tannin composition of the stems analogous to that of the berry skin. This was consistent with the observations of Souquet et al. (2000) for the cultivar Merlot, with condensed tannins in stems around 80% catechin terminal subunits with >15% epicatechin and ~5% epicatechin-gallate. Extension subunits were about 75% epicatechin with epicatechin-gallate around 17%, catechin around 6.5% and the remainder epigallocatechin (Souquet et al., 2000). In grape stems, the average polymer length across a range of cultivars was around 8-10 subunits compared to around 10 in seeds and 30 in skins (Souquet et al., 2000).

Tannins are also reported in leaves of Vitis vinifera L. with catechin, epicatechin and epicatechin-gallate detected as well as the dimers B1 to B4, a trimer and tetramer (Bourziex et al., 1986; Boucheny & Brum-Bousquet, 1990). The most abundant compound in grapevine leaves was the B1 dimer (see Figure 1.5), with proportionally more of the monomers and terminal subunits being catechin. These authors also reported that the amount of proanthocyanidin material extracted from grapevine leaves decreased with increasing leaf maturity (Bourziex et al., 1986; Boucheny & Brum-Bousquet, 1990).

To date there have been no detailed studies of the flavan-3-ol and proanthocyanidin composition during early berry development and no investigation of both seeds and skins of the same fruit throughout development and few studies of the proanthocyanidin
composition of other grapevine tissues. This project examines the accumulation of flavan-3-ols and proanthocyanidins in vegetative and reproductive tissues throughout the growing season until commercial harvest in the major Australian winegrape cultivars, Shiraz and Chardonnay.

**3.2. Materials and Methods**

**3.2.1. Sampling of Grape Tissues**

Grape berry tannins were analysed in whole berries of Shiraz and Chardonnay, as well as Shiraz seeds during the first experimental season, 1999-2000. In the second season, 2000-2001 only Shiraz berries were analysed and these were dissected into seeds and skin and each component analysed separately. Tannins were also analysed in a range of vegetative tissues in Shiraz grapevines collected during the second season. Chardonnay and Shiraz berries were collected as detailed in Section 2.3.3. In the first season (1999-2000) approximately 50 whole berries each of Shiraz and Chardonnay were analysed from each sampling date (Table 2.1). These were frozen in liquid nitrogen in the field and stored at -80°C until analysed. In addition, seeds were dissected out of the Shiraz berries in the field from fruit-set until harvest during the 1999-2000 season. In the second season (2000-2001) Shiraz berries were dissected into seeds and skins in the field and each component frozen separately in liquid nitrogen and stored at -80°C until analysed. A sample of Shiraz berry flesh was also collected after veraison during the 2000-2001 season (01 February 2001; +3V).

Shiraz leaves were collected at five stages of development. The first stage was a newly emerged leaf less than 2 cm² and 0.09 g, densely villous with pigmented leaf margins. The second Stage was the next oldest leaf after Stage 1, approximately 5-6 cm² and
weighing 0.22 g, becoming glabrous on the dorsal surface of the leaf lobes. Shiraz leaf Stage 3 was approximately 15 cm² and 0.44 g, with noticeably reduced trichome density and a faint trace of anthocyanin pigment remaining in leaf epidermis. Stage 4 was an expanded leaf around 40 cm² and 0.99 g with glabrous dorsal, but villous ventral surface with a translucent appearance and no trace of pigmentation. The final leaf stage, Stage 5, was a fully expanded mature leaf approximately 160 cm² in area, weighing 3.62 g and no longer translucent. In addition, tendrils were also collected from Shiraz vines at flowering during the 2000-2001 season (16 November 2000). All tissues were frozen in liquid nitrogen and stored at -80°C until analysed.

3.2.2. Extraction of Flavan-3-ols and Proanthocyanidins

A number of methods have been proposed for the extraction of tannins from plant material. The solvation and diffusion of these compounds from the plant tissues is greatly increased if they are very soluble in the extractant, the concentration in the solvent relatively low and the temperature high. Further, extraction is generally slower from dried samples than from fresh material (Waterman and Mole, 1994). Several factors affecting the yield of tannins from plant material were examined preliminary to the analysis of experimental samples. As discussed above (Section 2.2.3) all analyses here were conducted on fresh samples and to increase extractability all samples were homogenised. In addition, the influence of solvent, time and temperature on yield were investigated.

A variety of solvents have been previously used to extract phenolic compounds from plant tissues, these have tended to utilise organic solvents such as ethyl acetate and diethyl ether (Ferrández de Simon et al., 1990; Pekic et al., 1998), but also aqueous alcoholic solvents such as ethanol (Kantz & Singleton, 1990; Alonso et al., 1991) and methanol (Cork & Krockenberger, 1991; Constantinides & Fownes, 1994; Sun et al.,
1998, Jordão et al., 2001a). In a survey of different solvents, Kallithraka et al. (1995) showed that methanolic extracts yielded proportionally more monomers, while ethanol tended to extract gallic acid. However, the most efficacious solvent system for the extraction of total tannins appears to be aqueous acetone ranging from 50% to 80% with the most popular composition being 70% acetone. This solvent has been used extensively in the analysis of grape proanthocyanidins (Cork & Krockenberger, 1991; Kallithraka et al., 1995; Kennedy et al., 2000a; Souquet et al., 2000; Kennedy et al., 2001).

Several durations of extraction were trialed as were several combinations of re-extraction and heating or cooling. Extraction for 12 and 24 hours in 70% acetone yielded the highest levels of both proanthocyanidins and catechins from all grape tissues. Subsequent extraction of the residue in fresh solvent yielded less than 10% of the initial 24 hour extraction, a third extraction of the same material yielded no detectable tannins. Extraction for 2 hours yielded around 70-80% of the yield from a 24 hour extraction. A second extraction for 2 hours of that material yielded approximately 10% of the yield from the initial extraction as did the third extraction. At this point repeated short extractions were considered too labour intensive and abandoned in favour of a single overnight extraction (15-20 hours). These extractions were carried out in a ten times volume (v/w) of solvent. The minimal yield with repeated extraction of the grape tissue residue after a 24 hour extraction suggested this to be a suitable volume to achieve a sufficiently steep diffusion gradient and was consistent with volumes reported elsewhere (Waterman & Mole, 1994).

The effect of temperature on the extraction of tannins from grape seeds was also tested. Commonly, tannin extracts have been made at room temperature, although both high and low temperature conditions have been utilised (Waterman & Mole, 1994). Extraction at
low temperature has been shown to increase the stability of extracted tannins (Cork & Krockenberger, 1991). Here, the frozen tissue (0.1 g) was extracted in 1.0 mL of 70% acetone overnight in a coolroom (4°C), this treatment made no difference to the yield of catechins and proanthocyanidins from grape seeds or skins. Further frozen samples were then heated to 50°C for 20 minutes followed by overnight incubation at room temperature. This method was shown to reduce catechins and proanthocyanidins by as much as 50% and resulted in large variations between samples during the subsequent HPLC analysis. In light of these observations, further temperature treatments seemed of dubious value.

In addition, light has also been reported to decrease tannin stability (Cork & Krockenberger, 1991) so extractions are commonly made in darkness. Some mixing has also been employed, almost universally, in the extraction of flavonoids and these practices were adopted here, as was the addition of an anti-oxidant in the form of 0.1% (w/v) sodium ascorbate (Kallithraka et al., 1995).

Each of these parameters was shown to effect the yield of proanthocyanidins from plant tissues. In addition to these considerations, it was also necessary to be able to compare results arising from this research with those of other workers in the field. For these reasons, and consistent with the investigations described above, all samples analysed here were extracted overnight in 70% acetone (v/v) plus 0.1% ascorbate (w/v) on a spinning wheel operating at 30 revolutions per minute in darkness at room temperature.

3.2.3. Analysis of Tannins

The acetone extract of grape seeds and skin contains both flavan-3-ol free monomers and the polymeric proanthocyanidins. The concentration of free monomers was determined
by HPLC analysis of the acetone extract. Polymeric proanthocyanidins have, in the past, been determined by acid hydrolysis of the proanthocyanidins to yield anthocyanidins these measured by visible absorbance spectroscopy (Swain & Hillis, 1959; Czochanska et al., 1980). While this method can differentiate procyanidins from prodelphinidins, its limitation is that both catechin and epicatechin generate cyanidin upon acid hydrolysis, while epigallocatechin yields delphinidin (Swain & Hillis, 1959; Butler, 1982; Czochanska et al., 1980). A less stringent degradation of the proanthocyanidin polymer releases the terminal and extension subunits, however the extension subunits are then present as reactive carbo-cationic species able to reform polymers or undergo separate side reactions (Botha et al., 1982; Stafford & Lester, 1984; Ricardo ad Silva et al., 1991b; Haslam, 1998). Thus, the cleavage of polymers has been conducted in the presence of a strong nucleophile. This has commonly involved acid catalysis with benzyl mercaptan (Hemingway et al., 1982; Porter, 1988; Souquet et al., 1996a; Sun et al., 1998). However, use of this thiolysis method has declined in many laboratories because of health and safety issues associated with the use of mercapto derivatives. In addition to the α-thiol, phloroglucinol is a strong nucleophile that has been employed in this field and a method was recently optimised for use with grape extracts (Kennedy & Jones, 2001). This is the method that has been used here to determine the subunit composition of proanthocyanidins.

Acid-catalysed cleavage of the proanthocyanidins in the acetone extract released the terminal subunits from the proanthocyanidin polymer (Figure 1.7) adding to the pool of monomers. The concentration of terminal subunits was determined by subtracting the concentration of monomers in the uncleaved sample from the monomers in the cleaved sample. Acid-catalysed cleavage of the proanthocyanidin polymer in the presence of
excess phloroglucinol (Kennedy & Jones, 2001) replaces the interflavan bond with a phloroglucinol moiety at the 4-position of the C-ring (Figure 1.7), from which the concentration of extension subunits was determined. The mean degree of polymerisation (mDP) was determined from the ratio of the total terminal plus extension subunits to terminal subunits as described by Butler (1982) and Guyot et al. (1999).

Thus, to extract and identify proanthocyanidins in grape tissues, frozen samples were ground to a fine powder under liquid N₂ and three separate 0.1 g aliquots (replicates) were extracted in darkness in 2 mL screw-top Eppendorf tubes for 24 hours with 70% acetone (1.0 mL) containing 0.1% ascorbate to prevent oxidation of polyphenols (Kallithraka et al., 1995). Samples were then centrifuged (15 min, 13,000 x g) and two 200 μL aliquots of the supernatant of each replicate were transferred to fresh tubes and dried down under vacuum at 35°C for 60 minutes. One of these aliquots was resuspended in 100 μL methanol acidified with 1% HCl then neutralised with 100 μL sodium acetate (200 mM, pH 7.5) for the analysis of free monomers. The other aliquot underwent acid-catalysed cleavage of the proanthocyanidins in the presence of excess phloroglucinol following the method of Kennedy & Jones (2001). Briefly, the dried sample was resuspended in 100 μL of phloroglucinol buffer (0.25 g ascorbate, 1.25 g phloroglucinol, 215 μL conc. HCl, 25 mL methanol) and incubated at 50°C for 20 minutes, then neutralised with 100 μL of sodium acetate (200 mM, pH 7.5) and centrifuged (15 min, 13,000 x g).

To determine the amount of proanthocyanidin remaining in the seed and skin residues after acetone extraction, the residue also underwent acid-catalysed cleavage in the presence of excess phloroglucinol (Matthews et al., 1997; Kennedy & Jones, 2001). The remainder of the supernatant from the acetone extraction was removed and the residue
dried under vacuum at 35°C for 60 minutes. The residue was then resuspended in 500 μL of the phloroglucinol reagent, incubated as above, neutralised with 500 μL of sodium acetate and centrifuged (15 min, 13,000 × g). The supernatant after centrifugation was then used for the determination of flavan-3-ols and proanthocyanidin subunits.

3.2.4. HPLC Method Development & Separation of Proanthocyanidins

Historically, chromatography on both paper and cellulose thin layers has been used to determine the composition of flavonoids in plant extracts (Bate-Smith, 1954; Markham, 1982; Anderson & Francis, 1985). While good separation and identification have been possible, difficulty has arisen in quantifying components using these methods. While quantitation has been possible using absorbance spectroscopy, high-performance liquid-chromatography (HPLC) employs both chromatographic separation and spectral determination of compounds and has been used extensively in the study of flavonoids (Williams et al., 1978; McCloskey & Yengoyan, 1981; Daigle & Conkerton, 1983; Nagel, 1985; Lazarus et al., 1999; Peng et al., 2001).

Numerous published separations using a range of solvents are available and several of these have been trialed here. Attempts at separation using acetic acid with a methanol gradient (Foo & Porter, 1983) proved relatively efficacious, but it took some time to develop a suitable method. Initially, a VydaTM (USA) C-18 reversed-phase column (5μm, 250 mm × 4 mm) was used, however after several months resolution decreased and repeatable results could not be achieved despite extensive efforts varying acid concentration (pH) and type (acetic, phosphoric, formic), temperature conditions, flow rate and mobile phase (methanol, ethanol, acetonitrile). At this point a Merck LiCrospherTM column (5μm, 250 mm × 4 mm) was borrowed from another research
group working on plant polyphenols and showed good potential for separation. A LiCrospher™ column was purchased and method development recommenced. A suitable separation for the flavan-3-ol terminal subunits and the phloroglucinol adducts of the extension subunits, produced during acid-catalysed cleavage of proanthocyanidins, was developed.

However, this method proved unsuitable for the separation of free monomers in the acetone extract as the polymer fraction routinely coeluted with epicatechin-gallate. Alternate conditions of acid, mobile phase and temperature were tested culminating in a method that employed phosphoric acid with an acetonitrile gradient. Baseline separation of the monomers from the polymer fraction was achieved with the aim of quantifying the unhydrolysed polymer fraction in the acetone extract. It was envisaged that this would enable an estimation of the percent conversion during acid-catalysed cleavage of the polymer and determination of the fraction resistant to cleavage under the conditions used here. This turned out to be fraught with difficulty as the polymer peak was later determined to contain a significant level of contamination with other phenolic compounds. At this point it was considered that the benefit in knowledge did not justify the effort to identify and quantify all of the components of the polymer fraction as the objective of the project had been to determine flux through the pathway with respect to the expression pattern of genes encoding biosynthetic enzymes observed by Boss et al. (1996a). Nevertheless, the method developed here was used to identify and quantify free monomers in the acetone extract of grapevine tissues.

A 200 µL aliquot each of the acetone extract, the acetone extract after acid-catalysed cleavage and, where applicable, the acid-cleaved residue of samples, was transferred to Sunbroker™ 250 µL reduced volume HPLC autosampler vials. Samples were run on a
Hewlett Packard HP 1100 high-performance liquid-chromatograph (HPLC) using a Merck (Germany) LiChrospher\textsuperscript{TM} C-18 (5\,\mu m, 250 mm $\times$ 4 mm) analytical column protected by an SGE (Australia) C-18 guard column.

A separate reversed-phase HPLC method was developed for the cleaved and uncleaved proanthocyanidin samples. Uncleaved samples: solvent A, 0.2\% (v/v) phosphoric acid, solvent B, 4:1 (v/v) acetonitrile:0.2\% phosphoric acid (gradient of solvent B: zero min, 0\%; 5 min, 10\%; 40 min, 10\%; 55 min, 17\%; 65 min, 19\%; 75 min, 19\%; 80 min, 100\%; 85 min, 100\%; 86 min, 0\%). Cleaved samples: solvent A, 0.2\% (v/v) acetic acid, solvent B, methanol (gradient of solvent B: zero min, 1\%; 40 min, 1\%; 120 min 30\%; 120.1 min, 100\%; 125 min, 100\%; 126 min, 1\%). For both methods, 25 $\mu$L of sample was injected and run at 25$^\circ$C with a flow rate of 1 mL/minute. Concentrations of free monomers and hydrolysed terminal subunits were determined from standard curves prepared from commercial standards of catechin, epicatechin and epicatechin-gallate obtained from Extrasynthese\textsuperscript{TM} (France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy & Jones, 2001). Polymer length was calculated as the mean degree of polymerisation (mDP), whereby terminal and extension subunits were summed and divided by terminal subunits (Butler, 1982; Guyot \textit{et al.}, 1999).

\section*{3.3. Results}

\subsection*{3.3.1. Identification of Flavan-3-ols and Proanthocyanidin Subunits}

Free monomers from the acetone extract of both seeds and skin from grape berries collected during the second experimental season (2000-2001), as well as whole berries from the first experimental season (1999-2000) were identified by comparison of their
elution time and absorbance spectra with those of commercial standards. Commercial standards were purchased from Extrasynthese™ (France) and chromatographed separately to determine elution times and to generate a library of absorption spectra. Figure 3.1 shows the separation of an acetone extract from grape seeds collected one week pre-veraison. The elution times of the free monomers catechin, epicatechin and epicatechin-gallate were consistent with those of the commercial standards. The peak eluting at 80 minutes contains polymers plus other phenolic compounds.

Following acid-catalysed cleavage of the proanthocyanidins in the grape seed, phloroglucinol adducts of the proanthocyanidin extension subunits were also observed in the HPLC analysis. Figure 3.2 shows peaks corresponding to the commercial standards of monomers and represent the sum of free monomers and terminal subunits released by acid-catalysed cleavage of proanthocyanidins. In addition, peaks corresponding to the phloroglucinol adducts of the extension subunits were also present (Figure 3.2). These
Figure 3.2. HPLC separation of Shiraz seed proanthocyanidin subunits one week pre-veraison (04 January 2000). Terminal and extension subunits after acid-catalysed cleavage in the presence of excess phloroglucinol. Terminal subunits catechin, epicatechin and epicatechin-gallate and phloroglucinol adducts of extension subunits were identified by their retention times with respect to the terminal subunits and by comparison of their absorbance spectra and retention times with those of purified phloroglucinol adducts provided Dr. Jim Kennedy (University of Adelaide; Kennedy & Jones, 2001).

In the separation of both the free monomers and proanthocyanidin subunits, the ascorbate added to the extraction solvent was observed to elute immediately after injection of the sample. In addition, excess phloroglucinol from the acid-catalysed cleavage was also observed to elute early in the separation (Figures 3.1 & 3.2). At the end of the separations of both the monomers and proanthocyanidin subunits a large peak was observed. It was thought that in the uncleaved sample this peak represented the polymeric fraction of the acetone extract, while in the samples following acid-catalysed cleavage this peak represented proanthocyanidins that were resistant to cleavage under the conditions used here. Consistent with this notion was the observation that the 'polymer' peak is
substantially larger in the uncleaved sample. Further, the HPLC method of Peng et al. (2001) utilised a similar solvent system and gradient to that employed here to separate the free monomers and identified this late eluting peak as containing “predominantly polymeric procyanidins”. Extensive discussions on this matter with Dr. Jim Kennedy concluded that with the method of extraction and sample preparation used here it would be difficult to conclude that this peak represented only proanthocyanidin polymers. The main contaminants likely to be present in this fraction were thought to be hydroxycinnamates and may represent a substantial fraction of this late eluting peak.

When a number of absorbance spectra are taken through this late eluting peak, it is apparent this does not represent a pure compound (Figure 3.3). Figure 3.3A shows five spectra through this late eluting peak in the uncleaved sample. Differences between these spectra suggest several compounds coelute under the chromatographic conditions at this time. Figure 3.3B shows spectra through the late eluting peak after acid-catalysed cleavage of proanthocyanidins. Differences between the spectra in Figure 3.3A and 3.3B suggest that some of this material has been modified.
Figure 3.4. HPLC separation of Shiraz skin proanthocyanidin subunits one week pre-veraison (04 January 2000). Terminal and extension subunits after acid-catalysed cleavage in the presence of excess phloroglucinol. Terminal subunits catechin, epicatechin and epicatechin-gallate and phloroglucinol adducts of extension subunits.

during catalysis of the proanthocyanidins. Similarities between the spectra in this peak before and after hydrolysis support the notion that some of this material may be resistant to cleavage under the conditions employed here.

The chromatographic separation of skin tannin extracts showed a similar pattern to that of the seed extracts with the exception of the acid-cleaved samples. In the cleaved skin samples, phloroglucinol adducts of epigallocatechin were also detected (Figure 3.4). As with the analysis of the seeds. Peaks representing ascorbate and phloroglucinol were observed to elute early in the separation, while a large peak eluting near the end of the run, may represent those elements of the grape skin polymer that were resistant to acid-catalysed cleavage by the method utilised here.
3.1.2. Whole Berry Analysis of Tannins 1999-2000

Initially, it was envisaged that a rapid whole berry analysis of tannins would be a useful tool for industry application. Thus, in the first experimental season, tannins were extracted from homogenised whole berries as described above (Section 3.2.2). Further, one of the aims of this project was to clone and characterise the gene encoding the biosynthetic enzyme leucoanthocyanidin reductase (LAR) that catalyses the reduction of leucoanthocyanidins to flavan-3-ols (Figure 1.6). Thus, the HPLC analysis primarily sought to identify products of LAR activity. Our current understanding of flavonoid biosynthesis suggested that only the flavan-3-ol free monomers and proanthocyanidin terminal subunits were products of LAR activity, thus the flavan-3-ols identified by HPLC subsequent to acid-catalysed cleavage in the presence of excess phloroglucinol would represent the sum of flux through this part of the pathway. Establishing the temporal pattern of accumulation of the products of LAR would focus efforts to clone the gene encoding this enzyme, assuming that this part of the pathway was transcriptionally regulated as appears to be the case with the expression of other genes involved in anthocyanin biosynthesis (Martin & Gerats, 1993; Sparvoli et al., 1994; Boss et al., 1996a).

Thus, the first analyses of tannins in this project, which were conducted on whole berries of the Chardonnay cultivar, used only acid-catalysed cleavage to determine the products of LAR. It readily became apparent, that rather more information regarding the general nature of tannin biosynthesis, accumulation and composition could be gleaned by additionally separating free monomers in the acetone extract thereby enabling a determination of mDP as well as giving an indication of the nuances of polymer formation during berry development. Furthermore, the distribution of flavan-3-ols
between monomers and polymeric terminal subunits has an organoleptic impact on the final wine, particularly with respect to bitterness and astringency (Horowitz, 1964; Noble, 1984; Glories, 1988; Thorngate, 1997).

Figure 3.5 shows the pattern of total extension subunit and flavan-3-ol accumulation in Chardonnay berries collected during the 1999-2000 season. On a per berry basis extension subunits accumulated steadily from flowering (-9V) towards veraison reaching approximately 75% of the maximum level two weeks pre-veraison (Figure 3.5A). This was followed by a further increase subsequent to veraison reaching a maximum one to two weeks post-veraison, then declining towards harvest. When expressed on a per gram fresh weight of berry basis, there was an increase in the concentration of extension subunits immediately following flowering (Figure 3.5B) with a decline from around fruit-set towards harvest, the greatest decrease occurring during the main period of berry growth (Figure 2.13). Total flavan-3-ols followed a similar pattern to that of the extension

![Figure 3.5](image)

Figure 3.5. Tannins extracted from Chardonnay whole berries (1999-2000) with SEM (n=3). A) Total extension subunits (mg/berry); B) Total extension subunits (mg/g fresh weight of berry); C) Total free monomers plus terminal subunits (mg/berry) referred to as total flavan-3-ols; D) Total free monomers plus terminal subunits (mg/g fresh weight of berry)
subunits, albeit at a lower level. Flavan-3-ols also reached a maximum per berry one to two weeks post-veraison followed by a decline (Figure 3.5C) with the concentration of flavan-3-ols highest around fruit-set (Figure 3.5D).

The pattern of total extension subunit and flavan-3-ol accumulation in Shiraz berries collected during the 1999-2000 season is shown in Figure 3.6. The overall trend in tannin accumulation in Shiraz was the same as that observed in the Chardonnay cultivar. Extension subunits per berry began to increase post-flowering (-9V) reaching a plateau around four weeks pre-veraison, which was slightly earlier than observed in the Chardonnay berries. This level remained relatively constant until veraison when a further increase in extension subunits was observed (Figure 3.6A). This level then decreased post-veraison to a level similar to that immediately pre-veraison. This level was maintained over the following three weeks then decreased towards harvest. The concentration of extension subunits in Shiraz over the 1999-2000 season showed the

Figure 3.6. Tannins extracted from Shiraz whole berries (1999-2000) with SEM (n=3). A). Total extension subunits (mg/berry); B). Total extension subunits (mg/g fresh weight of berry); C). Total free monomers plus terminal subunits (mg/berry); D). Total free monomers plus terminal subunits (mg/g fresh weight of berry)
same pattern as seen in Chardonnay, a steady increase following flowering reaching a maximum concentration seven weeks pre-veraison then declining rapidly during the first phase of berry growth (Figure 3.6B). This level was observed to vary slightly during the remainder of the season, but the overall trend was a decline in extension subunits towards harvest.

Similarly with the level of flavan-3-ols per berry, in Shiraz the pattern of an increase to around four weeks pre-veraison reflected that observed in Chardonnay. However, while Chardonnay berries continued to steadily accumulate flavan-3-ols from four weeks pre-veraison towards a maximum one week post-veraison, in Shiraz the level was more or less constant until veraison (Figure 3.6C). In the week following veraison, a sharp increase in flavan-3-ols per berry was observed in Shiraz, also reaching a maximum one week post-veraison. This level was relatively stable for the following two weeks then declined towards harvest. In Figure 3.6D the decline in flavan-3-ols per gram fresh weight of berry from seven to three weeks pre-veraison also reflects the first phase of berry growth (Figure 2.13). Post-veraison, the dramatic increase observed on a per berry basis is also observed as an increase in flavan-3-ol concentration. This increase occurs during the second phase of berry growth immediately post-veraison and must represent a period of intense flavan-3-ol synthesis at this time.

3.1.3. Tannin Composition of Whole Berries 1999-2000

Extension subunit composition in Chardonnay berries throughout berry development was predominantly epicatechin. Around veraison 70% of total extension subunits were epicatechin with epicatechin-gallate accounting for a further 25% and catechin making up the remaining 5% of total extension subunits (Figure 3.7A). While analysing samples collected during 1999-2000, epigallocatechin extension subunits were not observed. This
was partly due to the absence of a suitable standard, which when rectified indicated that these compounds were coeluting with other compounds requiring a modification of the original method to separate the epigallocatechin-phloroglucinol adducts from these non-tannin contaminants in the following season. In contrast, the composition of flavan-3-ols in Chardonnay berries showed similar levels of catechin, epicatechin and epicatechin-gallate throughout the season, although a slight increase in the proportion of epicatechin-gallate was observed post-veraison (Figure 3.7B).

![Graph A](image_url)

**Figure 3.7.** Subunit composition of Chardonnay whole berries (1999-2000) after acid-catalysed cleavage of proanthocyanidins. **A.** Extension subunit composition (mg/berry); **B.** Flavan-3-ol composition comprising free monomer and terminal subunits (mg/berry)
In berries of the Shiraz cultivar the composition of extension subunits was similar to that observed in Chardonnay berries, with epicatechin comprising the bulk of extension subunits (Figure 3.8A). Epicatechin subunits accounted for more than 80% of total extension subunits at veraison, with epicatechin-gallate extension subunits around 15% and catechin less than 5% of the total at that time. In contrast to the Chardonnay berries, the flavan-3-ol composition in Shiraz, while equally catechin, epicatechin and epicatechin-gallate prior to veraison, exhibited a marked increase in the proportion of epicatechin post-veraison (Figure 3.8B). While all flavan-3-ols were observed to increase

![Graph A](image.A.png)

![Graph B](image.B.png)

Figure 3.8. Proanthocyanidin subunit composition of whole Shiraz berries (1999-2000). A). Extension subunit composition (mg/berry); B). Flavan-3-ol composition, includes free monomers and terminal subunits (mg/berry)
post-veraison, the increase in epicatechin in the first week post-veraison represented an eight-fold increase compared with a three- to five-fold increase in catechin and epicatechin-gallate in the week following veraison.

While the analysis of Chardonnay berries from the 1999-2000 season only identified total flavan-3-ols and extension subunits following the cleavage of proanthocyanidins, free monomer content and composition was analysed in Shiraz berries collected during that season (Figure 3.9).

This analysis showed that the increase in total flavan-3-ols post-veraison was due to an increase in both the terminal subunits and the free monomers (Figures 3.9A & 3.9B). Strikingly, the pattern of free monomer accumulation was virtually identical to that of total flavan-3-ols (Figure 3.8). In both the terminal subunits and free monomers, the majority of the total increase is due to an increase in epicatechin, although catechin free monomers were also seen to increase substantially post-veraison (Figure 3.9B). What is not
apparent from this analysis is the relative contribution of the different tissues in the grape berry that contain tannins to this overall picture of tannin accumulation and composition in the whole berry.

3.1.4. Shiraz Seed Tannins 1999-2000

During the 1999-2000 season, seeds were dissected out from Shiraz berries and the tannin composition was determined from the acetone extract both before and after acid-catalysed cleavage of the proanthocyanidins, enabling the separate determination of free monomers, terminal and extension subunits. Figure 3.10 shows proanthocyanidin accumulation in grape seeds during berry development in the 1999-2000 season. Extension subunits accumulated in two phases, initially from fruit-set until three weeks pre-veraison, then a further phase occurred from one to two weeks pre-veraison until two weeks post-veraison (Figure 3.10A). From two
weeks post-veraison until harvest there was a decline of approximately 20% of total extension subunits. This general pattern early in seed development parallels that of the whole berry (Figure 3.6A), however seed extension units do not increase as dramatically post-veraison suggesting this increase occurs in the skin of the berry. Post-veraison the seed extension subunit decrease appears greater than that of the whole berry and this also suggests extension subunits may be synthesised in the skin at this time.

The pattern of terminal subunit composition was similar to that of extension subunits, however the initial phase of accumulation represented a smaller increase in subunits and the later phase a proportionally greater increase (Figure 3.10B). The highest level of terminal subunits, on a per berry basis, occurred two to three weeks post-veraison, followed by a decline towards harvest.

Free monomers in grape seeds in the 1999-2000 season increased slowly from fruit-set until two weeks pre-veraison when the rate of accumulation increased markedly (Figure 3.10C). Free monomers increased to a peak three weeks post-veraison whereafter a steady decline until harvest was observed. When considered together, the increase in terminal subunits and free monomers in the seed immediately post-veraison account for the majority of the increase in flavan-3-ols in the berry at this time.

3.1.5. Shiraz Seed Tannin Composition 1999-2000

Extension subunit composition in Shiraz seeds from the 1999-2000 season showed that epicatechin was the main extension subunit (Figure 3.11A), which was similar to the pattern observed for the whole berry (Figure 3.8A). Extension subunits in the seed represent only 30 to 40% of the total in the berry around veraison suggesting polymers in the seed to be shorter than those in the skin.
While terminal subunit analysis of the seed also showed epicatechin to be the predominant subunit, these were at most two-fold the level of catechin and epicatechin-gallate. Compare this with the ratios in the whole berry (Figure 3.8) and it becomes apparent that much of the disproportionate increase in epicatechin terminal subunits must
occur in the skin. Although it must also be considered that terminal subunits in the seed, at around 5-6 mg/berry two weeks post-veraison (Figure 3.9B), account for the bulk of terminal subunits in the whole berry, around 8 mg/berry two weeks post-veraison (Figure 3.8B). These observations further suggest that polymers in the seed must be substantially shorter than those in the skin, through a combination of fewer extension and more terminal subunits.

Similarly with the free monomer composition in the seeds of Shiraz berries, the post veraison increase in free monomers occurs largely in the skin with the level of free monomers detected in the seed around 80% of total free monomers in the whole berry (Figure 3.8C & Figure 3.9C). Also in common with the observed pattern in terminal subunits, the increase in epicatechin monomers was proportionally less than for the whole berry suggesting some epicatechin monomer formation in the skin at this time.


In the second experimental season, the general pattern of seed tannin accumulation was similar to that of the previous season (1999-2000). Figure 3.12 shows the level of extension and terminal subunits as well as free monomers calculated as mg/berry and mg/g fresh-weight of seed for the 2000-2001 season. Extension subunits per berry showed a rapid increase from fruit-set until four weeks pre-veraison (Figure 3.12A), whereafter the level remained relatively constant until two weeks pre-veraison when a further increase was observed over the following week. This was the highest level of extension subunits observed (5.07 mg/berry) and was around three times higher than either terminal subunits or monomers. There was a slight decrease in extension subunits at veraison whereafter the level remained relatively constant until eight weeks post veraison (3.16 mg/berry) with a decline to 1.57 mg/berry in the final week (Figure
3.12A). When viewed on a fresh-weight basis, extension subunits increased substantially in the first week after fruit-set and reached a maximum four weeks pre-veraison, then gradually declined until harvest (Figure 3.12B). A similar pattern was observed for terminal subunits per berry with an increase immediately following fruit-set (Figure 3.12C), with a second period of accumulation was observed from two weeks pre-veraison

![Graphs of extension and terminal subunits](image)

Figure 3.12. Total flavan-3-ols and proanthocyanidin subunits in Shiraz seeds 2000-2001 A. Extension subunits (mg/berry); B. Extension subunits (mg/g fresh weight of seed); C. Terminal subunits (mg/berry); D. Terminal subunits (mg/g fresh weight of seed); E. Free monomers (mg/berry); F. Free monomers (mg/g fresh weight of seed). Three separate aliquots of each sample were extracted, cleaved and analysed by HPLC. The data presented is the mean (+SEM) of the triplicate extractions.

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until veraison, thereafter declining towards harvest. The pattern per gram fresh-weight showed a more gradual increase towards veraison, after the initial sudden increase immediately following fruit-set (Figure 3.12D). The brief increase in terminal subunits around veraison was exaggerated in the pattern of free monomer accumulation both on a per-berry and per-gram fresh-weight basis (Figure 3.12E, 3.12F). The observed pattern of monomer accumulation was a slow increase until three weeks pre-veraison, when an eight-fold increase was observed between that time and the peak of monomer accumulation one week post-veraison. This was followed immediately by a sharp decline over the succeeding three weeks and a steady decline from that point until harvest.

3.1.7. Shiraz Seed Tannin Composition 2000-2001

Figure 3.13 shows the proanthocyanidin composition of seeds, on a per berry basis, throughout berry development. In grape seeds epicatechin was the major constituent representing around 65% of extension subunits, with epicatechin-gallate accounting for around 25% of extension subunits, while catechin represented less than 10% (Figure 3.13A). The relative proportion of each extension subunit remained relatively constant throughout berry development.

In comparison, there was a noticeable change in the relative proportions of catechin, epicatechin and epicatechin-gallate terminal subunits in the seed during berry development (Figure 3.13B). During the period of apparent synthesis of terminal subunits immediately prior to veraison, proportionally more catechin was produced than either epicatechin or epicatechin-gallate. At veraison, epicatechin represented 20% of total terminal subunits with catechin and epicatechin-gallate both around 40%. Catechin decreased post-veraison while epicatechin increased, after which the relative proportions
of terminal subunits varied little with epicatechin accounting for 40% and catechin and epicatechin-gallate both around 30% of total terminal subunits.

In the early period of seed development, the free monomer composition was mainly catechin. By three weeks pre-veraison, seed monomer composition was equally catechin, epicatechin and epicatechin-gallate (Figure 3.13C). From one week pre-veraison the level

Figure 3.13. 2000-2001 Proanthocyanidin composition of Shiraz seeds between fruit-set and harvest showing levels of individual subunits expressed as mg/berry (±SEM; n=3). A). Extension subunit composition; B). Terminal subunit composition; C). Free monomer composition
of all monomers began to increase, with a four-fold increase in epicatechin and a three-fold increase in catechin, but only a two-fold increase in epicatechin-gallate to a maximum one week post-veraison. After this peak, the concentration of all monomers declined. Both catechin and epicatechin-gallate decreased by 50% over the following two weeks, however epicatechin decreased more slowly and there was proportionally more epicatechin than other monomers until around eight weeks post-veraison. For the last two weeks until harvest the levels of catechin and epicatechin were the same, with epicatechin-gallate around 5% of total monomers.

3.1.8. Shiraz Skin Tannins 2000-2001

On a per berry basis, extension subunits in skin showed a steady increase from fruit-set until two weeks pre-veraison at which point a sharp increase was observed (Figure 3.14A). The greatest increase in extension units was recorded one to two weeks before veraison, when the level doubled from 1.7 to 3.4 mg/berry. Extension subunits per berry then remained static for the following two weeks when there was a further increase to the maximum level observed, around 4.0 mg/berry. This was followed by a steady decline to around five weeks post-veraison, after which the level was around 1.5 to 2.0 mg/berry. Considered on a per gram fresh weight basis, a substantial concentration of extension subunits was present in the developing berries at fruit-set (Figure 3.14B). The level of extension subunits per gram declined immediately after fruit-set, then increased steadily for the next four weeks with a sharp increase two weeks pre-veraison. A steady decline in extension subunits per gram was observed from one week pre-veraison until harvest.
Terminal subunits per berry exhibited a steady increase from fruit-set until two weeks pre-veraison when a sharp increase was seen, doubling terminal subunit level to 0.09 mg/berry. Subsequently there was a lag phase when the level of terminal subunits remained static for a two-week period followed by a further increase in terminal subunits to the maximum of 0.13 mg/berry. From this point terminal subunits declined steadily to

Figure 3.14. 2000-2001 Proanthocyanidin composition of Shiraz seeds between fruit-set and harvest showing levels of individual subunits expressed as mg/berry (±SEM; n=3). A). Extension subunit composition; B). Terminal subunit composition; C). Free monomer composition
around 50% of the maximum, thereafter remaining relatively constant at around 0.06 mg/berry until harvest (Figure 3.14C). As with the extension subunits, there was a substantial concentration of terminal subunits at fruit-set. This point represents the highest concentration of terminal subunits per gram of skin at any point during berry development (Figure 3.14D). There was a dramatic decrease as the berry started to develop, followed by a steady increase towards veraison. Another noticeable increase was observed immediately prior to veraison, whereafter the level remained relatively constant at around 0.45 mg/g fresh weight until three weeks post-veraison. The concentration of terminal subunits in the skin then declined to approximately 0.25 mg/g fresh weight at five weeks post-veraison, remaining constant around that level until harvest.

Monomer accumulation in the skin showed a steady increase from fruit-set to a peak of 0.04 mg/berry two weeks pre-veraison declining to around 0.02 mg/berry at four weeks post-veraison after which the level was relatively constant (Figure 3.14E). On a per gram fresh weight basis the highest level of monomers in skin also occurred at fruit-set followed by a sharp decrease from seven to six weeks pre-veraison. A less dramatic decrease was observed over the following week and then a small increase (Figure 3.14F). From four weeks pre-veraison the level was constant for two weeks then began a gradual decline until four weeks post-veraison, at which level it remained static until harvest.

3.1.9. Shiraz Skin Tannin Composition 2000-2001

In grape skins, epigallocatechin extension subunits were observed in addition to the catechin, epicatechin and epicatechin-gallate subunits detected in the seeds (Figure 3.15A). Extension subunit composition in grape skins was primarily epicatechin and epigallocatechin with the former slightly in excess and levels of both some twenty-fold greater than either catechin or epicatechin-gallate. The levels of epicatechin and
epigallocatechin showed a two-fold increase during the main period of synthesis one to two weeks prior to veraison. Post-veraison, both epicatechin and epigallocatechin extension subunits declined by 50% towards harvest, while the levels of catechin and epicatechin-gallate were virtually unchanged throughout berry development.

![Graph showing Shiraz skin composition between fruit-set and harvest showing levels of individual subunits and monomers expressed as mg/berry (±SEM; n=3). A). Extension subunit composition; B). Terminal subunit composition; C). Free monomer composition](image-url)
The composition of terminal subunits in the skins was primarily catechin, with low levels of epicatechin and epicatechin-gallate observed (Figure 3.15B). There was a two-fold increase in catechin during the period of synthesis commencing just prior to veraison and little change in the amount of epicatechin and epicatechin-gallate.

In the skins of the grape berry, the free monomer composition was also predominantly catechin (Figure 3.15C). Catechin accumulated steadily from flowering reaching a peak around two weeks prior to veraison. Subsequently, a decline accounting for around 50% of catechin monomers was observed. While grape skin monomers between fruit-set and two weeks post-veraison were mainly catechin, after this time there were similar levels of catechin and epicatechin due to a two-fold increase in epicatechin free monomers. The level of epicatechin-gallate monomers was low and remained relatively constant throughout berry development. The decrease in catechin free monomers in the grape skin coincided with an increase in catechin terminal subunits.

3.1.10. Shiraz Flesh Tannins 2000-2001

Tannins were extracted from a sample of grape berry flesh after veraison (01 February 2001; +3V) during the 2000-2001 season. Generally the levels of tannins detected in the flesh were lower than the level detected in the skin. While the levels of flavan-3-ols were similar between the flesh and skin, extension subunits and total tannins were 10-fold lower in the flesh than the skin and almost 100-fold lower than in the seed on a fresh weight basis.
Table 3.1. Tannins in the flesh of Shiraz grapes three weeks post-veraison (01 February 2001). Flavan-3-ol subunits, comprising proanthocyanidin terminal subunits and free monomers (mg/g fresh weight of flesh ± SEM; n=3), and proanthocyanidin extension subunits (phloroglucinol adducts) are shown, as well as total flavan-3-ols and extension subunits and total tannins in the flesh (mg/g fresh weight of flesh ± SEM; n=3)

<table>
<thead>
<tr>
<th></th>
<th>mg/g fresh weight</th>
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<tbody>
<tr>
<td>Catechin</td>
<td>0.049±0.013</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.229±0.026</td>
</tr>
<tr>
<td>Epicatechin-gallate</td>
<td>0.014±0.001</td>
</tr>
<tr>
<td>Catechin-phloroglucinol</td>
<td>0.010±0.001</td>
</tr>
<tr>
<td>Epicatechin-phloroglucinol</td>
<td>0.326±0.066</td>
</tr>
<tr>
<td>Epicatechin-gallate-phloroglucinol</td>
<td>0.030±0.004</td>
</tr>
<tr>
<td>Epigallocatechin-phloroglucinol</td>
<td>0.040±0.008</td>
</tr>
<tr>
<td>Free Monomer + Terminal Units</td>
<td>0.292±0.017</td>
</tr>
<tr>
<td>Extension Units</td>
<td>0.406±0.060</td>
</tr>
<tr>
<td>Total Tannins</td>
<td>0.698±0.044</td>
</tr>
</tbody>
</table>

Table 3.1 shows the content and composition of tannins in the flesh of Shiraz berries post-veraison. The sum of free monomers and terminal subunits, as products of LAR, is shown and of these the dominant compound was epicatechin accounting for around 78% of total flavan-3-ols. Extension subunit composition in the flesh was also predominantly epicatechin with around 80% of extension subunits being epicatechin. Epicatechin-gallate and epigallocatechin extension subunits were present at more or less equal levels, combined accounting for around 17.5% of the total, while catechin made up the remaining 2.5% of total extension subunits (Table 3.1).

3.1.11. Direct Acid-Catalysis of Seed & Skin Residues 2000-2001

The post-veraison decrease in tannin, in both seeds and skins, may have been the result of less efficient extraction from these tissues as the grapes ripened. While repeated extraction of the residues with acetone released only trace amounts of additional
proanthocyanidins (data not shown), direct acid-catalysed cleavage of the residue did yield significant amounts of proanthocyanidins. The amount of extension and terminal subunits released with direct acid-catalysed cleavage of the seed residue increased from veraison to harvest (Figure 3.16A, 3.16B). Acid-catalysis of the seed residue showed a three-fold increase in terminal subunits and an eight-fold increase in the yield of extension subunits from veraison to eight weeks post-veraison. The level of extension subunits in the residue at veraison was around 3% of that in the extract. By eight weeks post-veraison, levels of extension subunits in the residue were equivalent to 30% of the

![Figure 3.16](image_url)

Figure 3.16. Extension and terminal subunit levels present in the acid-cleaved acetone extract (□-SEM) of Shiraz grape seeds and skin and released from the residue by direct acid-catalysed cleavage following acetone extraction (■+SEM), at veraison, six and eight weeks post-veraison. A. Seed extension subunit yield from the acetone extract after acid-catalysis and from direct acid-catalysis of the residue; B. Seed terminal subunits; C. Skin extension subunits; D. Skin terminal subunits
extractable proanthocyanidins in grape seeds. Terminal subunits in the residue were also around 3% of the level in the extract at veraison, but equivalent to 20% by eight weeks post-veraison.

Conversely, acid-catalysis of Shiraz skin residues showed a decrease in the yield of proanthocyanidins with increasing maturity (Figure 3.16C, 3.16D). Direct acid-catalysis of the skin residue at veraison and eight weeks post-veraison showed a three-fold decrease in extension subunits in the residue and a two-fold decrease in terminal subunits in the residue during this time. In the skin at veraison, the level of extension subunits in the residue represented approximately 25% of that in the acetone extract while the level of terminal subunits was almost 50% of that in the acetone extract. By eight weeks post-veraison extension subunits in the residue were equivalent to 15% of the level in the acetone extract and terminal subunits had decreased to around 20%.

3.1.12. Polymer Length (mDP) in Grape Berries

In Shiraz seeds, mean proanthocyanidin polymer length (mDP) was relatively constant throughout berry development (2000-2001) at around five subunits (Figure 3.17A). From fruit-set to one week pre-veraison, polymer length remained between five and six subunits. An increase in terminal subunits at one week pre-veraison temporarily exceeded the accumulation in extension subunits resulting in a slight fall in polymer length to around four subunits between one week pre-veraison and veraison either through increased extension subunits, or through loss of terminal subunits, as appeared to be the case (Figure 3.13C). Following this decrease, mDP increased to five subunits and this level was maintained from two weeks post-veraison until harvest. In the 1999-2000 season, seed proanthocyanidin polymer length was slightly higher pre-veraison at around
6.5 subunits and declined towards veraison (Figure 3.17A). From that time until harvest polymer length remained between 3.5 and 4.0 subunits.

Shiraz skin polymers (2000-2001) increased in length during the phase of berry development immediately after fruit-set from around 25 to 40 subunits. Mean degree of polymerisation then remained relatively constant at around 30-40 subunits until veraison (Figure 3.17B). After veraison, mDP was seen to decrease gradually towards 30 subunits at four weeks post-veraison.

From that time, polymer length was around 25 to 30 subunits dropping to 20 subunits at harvest.

3.1.13. Extractable Tannins at Commercial Harvest

Commercial harvest of winegrapes normally occurs between 20-24°Brix (Boulton et al., 1998). In the Willunga vineyard at the end of the 1999-2000 season, the Chardonnay grapes were harvested six weeks post-veraison at 23.2°Brix, while the Shiraz was harvested seven weeks post-veraison at 23.5°Brix. At harvest total tannins in the
Chardonnay fruit was around 5.63 mg/berry (Table 3.2) with extension units comprising around 78% of the total. In the Shiraz grapes the total acetone extractable tannins in the berry was 4.88 mg/berry also with extension subunits accounting for 78% of the total (Table 3.1). Of the total extractable tannins 59% was extracted from the seed where extension subunits only accounted for around 68% of the total. With the breakdown of flavan-3-ols into free monomers and terminal subunits it is apparent the latter represent the bulk of these resulting in a mean polymer length in the seed at this time of around 4 subunits (see also Figure 3.17A).

In Shiraz grape berries in the following season, commercial harvest occurred nine weeks post veraison (29°Brix), which was approximately a week later than was usual for this vineyard and the fruit were noticeably shriveled. One week earlier at eight weeks post-

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<tr>
<td><strong>Whole Berry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension Subunits</td>
<td>4.36</td>
<td>3.78</td>
<td>4.47</td>
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<tr>
<td>Terminal Subunits</td>
<td>0.62</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Free Monomers</td>
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<tr>
<td>Total Flavan-3-ols</td>
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<td>1.11</td>
<td>1.24</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>5.63</strong></td>
<td><strong>4.88</strong></td>
<td><strong>5.71</strong></td>
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<tr>
<td><strong>Seed</strong></td>
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<tr>
<td>Extension Subunits</td>
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<td>3.16</td>
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<tr>
<td>Terminal Subunits</td>
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<tr>
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<td>0.28</td>
<td></td>
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<tr>
<td>Total Flavan-3-ols</td>
<td>0.93</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2.87</strong></td>
<td><strong>4.32</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Skin</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extension Subunits</td>
<td></td>
<td>1.31</td>
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<td>Free Monomers</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total Flavan-3-ols</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>1.39</strong></td>
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veraison, total soluble solids were 24.9\degree Brix and total extractable tannin in the berry was 5.71 mg/berry. Once again 78\% of this total was attributed to extension subunits, with terminal subunits comprising approximately 17\% and free monomers 5\% (Table 3.2). However, in the second season (2000-2001) seed tannins accounted for 75\% of total tannins. From the seed derived material the average polymer length was 4.6 subunits, while grape skin polymer length at this time was 22.8 subunits, with extension subunits in the skin accounting for almost 95\% of the extractable tannin in that tissue (Figure 3.17).

3.1.14. Tannins in Grapevine Leaves and Tendrils

![Graph A](image1.png)

![Graph B](image2.png)

Figure 3.18. Composition of flavan-3-ols in Shiraz leaves at five different stages of development as described in Section 3.2.1. A). Free monomers and terminal proanthocyanidin subunits expressed as flavan-3-ols (mg/g fresh weight of leaf +SEM; n=3); B). Free monomers and terminal subunits expressed as flavan-3-ols (mg/leaf +SEM; n=3)

Tannins were also extracted from the leaves of the grapevine (*Vitis vinifera* L. cv. Shiraz) with acetone (70\% v/v) and this acetone extract was subjected to acid catalysed cleavage of the proanthocyanidins in the presence of a molar excess of phloroglucinol. The composition of free monomers, and terminal subunits released through acid-catalysed cleavage of proanthocyanidins in the acetone extract, collectively termed flavan-3-ols, is presented in Figure 3.18.
Composition of flavan-3-ols in newly emerged and expanding leaves was predominantly epicatechin, while in the more mature leaves, Stages 3 and 4 as well as the fully expanded mature leaf, Stage 5, there were similar levels of each of the flavan-3-ols, catechin, epicatechin and epicatechin-gallate. The concentration of flavan-3-ols was highest in the youngest leaves, decreasing with leaf age (Figure 3.18A). However the total per leaf was seen to increase throughout leaf expansion suggesting ongoing synthesis (Figure 3.18B).

![Graph A](image)

![Graph B](image)

Figure 3.19. Composition of proanthocyanidin extension subunits in Shiraz leaves at five stages of development as described in Section 3.2.1. A). Extension subunits (mg/g fresh weight of leaf +SEM; n=3); B). Extension subunits (mg/leaf +SEM; n=3).

Extension subunits released from Shiraz leaf extracts during acid-catalysed cleavage had a similar composition to that of the skin of Shiraz berries with epigallocatechin present in addition to catechin, epicatechin and epicatechin-gallate (Figure 3.19). On a per gram fresh weight of leaf basis, extension subunit concentration increases with increasing leaf size and age from the newly emergent leaf until leaf Stage 4 (Figure 3.19A). However, in the fully expanded leaf, the concentration is lower than at any other time during leaf expansion, likely as a result of massive leaf expansion. The level of extension subunit adducts in Shiraz leaves on a per leaf basis appears very low in the newly emergent leaf compared to
the mature leaf (catechin 0.0022±0.0008; epicatechin 0.0223±0.0043; epicatechin-gallate 0.0052±0.0006; epigallocatechin 0.0075±0.0041 mg/leaf). While the concentration of extension subunits was seen to decrease substantially in the leaf as the leaf matured (Figure 3.19A) the total amount per leaf continued to increase (Figure 3.19B), indicative of ongoing extension subunit synthesis in the leaves.

This general pattern in both flavan-3-ols and extension subunits is confirmed in Figure 3.20, which shows total proanthocyanidins subunits in each of the five Shiraz leaf stages. The amount of both flavan-3-ols and extension subunits in leaves increased as the leaf expanded and matured. The level of proanthocyanidins in the newly emerged leaves (flavan-3-ols 0.012; extension subunits 0.037; total tannins 0.044 mg/leaf) was around 200-fold lower than that in the fully matured leaf. On a per gram fresh weight basis, the mature leaf contained a total of around 3.8 mg of total tannin, in the skin of a ripe grape berry the concentration was around 5.1 mg/g fresh weight of skin, while in the seed at commercial harvest the level was around 27.8 mg per gram fresh weight of seed.

Figure 3.20. Total proanthocyanidin subunits in Shiraz leaves at five stages of expansion from newly emerged to fully mature as described in Section 3.2.1. Total flavan-3-ols, total extension subunits and total tannins (mg/leaf +SEM; n=3) are shown
In addition to the leaves of the grapevine, tendrils were also collected and tannins extracted and analysed after acid-catalysed cleavage of proanthocyanidins. The results of this analysis can be seen in Table 3.3. The flavan-3-ols were largely epicatechin and epicatechin-gallate, each a little over 40% with catechin around 10-15% of the total. Extension subunits in the tendrils were predominantly epicatechin at around 75% of the total with the other extension subunits more or less equal (catechin 8%; epicatechin gallate 5%; epigallocatechin 12.5%). The level of total proanthocyanidin subunits in the tendril was similar to that observed in the skin of the mature berry, although the proportion of free monomers and terminal subunits (flavan-3-ols) was substantially greater. The relative proportion of extension subunits in the acetone extract of the tendrils was substantially less than that observed in either the seeds or skin of the berries or in the leaves.

Table 3.3. Tannin composition of Shiraz tendrils showing flavan-3-ol subunits, comprising proanthocyanidin terminal subunits and free monomers (mg/g fresh weight of flesh ± SEM; n=3). Also shown are proanthocyanidin extension subunits (phloroglucinol adducts) as well as total flavan-3-ols, total extension subunits and total tannins in the flesh of Shiraz grapes (mg/g fresh weight of flesh ± SEM; n=3)

<table>
<thead>
<tr>
<th></th>
<th>mg/g fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin</td>
<td>0.255 ± 0.042</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>0.835 ± 0.102</td>
</tr>
<tr>
<td>Epicatechin-gallate</td>
<td>0.852 ± 0.045</td>
</tr>
<tr>
<td>Catechin-phloroglucinol</td>
<td>0.336 ± 0.029</td>
</tr>
<tr>
<td>Epicatechin-phloroglucinol</td>
<td>2.986 ± 0.326</td>
</tr>
<tr>
<td>Epicatechin-gallate-phloroglucinol</td>
<td>0.212 ± 0.017</td>
</tr>
<tr>
<td>Epigallocatechin-phloroglucinol</td>
<td>0.514 ± 0.025</td>
</tr>
<tr>
<td>Total Flavan-3-ols</td>
<td>1.942 ± 0.189</td>
</tr>
<tr>
<td>Total Extension Subunits</td>
<td>4.048 ± 0.396</td>
</tr>
<tr>
<td>Total Tannins</td>
<td>5.990 ± 0.579</td>
</tr>
</tbody>
</table>
3.4. Discussion

Tannins extracted from whole berries of the *Vitis vinifera* cultivars, Shiraz and Chardonnay showed very similar patterns and levels of accumulation. In both cultivars, the total flavan-3-ols and extension subunits per berry reached their maxima around one week post-veraison, followed by a gradual decline towards the time when the fruit was harvested. The main difference between the two cultivars was that the total levels of tannins were slightly higher in Shiraz berries. In terms of their composition, both Chardonnay and Shiraz extension subunits were primarily epicatechin. In Chardonnay, flavan-3-ols in the whole berry were equally catechin, epicatechin and epicatechin-gallate, while in Shiraz, particularly post-veraison, there was proportionally more epicatechin. The similarity in the pattern and timing of tannin accumulation across the two cultivars studied here suggests that tannin biosynthesis in grape berries is a developmental process and likely the pattern throughout the Vitaceae. However, the differences in total tannins and tannin composition observed here indicates the possibility of considerable variability in composition across the family. While the extent of this diversity has yet to be established, there is present the potential to alter wine tannin profiles through a variety of strategies. These might include blending of wines from cultivars with novel or desirable tannin profiles, purifying tannins from such cultivars for use as a wine additive as well as the strategic breeding or engineering of new varieties with idealised or novel tannin profiles.

Much of the quality of red wine derived from its tannin content and composition is dependent on the distribution between monomers and polymers, on the length of polymers and on their associations with other compounds, which effects their stability and solubility in the wine. HPLC analysis of the acetone extract of grape tannins before
as well as after acid-catalysed cleavage partitions the flavan-3-ol fraction into polymeric terminal subunits and free monomers, the monomers being significantly more bitter in the subsequent wine (Noble, 1994). This analysis of Shiraz whole berry extracts showed that a large proportion of the flavan-3-ols present pre-veraison were terminal subunits. This analysis also suggested that the post-veraison increase in flavan-3-ols was equally distributed between terminal subunits and free monomers generally. While both of these decrease post-veraison, the inference from previous studies and this analysis is that monomers become incorporated into polymers and that the extractability of polymers from the grape decreases with increasing grape maturity as discussed below. What is not apparent from the whole berry analysis is the relative contribution of the seed and the skin to the pool of tannic material extracted from the whole berry.

In grape seeds, tannin biosynthesis and accumulation commenced with seed development. From the first time that seeds could be readily dissected from the remainder of the berry, one week post-flowering, significant levels of proanthocyanidins could be detected suggesting that tannin biosynthesis commences in the earliest stages of seed development. The tannin composition of grape seeds consisted primarily of epicatechin extension subunits the majority of which were formed in the three weeks following fruit-set. The bulk of tannin synthesis in grape seeds occurred immediately after fruit set and was complete five weeks before veraison. Both extension and terminal subunits increased at this time and little free monomer was detected (Figure 3.10). A second phase of tannin accumulation commenced just prior to veraison and the maximum levels of proanthocyanidins were observed at veraison. These observations are consistent with and expand upon previous reports that suggest a substantial amount of proanthocyanidin material is present in grape seeds prior to veraison (Romeyer et al., 1986; Katalinic &
Males, 1997; Kennedy et al., 2000a; Kennedy et al., 2000b; Jordão et al., 2001b). The second phase of proanthocyanidin accumulation in seeds was the result of an increase in terminal subunits and monomers. A similar increase in both terminal subunits and free monomers was reported by Kennedy et al. (2000b) with comparable proportions of individual subunits, although the timing was one to two weeks later than that reported here. If free monomers were being incorporated into polymers, then the level of terminal subunits would be expected to continue to increase as free monomers decreased. Such being the case polymer length would decrease, as observed by Kennedy et al. (2000b). While we observed such a decrease in polymer length in the previous season (1999-2000), it was not seen during 2000-2001. This suggests that a substantial increase in extension subunits occurred at this time that was sufficient to maintain a constant polymer length.

The post-veraison decrease in both terminal subunits and free monomers that was observed over two seasons appears to represent a net loss of flavan-3-ols. Similar increases in monomers around veraison followed by a significant decrease post-veraison have been previously reported (Romeyer et al., 1986; Kennedy et al., 2000b). The decrease in monomers was accompanied by a decrease in proanthocyanidin polymer length suggesting their conversion to terminal subunits. However, at this time both terminal and extension subunits also declined. This decrease in proanthocyanidins post-veraison has been widely reported and while turnover or degradation are possible explanations, no direct evidence of such metabolism has been reported in plants. Several authors have suggested this decrease could be attributed to reduced extractability resulting from the conjugation of proanthocyanidins with other cellular components (Amrani-Joutei et al., 1994; Cheynier et al., 1997; Saint-Cricq de Gaulejac et al., 1997).
Further, Kennedy et al. (2000b) asserted that oxidative cross-linking of polymers would decrease their extractability. The inference drawn from these observations is that a substantial amount of proanthocyanidin remains in the residue after extraction and the proportion of non-extractable material changes during berry development (Figure 3.16).

Direct acid-catalysis of the grape seed residue remaining after extraction with acetone supported this hypothesis as an increase in the yield of both terminal and extension subunits was observed in the residue post-veraison (Figure 3.16). The yield of proanthocyanidin extension subunits from the residue increased with maturity to the extent that the sum of extractable extension subunits and those released from direct acid-catalysis of the residue remained constant after veraison. These results suggest that association of proanthocyanidins with the residue accounts for the decrease in extension subunits post-veraison. However, the sum of extractable and residue derived extension subunits (around 4.0 mg/berry) was at no time equivalent to the maximum level of extension subunits at one week pre-veraison (around 5.0 mg/berry). Thus, assuming the maximum observed at one week pre-veraison represents total extension subunit production in the seed, some 20% of extension subunits remain unaccounted for.

While there was an increase in terminal subunits released from the seed residue post-veraison, these represented significantly less than the observed decrease in terminal subunits in the acetone extract. As with the analysis of extension subunits released from direct acid-catalysis of the grape seed residue, a substantial portion of terminal subunits remain unaccounted for.

The formation of branched or oxidatively linked polymers might well account for these 'lost' extension and terminal subunits, as well as the decrease in free monomers that do not appear as terminal subunits. Branched polymers would require the formation of 4-6
interflavan linkages in addition to the more common 4-8 linked proanthocyanidin dimers. The proanthocyanidin dimer B5 contains the 4-6 dimeric vinculum and has been reported as a significant component of grape seed tannins (Santos-Buelga et al., 1995; Souquet et al., 1996a; Saint-Cricq de Gaulejac et al., 1997; de Freitas & Glories, 1999). Further, such linkages are more resistant to hydrolysis than 4-8 linkages (Hemingway & McGraw, 1983; Ricardo da Silva et al., 1991b). In addition, oxidative coupling (Geissman & Crout, 1969) of adjacent proanthocyanidin subunits generates xanthylum salts, also resistant to hydrolysis (Jurd & Somers, 1970). Xanthylum derivatives (Singleton & Esau, 1969; Hrazdina & Borzell, 1971) and the free radicals implicated in the formation of oxidative linkages (Kennedy et al., 2000b) have previously been reported in grape seeds. Thus, during the acid-catalysed cleavage of seedcoat polymers, 4-6 linked dimers and xanthylum derivatives would be released, but not identified by this analysis as not corresponding to any of the known monomers or phloroglucinol adducts. While several candidate peaks were identified during HPLC analysis, these have yet to be isolated and their structures confirmed by NMR.

At harvest, the Shiraz seed mDP was, on average, between four and five subunits across two seasons. Previous studies have reported seed mDP in Cabernet Sauvignon as high as 16 subunits with Cabernet franc and Carignan around 9 subunits (Moutounet et al., 1996) and Alicante Bouchet at around 6-7 subunits (Frieur et al., 1994). Other workers (de Freitas et al., 2000; Kennedy et al., 2000a; Kennedy et al., 2000b) have also observed a decrease in grape seed polymer length during maturation, however this was not observed here to any appreciable extent. The most likely explanation for this observation appears to be seasonal variation in tannin composition. Considerable seasonal variation in the tannin content of seeds has been reported (Katalinic & Males, 1997; de Freitas & Glories,
1999). Data collected in the previous season from the same site (1999-2000), and analysed by the same method, showed a higher mDP pre-veraison (6.5 subunits), decreasing with maturity to a lower level than that observed in the 2000-2001 season. This represented a decrease of similar magnitude, approximately 40%, to that reported by Kennedy et al. (2000b). In the previous season (1999-2000), the same general pattern of accumulation was observed with an initial burst in synthesis occurring a few weeks after fruit-set followed by a second phase around veraison. However, the initial period of accumulation was much smaller, around 45% of the maximum observed that season compared with 72% during the 2000-2001 season. The second phase of tannin accumulation in the previous season commenced later, one week pre-veraison, reaching a maximum two to three weeks post-veraison. In the early phase of accumulation, the relative proportion of terminal subunits was lower than in 2000-2001, but the increase during the second phase was proportionally greater, resulting in a higher mDP early in development followed by a noticeable decrease. In addition to the variation in the timing of seed tannin accumulation between seasons, there was also a significant difference in total tannins derived from the seeds as well as seed number per berry. This is supported by the observations of Harbertson et al. (2002), which reported variability in total seed tannin being influenced more by seed number per berry than by tannin per seed.

A final consideration on seed tannin extractability and differences between the results presented here and elsewhere concerns sample preparation. Tannin analysis of grape seeds was conducted on ground frozen seeds as opposed to whole seeds. It is possible that the homogenisation process exposed grape seed tannins to proteins associated with embryonic material resulting in a degree of protein fining. Such an occurrence has not previously been studied or documented. Two factors likely to impact upon such a
phenomenon are the nature of the extraction solvent (70% acetone), which precipitates protein (Sambrook et al., 1989). In addition, recent research has shown that tannin extractability from seeds increases with cracking or disruption of the seed and seed coat (pers. comm. Dr. Steve Price, Ph.D.).

The proanthocyanidin content of the pulp or flesh of grape berries has in the past been largely neglected. Generally the flesh has been considered not to contain any proanthocyanidin material (Souquet et al., 1996b), although trace amounts of proanthocyanidins have been reported in the vascular traces of the fruit (Lea et al., 1979). However, flavan-3-ols and proanthocyanidins have previously been reported in the flesh of grape berries, albeit white cultivars (Lee and Jaworski, 1989). Here it is reported that in the flesh of Shiraz berries, a low level of proanthocyanidins has been detected. The total tannic material detected in the flesh was around 0.7 mg per gram fresh weight of flesh (Table 3.1). With the weight of berry flesh at that time (+3V) being around 0.23 grams, the amount per berry was approximately 0.16 mg total tannins in the berry flesh. In comparison, total tannins in the seed at this time was around 5.9 mg/berry (51.9 mg/g fresh weight of seed) and in the skin 3.2 mg per berry (12.9 mg/g fresh weight of skin). Thus, the flesh contributed less than 2% of total berry tannins. It is likely that the levels detected in the flesh are attributable to the berry vasculature or the result of contamination with seed or skin material during dissection in the field.

In grape skins the concentration of proanthocyanidin subunits was relatively high at fruit-set. For free monomers and terminal subunits, this was the highest concentration observed during berry development, with the concentration of extension subunits almost as great as that in the seed at this time. This may be partly due to the limitations of field dissection at this early stage as it was difficult to separate skin from flesh, resulting in an
arbitrary separation into seed and not-seed. This fraction would have included the ovary wall, a tissue known to contain proanthocyanidins (Fougère-Rifot, et al., 1995a). The pattern of accumulation in grape skins showed that free monomers, notably catechin, appeared prior to veraison, but only at relatively low levels. These began to decrease towards veraison coincident with a substantial increase in catechin terminal subunits that greatly exceeded the pool of catechin free monomers. This strongly suggests an upregulation of the biosynthesis of catechin at this time, although expression studies of the gene encoding the biosynthetic enzyme (leucoanthocyanidin reductase) and assays of enzyme activity will be required to confirm this. Concurrent with this apparent period of flavan-3-ol biosynthesis, was an increase in extension subunits suggesting these too were being synthesised. Thus, there was an accumulation of proanthocyanidins during the pre-veraison phase of berry development when anthocyanin synthesis had not commenced. This could account for the pattern of expression of flavonoid pathway genes reported by Boss et al. (1996a) since the early part of the pathway is required for the synthesis of both anthocyanins and tannins. This suggests that expression of genes in the flavonoid pathway may occur pre-veraison for the synthesis of tannins, and possibly flavonoids, whereas expression post-veraison is required for anthocyanin biosynthesis.

In grape skins, the increase in both extension and terminal subunits was coincident and the rates of accumulation complimentary, such that polymer length remained virtually unchanged from just after fruit-set until just after veraison. Post-veraison, polymer length gradually declined and this was due to the rate of extension subunit decrease exceeding that of terminal subunits. Such a decrease in proanthocyanidins post-veraison has been previously reported (Czochanska et al., 1979; de Freitas & Glories, 1999; Kennedy et al., 2001). While Czochanska et al. (1979) observed polymer length to decrease towards
harvest, the findings of de Freitas & Glories (1999) suggest a decrease in mDP post-veraison in one cultivar (Ugni blanc) and an increase in another (Semillon). Kennedy et al. (2001) also reported mDP increasing towards maturity in Shiraz. However, the post-veraison increase observed by Kennedy et al. (2001) was only to a level similar to that reported here at a similar level of ripeness as defined by sugar accumulation (°Brix). The harvest mDP of proanthocyanidins in Shiraz skin reported here to be around 25-30 subunits, is comparable with those reported elsewhere (Moutounet et al., 1996)

Decreasing proanthocyanidin extraction from grape skins post-veraison has been attributed to tannins forming associations with other cellular components such as cell wall polysaccharides, lignins and proteins (Amrani-Joutei et al., 1994; Escribano-Bailon et al., 1995; Cheynier et al., 1997; Saint-Cricq de Gaulejac et al., 1997). The association of grape skin proanthocyanidins with components of the grape skin residue, as seen with grape seeds, might account for the apparent decline in skin tannins. Direct acid catalysis of the grape skin residue realised a substantial yield of proanthocyanidins, however the level of both extension and terminal subunits in the residue decreased during ripening.

The formation of 4-6 linkages in grape skins might also be an explanation for the apparent decrease of grape skin proanthocyanidins as mooted for grape seeds. Reports to date suggest 4-6 linkages do not occur in grape skins to any appreciable extent with only trace amounts of the B5 dimer detected in the skin while it was one of the most prevalent in seeds (de Freitas & Glories, 1999). However, Cheynier et al. (1997) reported that proanthocyanidins with 4-6 linkages bound much more readily to proteins than those with predominantly 4-8 linkages. This could account not only for the absence of 4-6 linked dimers, but for a substantial portion of the apparent loss of proanthocyanidins in grape skins during ripening. Thus, it would appear that the overall decline in grape skin
proanthocyanidins between veraison and harvest could only be accounted for by proanthocyanidins in the skins forming increasingly stable associations with other cellular components. Furthermore, the nature of these associations renders them resistant to dissociation by the methods utilised here.

Around the time of veraison, there was a proportional increase in epigallocatechin extension subunits in the skin (Figure 7A). This increase in flavonoid intermediates that are trihydroxylated on the B-ring (Figure 1), occurs immediately prior to veraison when the anthocyanin pigments accumulate in the grape skin, these being primarily the trihydroxylated malvidin glycosides (Mazza & Miniati, 1993). This suggests that the accumulation of tannins and anthocyanins in grape skins is a coordinated process, although it has yet to be established whether these compounds occur in the same cells.

It is clear that tannins in the seeds and skin of *Vitis vinifera* differ biochemically, seed proanthocyanidins were short (mDP ~5) with subunit composition in both terminal and extension subunits consisting of a mixture of flavan-3-ols. In skin, the proanthocyanidins were longer (mDP ~40) consisting mostly of catechin terminal subunits and epicatechin and epicatechin-gallate extension subunits. The results presented here are consistent with much of the published literature for grape seed and skin tannins and confirm the suggestion that the bulk of tannin synthesis occurs prior to veraison. Most of this synthesis occurs in the seeds and it is now apparent that accumulation of tannins in the seeds is independent of that in the skins. It was also observed that early accumulation of flavan-3-ols and proanthocyanidins in the skins was consistent with the reported expression pattern of genes coding for enzymes in the flavonoid biosynthetic pathway prior to veraison. This suggests early expression of the pathway is at least related to the synthesis of extension subunits in the first three to four weeks of berry development.
Expression studies of the gene encoding the biosynthetic enzyme, leucoanthocyanidin reductase, should correlate with the observed pattern of monomer and terminal subunit accumulation presented here. For this purpose, the tissue most actively synthesising flavan-3-ols, that of young berries immediately following fruit-set, would present the best opportunity to clone the genes encoding biosynthetic enzymes.
Chapter 4. Flavonol Synthesis and Expression of Flavonol Synthase Genes in *Vitis vinifera* L.

4.1. Introduction

Flavonols are products of the flavonoid biosynthetic pathway, which also gives rise to anthocyanins and to condensed tannins in grapevines (Stafford, 1990; Darne, 1993). Flavonols in plants have physiological functions ranging from microbial interactions to pollen fertility (Kocs et al., 1994; Taylor, 1995). However, their most widespread roles appear to be as UV protectants (Flint et al., 1985; Smith & Markham, 1998), and as copigments in flowers and fruit (Asen et al., 1972; Scheffeldt & Hrazdina, 1978). Copigmentation is a non-covalent association between a co-factor (eg. flavonols) and anthocyanins that confers stability on the coloured form of the anthocyanin molecule resulting in increased colour or altered hue (Hoshino et al., 1980; Osawa, 1982).

Of the three main flavonols kaempferol, quercetin and myricetin, mainly quercetin-3-O-glucoside and -3-O-glucuronide are found in grape berries (Cheynier & Rigaud, 1986; Price et al., 1995). Flavonols have also been reported in the leaves (Hmamouchi et al., 1996) and stems of grapevines (Souquet et al., 2000; Jordão et al., 2001b). Flavonols occur in the upper epidermis of plant organs, consistent with their role in UV protection (Flint et al., 1985; Beggs et al., 1987), while anthocyanins are present in the cells of the lower epidermis (Asen, 1975). However, for copigmentation to occur, anthocyanins and flavonols would have to be present in the same cells. Hrazdina and Moskowitz (1980; Moskowitz & Hrazdina, 1981) identified both flavonols and anthocyanins in vacuoles of the outer epidermis of the grape, but were not convinced these were associated as
copigments. Nevertheless, flavonols may play a more significant role as copigments in wine.

The colour of young red wines is due to anthocyanin pigments extracted from the grape skin, but these compounds are unstable and much of the initial colour is lost during fermentation and maturation (Somers & Evans, 1979; Somers & Verette, 1988). The colour of older wines results from increasingly stable associations between anthocyanins and other flavonoid compounds to form pigmented polymers (Nagel & Wulf, 1979; McCloskey & Yengoyan, 1981; Somers & Verette, 1988; Malien-Aubert et al., 2002; Mateus et al., 2002a). Pigmented polymers form slowly in the wine and anthocyanin-copigment complexes are considered intermediates that not only maintain the anthocyanins in the wine, but align them in favorable orientations for the formation of more stable associations (Brouillard & Dangles, 1994; Boulton, 2001). The most stable copigment associations occur between the flavonols quercetin and quercetin-3-O-glucoside and the main anthocyanin in red wines, malvidin-3-O-glucoside (Baranac et al., 1997; Lambert, 2002). These observations suggest that the critical factor in determining the extent of colour in mature wine is the concentration of anthocyanins and copigments at harvest (Lambert, 2002). Thus, any process that increases the anthocyanin or flavonol content in grapes would be a valuable contribution to wine quality.

Previous investigations into grape flavonols have largely focused on the flavonol composition of the ripe fruit (Cheynier & Rigaud, 1986) and the effects of sun exposure (Price et al., 1995). Haselgrove et al. (2000) examined flavonols earlier in berry development, reporting relatively high levels (0.15 mg/berry) at veraison, the onset of ripening, with little change towards harvest. Furthermore, while many genes involved in flavonoid biosynthesis have been cloned in Vitis vinifera (Sparvoli et al., 1994), this has
not been the case for flavonol synthase (FLS), the gene that encodes the biosynthetic enzyme converting dihydroflavonols to flavonols (Spribille & Forkmann, 1984; Lukacin et al., 2003). This gene has been cloned in other genera including Petunia, Arabidopsis, Solanum and Citrus (Holton et al., 1993b; Pelletier et al., 1997; van Eldik et al., 1997; Moriguchi et al., 2002).

Here, the cloning and characterisation of genes encoding flavonol synthase from grapevine together with a study of flavonol accumulation in grapes during berry development are presented.

4.2. Materials and Methods

4.2.1. Sampling of Grape Tissues

Grapevine tissues from Vitis vinifera L. cultivars Shiraz and Chardonnay were collected from a commercial vineyard in Willunga, South Australia as described in Section 2.2.1. The developing fruit was sampled at weekly intervals (Table 2.1) over three seasons for Shiraz, and a single season for Chardonnay. Leaves from Shiraz grapevines were collected as described in Section 3.2.1 and early vegetative stages from bud-burst towards flowering were collected from Shiraz grapevines during the 2000-2001 season. At bud-burst the emerging buds were excised from the canes. On successive weeks the developing shoots were collected, the growth stages of the developing tissues were determined according to the modified Eichorn-Lorenz (E-L) scheme (Coombe, 1995). Samples corresponded to E-L Stage 4 (buds), E-L 7 (1st separated leaf; -16V), E-L 9 (shoot with 2-3 leaves separated), E-L 12 (shoot with 4-5 leaves separated), E-L 13 (developing inflorescence; -14V), E-L 14 (expanding inflorescence; -13V) and E-L 17.
(unopened flowers; -10V). All samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

Roots of Shiraz vines were collected from glasshouse grown potted vines. Plants were removed from pots and the soil shaken free from the root ball. The roots were then washed to remove the remainder of the soil and blotted dry. Root samples of 1-2 cm in length were cut from the root tips, these were frozen immediately in liquid nitrogen and stored at -80°C.

4.2.2. Extraction and Identification of Flavonols

In order to determine the pattern of flavonol accumulation in the grape berry and to target cloning of a grapevine homologue of flavonol synthase the flavonol content of the fruit was investigated. The flavonol synthase enzyme catalyses the desaturation of the dihydroflavonol (see Figure 1.6) to produce the flavonols Spribille & Forkmann, 1984; Forkmann et al., 1986; Holton et al., 1993b). However, the flavonol aglycones (Figure 1.3) rarely occur in plant tissues, with the majority of flavonols present as glycosides, commonly glucosides and glucuronides (Markham, 1989). Thus, flavonol accumulation was determined as the accumulation of the flavonol glycosides. While the aglycones are relatively non-polar and readily extracted in organic solvents such as methanol, ethanol or acetone (Markham, 1975), the glycosides are comparatively more polar and aqueous combinations of these solvents are more effective in extracting flavonol glycosides (Markham, 1982). While a range of solvents are suitable for extraction of flavonol glycosides from plant material, it was considered prudent to utilise here the method employed by others investigating flavonol composition in grapevines to enable valid comparisons between experimental outcomes. While Cheynier and Rigaud (1986) used
methanol (0.1% HCl) as a solvent, subsequent investigations (Price et al., 1995; Haselgrove et al., 2000) have employed acidulated ethanol. Thus, for the HPLC analysis of flavonol composition in grapevines, samples collected in the field, frozen and stored at -80°C were ground to a fine powder under liquid nitrogen. Three separate aliquots (analysis replicates) of 0.1 g were extracted in 1.0 mL of acidulated ethanol (Price et al., 1995) for 5 minutes at 70°C followed by 20 minutes at room temperature with occasional vortexing. Extracts were then centrifuged (15 min at 13,000 × g) and 200 μL of the supernatant transferred to HPLC auto-sampler vials. A range of extraction durations and temperatures were trialed to determine effectiveness of this method. It was observed that heating for five minutes yielded the same level of flavon glycosides as a 12 hour extraction at room temperature and considerably more flavonols than 20 or 60 minute extractions at room temperature. Extraction at low temperature (0°C) was not effective, even after 12 hours. Repeated extraction was effective for extracts made at room temperature for 20 and 60 minutes, but no additional flavonols were detected in extracts that had initially been heated at 70°C for five minutes. When triplicate samples were allowed to return to room temperature after extraction at 70°C and prior to transfer to autosampler vials, more consistent results were observed suggesting some loss of solvent through evaporation.

While both Price et al. (1995) and Haselgrove (1997) employed a method for HPLC separation of the flavonol glycosides that utilised phosphoric acid and with an acetonitrile gradient, issues associated with the disposal of acetonitrile waste encouraged the adoption of acetic acid with a methanol gradient (Cheynier & Rigaud, 1986). Samples were analysed using a Hewlett Packard HP 1100 high-performance liquid chromatograph (HPLC) with two Merck (Germany) Chromolith™ analytical columns in series protected
by an SGE (Australia) C-18 guard column. The separation used 0.2% acetic acid (solvent A) with a methanol (solvent B) gradient (gradient of solvent B: zero min, 24%; 10 min, 28%; 10.1 min, 100%; 13 min, 100%; 13.1 min 24%) with a flow rate of 5 mL/minute. The major flavonol peak was identified by comparison of the elution time and absorbance spectra with a commercial standard of quercetin-3-O-glucoside obtained from Extrasynthese™ (France). In addition, the main flavonol peak was collected and analysed by mass spectroscopy (ESI-MS). The integrated absorbance at 353 nm was used to determine the concentration of flavonol glycosides in each sample (25 μL injection) expressed as quercetin-3-O-glucoside equivalents. Where present, kaempferol-3-O-glucoside was identified by comparison of elution time and absorbance spectra with a kaempferol-3-O-glucoside standard from Extrasynthese™ (France). Concentrations of quercetin-3-O-glucoside and kaempferol-3-O-glucoside equivalents were calculated from a standard curve prepared from commercial standards.

4.1.3. Cloning of Flavonol Synthase

For the cloning and expression of flavonol synthase genes grapevine material frozen in liquid nitrogen in the field and stored at −80°C, was ground to a fine powder in liquid nitrogen using a pre-frozen mortar and pestle. Genomic DNA was extracted from frozen Stage 2 leaf tissue following the method of Thomas et al. (1993).

Total RNA was extracted from 1.0 g of the frozen powdered tissue in 7.0 mL of hot borate buffer as described by Wan and Wilkins (1994). Final pelleted RNAs were resuspended in Tris-EDTA (TE) and stored at −40°C in a solution containing 1/10th volume sodium acetate and 3 volumes of 100% ethanol. Two 5.0 μg aliquots of total RNA for each sample were pelleted by centrifugation at 13,000 × g for 20 minutes at 4°C,
the pellet was rinsed in 70% cold ethanol and allowed to air dry. First strand cDNA synthesis was performed using the 3'-RACE adapter primer and Superscript II™ (Invitrogen Life Technologies, Australia) using methods recommended by the supplier in a final reaction volume of 25 μL. After heat inactivation of the reverse transcriptase, two reactions were combined to give a working 50 μL stock and stored at −20°C.

cDNA clones encoding flavonol synthase (FLS) were obtained by PCR of cDNA prepared from RNA isolated from Shiraz flowers. Degenerate primers were designed based on FLS sequences for other dicotyledonous plants in the GENBANK database. The forward primer was 5'-GC/AAATTCGAA/GAA/GAA/GC-3' (FLS9), designed to the protein sequence EN/KEQPA and the reverse primer was 5'-ACIGGCCAIC/GA/TCATCG/TIGTC/TIT-3' (FLS6), designed to the protein sequence KTRMSWPV. The PCR was carried out according to the method of Frohman et al. (1988) and a predicted band of approximately 800bp was obtained and cloned into the pGEM-T Easy vector from Promega™ (Australia). A number of independent clones were sequenced and one clone (FLS36) was sequenced in both directions and found to encode a 797bp fragment with high homology to FLS sequences in the database. This sequence was named as pVvFLSl (GenBank Accession No. AY257978).

A second gene fragment encoding FLS was identified by PCR from grapevine genomic DNA. The equivalent cDNA was amplified from the flower cDNA using a gene-specific primer based on the genomic sequence. Flower cDNA was amplified by PCR using the specific primer 5'-AATCCTCCTTACAGGGATGC-3' (FLS10) and the oligo-dT adapter primer B26 described by Frohman et al. (1988). This yielded a band of approximately 700bp that was cloned and sequenced as described above to yield a 706bp
cDNA fragment (FLS26), also encoding FLS. This second sequence was named pVvFLS2 (GenBank Accession No. AY257979).

4.1.4. Expression of Flavonol Synthase Genes

Gene expression was determined by real-time PCR analysis. cDNA syntheses for all RNA samples to be analysed were performed simultaneously. Reagents required for the reverse transcription (RT) reactions were made as a master mix and aliquoted into each reaction in a single step to avoid pipetting errors that may have affected the efficiency of the RT reactions in different samples.

Quantitative real-time PCR was performed on a Rotor-Gene 2000™ (Corbett Research, Australia) real-time PCR machine using SYBR green as the system of detection for double stranded PCR products. Primers homologous to the grapevine cDNAs encoding VvFLS1, VvFLS2 and VvUbiquitin1 were designed using primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The primer pairs VvFLS1 Forward: 5’-CAGGGCCTTGCAGGTTTTAG-3’, VvFLS1 Reverse: 5’-GGGTCTTCTCCTTGTTCACG-3’ and VvFLS2 Forward 5’-TGAGCTGGCTATAGGTCCTC-3’ and VvFLS2 Reverse 5’-TGCATGTACACTGGAAAAAGG-3’ were used to generate PCR products of 154bp and 195bp respectively. The VvUbiquitin1 transcript was detected using the primers VvUbiquitin1 Reverse: 5’-AACCTCCAATCCAGTCATCTAC-3’ and VvUbiquitin1 Forward: 5’-GTGGTATTATTGAGCCATCCT-3’ which amplify a product of 182base pairs.

All primer pairs were used under identical conditions, 95°C for 5 minutes followed by 35 rounds of amplification (95°C/30sec, 57°C/30sec, 72°C/30sec). Each PCR reaction
contained 1 × SYBR Green PCR Master Mix™ (Applied Biosystems, U.K.), 30 nmoles of each primer and 1/50th volume of cDNA stock in a final reaction volume of 25 μL. The VvFLS1 and VvFLS2 mRNAs was quantified as a proportion of VvUbiquitin1 using the Rotor Gene 2000™ software.

4.1.5. Southern and Northern Blot Analysis

Southern and Northern blot analysis was performed as described by Sambrook et al. (1989) using 0.4 M and 50 mM NaOH respectively as transfer buffer. Nucleic acids were transferred onto Zeta-Probe™ nylon membranes (Bio-Rad, Australia) and fixed as recommended by the supplier. Membranes were prehybridised in 7% SDS, 0.25 M Sodium Phosphate (pH 7.0), 1.0 mM EDTA at 65°C for 2 hours. DNA fragments specific for VvFLS1 and VvFLS2 were excised from pGEM T-Easy™ (Promega, Australia) clones by digestion with EcoRI according to the instructions of the supplier. Vector and insert fragments were resolved on 1% agarose gels and the insert bands removed under UV light after staining with ethidium bromide. Insert DNA was purified from the gel fragment using a QIAquick™ gel extraction kit (QIAGEN, Australia) according to the manufacturers instructions. Inserts were radioactively labeled using a Rediprime™II labeling kit (Amersham Biosciences, Australia), added to the prehybridisation solution after heat denaturation and hybridised overnight at 65°C. Membranes were rinsed in 2 × SSC, 0.1% SDS at room temperature before being washed in 1.0 × SSC, 0.1% SDS for 20 minutes at 65°C and 0.1 × SSC, 0.1%SDS for 20 minutes. Membranes were then rinsed in 2 × SSC at room temperature before being exposed to Biomax™ (MS) film (Eastman Kodak Co., USA) at −80°C with appropriate intensifying screens.
4.3. Results

4.3.1. Cloning of Flavonol Synthase Genes

The flavonoid pathway produces a range of secondary compounds including anthocyanins, flavonols and tannins. The first step in the branch of this pathway that leads to the synthesis of the flavonol derivatives is catalysed by the enzyme flavonol synthase (FLS), a dioxygenase that catalyses the desaturation of the ‘C’ ring of dihydroflavonols (Stafford, 1990). If FLS is transcriptionally regulated as appears to be the case with other flavonoid pathway enzymes (Boss et al., 1996a), it is anticipated that flavonol accumulation would be preceded by expression of the gene encoding FLS. To determine expression levels of the genes encoding FLS in grapevine tissues, we first isolated cDNA clones encoding FLS to provide gene probes. RNA extracted from Shiraz flowers was used to make cDNA by reverse transcription and this was amplified using the polymerase chain reaction (PCR) and the DNA fragments obtained were cloned and sequenced in both directions. This yielded two distinct grapevine FLS clones: pVvFLS1, a 797bp fragment of FLS encoding 265 amino acids in the open reading frame of the gene, and pVvFLS2, a 706bp fragment encoding 185 amino acids at the C-terminal end of the protein plus a 3'-untranslated region of 151bp. The two fragments shared a 425bp overlap and pVvFLS1 and pVvFLS2 had 79% sequence identity at the nucleotide level in this overlapping region. The two grapevine FLS sequences have been deposited in the GenBank database (Accession numbers AY257978 and AY257979). The two grape FLS genes had high protein sequence homology to other FLS genes in the two conserved dioxygenase domains (data not shown), indicating that they are part of the dioxygenase family (van Eldik et al., 1997; Moriguchi et al., 2002). Both grape sequences had high homology to FLS genes in the databases and the nearest matches at the amino acid level.
were to Petunia FLS (Z22543; 73% identity to pVvFLS1, 74% identity to pVvFLS2) and Cirrus FLS (BAA36554; 73% identity to pVvFLS1, 70% identity to pVvFLS2). A number of other degenerate primers were designed based on plant FLS sequences and used in PCR of the cDNA from grape leaves and flowers, however no additional FLS genes were identified.

4.1.2. Southern Blot Analysis

Southern blots of Shiraz genomic DNA restricted with HindIII and hybridised with either the VvFLS1 or VvFLS2 probes demonstrated that each probe could bind to four distinct bands of the HindIII restricted DNA (Figure 4.1). Analysis of the two cDNA sequences shows that HindIII cuts the VvFLS1 clone at least twice and the VvFLS2 clone at least once. As both clones are only partial cDNA sequences it is not known if the remaining coding sequence or the introns of the respective genomic sequences contain further HindIII sites. Given the high stringency washing conditions used in this procedure, it is unlikely that either probe could cross hybridise to the target genomic sequence of the other. In the absence of further analysis we cannot absolutely confirm that the two sequences exist as single copy genes in the Shiraz genome. However, given the fact that no other FLS-like clones were identified during the cloning of the VvFLS cDNAs and that HindIII does cut the FLS sequences, this is the most likely conclusion.

![Figure 4.1. High stringency wash (0.1 × SSC @ 65°C) of Southern blot of Vitis vinifera clones of the flavonol synthase gene, VvFLS1 and VvFLS2 restricted with HindIII.](image)
4.1.3. Identification of Grapevine Flavonols

Flavonols in the *Vitis vinifera* L. cultivars Chardonnay and Shiraz were identified initially by comparison of their elution times and absorbance spectra with commercially available standards of quercetin-3-*O*-glucoside or kaempferol-3-*O*-glucoside. Figure 4.2A shows the separation of these standards using the HPLC conditions described above. Typically, a HPLC separation of grape tissue extracts (Figure 4.2B) showed two peaks corresponding to the quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside standards, based on their elution times and absorbance spectra. In all samples, the peak corresponding to quercetin-3-*O*-glucoside accounted for most of the flavonols. The separation

Figure 4.2. Identification of flavonols in *Vitis vinifera* by HPLC-MS (*A*<sub>355</sub>) A. HPLC separation of commercial standards of quercetin-3-glucoside and kaempferol-3-glucoside (25 μL injection, 50 μg/mL); B. HPLC separation of extract from Shiraz flowers (-8V, 16 November 2000; 25μL injection); C. Mass spectrum of the major flavonol peak in *Vitis vinifera* tissues corresponding to quercetin-glucoside; three ionisation products were apparent, quercetin (M+H<sup>+</sup>, m/z 303.2), quercetin-3-*O*-glucoside (M+H<sup>+</sup>, m/z 465.2) and quercetin-3-*O*-glucuronide (M+H<sup>+</sup>, m/z 479.0).
presented here (Figure 4.2A), is that of Shiraz flowers, nine weeks pre-veraison and was the only time during Shiraz berry development when the kaempferol glycoside could be detected. Kaempferol derivatives were not detected in Chardonnay berries or flowers. The major flavonol peak, corresponding to quercetin-3-\(O\)-glucoside was collected and analysed by mass spectrometry. The mass spectra from this peak (Figure 4.2C) showed three ionisation products present, corresponding to the aglycone quercetin, quercetin-3-\(O\)-glucoside and quercetin-3-\(O\)-glucuronide.

HPLC analysis did not detect the flavonol aglycone, quercetin. While a peak was present in the HPLC separation at the approximate time that quercetin eluted (elution time), spectral analysis of this peak was unable to identify any flavonol. The absorbance spectra of a commercial standard of the flavonol aglycone, quercetin is presented in Figure 4.3A. The absorbance spectra of flavonols has a distinctive absorbance maxima at 353 nm. Five absorbance spectra through the late eluting peak in the HPLC separation of grape samples does

![Absorbance spectra](image)

**Figure 4.3.** Absorbance spectra of commercial quercetin standard (A) and five absorbance spectra through the peak in a grape skin extract that elutes at a similar time to that of the quercetin standard (B).
not show any spectra consistent with that of the flavonols (Figure 4.3B).

4.1.4. Berry Development, Flavonol Accumulation and FLS Expression in Chardonnay Berries

Samples of Chardonnay grapes were collected during the 1999-2000 growing season. The pattern of berry development and the accumulation of flavonols and expression of \( VvFLS1 \), the gene encoding a grapevine flavonol synthase, during the 1999-2000 season are presented in Figure 4.4. Flowering occurred in the week of 21 October 1999 and veraison occurred nine weeks later, around 23 December 1999 (Figure 4.4A). Six weeks after veraison, the Chardonnay was harvested at 23.2°Brix on 03 February 2000. Berry weight increased slowly from flowering until six weeks pre-veraison, however this represented a 12-fold increase in berry weight (Figure 4.4A). From six weeks pre-veraison berry weight increased steadily until one week pre-veraison, then remained static until veraison when berry growth recommenced for a further three weeks. During this time the level of sugar in the berry, measured as total soluble solids (°Brix), increased steadily (Figure 4.4A). Berry weight and total soluble solids were constant for the next two weeks, while in the final week before harvest, there was a decrease in berry weight and an increase in total soluble solids. This observation was consistent with water loss from the ripe fruit.

In Chardonnay berries, only glycosides of quercetin were detected. At flowering there was a high concentration of flavonols (1.46±0.130 mg/g fresh weight), however as the berry began to develop there was a 5-fold decrease in flavonol concentration (Figure 4.4B). This decline continued until two weeks pre-veraison, after which time the level of flavonols per gram of tissue remained relatively constant until harvest (0.03±0.005 mg/g).
During this time, the berry was increasing in size suggesting an overall increase in total flavonols in the berry. This was the pattern observed on a per berry basis (Figure 4.4C), where total quercetin glycosides per berry were relatively constant for the first few weeks (0.009 mg/berry) followed by a two-fold increase at five weeks pre-veraison (0.019 mg/berry). This level was maintained until one week pre-veraison when flavonols began to accumulate once more. At one week post-veraison, quercetin glycosides increased again to the maximum level observed in the berry during development (0.035 mg/berry). From one week post-veraison the flavonol level remained relatively constant until harvest.

The expression of a grapevine gene encoding the flavonol...
synthase biosynthetic enzyme ($VvFLSI$) in Chardonnay berries during the 1999-2000 season is shown in Figure 4.4D relative to an internal control ($VvUbiquitin1$) whose expression was constant throughout berry development (data not shown). In flowers, nine weeks pre-veraison, a high level of $VvFLSI$ expression was observed, which declined markedly after flowering and remained low until veraison. After veraison, the level of $VvFLSI$ expression increased steadily towards harvest with a 10-fold increase in $VvFLSI$ expression during this time. The final level of $VvFLSI$ expression in grape berries at harvest was similar to that at flowering. The expression of $VvFLS2$ was not determined in Chardonnay berries.

4.1.5. Berry Development, Flavonol Accumulation and FLS Expression in Shiraz Berries

Primarily, results from the 2000-2001 season are presented here with the previous and successive seasons included for comparison. Shiraz grapes were collected over three seasons; 1999-2000, 2000-2001 and 2001-2002. Table 4.1 shows the flowering and veraison dates as well as the date on which berries attained 24°Brix for Shiraz grapes over the three successive seasons. Berry weight, skin weight and flavonol levels are also indicated. The patterns of berry development, flavonol accumulation and expression of $VvFLSI$, a grapevine gene encoding flavonol synthase during the 2000-2001 season are presented in Figure 4.5. Berry growth during the 2000-2001 season was measured as berry weight and showed a six-fold increase from seven weeks pre-veraison (fruit-set) until four weeks pre-veraison when berry growth slowed (Figure 4.5A). Berry weight reached a maximum five weeks post-veraison then declined until commercial harvest at nine weeks post-veraison. From veraison, sugars in the berry, measured as total soluble solids (°Brix), also increased steadily reaching 29.3°Brix at commercial harvest. This
Table 4.1. Seasonal variation in the timing of flowering, veraison and ripening in *Vitis vinifera* L. cv. Shiraz over three successive seasons, at Willunga, SA. The date when berries reached approximately 24°Brix is shown. Berry weight, skin weight and levels of quercetin glycosides (flavonols) at 24°Brix is shown for each season expressed per gram fresh weight of skin and per berry (±SEM, n=3)

<table>
<thead>
<tr>
<th>Season</th>
<th>Flowering</th>
<th>Veraison = 24°Brix</th>
<th>Berry weight (g)</th>
<th>Skin weight (g)</th>
<th>Flavonols at 24°Brix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999-2000</td>
<td>04-Nov-99</td>
<td>06-Jan-00</td>
<td>1.36</td>
<td>-</td>
<td>0.046±0.001</td>
</tr>
<tr>
<td>2000-2001</td>
<td>16-Nov-00</td>
<td>11-Jan-01</td>
<td>1.27</td>
<td>0.39</td>
<td>0.086±0.002</td>
</tr>
<tr>
<td>2001-2002</td>
<td>23-Nov-01</td>
<td>31-Jan-02</td>
<td>1.16</td>
<td>0.44</td>
<td>0.071±0.009</td>
</tr>
</tbody>
</table>


increase in total soluble solids from five weeks post-veraison coincided with a decrease in berry weight, which was consistent with previous reports of berry shrivel in ripe Shiraz (McCarthy, 1999; McCarthy & Coombe, 1999).

The concentration of quercetin glycosides during berry development was highest in the unopened Shiraz flowers, nine weeks pre-veraison (1.60±0.050 mg/g fresh weight, Figure 4.5B). At flowering (50% cap-fall) the following week, the level had fallen to 1.31±0.069 mg/g and in the following week to fruit-set, a substantial decrease was observed to 0.17±0.005 mg/g, followed by a smaller decline in the next week. From this time until harvest the flavonol concentration remained relatively constant (0.08±0.004 mg/g fresh weight of skin). On a per berry basis (Figure 4.5C), there was an initial increase in total quercetin glycosides in the developing berries from fruit-set (0.011±0.001 mg/berry) until five weeks pre-veraison. Although some fluctuations were observed, this level was maintained until veraison (0.013±0.001 mg/berry). At veraison, flavonols in the Shiraz berry began to increase and continued to do so until six weeks post-veraison.
(0.049±0.001 mg/berry). For the following two weeks flavonols appeared to decline, however in the final week, total flavonols in the berry increased again.

The expression of the gene encoding the flavonol synthase biosynthetic enzyme (VvFLSI) during berry development in the 2000-2001 season is shown in Figure 4.5D relative to the expression of VvUbiquitin1 whose expression remained constant throughout berry development (data not shown). In flowers prior to cap-fall, a high level of VvFLSI expression was observed, which declined markedly at flowering (eight weeks pre-veraison) and during the following week, remaining at a low level from six weeks pre-veraison until veraison. After veraison, the level of VvFLSI expression began to increase slowly, with a noticeable increase three weeks post-veraison. This was followed by an additional increase from four to six weeks post-veraison. From two to six weeks post-veraison there

![Berry development, flavonol accumulation and flavonol synthase gene expression in Vitis vinifera L. cv. Shiraz. Flowering occurred around 16 November 2000 (8 weeks pre-veraison) with veraison around 11 January 2001 and commercial harvest on 13 March 2001 (9 weeks post-veraison). A). Shiraz berry development, berry weight (△) and total soluble solids expressed as °Brix (●); B). Quercetin glycosides (mg/g fresh weight of skin ±SEM); C). Quercetin glycosides (mg/berry ±SEM); D). Expression of the flavonol synthase gene (VvFLSI) in developing grape skins relative to the expression of VvUbiquitin1.](image-url)
was a 10-fold increase in \textit{VvFLSI} expression. This was followed by a decline and then a further increase over the final two weeks of berry development until harvest. The final level of \textit{VvFLSI} expression was marginally higher than that seen in unopened flowers.

\textit{VvFLS2} was constitutively expressed in all the developing berry skin samples but at levels $10^4$-$10^5$ times lower than that of \textit{VvFLSI} in corresponding samples. The expression of \textit{VvFLS2} did not vary in any of the skin samples that were assayed and was thus incongruent with the observed pattern of flavonol accumulation. These results were confirmed by Northern blot analysis (data not presented).

### 4.1.6. Seasonal Variation in Flavonol Accumulation in Shiraz

Figure 4.6 shows the variation in total soluble solids (°Brix) and flavonols (quercetin glycosides) across three seasons, 1999-2000, 2000-2001 and 2001-2002 in \textit{Vitis vinifera} L. cv. Shiraz berries. In the 1999-2000 season, flowering occurred during the week of 04 November 1999 with veraison nine weeks later around 06 January 2000 and commercial harvest seven weeks later on 24 February 2000 (Table 4.1). In the 2000-2001 season, flowering occurred two weeks later than in the 1999-2000 season, during the week of 16 November 2000. Veraison occurred eight weeks after flowering, around 11 January 2001, with commercial harvest after a further nine weeks on 13 March 2001. During the 2001-2002 season flowering occurred later than in either of the preceding seasons, around 23 November 2001. In addition, the time between flowering and veraison was longer than in the previous seasons at ten weeks, veraison occurring at the end of January (31 January 2002). However, in the 2001-2002 season the time between veraison and commercial harvest was the shortest recorded during these investigations at six weeks. Shiraz grapes were harvested at 26.4 °Brix on 15 March 2002 (Table 4.1).
Ripening of the fruit progressed in similar fashion each season with sugar accumulation (°Brix) increasing from veraison towards harvest, reaching 24°Brix between five and seven weeks post-veraison (Table 4.1, Figure 4.6A). The pattern of flavonol (quercetin glycoside) accumulation that was observed in Shiraz grape skin during the 2000-2001 season was observed over all three of the seasons studied here (Figure 4.6B). When examined on a per berry basis, total quercetin glycosides per berry increased steadily from flowering/fruit-set through veraison towards harvest. In addition, there was a significant increase in flavonol accumulation commencing at around two to six weeks post-veraison.

Towards harvest, there was some variation in the levels of flavonols between seasons. When considered at similar levels of ripeness (24°Brix), the highest level of flavonols per berry was observed in the first season (1999-2000), while the lowest level was observed in the final season (2001-2002). However, it should be noted that berry weight at this time was also highest in the first season and lowest in the third season (Table 4.1).
In the two seasons where skin was collected (2000-2001 and 2001-2002), the level of flavonols (mg/g fresh weight of skin) was higher in 2000-2001 than in the following season. To some extent this is reflected in the level of flavonols per berry (Table 4.1), but it should also be noted that in the earlier season skin weight as a percentage of berry weight was lower (30.1%) than in 2001-2002 (37.9%).

4.1.7. Flavonol Accumulation and FLS Expression in other Tissues of Shiraz

Figure 4.7 shows the comparative levels of flavonols present in other tissues of the grapevine and expression of the gene encoding flavonol synthase (VvFLSI) in the same tissues. Immediately after bud-burst, at the beginning of the growing season for grapevines (E-L stage 4), a sample of the emerging buds was taken with successive developmental stages of young shoots made at weekly intervals. All of these tissues had high levels of quercetin glucosides, but kaempferol glucoside was not detected. The developing inflorescence (E-L stage 13) had the highest flavonol concentration of any stage in berry development (2.111±0.063 mg/g) and, accordantly, the highest level of VvFLSI expression (Figure 4.7B). The decline observed in the level of quercetin glycosides in the developing fruit (Figure 4.5B) was more dramatic when the initial level in the inflorescence is considered. A high level of flavonols was also detected in the anthers of Shiraz flowers (1.200±0.025 mg/g). Compared with the vegetative tissues, the levels of quercetin derivatives in the berry three weeks post-veraison and at harvest, were low (Figure 4.7A). There were no flavonols detected in either the seeds or the flesh of Shiraz berries sampled three weeks post-veraison, although a trace of VvFLSI expression could be detected in flesh, while there were significant levels in the skin throughout development as described above. Grapevine tendrils and the pedicel of the berry also contained high levels of flavonols, with kaempferol glucoside also detected in these
tissues, around 5% of total flavonols in tendril and 2% in the pedicel. The level of flavonols in newly opened buds, tendrils, pedicels and developing inflorescences was significantly higher than at any time during berry development from fruit-set until harvest. Although there were clearly detectable levels of VvFLS1 in emerging buds, tendrils and developing flowers, very little VvFLS1 expression was detected in the pedicel or root, despite the presence of flavonols in these tissues. There was no detectable expression of VvFLS2 in any of the vegetative tissues analysed here.

Shiraz leaves had a high concentration of flavonols, around 2.5 times greater than that observed in the flowers. In berries, almost all of the flavonols were quercetin glucosides, but Shiraz leaves contained both quercetin and kaempferol glucosides (Figure 4.7A). In leaves, approximately 10% of total flavonols were kaempferol glucoside. A high level of flavonols was observed in newly emergent Shiraz leaves (quercetin glycoside 3.16±0.27 mg/g; kaempferol glycoside 0.39±0.04 mg/g fresh weight). As the leaf expanded this level declined as seen in the second leaf stage (Figure 4.7A). The level of flavonols increased again as the leaf continued to expand reaching a level similar to that in the youngest leaf. However, the level of kaempferol glucoside in the older leaves was lower than that observed in the youngest leaf. Roots from glasshouse grown Shiraz vines contained low levels of flavonols compared to other vegetative tissues, but similar levels to the skin (0.069±0.004 mg/g).

The VvFLS1, but not VvFLS2, mRNA was detected at all stages of leaf development with the transcript becoming gradually more abundant as the leaves matured. This level of transcript expression was commensurate with the ongoing accumulation of flavonols in the expanding leaf.
Figure 4.7. Flavonol concentration and expression of a gene encoding flavon synthase in tissues of *Vitis vinifera* L. cv. Shiraz. A). Levels of quercetin (■) and kaempferol (□) glucosides (mg/g ±SEM) in Shiraz tissues; B). Expression of *VvFLS1* relative to the expression of *VvUbiquitin1* in grapevine tissues (■). Note: Expression of *VvFLS1* in anthers was not determined due to difficulty isolating RNA from that tissue. Very low levels of *VvFLS1* expression were detected in pedicel (0.005) and post-veraison seed (0.001). No expression of *VvFLS1* was detected in grapevine roots. Post-veraison samples: 01 February 2001 (3 weeks post-veraison); Harvest: 13 March 2001 (9 weeks post-veraison). Developmental stages defined according to the Eichorn-Lorenz (E-L) system (Coombe, 1995), buds (E-L 4), expanding inflorescence (E-L 13; 14 weeks pre-veraison), flowers pre-capfall (E-L 17; 10 weeks pre-veraison).
4.4. Discussion

Flavonols and anthocyanins are predominantly synthesised in the skin of red grapes whereas tannins are present in both the skin and seeds (Mazza & Miniati, 1993; Souquet et al., 1996). No flavonols were detected in the seeds, nor in the flesh, of Shiraz grapes (Figure 4.7) suggesting that flavonols are only present in the skin, which was consistent with previous reports (Souquet et al., 1996).

In *Vitis vinifera* L. cv. Shiraz grapes at harvest over three seasons, the major flavonols detected were quercetin-3-glucoside and quercetin-3-glucuronide (Figure 4.2). In Chardonnay grapes the major flavonols were also quercetin glucosides (data not shown). In neither Chardonnay nor Shiraz, were flavonols other than quercetin glycosides detected at harvest, although kaempferol glucosides were detected in Shiraz at flowering.

We did not detect the flavonol aglycones quercetin and kaempferol in any of the grape berry samples analysed. Ribereau-Gayon (1964) reported the main flavonols in grapes as being the 3-glucosides of myricetin, quercetin and kaempferol, as well as the 3-glucuronide of quercetin. In addition to these, Cheynier and Rigaud (1986) also reported the glucuronides of kaempferol and myricetin, the glucosylgalactoside and glucosylxyloside of quercetin, and the galactoside and glucosylarabinoside of kaempferol, as well as the 3-glucoside of isorhamnetin, a 3' methylated quercetin derivative. Of these compounds, the glucoside and glucuronide of quercetin represented by far the greater proportion of flavonols in the fruit of *Vitis vinifera* L. cv. Cinsault (Cheynier & Rigaud, 1986). Macheix et al. (1990) later quantified the level of quercetin derivatives in grape berries, reporting levels to range from less than 0.01 to 0.1 mg/g fresh weight of skin. Price et al. (1995) also reported quercetin glucoside and glucuronide as the largest flavonol components in grape skin, with around 0.065 mg/g fresh weight of
skin in *Vitis vinifera* L. cv. Pinot noir at harvest (23°Brix). Subsequently, Haselgrove *et al.* (2000) reported levels of around 0.065 mg/g fresh weight of berry (0.056 mg/berry;) in Shiraz at harvest (24°Brix).

The results presented here for Chardonnay and Shiraz berries at commercial ripeness are comparable with previous reports. In the 1999-2000 season, the level of quercetin glycosides in Shiraz (0.034±0.001 mg/g of berry fresh weight; 0.046±0.001 mg/berry; n=3) and Chardonnay (0.031±0.003 mg/g of berry fresh weight; 0.047±0.005 mg/berry; n=3) were not significantly different on either a per gram (p=0.133) or a per berry (p=0.478) basis. These levels are slightly lower than those reported by Haselgrove *et al.* (2000) for whole Shiraz berries. The level of flavonols in Shiraz skin ranged from 0.071-0.086 mg/g fresh weight of skin at approximately 24°Brix, which was slightly higher than reported by Price *et al.* (1995) in Pinot noir, but falling within the general range reported by Macheix *et al.* (1990). Over three seasons, there were similar levels of flavonol glycosides in Shiraz berries (mg/berry) at a similar level of ripeness (24°Brix, Table 4.1).

In both Chardonnay and Shiraz berries, similar patterns of accumulation were observed. High flavonol concentrations (mg/g of berry fresh weight) were recorded around flowering, followed by a decrease as the grape berry increased in size. However, after the initial decrease following flowering and fruit-set, the per gram level of quercetin glucosides in the fruit remained constant until harvest despite substantial increases in berry size. This indicated ongoing biosynthesis of flavonols during berry development and this was confirmed by the increase in flavonols per berry. The pattern of flavonol accumulation per berry suggests two main periods of flavonol synthesis, the first occurring around flowering and the second occurring after veraison. The post-veraison phase represented the greatest increase in flavonols per berry, with a substantial increase
observed in all three seasons commencing three to four weeks post-veraison (Figure 4). Despite minor variations in timing and amplitude, the similarity across seasons establishes a convincing pattern for flavonol accumulation in grape berries that has not previously been reported. The increase observed in the final weeks of berry development was not observed by Haselgrove et al. (2000), although McDonald et al. (1998) reported that the levels of flavonols were higher in wines made from very ripe fruit, which is consistent with what we have observed here. Towards the end of berry development there was a notable increase in flavonol content during the final weeks of ripening. The post-veraison increase in flavonols occurred after the main period of anthocyanin biosynthesis, which occurred in the 2-3 weeks immediately post-veraison (data not shown). This increase in flavonols post-veraison might be related to copigmentation or to the role of flavonols as UV protectants, or involved in preventing the photobleaching of anthocyanins reported by Yamasaki et al. (1996).

At flowering, significant amounts of flavonols were present in both Chardonnay and Shiraz grapes. This led us to examine the flavonol content in developing inflorescences and other vegetative tissues of *Vitis vinifera* L. cv. Shiraz. The level of flavonols in flowers was 10-15 times higher than that observed in the skin of ripe grapes, but was highest in the developing inflorescence (Figure 4.7A). Such high levels of flavonols would be consistent with a role for flavonols in UV protection and of particular importance in reproductive tissues (Lodish *et al.*, 1995). High levels of flavonols were also observed in the anthers collected from Shiraz flowers and this might account for a considerable portion of the flavonols in the flower. The high level of quercetin glycosides in anthers suggests that flavonols might also play a role in grapevine pollen fertility as reported in tobacco and petunia (Ylstra *et al.*, 1992; Ylstra *et al.*, 1994).
The bunch rachis of Vitis vinifera cv. Merlot has previously been shown to contain primarily quercetin-3-glucoside, around 0.2 mg/g fresh weight, with quercetin glucuronide less than 10% of that amount and small amounts of the myricetin and kaempferol glycosides (Souquet et al., 2000). While the relative proportions observed here are similar to those reported in Merlot stems by Souquet et al. (2000), the level of flavonols in Shiraz pedicels was around two-fold greater (0.43 mg/g) and in tendrils, six-fold higher (1.23 mg/g). Given the proposed role of these compounds in UV protection, the European (France) location of the previous work might well account for lower flavonol content compared to vines grown in South Australia.

Previously, the flavonol composition of grapevine leaves has also been studied. Mature grapevine leaves growing in Morocco were shown to contain all three flavonols, myricetin, quercetin and kaempferol, with quercetin derivatives accounting for 80-100% of the flavonol content (Imamouchi et al., 1996). Leaves of the four Vitis cultivars, Alicante, Carignan, Cinsault and Grenache noir, were examined with leaves of the latter two containing only trace amounts of myricetin and kaempferol. Total flavonols in grapevine leaves ranged from 8.1 mg/g dry weight in Grenache noir, to 38.6 mg/g dry weight in Alicante (Hmamouchi et al., 1996). In Shiraz, we observed both quercetin and kaempferol glycosides with total flavonols around 3.5 mg/g fresh weight (Figure 4A). Based on a water content of grape leaves of 90% or greater (Patakas et al., 1997), the levels reported here in Shiraz are comparable with those reported in cultivars grown in Morocco.

The flavonol synthase (FLS) enzyme forms a branchpoint in the flavonoid pathway and genes encoding FLS have now been isolated from a number of plants. Holton et al. (1993b) confirmed the function of the petunia FLS gene by expressing it in yeast and
demonstrating that extracts of the yeast catalysed the conversion of dihydroquercetin to quercetin in the presence of the appropriate cofactors. The grapevine cDNAs *VvFLSI* and *VvFLS2* both had high sequence homology to the petunia and other FLS genes indicating that they most likely encode FLS in grapevine. Previously, expression of genes encoding FLS has been detected in flowers but there have been few studies of flavonol synthesis in fruit. The petunia FLS gene was highly expressed in early developmental stages of flowers, but was not detected in leaves of petunia (Holton *et al*., 1993b). In potato, van Eldik *et al.* (1997) also found FLS expressed during flower development, particularly in the pistil, anther and petal tissues. Moriguchi *et al.* (2002) detected citrus FLS expression in flowers and young leaves but also in the juice sacs and peel of the fruit throughout fruit development. While little expression of *VvFLS2* was detected in any grapevine tissues, *VvFLSI* was expressed in the buds, leaves and tendrils of the grapevine as well as in the flowers, developing fruit and the skin of ripening berries of both Chardonnay and Shiraz.

The pattern of FLS gene expression in both Chardonnay and Shiraz berries between flowering and veraison was commensurate with the pattern of flavonol accumulation in the berry during that time. Expression of the *VvFLSI* gene at a high level at flowering suggests a period of intense flavonol biosynthesis coinciding with the high concentrations of flavonols detected in these tissues. The concentration of flavonols was observed to decrease substantially after flowering, due to a 20-fold increase in berry size by weight, and *VvFLSI* expression also decreased after flowering.

The concentration of flavonols (mg/g fresh weight) was relatively constant after veraison, but the amount of flavonols (mg/berry) increased, suggesting continued synthesis during the second phase of berry growth. Expression of *VvFLSI* increased around veraison and particularly during the period from two weeks post-veraison until ripening was complete.
This expression of \textit{VvFLSI} during ripening coincided with the increase in flavonols per berry in Shiraz (Figure 4.5D), although this was less noticeable in ripening Chardonnay berries (Figure 4.4D).

The level of flavonols in newly opened buds, tendrils, pedicels and developing inflorescences of Shiraz was significantly higher than at any time during berry development from fruit-set until harvest. Although there were clearly detectable levels of \textit{VvFLSI} in emerging buds, tendrils and developing flowers, very little \textit{VvFLSI} expression was detected in the pedicel or root. No expression of \textit{VvFLS2} was detected in these or any other vegetative tissue and it seems most likely that at the time of sampling, flavonol synthesis was complete in the grape pedicel and the root. Therefore, to determine expression of \textit{VvFLSI} in these tissues, samples would need to be taken at different times during their development. An alternative, albeit less likely, explanation for the absence of \textit{VvFLSI} expression in these tissues is that another FLS gene exists in grapevines that we are yet to identify and that expression of this gene accounts for flavonol synthesis in these tissues. This seems less likely as we were unable to amplify additional gene homologues by PCR with a range of degenerate primers and only two bands homologous to the \textit{VvFLSI} and \textit{VvFLS2} probes were detected by Southern blot analysis.

Studies of anthocyanin biosynthesis in grapevines showed that genes encoding biosynthetic enzymes for much of the flavonoid pathway were expressed post-veraison, when anthocyanin synthesis occurs, but also early in berry development (Boss \textit{et al.}, 1996a). This was considered particularly interesting, as expression of the pathway was not anticipated pre-veraison when anthocyanins were not being produced. Furthermore, Boss \textit{et al.} (1996b) observed that much of the pathway was also expressed in other grapevine tissues that did not normally accumulate anthocyanins. An explanation
consistent with these observations would be that flavonoid compounds other than anthocyanins were being synthesised in these tissues. We have previously shown substantial quantities of tannins (flavan-3-ols and proanthocyanidins) to be present in Shiraz berries from the earliest phase of berry development and increased towards veraison (Chapter 3). It is now clear that a significant level of flavonol biosynthesis is also occurring during berry development. Together, the spatial and temporal pattern of flavonol and tannin accumulation in grapes and expression of the FLS gene form the strongest indication yet that the expression of genes involved in flavonoid biosynthesis reported by Boss et al. (1996a; 1996b) is related to flavonol and tannins biosynthesis in grapevines.

To date, this is the most comprehensive study of flavonols in grapevines in that it has quantified flavonols throughout berry development. The pervasive nature of flavonols in the grapevine, and particularly the very high levels in juvenile and reproductive tissue, as well as the high levels in leaves are strong indications that the role of grapevine flavonols is UV protection. Such being the case, and the correlation between sun exposure and flavonol content reported by Price et al. (1995) and Haselgrove et al. (2000), viticultural practices that increase the sun exposure might significantly increase the flavonol content of the ripe fruit and the subsequent copigment pool in the wine.

The level of flavonols in grapes is relatively low compared to the anthocyanins and other potential copigments such as proanthocyanidins, with flavonols comprising as little as one to ten percent of total flavonoids depending on variety, site and season. The role of flavonoids in contributing to colour stability and the organoleptic properties of wine has yet to be established. However, our studies suggest that two periods of flavonol synthesis occur during berry development, around flowering and in the latter phases of ripening.
Manipulation of conditions in the vineyard at those times, such as bunch exposure to light, may have significant effects on flavonol synthesis and wine quality.
Chapter 5. Effect of Shading on Flavonoid Biosynthesis in *Vitis vinifera* L. cv. Shiraz

5.1. Introduction

The flavonoid composition of grapes has attracted much attention as an important contributor to wine quality (Glories, 1988). The three main classes of flavonoids found in grapes and wine are the anthocyanins, flavonols and condensed tannins. Anthocyanins are the pigmented compounds responsible for the colour of red wine and grapes (Ribereau-Gayon & Glories, 1986). Flavonols are thought to act as UV protectants (Flint *et al.*, 1985; Smith & Markham, 1998) and free radical scavengers (Markham *et al.*, 1998), and although not red in appearance they are thought to contribute to wine colour as copigments for anthocyanins (Asen *et al.*, 1972; Scheffeldt & Hrazdina, 1978). While the role of condensed tannins in the grape is uncertain, their astringency is thought to act as a feeding deterrent to herbivorous animals and insects (Feeny, 1976; Harborne & Grayer, 1993). In wine, condensed tannins contribute mouthfeel to red wines, as well as colour stability by forming complexes with anthocyanins (Timberlake & Bridle, 1976; Gawel, 1998; Malien-Aubert *et al.*, 2002; Mateus *et al.*, 2002b; Vidal *et al.*, 2002). Of the three classes of flavonoids, tannins are present in the greatest proportion in grapes, followed by anthocyanins, with flavonols being present at relatively low levels (Souquet *et al.*, 1996).

Of the many environmental and management factors that affect the flavonoid composition of grapes, exposure is regarded as one of the major influences (Winkler *et al.*, 1974; Archer & Strauss, 1989; Jackson & Lombard, 1993). The consensus has been that low light reduces colour and other flavonoids, while increased light increases
flavonoid content (Wicks & Kliewer, 1983; Dokoozlian & Kliewer, 1996). However, some authors have reported no change with light treatments (Hunter et al., 1995), while others had observed high light to result in decreased colour (Bergqvist et al., 2001).

Many explanations for these differences have been suggested, including differences in cultivar, site and season as well as different sampling and analytical techniques. In addition, many shading treatments have had an impact upon other microclimate parameters in the fruit zone, particularly temperature, which has been shown to significantly affect flavonoid accumulation and berry development (Kliewer, 1970; Jackson & Lombard, 1993). Some treatments have also altered the foliage exposure or the fruit to leaf ratio, affecting photosynthetic carbon assimilation or partitioning of assimilate to the fruit (Peterson & Smart, 1975; Rojas-Lara & Morrison, 1989; Morrison & Noble, 1990).

Where treatments have tried to shade fruit without altering berry temperature or leaf exposure, the anthocyanin content of the fruit was reduced in some cases (Wicks & Kliewer, 1983; Gao & Cahoon, 1994; Dokoozlian & Kliewer, 1996). Other authors have reported no change in the anthocyanin content of the fruit with shading, but a significant decrease in the flavonol content of the fruit and lower anthocyanins in the wine (Price et al., 1995; Haselgrove et al., 2000). In addition, shading has also been reported to alter the anthocyanin composition, with a shift towards acylated anthocyanins in the shaded fruit (Gao & Cahoon, 1994; Haselgrove et al., 2000). Thus, while a body of research exists on the effects of sun-exposure on grape flavonoid composition, the diversity of outcomes has failed to clarify the relationship between light and flavonoid biosynthesis in grapevines. In addition, many of these investigations have been limited to ripe or ripening fruit over as little as a single growing season. Recent studies have shown large seasonal variation in
flavonoid composition (Revilla et al., 1997; de Freitas & Glories, 1999; Guidoni et al., 2002), and indicate that much of the accumulation of tannins in the grape occurs prior to veraison (Kennedy et al., 2000a; Kennedy et al., 2000b). High levels of flavonols have also been reported in the fruit at veraison as well as at flowering and early in berry development (Haselgrove, 1997). To gain a greater understanding of the effect of shading on flavonoid biosynthesis in grapes with a view to managing the flavonoid content and composition of the fruit, and ultimately the wine, it is necessary to examine the entirety of berry development across several seasons.

In this work, we sought to separate the effects of light and temperature and to explore the impact of light exclusion on flavonoid biosynthesis in the major Australian red wine grape cultivar Shiraz throughout berry development over three successive seasons.

5.2. Materials and Methods

5.2.1.1. Shading Treatments

In addition to the shading treatments described previously (Section 2.2.2), a modest trial also investigated the inducibility of flavonol accumulation and VvFLS1 expression. When the boxes were repositioned during the 2000-2001 season to enclose the developing bunches while allowing further development of the shoot, shaded tissue samples were collected. Three days following this operation, samples were also collected of the newly exposed leaves and inflorescences to determine flavonol accumulation and VvFLS1 expression in these tissues following exposure to light.
5.2.1.2. Analysis of Flavonoids in Shaded Berries

Tannins in whole berries, seeds and skins of grapes were extracted and analysed by HPLC as described in Section 3.2.2 and Section 3.2.3, while flavonols were extracted and analysed by HPLC as described in Section 4.2.2. For HPLC analysis of anthocyanins, the frozen samples were ground to a fine powder under liquid nitrogen. Three separate aliquots (analysis replicates) of 0.1-g were extracted in 1.0 mL of acidified methanol (1% HCl v/v; 3 mL conc. HCL + 97 mL methanol; Harborne, 1958) for one hour in darkness at room temperature with occasional vortexing. Extracts were then centrifuged (15 min at 13,000 x g) and 500 µL of the supernatant transferred to HPLC auto-sampler vials.

Samples were analysed using a Hewlett Packard HP 1100 high-performance liquid chromatograph (HPLC) with a Merck (Germany) LiCrospher™ analytical column protected by an SGE (Australia) C-18 guard column. The separation used water (solvent A) with a methanol (solvent B) and perchloric acid 1.5% v/v (solvent C) gradient. The gradient was: zero min, 25% B, 15% C; 10 min, 30% B, 15% C; 40 min, 35% B, 15% C; 45 min, 45% B, 30% C; 105 min, 55% B, 30% C; 107 min 100 % B, zero % C; 112 min, 100% B, zero % C; 114 min, 25% B, 15% C.

The anthocyanin peaks were identified by comparison of their elution order with the published separation of Wulf and Nagel (1978) and comparison of the elution time and absorbance spectra with a commercial standard of malvidin-3-glucoside (Extrasyntese, France). The integrated absorbance at 520 nm was used to determine the concentration of individual anthocyanins in each sample (100 µL injection) expressed as malvidin-3-glucoside equivalents calculated from a calibration function prepared from the commercial standard.
Gene expression was determined by real-time PCR analysis. For the isolation of RNA, grapevine material frozen in liquid nitrogen in the field was ground to a fine powder in liquid nitrogen using a pre-frozen mortar and pestle and stored at -80°C. Total RNA was extracted from the frozen powdered tissue and cDNA was prepared from the RNA as described in Section 4.2.3. cDNA syntheses for all RNA samples to be analysed were performed simultaneously. Reagents required for the reverse transcription (RT) reactions were made as a master mix and aliquoted into each reaction in a single step to avoid pipetting errors that may have affected the efficiency of the RT reactions in different samples.

Quantitative real-time PCR was performed on a Rotor-Gene 2000™ (Corbett Research, Australia) real-time PCR machine using SYBR green as the system of detection for double stranded PCR products. Primers homologous to the grapevine cDNAs encoding VvFLS1 (AY257978), VvUFGT (X75968) and VvUbiquitin1 (TC9998) were designed using primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3 WWW.cgi). The primer pairs VvFLS1 Forward: 5'-CAGGGCTTGCAGGTTTTTAG- 3', VvFLS1 Reverse: 5'-GGGTCTTCTCCTTGTTCACG- 3' and VvUFGT Forward: 5' - TAACACATTGTGGATGGAACTCAT- 3' and VvUFGT Reverse: 5' - ACCTTCAATTCTCACTCCAATCTC- 3' were used to generate PCR products of 154bp and 140bp respectively for FLS and UFGT. The VvUbiquitin1 transcript was detected using the primers VvUbiquitin Forward: 5'-GTGGATTATTGAGCCCATCCT- 3' and VvUbiquitin Reverse: 5'-AACCTCCAATCCAGTCATCTC- 3' which amplify a product of 182base pairs.
All primer pairs were used under identical conditions, 95°C for 5 minutes followed by 35 rounds of amplification (95°C/30sec, 57°C/30sec, 72°C/30sec). Each PCR reaction contained 1 × SYBR Green PCR Master Mix™ (Applied Biosystems, UK), 30 nmoles of each primer and 1/50th volume of cDNA stock in a final reaction volume of 25 μL. The VvFLSI and VvUFGT mRNAs were quantified as a proportion of VvUbiquitin1 using the Rotor Gene 2000™ software. Expression of VvFLSI and VvUFGT mRNA is presented as a ratio of expression of VvFLSI and VvUFGT to expression of VvUbiquitin1 and therefore has no units.

5.3. Results

5.3.1.1. Effect of Shading on Berry Development

In the first experimental season (1999-2000) flowering occurred nine weeks pre-veraison, with fruit set occurring one week later (eight weeks pre-veraison). Berry weight increased steadily from fruit-set towards veraison in both the shaded and exposed fruit. At veraison, shaded berries weighed less than the exposed fruit and shading had a noticeable effect on berry weight post-veraison (Figure 5.1A) with the weight of shaded berries substantially lower than that of the exposed fruit. At harvest, shaded berries weighed 35% less than the exposed berries. Despite the difference in berry weight, shaded berries ripened at a similar rate to the exposed fruit and reached the same level of total soluble solids at harvest, around 23.5°Brix (Figure 5.1B).
In the second season (2000-2001), flowering occurred eight weeks pre-veraison with fruit-set the following week (seven weeks pre-veraison). The difference in berry weight between shaded and exposed fruit (Figure 5.1C) was less noticeable than that observed during the 1999-2000 season (Figure 5.1A). Pre-veraison, berry weight in the shaded fruit was lower than that in the exposed fruit (Figure 5.1C). However, post-veraison increases

Figure 5.1. Grape berry development in Vitis vinifera cv. Shiraz over three seasons. A). Berry weight for exposed (●) and shaded (□) fruit; B) Total soluble solids expressed as °Brix for exposed (●) and shaded (□) fruit in the 1999-2000 season. Flowering occurred during the week of 04 November 1999 (9 weeks pre-veraison) with veraison around 06 January 2000 and commercial harvest on 24 February 2000 (7 weeks post-veraison). C) Berry weight for exposed (●) and shaded (□) fruit; D) Total soluble solids expressed as °Brix for exposed (●) and shaded (□) fruit in the 2000-2001 season. Flowering occurred 8 weeks pre-veraison (16 November 2000), with veraison around 11 January 2001, the fruit was harvested 13 March 2001 (9 weeks post-veraison). E) Berry weight for exposed (●) and shaded (□) fruit; F) Total soluble solids expressed as °Brix for exposed (●) and shaded (□) fruit in the 2001-2002 season. Flowering occurred 10 weeks pre-veraison (23 November 2001), with veraison around 31 January 2001, the fruit was harvested 15 March 2001 (6 weeks post-veraison).
in the berry weight of shaded fruit resulted in similar weights by five weeks post-veraison and at harvest, berry weights were similar between shaded and exposed fruit (approximately 0.96 g/berry). There was also no difference in total soluble solids between shaded and exposed fruit throughout ripening during the 2000-2001 season (Figure 5.1D), similar to the trend for sugar accumulation observed during the previous season (Figure 5.1B).

During the third experimental season (2001-2002), flowering occurred 10 weeks pre-veraison with fruit-set occurring two weeks later at eight weeks pre-veraison. The increase in berry weight in the early part of berry development (pre-veraison) was more gradual than observed in previous seasons (Figure 5.1E). There was virtually no difference in berry weight between the shaded and exposed fruit throughout development during the 2001-2002 season with berry weight increasing steadily from fruit-set towards harvest. Similarly, there was no discernible difference in the level of total soluble solids (°Brix) between the exposed and shaded berries (Figure 5.1F) during berry development in the 2001-2002 season.

5.3.1.2. Anthocyanin Accumulation in Shaded Grapes

Figure 5.2 shows the pattern of anthocyanin accumulation in Shiraz berries over three successive seasons (1999-2000, 2000-2001 and 2001-2002) on both a mg/g fresh weight and a mg/berry basis. The total anthocyanin content was calculated as the sum of the individual compounds determined by HPLC and expressed as malvidin-3-glucoside equivalents. In the first experimental season (1999-2000) anthocyanins accumulated slowly from veraison, followed by a rapid increase in concentration between two and four weeks post-veraison (Figure 5.2A). Anthocyanins accumulated more slowly at first in the
exposed fruit than in the shaded fruit, but at commercial harvest there was no significant difference ($\alpha=0.05$; $n=3$) in the concentration of anthocyanins between shaded and control fruit. On a per berry basis (Figure 5.2B), the pattern was similar to that observed per gram of berry weight.

In the second experimental season (2000-2001), there was a significant difference ($\alpha=0.05$, $n=3$) in the concentration of anthocyanins between the shaded and exposed (control) fruit, with the exposed fruit having a higher level of anthocyanins per gram

![Figure 5.2. Total anthocyanin accumulation in Vitis vinifera cv. Shiraz berries over three seasons in Willunga, SA. A) Total anthocyanins (mg/g fresh weight berry) in exposed (•) and shaded (□) fruit in the 1999-2000 season; B) total anthocyanins (mg/berry) in exposed (•) and shaded (□) fruit in the 1999-2000 season; C) Total anthocyanins (mg/g fresh weight berry) in exposed (•) and shaded (□) fruit in the 2000-2001 season; D) total anthocyanins (mg/berry) in exposed (•) and shaded (□) fruit in the 2000-2001 season; E) Total anthocyanins (mg/g fresh weight berry) in exposed (•) and shaded (□) fruit in the 2001-2002 season; F) total anthocyanins (mg/berry) in exposed (•) and shaded (□) fruit in the 2001-2002 season.]
fresh weight of skin than the shaded fruit (Figure 5.2C). In the exposed fruit, anthocyanins accumulated steadily from one to two weeks post-veraison, then more rapidly over the following week to three weeks post-veraison when the highest concentration was recorded (Figure 5.2C). This level per gram fresh weight of berry was maintained until harvest. However, on a per berry basis, anthocyanin accumulation continued as the berry increased in size until five weeks post-veraison as shown in Figure 5.2D. On a per berry basis a decline in total anthocyanins was observed from seven weeks post-veraison until harvest. This decline was also observed in the shaded fruit, although the maximum observed was lower. In addition, the peak in anthocyanin accumulation per gram fresh weight in the shaded fruit occurred one to two weeks later than observed in the exposed berries. The level of total anthocyanins per berry was also significantly lower in the shaded fruit during the second season (Figure 5.2D). While the pattern of anthocyanin accumulation was similar between the 1999-2000 and 2000-2001 seasons, the level of total anthocyanins per gram fresh weight of berry and per berry was substantially lower than in the previous season, in both exposed and shaded fruit. This level was also lower than that observed in the following season (2001-2002).

In the third experimental season (2001-2002), there was no difference between exposed and shaded fruit in either the amount of anthocyanins per gram fresh weight of berry skin (Figure 5.2E), or per berry (Figure 5.2F). The pattern of anthocyanin accumulation per gram fresh weight of berry and per berry was similar to that observed in previous seasons with the bulk of accumulation occurring during the first three to four weeks following veraison. However, on a per gram fresh weight basis, anthocyanin accumulation in the shaded fruit continued steadily until harvest whereas in the exposed fruit, accumulation slowed towards harvest.
The anthocyanin composition of Shiraz berries at 24 °Brix was determined by HPLC and the mean percent composition compared between treatments and seasons for each of the anthocyanins commonly detected in Shiraz using Student’s t-test (α=0.05; n=3) (Table 5.1). In the first experimental season (1999-2000), there was no significant effect of shading on total anthocyanins and there was no apparent change in the relative proportions of non-acylated, acetyl- and coumaroylglucosides.

In the second experimental season (2000-2001), there was a significant difference in the level of total anthocyanins in Shiraz berries between treatments (Figure 5.2) as well as substantial shifts in anthocyanin composition with shading (Table 5.1). While all anthocyanins were lower in the shaded fruit, the decrease in non-acylated and

| Table 5.1. Mean percentage of anthocyanins in Shiraz berries at 24 °Brix across three seasons. The effect of shading on the level of each anthocyanin is shown; NS indicates no significant difference, while increase or decrease indicate a significant difference in the level of anthocyanin (α=0.05; n=3). Superscripts indicate differences between seasons in the level of each of the anthocyanins present in Shiraz berries for shaded and exposed fruit |
|---|---|---|---|---|---|---|---|---|---|---|
| **Glucoside** | **Acetylglucoside** | **Coumaroylglucoside** |
| **Season** | **Shaded** | **Exposed** | **Effect of Shading** | **Shaded** | **Exposed** | **Effect of Shading** | **Shaded** | **Exposed** | **Effect of Shading** |
| Malvidin | 1999-2000 | 24.06° | 23.80° | NS | 6.13° | 7.05° | NS | 11.66° | 12.62° | NS |
| 2000-2001 | 20.51° | 31.54° | decrease | 7.81° | 13.45° | decrease | 31.21° | 24.54° | increase |
| 2001-2002 | 34.68° | 35.87° | decrease | 5.22° | 6.40° | decrease | 8.93° | 9.45° | decrease |
| Petunidin | 1999-2000 | 7.06° | 7.48° | NS | 1.50° | 1.77° | NS | 3.5° | 4.07° | increase |
| 2000-2001 | 3.84° | 5.13° | decrease | 0.77° | 1.27° | decrease | 4.62° | 3.12° | increase |
| 2001-2002 | 9.39° | 9.97° | decrease | 1.27° | 1.59° | decrease | 1.88° | 2.01° | decrease |
| Delphinidin | 1999-2000 | 6.2° | 7.72° | NS | 1.65° | 1.69° | NS | 2.81° | 3.95° | decrease |
| 2000-2001 | 4.32° | 4.97° | NS | 0.89° | 0.89° | NS | 4.91° | 3.63° | increase |
| 2001-2002 | 9.05° | 10.57° | decrease | 1.16° | 1.50° | decrease | 2.08° | 2.48° | decrease |
| 2000-2001 | 6.07° | 3.64° | increase | 2.03° | 1.96° | NS | 10.73° | 4.67° | increase |
| 2001-2002 | 15.24° | 10.56° | increase | 2.14° | 1.83° | increase | 5.44° | 4.30° | increase |
| Cyanidin | 1999-2000 | 2.98° | 2.95° | NS | 1.03° | 1.36° | decrease | 2.25° | 2.76° | NS |
| 2000-2001 | 0.93° | 0.50° | increase | 0.27° | 0.15° | increase | 1.09° | 0.51° | increase |
| 2001-2002 | 2.38° | 2.61° | NS | 0.28° | 0.28° | NS | 0.68° | 0.59° | increase |
| Total | 1999-2000 | 54.97° | 51.76° | NS | 14.84° | 16.29° | NS | 30.19° | 31.95° | NS |
| 2000-2001 | 35.68° | 45.81° | decrease | 11.76° | 17.72° | decrease | 52.56° | 36.47° | increase |
| 2001-2002 | 70.93° | 69.38° | increase | 10.06° | 11.60° | decrease | 19.07° | 18.82° | NS |
acetylglucosides in the shaded fruit was greater than the change in coumaroylglucosides, so the proportion of total coumaroylated glucosides increased, while the proportion of non-acylated and acetylglucosides decreased. This general pattern was the same for the glucosides of malvidin and petunidin, and while there was no change in the delphinidin non-acylated and acetylglucosides, the coumaroylglucoside of delphinidin also increased with shading during the 2000-2001 season. In contrast to delphinidin, petunidin and malvidin, there was a general increase in the relative proportions of the glucosides of cyanidin and peonidin, with peonidin non-acylated glucosides and coumaroylglucosides in the shaded fruit consistently higher across all three seasons (Table 5.1).

In the third experimental season (2001-2002), while there was no change in total anthocyanins with shading, significant changes in percent composition were again observed (Table 5.1). The general pattern was very similar to that of the previous season (2000-2001), with the proportion of total acetylglucosides decreased in the shaded fruit, although there was no change in total coumaroylglucosides and a slight increase in total non-acylated glucosides. All of the glucosides of malvidin, petunidin and delphinidin were lower in the shaded fruit while the glucosides of peonidin were significantly higher and those of cyanidin generally unchanged.

These observations indicate a clear difference in the response of cyanidin and peonidin based anthocyanins to shading compared to delphinidin, petunidin and malvidin. Cyanidin and peonidin differ from delphinidin, petunidin and malvidin in the hydroxylation pattern of the flavonoid “B” ring (Holton & Cornish, 1995). Therefore, the percent of dihydroxylated anthocyanins was compared with the proportion of anthocyanins with three hydroxyl moieties on the “B” ring (Table 5.2). In each of the three seasons there was a significant difference (p<0.005; n=3) between treatments. The shaded fruit were
consistently lower in trihydroxylated anthocyanins and there was a higher proportion of anthocyanins with two hydroxyl moieties on the flavonoid “B” ring.

Significant differences were also observed between seasons in the mean percentage of anthocyanins (Table 5.1). Total non-acylated glucosides and coumaroylglucosides were significantly different across all three seasons in both shaded and exposed fruit, but there was no consistent pattern in the changes in total acetylglucosides, between seasons. In the second season (2000-2001) when total anthocyanins were significantly lower in the shaded berries, there were proportionally less total non-acylated glucosides, little difference in the level of acetylglucosides and a significantly higher percentage of coumaroylated anthocyanins in the shaded fruit. Generally, non-acylated and acetylglucosides were lower in the second season, although the pattern of malvidin non-acylated and acetylglucosides was less consistent between seasons. Malvidin acetyl- and coumaroylglucosides were proportionally higher in the second season in both the shaded and exposed fruit. Otherwise, there was not a consistent pattern in the differences in coumaroylated anthocyanins between seasons.

Table 5.2. Effect of shading and season on the pattern of “B” ring hydroxyl substitution of anthocyanins in grapes at 24 Brix. Increase/Decrease indicates a significant difference in mean percentage of dihydroxylated (3’,4’-OH; cyanidin, peonidin) and trihydroxylated (3’,4’,5’-OH; delphinidin, petunidin, malvidin) anthocyanins (p<0.005; n=3). Superscripts indicate significant difference in mean percentage of dihydroxylated and trihydroxylated anthocyanins between seasons (p<0.0001; n=3)

<table>
<thead>
<tr>
<th>&quot;B&quot; Ring Substitution</th>
<th>Season</th>
<th>Shaded</th>
<th>Exposed</th>
<th>Effect of Shading</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’-5’-OH</td>
<td>1999-2000</td>
<td>35.42a</td>
<td>29.87b</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>2000-2001</td>
<td>21.13b</td>
<td>11.43b</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>2001-2002</td>
<td>26.36c</td>
<td>20.17c</td>
<td>Increase</td>
</tr>
<tr>
<td>3’-4’-5’-OH</td>
<td>1999-2000</td>
<td>64.58a</td>
<td>70.13a</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>2000-2001</td>
<td>78.87b</td>
<td>88.57b</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>2001-2002</td>
<td>73.64c</td>
<td>79.83c</td>
<td>Decrease</td>
</tr>
</tbody>
</table>
There were also significant differences in the proportions of di- and trihydroxylated anthocyanins between seasons (Table 5.2). While there was a significant difference between all three seasons (p<0.0001; n=3), the most obvious difference was the substantially lower proportion of dihydroxylated anthocyanins (cyanidin and peonidin) and greater proportion of trihydroxylated anthocyanins (delphinidin, petunidin and malvidin) in both the shaded and exposed fruit in the second season (2000-2001). In absolute terms, this difference represents a decrease in cyanidin and peonidin glucosides per gram fresh weight of skin and an increase in delphinidin, petunidin and malvidin glucosides (raw data not shown).

The pattern of methyl substitution was also significantly different between seasons with the levels of the unmethylated anthocyanins, cyanidin and delphinidin, and peonidin and petunidin, with a single methyl group on the "B" ring, both lower in the 2000-2001 season (Table 5.3). Whereas malvidin was significantly higher in the second season (2000-2001) in both the shaded and exposed (control) fruit (p<0.05; n=3).

Table 5.3. Effect of shading and season on the pattern of "B" ring methoxyl substitution of anthocyanins in the skin of Shiraz grapes at 24°Brix. Increase/Decrease indicates a significant difference in mean percentage of unmethylated (cyanidin and delphinidin glucosides), 3'-methylated (peonidin glucosides) and 3',5'-d-methylated (petunidin and malvidin glucosides) anthocyanins (p<0.05; n=3). Superscripts indicate significant difference between season (p<0.05; n=3).

<table>
<thead>
<tr>
<th>&quot;B&quot; Ring Substitution</th>
<th>Season</th>
<th>Shaded</th>
<th>Exposed</th>
<th>Effect of Shading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsubstituted</td>
<td>1999-2000</td>
<td>16.93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2000-2001</td>
<td>12.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2001-2002</td>
<td>15.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Decrease</td>
</tr>
<tr>
<td>3'-CH&lt;sub&gt;3&lt;/sub&gt;OH</td>
<td>1999-2000</td>
<td>41.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>2000-2001</td>
<td>28.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Increase</td>
</tr>
<tr>
<td></td>
<td>2001-2002</td>
<td>35.35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.26c</td>
<td>Increase</td>
</tr>
<tr>
<td>3',5'-CH&lt;sub&gt;3&lt;/sub&gt;OH</td>
<td>1999-2000</td>
<td>41.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>2000-2001</td>
<td>59.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Decrease</td>
</tr>
<tr>
<td></td>
<td>2001-2002</td>
<td>48.82&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.71&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Decrease</td>
</tr>
</tbody>
</table>
5.1.1.3. Tannin Accumulation in Shaded Grapes

The tannin composition of fruit from the first experimental season was determined from whole berries. In the shaded fruit, the level of free monomers per gram fresh weight of berry was initially slightly lower than that in the exposed fruit (Figure 5.3A). From veraison onwards there was an increase in monomers in both the shaded and exposed fruit, both reaching similar maxima one to two weeks post-veraison, thereafter declining towards harvest. Expressed on a per berry basis the difference in free monomers between exposed and shaded fruit was more apparent, particularly the greater increase in free

![Figure 5.3. Tannin accumulation in Vitis vinifera cv. Shiraz whole berries 1999-2000 in exposed (●) and shaded (□) fruit. A) Free monomers mg/g fresh weight of berry (±SEM); B) free monomers mg/berry (±SEM); C) Terminal subunits mg/g fresh weight of berry (±SEM); D) terminal subunits mg/berry (±SEM); E) extension subunits mg/g fresh weight of berry (±SEM); F) extension subunits mg/berry (±SEM)
monomers in the exposed fruit at veraison (Figure 5.3B). This lower total per berry in the shaded fruit was consistent with the lower berry weight in the shaded fruit observed during that season. At commercial harvest, the levels per gram and per berry in both the shaded and exposed fruit were similar.

In the shaded fruit, the level of terminal subunits per gram fresh weight of berry was lower than that observed in the exposed fruit (Figure 5.3C). This difference became apparent from around fruit-set, with the berries shaded from flowering during that season. The maximum level of terminal subunits in the exposed fruit occurred around six weeks pre-veraison, in the shaded fruit the maximum occurred at around fruit-set, eight weeks pre-veraison. From their respective maxima, the levels in both shaded and exposed fruit declined steadily towards veraison, remaining relatively constant thereafter at similar levels. This was not reflected in the per berry presentation (Figure 5.3D) as the difference in berry weight post-veraison resulted in a large difference in terminal subunits per berry between treatments. Nevertheless, at commercial harvest, the substantially greater level of terminal subunits per berry in the exposed fruit had decreased to a level similar to that in the shaded fruit.

This pattern was virtually the same as that observed for the extension subunits between the shaded and exposed whole berries during the 1999-2000 season (Figure 5.3E, 5.3F). On a per gram fresh weight basis the highest concentration of extension subunits occurred around fruit-set and declined towards veraison, remaining relatively constant from that time until harvest (Figure 5.3E). On a per berry basis, extension subunits began to increase with increasing berry weight (Figure 5.1). The increase in extension subunits per berry was greater in the exposed fruit (Figure 5.3F). From six to four weeks pre-veraison extension subunits increased in both shaded and exposed fruit, although the increase was
greater in the exposed fruit. After that time the total in the shaded berries remained relatively stable until veraison when a further increase was observed to two weeks post-veraison. This level was maintained until harvest. In the exposed fruit there was a greater increase at veraison than in the shaded whole berries, followed by a substantial decline in extension subunits towards harvest, but again at harvest similar levels were observed (Figure 5.3F).

In the second experimental season (2000-2001) tannins were determined in the seeds and skins separately. The levels of free monomers, terminal and extension subunits in the

Figure 5.4. Tannin accumulation in Vitis vinifera cv. Shiraz seeds in the 2000-2001 season in exposed (♦) and shaded (○) fruit. A) Free monomers mg/g fresh weight of seed (±SEM); B) free monomers mg/berry (±SEM); C) Terminal subunits mg/g fresh weight of seed (±SEM); D) terminal subunits mg/berry (±SEM); E) extension subunits mg/g fresh weight of seed (±SEM); F) extension subunits mg/berry (±SEM)
seeds of both shaded and exposed fruit during the 2000-2001 season are presented in Figure 5.4. During the second experimental season no difference was observed in the level of free imonomers in the seeds between treatments on either a per gram fresh weight of seed or a per berry basis (Figure 5.4A, 5.4B). In both shaded and exposed fruit free monomers accumulated slowly at first, then rapidly from around three weeks pre-veraison until one week post-veraison, thereafter declining steadily until around five weeks post-veraison. After this time, the levels remained relatively constant until commercial harvest.

Similarly, the pattern of terminal subunit accumulation in the seeds was virtually identical between the shaded and exposed fruit, although on a per gram basis it appeared that there was a slightly higher level of terminal subunits in the seeds of the shaded fruit (Figure 5.4C). However, this was not apparent on a per berry basis where no difference was observed in the level of seed terminal subunits between the shaded and exposed fruit (Figure 5.4D). On both a per gram and a per berry basis a substantial level of terminal subunits was present at fruit-set, the level was maintained until around two to three weeks pre-veraison when the level increased until around one week post-veraison. This was followed by a decline over the following two weeks, whereafter the levels remained relatively constant until the fruit was harvested.

As with the free monomers and terminal subunits, there was very little difference in the level of extension subunits in the seeds of the shaded and exposed fruit (Figure 5.4E, 5.4F). The level of extension subunits per gram of seed was highest in the exposed fruit by around four weeks pre-veraison thereafter the level declined towards harvest (Figure 5.4E). In the shaded fruit samples were not analysed until three weeks pre-veraison when the level was also relatively high, increasing slightly in the next sample (one week pre-
veraison) and then also declining towards harvest. On a per berry basis there were minor differences in the level of extension subunits in the seeds between the shaded and exposed fruit, but the trend was the same and the levels comparable (Figure 5.4F).

In Shiraz berry skins collected during the second experimental season (2000-2001), the level of free monomers was noticeably different between treatments prior to veraison. On a per gram fresh weight of skin basis the concentration was much lower in the shaded fruit pre-veraison (Figure 5.5A). However, an increase in free monomers per gram

Figure 5.5. Tannin accumulation in *Vitis vinifera* cv. Shiraz skin in the 2000-2001 season in exposed (+) and shaded (□) fruit. A) Free monomers mg/g fresh weight of skin (±SEM); B) free monomers mg/berry (±SEM); C) Terminal subunits mg/g fresh weight of skin (±SEM); D) terminal subunits mg/berry (±SEM); E) extension subunits mg/g fresh weight of skin (±SEM); F) extension subunits mg/berry (±SEM)
between one week pre-veraison and one week post-veraison made the levels indistinguishable. On a per berry basis, this difference pre-veraison was more apparent, but again the levels converged post-veraison (Figure 5.5B). In the exposed fruit, the level of free monomers per berry reached a maximum around two weeks pre-veraison, while in the shaded fruit, the maximum level observed was recorded five weeks post-veraison. On both a per gram and a per berry basis, the levels of free monomers in the skins of shaded and exposed fruit were comparable at commercial harvest.

Skin tannin terminal subunits in the shaded fruit were actually higher per gram fresh weight of skin than in the exposed fruit prior to veraison, with the maximum level observed around twice that in the exposed fruit (Figure 5.5C). However, this difference was not as obvious when the data were expressed on a per berry basis (Figure 5.5D). The level of terminal subunits in the skins of both shaded and exposed Shiraz berries reached a maximum around two weeks post-veraison. This level was comparable between treatments and declined to similar levels towards harvest.

The level of extension subunits in grape skins was also noticeably different between shaded and exposed fruit. While the difference per gram fresh weight of skin was relatively small (Figure 5.5E), the differences per berry were much more apparent (Figure 5.5F). The maximum level of extension subunits observed in the skin of the exposed fruit per berry was around twice that observed in the skin of the shaded fruit. However, as was observed with both the free monomers and the terminal subunits, by commercial harvest similar levels were observed between the shaded and exposed fruit.

While extension subunits were generally lower in the skin of the shaded fruit, there was a shift in the relative amounts of individual extension subunits based on the hydroxylation pattern of the “B” ring (Table 5.4). There was a proportionally greater decrease in the
trihydroxylated extension subunits, epigallocatechin, with shading than there was in the
dihydroxylated extension subunits, catechin, epicatechin and epicatechin-gallate (p<0.01; n=3). This decrease represented a 50% decrease in the concentration of epigallocatechin (mg/g fwt berry) with a commensurate increase in catechin and epicatechin in the skin of the shaded fruit (tannin compositional data for shaded fruit not presented). Epigallocatechin extension subunits were not detected in Shiraz seeds.

Table 5.4. Effect of shading and season on the pattern of “B” ring hydroxyl substitution of proanthocyanidin extension subunits in the skin of Shiraz grapes at 24°Brix. Increase/Decrease indicates a significant difference in mean percentage of dihydroxylated (3',4'-OH; catechin, epicatechin, epicatechin-gallate) and trihydroxylated (3',4',5'-OH; epigallocatechin) extension subunits (p<0.01; n=3).

<table>
<thead>
<tr>
<th>&quot;B&quot; Ring Substitution</th>
<th>Season</th>
<th>Shaded</th>
<th>Exposed</th>
<th>Effect of Shading</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-5'-OH</td>
<td>2000-2001</td>
<td>77.95</td>
<td>64.79</td>
<td>Increase</td>
</tr>
<tr>
<td>3'-4'-5'-OH</td>
<td>2000-2001</td>
<td>22.05</td>
<td>35.21</td>
<td>Decrease</td>
</tr>
</tbody>
</table>

Of the dihydroxylated procyanidins, there was also a slight, but significant (p<0.001, n=3) shift from catechin to epicatechin extension subunits and a greater shift towards epicatechin terminal subunits (p<0.0001; n=3) with shading. Epicatechin accounted for approximately 25% of terminal subunits in the exposed fruit, but almost 50% in the shaded fruit due to a decrease in the amount of catechin per gram fresh weight of berry skin (compositional data for shaded fruit not presented).

In addition to compositional shifts between catechin and epicatechin with shading, the effect of shading on galloylation was also examined. The proportion of galloylated terminal subunits were slightly higher in the shaded fruit (18%) than in the exposed fruit (14%) in the 2000-2001 season, however this difference was not statistically significant (p=0.594; n=3). In contrast, galloylated extension subunits in the shaded fruit were significantly higher (15%) than in the exposed fruit (8.7%; p<0.0001; n=3).
The small differences observed between shaded and exposed fruit in seed tannins and the larger differences in skin tannins are highlighted by the differences in polymer length (mDP) between treatments (Figure 5.6). In Shiraz seeds, polymer length was very similar between the shaded and exposed fruit as suggested by the similar levels of terminal and extension subunits (Figure 5.6A). However, in Shiraz skin, the slightly higher level of terminal subunits and lower extension subunit level in the shaded fruit combined to lower the mean degree of polymerisation (mDP) in the shaded fruit (Figure 5.6B).

5.1.1.4. Flavonol Accumulation in Shaded Grapes

Flavonol accumulation was measured as total quercetin glycosides (Section 4.2.2) over three successive seasons (1999-2000, 2000-2001 and 2001-2002) in the skin of shaded and exposed fruit (Figure 5.7). In the first season (1999-2000), when boxes were applied one week after flowering, high levels of flavonols per gram fresh weight of skin were present in berries of both exposed and shaded fruit in the early stages of berry development, declining as the berries grew (Figure 5.7A). The level of flavonols per gram fresh weight of skin was noticeably higher in the exposed fruit than in the shaded fruit at all stages of berry development and this was emphasised when the data were
presented on a per berry basis (Figure 5.7B). There was an increase in flavonols per berry 2-4 weeks after veraison in both the exposed and shaded fruit. In ripe fruit (~24°Brix), the concentration of flavonols in the skin of shaded fruit was 48% of that in exposed fruit.

In the second season (2000-2001), boxes were applied five weeks before flowering, when significant flavonol accumulation occurs (Section 4.3.5). The pattern of flavonol accumulation was broadly similar to the previous year in exposed fruit but no flavonols were detected in the shaded fruit at any stage of berry development (Figure 5.7C, 5.7D). On a per berry basis, flavonol accumulation was again observed after veraison in the

Figure 5.7. Flavonol accumulation in *Vitis vinifera* cv. Shiraz skins in exposed (●) and shaded (♦) fruit. A) Total flavonols (mg/g fresh weight berry) 1999-2000; B) total flavonols (mg/berry) 1999-2000; C) Total flavonols (mg/g fresh weight berry) 2000-2001; D) total flavonols (mg/berry) 2000-2001; E) Total flavonols (mg/g fresh weight berry) 2001-2002; F) total flavonols (mg/berry) 2001-2002
exposed fruit (Figure 5.7D) but even at harvest no flavonols could be detected in the shaded fruit.

In the third experimental season (2001-2002) a much larger number of boxes were applied, four weeks before flowering. Flavonols in the shaded fruit were again much lower than in exposed fruit, on both a per gram fresh weight and per berry basis (Figure 5.7E, 5.7F). In ripe grapes (~24°Brix), the concentration of flavonols in the skin of shaded fruit was only 10% of that in exposed fruit. An increase in flavonols per berry was observed in the exposed fruit later in ripening in all three seasons (Figure 5.7). In the shaded fruit, there was also some increase in flavonols per berry after veraison in the first experimental season (1999-2000) and to a lesser degree in the third season (2001-2002).

5.1.1.5. Expression of Genes Involved in Flavonol and Anthocyanin Biosynthesis in Shaded Berries

Expression of genes encoding enzymes involved in anthocyanin and flavonol biosynthesis was determined by real-time PCR. The levels of expression of the genes encoding UDP glucose: flavonoid-3-O-glucosyltransferase (UFGT), one of the key steps in anthocyanin synthesis, and flavonol synthase (FLS), the first step in flavonol synthesis, are presented relative to the expression of a consistently expressed gene, Ubiquitin. The levels, of expression of these genes was compared between the shaded and exposed fruit in the 2000-2001 season in Shiraz berry skin (Figure 5.8).

Expression of VvUFGT was very low before veraison but increased dramatically in the first 1-2 weeks after veraison, coincident with the onset of anthocyanin accumulation (Figure 5.2C). The level and pattern of expression of VvUFGT was similar in shaded and
exposed fruit, declining at the later stages of ripening as reported previously (Boss et al., 1996c).

In exposed fruit, the gene encoding flavonol synthase \((VvFLSI)\) was expressed early in berry development and again during ripening (Figure 5.8), consistent with the two phases of flavonol accumulation in the berries (Section 4.3.5). Expression of \(VvFLSI\) in shaded fruit was extremely low post-veraison, when expression was detected in exposed fruit (Figure 5.8). To determine the effect of light on flavonol synthesis early in berry development, the concentration of flavonol glycosides and expression of \(VvFLSI\) was also determined in developing inflorescences, leaves and bunches which had not been exposed to light (Table 5.5). Boxes were applied to spurs prior to budburst and transferred to the developing inflorescence in the dark once the shoot had emerged.

Leaves sampled from shoots grown in the dark had less than 3% of the flavonol concentration of equivalent exposed leaves and expression of \(VvFLSI\) was only 5% of that in exposed leaves (Table 5.5). In the developing inflorescence, the bunch one week after flowering and in the skin of ripe grapes grown in boxes throughout development, the
concentration of flavonols was very low or undetectable and the expression of \( VvFLSI \)
was also greatly reduced compared to equivalent tissues exposed to light (Table 5.5).

### Table 5.5. Concentration of flavonols and relative expression of the flavonol synthase gene \( VvFLSI \) in different tissues of Shiraz. The growth stages of the developing inflorescence were determined according to the modified Eichorn-Lorenz (E-L) scheme (Coombe, 1995) and were collected 7 weeks prior to flowering (EL#12), 4 weeks before flowering (EL#16) and 1 week after flowering (EL#28). Expanding leaves were at stage 3 as described in Section 3.2.1. Harvest skin is from ripe grapes. All samples were collected during the 2000-2001 season and mean flavonol concentration (±SEM, n=3) is presented. \( VvFLSI \) expression is the ratio of expression of \( VvFLSI \) divided by the level of expression of an internal control gene (\( VvUbiquitin1 \)).

<table>
<thead>
<tr>
<th></th>
<th>Flavonols (mg/g fwt tissue)</th>
<th>( VvFLSI ) Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shaded</td>
<td>Exposed</td>
</tr>
<tr>
<td>Leaf Stage 3</td>
<td>0.093 ± 0.012</td>
<td>3.323 ± 0.321</td>
</tr>
<tr>
<td>EL #12 Inflorescence</td>
<td>0.056 ± 0.016</td>
<td>1.529 ± 0.037</td>
</tr>
<tr>
<td>EL #16 Inflorescence</td>
<td>0.281 ± 0.021</td>
<td>1.407 ± 0.063</td>
</tr>
<tr>
<td>EL #28 Berries</td>
<td>0.000</td>
<td>0.172 ± 0.005</td>
</tr>
<tr>
<td>Harvest Skin</td>
<td>0.000</td>
<td>0.116 ± 0.007</td>
</tr>
</tbody>
</table>

5.1.1.6. Induction of Flavonol Biosynthesis

Developing inflorescences and grapevine leaves were sampled three days after the shading treatment had been removed. These were analysed by HPLC for flavonol content and by real-time PCR for expression of \( VvFLSI \). In shaded leaves at growth stage 3 (Section 3.2.1) the level of flavonols was less than 3% of that in the exposed leaves. After the shading treatment had been removed the level of flavonols in the induced leaves was around 39% that of normally exposed (control) leaves, or about 14-fold higher than the level in the shaded leaf (Table 5.6). While the level of flavonols in the newly exposed leaves had not reached the level in exposed leaves, the level of \( VvFLSI \) transcript was more than 6-fold greater than the level in the normally exposed leaves and 126-fold higher than shaded leaves.
Similarly, in the shaded inflorescence the level of flavonols was only 20% of that in the exposed inflorescences, while the level in the induced tissues after three days was around 45% of that in exposed inflorescence. Again, it was seen that the level of flavonols in the induced tissue had not reached that of the exposed inflorescence, but the level of VvFLSI transcript was almost 2-fold higher than that of the control and 10-fold higher than the level observed in the shaded inflorescences.

Table 5.6. Induction of flavonol biosynthesis in Vitis vinifera cv. Shiraz tissues. Tissues were shaded for ten weeks from bud-burst and then exposed to normal light for three days. Flavonol and VvFLSI levels shown as percentage of normally exposed (control) tissues. Growth stage of the developing inflorescence determined according to the modified Eichorn-Lorenz (E-L) scheme (Coombe, 1995) 4 weeks pre-flowering (EL#16). Expanding leaves at stage 3 (Section 3.2.1.) Samples were collected during the 2000-2001 season.

<table>
<thead>
<tr>
<th>% of Control Tissue</th>
<th>Flavonols</th>
<th>VvFLSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shaded Leaf, Stage 3</td>
<td>2.81</td>
<td>5.00</td>
</tr>
<tr>
<td>Induced Leaf, Stage 3</td>
<td>38.95</td>
<td>631.25</td>
</tr>
<tr>
<td>Shaded EL #16 Inflorescence</td>
<td>20.02</td>
<td>19.63</td>
</tr>
<tr>
<td>Induced EL #16 Inflorescence</td>
<td>45.21</td>
<td>192.52</td>
</tr>
</tbody>
</table>

5.4. Discussion

5.4.1.1. Shading Treatment

In order to investigate the effect of light exposure on flavonoid composition in grapes, developing Shiraz berries were shaded in polypropylene boxes designed to exclude light from the developing fruit without otherwise altering bunch microclimate or vine physiology (Figure 2.2). Trials of the boxes used for shading Shiraz berries suggest that the light environment around the developing fruit was substantially reduced. While the possibility exists that some reflected light may have entered the boxes, our measurements indicated that the light intensity inside of the boxes was less than 0.5% of incident light.
This was reinforced by measurements of chlorophyll in shaded and exposed fruit (Section 2.3.13) and supported by visual observation of the shaded bunches, which were yellow compared to the green berries of exposed bunches (Figure 2.38). From these results, we concluded that the levels of light entering the box as reflected light, in the absence of light transmission through the box material, were not sufficient in either quality or quantity to induce chlorophyll biosynthesis.

While the boxes were successfully able to exclude light from around the fruit, they did not modify the air temperature or relative humidity around the bunches (Section 2.2.1). Thus, it seems reasonable to conclude that differences in fruit composition between treatments would be due to shading rather than to altered temperature or relative humidity.

5.4.1.2. Berry Development in Shaded Grapes

In the first experimental season (1999-2000) there was a noticeable difference in berry weight between the shaded and exposed fruit (Figure 5.1A). However, in that season, the phenomenon known in Australia as “hen and chicken” was observed in Shiraz resulting in a high proportion of small under-developed berries as discussed in Section 2.4. However, there was no apparent difference in berry weight between the shaded and exposed fruit in successive seasons (2000-2001 and 2001-2002) it is likely that a disproportionate number of smaller underdeveloped berries were collected from the boxed samples in the first season. The combined data over three seasons (Figure 5.1) suggest that shading of the fruit did not have a consistent impact upon berry development nor on fruit weight at harvest.
The accumulation of sugars in the berry as measured by total soluble solids (°Brix) was also consistent between treatments and across three successive seasons. While minor differences were observed between samples, the trend and overall level was virtually the same in the shaded and exposed fruit (Figure 5.1). While a slight decrease in sugar accumulation with shading has been reported in the *Vitis* hybrid Reliance (Gao & Cahoon, 1994), the hybrid Seyval blanc was unaffected (Reynolds *et al.*, 1986). Previous work in the *Vitis vinifera* cultivars Shiraz and Merlot also showed no difference in sugar accumulation with bunch shading (Haselgrove *et al.*, 2000; Spayd *et al.*, 2002). However, differences in sugar accumulation have been reported in the *Vitis vinifera* cultivars Cabernet Sauvignon and Grenache from the north to south side of the rows (Bergqvist *et al.*, 2001). This may have been a reflection of difference in leaf sun exposure leading to reduced photosynthate assimilation, rather than altered berry exposure to light. The overall indication from the data presented here and that of other workers is consistent with the opinion of Coombe (1989), that sink activity and sugar accumulation in ripening grape berries is largely independent of external events.

5.4.1.3. Anthocyanins in Shaded Grapes

In many plants, such as apple and petunia, anthocyanin biosynthesis is light induced and tissues grown in the dark do not accumulate anthocyanins (Siegelman & Hendricks, 1958; Lancaster, 1992; Beggs & Welmann, 1994; Dong *et al.*, 1998; Katz & Weiss, 1999). Here we have seen that grapes grown in the absence of light did accumulate anthocyanins, which indicates that light is not an absolute requirement for anthocyanin biosynthesis in the berries of *Vitis vinifera* cv. Shiraz.
Across the three experimental seasons examined here, there was only a difference in total anthocyanins between treatments in one of those seasons. However, there were consistent differences in the composition of individual anthocyanins between treatments across all three seasons. The most obvious difference in anthocyanin composition between treatments was the shift from those anthocyanins with three hydroxyl groups on the "B" ring, the glycosides of malvidin, petunidin and delphinidin, to those with only two hydroxyls, based on peonidin and cyanidin. This observation suggests that the effect of shading is to reduce the activity of the flavonoid-3',5'-hydroxylase, or expression of the gene encoding that enzyme, or alternatively to increase the activity of flavonoid-3'-hydroxylase or upregulate expression of that gene. While genes encoding these enzymes have been cloned in other species (Holton et al., 1993a; Neilsen & Podivinsky, 1997; Shimada et al., 1999), there are currently no published sequences for these genes in *Vitis vinifera* and this aspect of anthocyanin biosynthesis in grapes has yet to be closely examined.

The shift in composition based on hydroxyl substitution of the "B" ring suggested that compositional differences based on the methylation pattern of the "B" ring should also be examined. There did appear to be an increase in the percentage of anthocyanins with a single methyl substitution and a decrease in the proportion of anthocyanins with two methyl moieties on the "B" ring across all seasons with shading (Table 5.3). However, this result may not indicate a change in anthocyanin methyltransferase (AMT) activity with shading, as decreased F3'5'H activity or increased F3'H activity could lead to a decrease in substrate for 5' methylation, which may result in a decrease in 5' methylated product.
This shift in anthocyanin composition based on “B” ring substitution with bunch exposure has not previously gained a great deal of attention. However, careful reading of the data presented by Spayd et al. (2002) shows a similar trend in Merlot, while Gao and Cahoon (1994) presented data that suggests a similar shift in the *Vitis* hybrid Reliance with shading. Previously, reports have tended to focus on shifts in the glycosylation pattern of the anthocyanins (Gao & Cahoon, 1994; Haselgrove et al., 2000), with a proportional shift from non-acylated glucosides to coumaroylglucosides with shading. Such a trend was observed here in the malvinid glucosides and total anthocyanin glucosides only in the second experimental season (Table 5.1). However, because of the shift based on the “B” ring hydroxylation pattern discussed here, it may not be useful to compare the relative proportions of anthocyanin glucosides, unless they are first separated by their pattern of “B” ring substitution. On this basis, a consistent pattern was again observed. The trihydroxylated anthocyanin non-acylated, acetyl-, and coumaroylglucosides were reduced on both a percentage and a mg/g fresh weight basis with shading, while the dihydroxylated non-acylated, acetyl-, and coumaroyl anthocyanin glucosides increased.

The only exception was in the second season (2000-2001), when the percentage of trihydroxylated coumaroyl derivatives increased, although in that season it was observed that all coumaroyl derivatives increased with shading. In the 2000-2001 season, the pattern of compositional change in both malvinid and total anthocyanins based on glycosylation was the same as that reported by Haselgrove et al. (2000). Non-acylated and acetylglucoside derivatives decreased with shading, while coumaroylglucosides increased. Gao & Cahoon (1994) also reported that the proportion of cyanidin non-acylated glucosides in Reliance grapes decreased with shading while the proportion of
acylated cyanidin glucosides increased. Haselgrove et al. (2000) suggested that increased light exposure lead to a decreasing proportion of coumaroyl derivatives in the fruit, however our results did not consistently support this conclusion leading us to seek an alternative explanation.

Of the three experimental seasons, the second (2000-2001) was significantly warmer than either of the other two seasons. Our data showed an increase in mean daily temperature during berry development and ripening over the next warmest season (1999-2000) of 4.2°C (p=0.0001) in November, 1.2°C (p=0.03) in December, and 3.1°C (p=0.008) in January.

Thus, the proportional increase in coumaroyl glucosides in the 2000-2001 season may be due to higher temperatures during that season. Similar results were presented by Spayd et al. (2002), who reported that cooling decreased coumaroyl derivatives, while heating substantially increased coumaroarylglucosides. The suggestion implicit in this observation is that either activity of the enzyme anthocyanin acyltransferase (AAT) or expression of the gene encoding AAT is increased with temperature. To date there are no *Vitis vinifera* clones of AAT and thus, no expression studies of the gene encoding this enzyme to determine whether AAT expression reflects these observations.

What is more interesting to note is that while anthocyanin coumaroarylglucosides were proportionally greater in the warmer season, coumaroarylglucosides were also higher in the shaded fruit in that season (2000-2001), even though overall colour was significantly lower in the shaded fruit. While Haselgrove et al. (2000) reported this shift towards coumaroylated anthocyanins with shading, they reported no change in total anthocyanins. It seems likely that the result observed by Haselgrove et al. (2000) was the result of their shading treatment inadvertently increasing berry temperature resulting in the shift
towards coumaroylated anthocyanins. Similarly, Price et al. (1995) reported no change in total anthocyanins in the fruit of *Vitis vinifera* cv. Pinot noir with shading, although this cultivar lacks acylated anthocyanins. In contrast, Gao & Cahoon (1994) observed both a shift in composition and a significant decrease in anthocyanins with shading. However, the absence of acylated glycosides of delphinidin, petunidin, peonidin and malvidin in this cultivar likely clouds the usefulness of these observations. Spayd et al. (2002), working on *Vitis vinifera* cv. Merlot reported no difference in total anthocyanins with shading in their first season, but a substantial decrease in the second season. If nothing else these observations highlight the ethereality of conclusions based on a single season’s observation and indicate the value of a long-term study in this field.

Generally, the indication is that higher temperature rather than light results in a shift towards coumaroylated anthocyanins, and that higher temperature results in decreased total anthocyanins. The mechanism for this is uncertain but supports the notion of anthocyanin degradation or inhibition of anthocyanin biosynthesis at high temperature (Buttrose et al., 1971; Pirie, 1977; Markakis, 1982; Crippen & Morrison, 1986a; Yamasaki et al., 1996). There is also a suggestion that at high temperature, the coumaroylated anthocyanins may be more stable than the non-acylated or acetylglucosides or more resistant to such degradative processes as may be taking place. However, the varying stabilities of individual grape anthocyanins has not been well documented. That a decrease in anthocyanin content is more pronounced in the shaded fruit suggests that in response to anthocyanin degradation at high temperature, supplementary anthocyanin biosynthesis is light inducible. Thus, there may be two systems regulating colour accumulation in grapes, an initial and constitutive system that generates a base level of anthocyanins and an inducible system that is light requiring.
The results presented here suggest that the effect of shading is to decrease the proportion of malvidin, petunidin and delphinidin glycosides relative to peonidin and cyanidin glycosides. It remains to be seen whether the effect of increased exposure is to reverse this trend. Furthermore, there is a strong indication that high temperature shifts the anthocyanin profile from non-acylated to coumaroylglucosides, irrespective of bunch exposure.

Thus, we might expect that exposed fruit in a warm climate would have a higher proportion of malvidin, petunidin and delphinidin coumaroyl derivatives, while cool, shaded fruit might have more peonidin and cyanidin non-acylated and acetyl glucosides. What has not yet been firmly established is the relative contribution of each of these components to final wine colour. However, research on the extractability of anthocyanins from the fruit into the wine suggests that the non-acylated and acetylglucosides are more readily extracted from the fruit than the coumaroylglucosides (Leone et al., 1984; Roggero et al., 1984).

5.4.1.4. Condensed Tannins in Shaded Grapes

While condensed tannin accumulation in grape berries has been extensively studied elsewhere (Czochanska et al., 1979; Katalinic & Males, 1997; de Freitas & Glories, 1999; Kennedy et al., 2000a; Kennedy et al., 2000b; Kennedy et al., 2001), the influence of light on tannin accumulation has not previously been investigated.

Over two seasons, 1999-2000 and 2000-2001, there was a noticeable difference in total condensed tannins. While in the first season tannin analysis was conducted on whole berry samples it was not possible to determine whether the changes observed were in the seed or the skin of the berry. However, in the subsequent season, these components were
analysed separately and it became apparent that there was no effect of shading on seed tannin content and composition. In contrast there were significant differences in the condensed tannin content of grape skins in the second season, 2000-2001. This suggests that the differences observed during the first season may have been due to changes in skin tannin content. In both seasons, substantial differences were observed in the levels of free monomers and extension subunits from early in berry development until several weeks post-veraison, but as the fruit approached maturity these differences became less pronounced. In the case of free monomers, the level in the exposed skin decreased slightly, while the level in shaded fruit continued to increase, with the levels in shaded and exposed fruit converging around three weeks post-veraison in both seasons. A similar pattern was observed in total extension subunits, whereby the level in the exposed fruit reached a higher total, but subsequently declined to a level similar to that in the shaded fruit at the time of harvest. In the case of the extension subunits, the level in the exposed fruit was much higher than in the shaded fruit and significant decrease was observed from a peak one to two weeks post-veraison until harvest.

Previous studies have suggested that the post-veraison decrease in condensed tannin in both seeds and skins is likely the result of reduced extractability (Cheynier et al., 1997; Saint Cricq de Gaulejac et al., 1997; Kennedy et al., 2000b; Section 3.4). This trend was apparent in the extension subunits of the exposed fruit, but there was no post-veraison decrease in extension subunits in the shaded fruit. The conclusion to be drawn from this observation is that even though the level of extractable tannin was similar at harvest in both the shaded and exposed fruit, more condensed tannin, particularly extension subunits, was produced in the exposed fruit. The lower level of extension subunits
observed in the shaded fruit also resulted in a lower mDP in the skins of shaded berries throughout berry development,

It is interesting to note that there was a difference in condensed tannins between treatments in both the 1999-2000 season and the 2000-2001 season, while there was only a difference in total anthocyanins between treatments in one of those seasons (2000-2001). This suggests that the mechanisms that control tannin and anthocyanin biosynthesis in the berry are independent.

So saying, it is worth noting that the observed shift in anthocyanin composition towards dihydroxylated anthocyanins based on cyanidin and peonidin and away from the glycosides of delphinidin, petunidin and malvidin was also observed in tannin composition. Specifically, this was observed in extension subunit composition where the proportion of trihydroxylated epigallocatechin was significantly reduced in the shaded fruit (Table 5.4).

There was no consistent effect of shading on terminal subunit composition. In the first season, terminal subunits in whole berries were substantially lower throughout most of berry development. However, in the second season there was virtually no difference in terminal subunits in either seeds of skin at any time during berry development.

The effect of shading on galloylation was to increase the relative proportion of galloylated epicatechin subunits in the skin. This effect was observed in both the terminal and extension subunits, although a two-fold increase in the percentage of galloylated extension subunits was considerably greater than the increase in the galloylated portion of the epicatechin terminal subunits.
While significant differences were observed in condensed tannin accumulation and composition between treatments, it is uncertain what implications these changes might have for winemaking. Potentially, bunch exposure could be a mechanism for managing winegrape tannins, although currently the optimum condensed tannin profile for a wine type has yet to be established.

5.4.1.5. Flavonols in Shaded Grapes

The influence of light on flavonol accumulation in plant tissues has previously been studied in a number of plant systems with exposure to UV-light shown to increase the level of flavonol glycosides in vegetative and reproductive tissues (Hrazdina & Parsons, 1982; Hartmann et al., 1998; Ryan et al., 1998; Vogt et al., 1999; Reay & Lancaster, 2001). This effect has also been reported in winegrapes, with exposed fruit higher in flavonol glycosides, while shaded fruit had lower flavonol content (Price et al., 1995; Haselgrove et al., 2000; Spayd et al., 2002). Because these studies examined fruit at harvest or during ripening, the effect of shading early in berry development remained uncertain. We have previously reported relatively high levels of flavonols in grape berries early in berry development (Section 4.3), so it is not clear whether lower levels of flavonols in the shaded fruit are caused by reduced biosynthesis or by degradation.

Here we have examined flavonol accumulation in shaded and exposed (control) fruit over three successive seasons. In each season the level of flavonols was significantly reduced in the shaded fruit, consistent with the previous reports (Price et al., 1995; Haselgrove et al., 2000; Spayd et al., 2002). In addition, it was observed that from the time that the shading treatment was applied, no further flavonol accumulation was detected in that fruit. This suggests that reduced biosynthesis, rather than degradation is responsible for the low levels of flavonols present in the shaded fruit. Furthermore, in the second
experimental season (2000-2001), when the shading treatment was applied five weeks before flowering, there were no detectable flavonols in the developing berries, which suggests that all flavonol biosynthesis in the Shiraz berries is light induced. This is in sharp contrast to anthocyanin and tannin synthesis, which was little effected by the shading treatments. This suggests that only the branch of the flavonoid pathway leading to flavonol synthesis is light-dependent.

Furthermore, while there was significant variation in total and individual anthocyanins between the three seasons studied here, the pattern of flavonol accumulation for the same fruit over the same three seasons showed very little variation (Figure 4.6). What has yet to be established is whether this level of flavonols (0.04-0.05 mg/berry) can be increased in more highly exposed fruit. Other authors have reported substantially greater levels in highly exposed grapes (Price et al., 1995; Haselgrove et al., 2000; Spayd et al., 2002).

5.4.1.6. UFGT and FLS Gene Expression in Shaded Grapes

The gene encoding UFGT appears to be a key step in anthocyanin synthesis, its expression correlating well with anthocyanin accumulation in grapes (Boss et al., 1996). Expression of VvUFGT was very low before veraison, but increased dramatically after veraison, when anthocyanin accumulation commenced, in agreement with previous studies (Boss et al., 1996c). The level of expression of VvUFGT was similar in shaded and exposed fruit (Figure 5.6), consistent with the similar levels of total anthocyanins (Figure 5.2). This suggests that shading had little effect on expression of genes involved in anthocyanin biosynthesis in the grape skin.

In contrast, expression of VvFLSI was greatly decreased in shaded leaves, developing inflorescences and ripening fruit (Table 5.5 and Figure 5.6). This gene encodes flavonol
synthase (FLS), the first enzyme in the branch of the flavonoid pathway leading to flavonol biosynthesis, and our results indicate that the expression of FLS is light-dependent in Shiraz leaves, inflorescences and fruit.

5.4.1.7. Induction of Flavonol Biosynthesis

When shaded leaves and developing inflorescences were exposed to light for a period of three days, these tissues began to accumulate flavonols and VvFLSI expression was upregulated. In fact, there was a 126-fold increase in VvFLSI expression compared to the level in the shaded leaves. Expression of VvFLSI also increased in developing inflorescences upon exposure, but this increase was not as great as in leaves. This is the first report of the induction of genes encoding flavonol synthase by light. Light induction of flavonols has been reported in pea and mustard (Hrazdina & Parsons, 1982; Beggs et al., 1987), but not in grapevines. The results presented here represent clear evidence of the light induction of flavonol biosynthesis and is the strongest indication yet that flavonols may play a UV-protection role in grapevines.

It appears that light differentially regulates expression of different genes in the flavonoid pathway in Shiraz. Further studies are required to elucidate the mechanisms by which light influences flavonol synthesis and expression of VvFLSI in Shiraz grapes while having no effect on parts of the flavonoid pathway that generate anthocyanins and tannins. Nevertheless, flavonols appear to be a good indicator of bunch exposure to light, in agreement with previous studies. Together, the results presented here and previous work suggests that flavonol synthesis in grape berries is highly light-dependent but that light does not greatly alter the total levels of anthocyanins and tannins in ripe Shiraz grapes. Thus, manipulating flavonol levels in grape berries through canopy management should be relatively straightforward. However, at this point it is not been equivocally
established whether flavonols contribute significantly to wine grape quality, either as potential copigments or by other mechanisms.
Chapter 6. General Discussion

The aim of this project (Section 1.5) was to investigate the apparent paradox identified in previous research (Boss et al., 1996a), that genes involved in anthocyanin biosynthesis were expressed at times during grape berry development when there was no synthesis of anthocyanins. The phenylpropanoid pathway (Figure 1.6) gives rise to a number of products other than anthocyanins and it was hypothesised that the accumulation of these compounds would coincide with expression of genes in the pathway early in berry development. Thus, we investigated the accumulation of two groups of these products, the flavonols and tannins in the developing fruit of the grape cultivars Shiraz and Chardonnay. In addition, we wanted to explore the effect of light on berry development and the accumulation of flavonoids in Shiraz grape berries.

6.1. Tannin Accumulation in Shiraz Berries

The flavan-3-ol and proanthocyanidin composition of seeds and skin of Shiraz grapes was determined by reversed-phase HPLC after acetone extraction and acid-catalysis in the presence of excess phloroglucinol. The main period of proanthocyanidin accumulation in grape seeds occurred immediately after fruit-set with maximum levels observed around veraison. Over two seasons there was variation in both proanthocyanidin content and the timing of proanthocyanidin accumulation in seeds. In the skin, proanthocyanidin accumulation occurred from fruit-set until 1-2 weeks after veraison. Proanthocyanidin subunit composition was different in seeds and skin and changed during berry development. Proanthocyanidin levels in both seeds and skin decreased between veraison and harvest. Additional proanthocyanidin subunits were released when the residues remaining after acetone extraction were subjected to direct acid-catalysis in
the presence of phloroglucinol. In the seeds, these accounted for much of the post-veraison decrease, but not in grape skin. At harvest, 75% of extractable berry proanthocyanidin was in the seeds. Accumulation of proanthocyanidins in the seeds appears to be independent of that in the skin, but in both tissues synthesis occurs early in berry development and maximum levels are reached around veraison.

Thus, the pattern of proanthocyanidin accumulation in Shiraz seeds and skin has been clearly established, however a number of areas remain ripe for further investigation. One of these is the post-veraison decline in proanthocyanidins, widely held to represent a decrease in extractability (Amrani-Joutei et al., 1994; Cheynier et al., 1997; Saint-Cricq de Gaulejac et al., 1997; Kennedy et al. 2000b). The release of additional proanthocyanidin subunits by direct acid-catalysis of seed residues suggests these had become associated with other seed coat components. This was not the case with the skin residues and the nature of the decrease in extractable tannins in the skin during ripening remains unclear. The proposition that compounds resistant to acid-catalysis are formed and that these were not identified by this analysis (Section 3.4) suggests that a more thorough investigation of proanthocyanidin-like compounds in the HPLC separation of catalysis products may give an indication of the fate of grape skin tannins post-veraison.

While the pattern of proanthocyanidin accumulation has been much clarified, the biochemistry of their formation remains less clear. Little or nothing is known of the process of polymerisation in condensed tannins, although it is generally thought that the chemistry of extension subunits inclines them to polymer formation as a chemical rather than an enzymatic process. Nevertheless, some pundits feel strongly that this is an enzymatic process and hold the ratios of subunits with differing stereochemistry as evidence. However, a body of work does indicate the ability of flavan-3-ols and flavan-
3,4-diols to polymerise non-enzymatically (Iurd, 1972; Delcour et al., 1983; Bakker & Timberlake, 1997; Saucier et al., 1997; Mateus et al., 2002b; Vidal et al., 2002). This work indicates that subunit ratios in the polymer are a function of favourable conformation in situations of steric hindrance and other constraints of attractive and opposing force. Per contra, the formation of dimers, particularly those comprising the 4-6 dimeric vinculum, in what appears to be a tissue specific manner, suggest that at least the dimerisation process may be enzymatic.

Mechanisms for tannin polymer formation have long been mooted, however the formation of flavan-3-ol monomers had until recently been considered a simple reduction of leucocyanidin to catechin or epicatechin, with the formation of the two isomers the result of an as yet unidentified epimerase. However, recent work (Xie et al., 2003) has challenged the conventional wisdom by demonstrating the formation of epicatechin from cyanidin. In addition, a gene encoding this anthocyanidin reductase was also cloned and functionality in carrying out this reaction was demonstrated. Coincidentally, similar work has recently reported cloning and characterisation of a gene encoding leucoanthocyanidin reductase (Tanner et al., 2003; Harvey et al., in prep.). Together these studies suggest two paths to condensed tannin formation, and indeed the subunit composition of tannin polymers may be determined by differential flux through the biosynthetic enzymes, leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR). Alternatively, the kinetics of the intermediate enzyme, leucoanthocyanidin dioxygenase (LDOX a.k.a. ANS) may be responsible for the predominance of epicatechin subunits in grape proanthocyanidins. However, much of the enzymology of flavonoid metabolism still remains to be thoroughly investigated.
Despite recent advances in our knowledge, the course of tannin biosynthesis remains unclear. Our thinking previously had terminal subunits synthesised from leucoanthocyanidin by LAR and extension subunits originating from leucoanthocyanidin (Figure 6.1A), with the involvement of an epimerase to generate the isomers catechin and epicatechin. The work of Xie et al. (2003) shows this is not the case, but rather that catechin and epicatechin have different origins, leading to a revision of our thinking about proanthocyanidin biosynthesis (Figure 6.1B). The results reported by Xie et al. (2003) suggest there may not be an epimerase, and in that light one must reconsider the origin of the extension subunits. Given the different origins of terminal subunits, it hardly seems fanciful that the extension subunits would have similar origins. Thus, the substrates for catechin and epicatechin monomers and terminal subunits, leucocyanidin and cyanidin respectively, would be the source of catechin and epicatechin extension subunits (Figure 6.1C). However, the absence of a hydroxyl moiety at the 4-position of “C” ring of cyanidin significantly weakens the argument for a purely chemical formation of proanthocyanidin polymers. This forces us to revisit the proposition of Haslam (1998) that a quinone methide intermediate in the LAR catalysed reaction is the extension subunit source. This, in the absence of such an intermediate in either the LDOX or ANR catalysed reactions, or a condensing enzyme, leaves Figure 6.1B as the most likely pathway for condensed tannin biosynthesis, based on our current knowledge.

Interestingly, recent research (Abrahams et al., 2003; Xie et al. 2003) also clarifies one of the enigmas of the pattern of gene expression reported by Boss et al. (1996a), which showed LDOX expressed early in berry development. The conventional pathway (Figure 6.1A) does not involve LDOX in tannin biosynthesis, but in the revised pathway (Figure 6.1B, 6.1C) LDOX and ANR are required for the synthesis of epicatechin.
Figure 6.1. Evolution of current thinking in proanthocyanidin biosynthesis: (A) Terminal subunits synthesised from leucocyanidin by LAR/Banyuls; extension subunits derived directly from leucocyanidin. (B) Subsequent to Xie et al. (2003), with catechin and epicatechin synthesised from leucocyanidin and cyanidin respectively by LAR (leucoanthocyanidin reductase) and ANR (anthocyanidin reductase). (C) Proposed origin of extension subunits with 2,3-trans (catechin) and 2,3-cis (epicatechin) stereochemistry from leucocyanidin and cyanidin respectively.
So far, this discussion has considered only the dihydroxylated flavan-3-ols, catechin and epicatechin, but epigallocatechin extension subunits are also detected in the grape skin. Based on the foregoing discussion, it seems likely that these are derived from the leucoanthocyanidin, delphinidin. It remains a mystery why epigallocatechin terminal subunits are not found in grape tissues, nor why the 2,3-\textit{trans} isomer, gallocatechin is not detected. Interestingly, the bulk of epigallocatechin subunits accumulate from veraison onwards, suggesting that there is an upregulation of F3'5'H at this time. To date, there has been no published work on the activity of F3'5'H or the expression of the gene encoding F3'5'H in grapevines, nor on the regulation of the F3'5'H gene. Once again, LDOX may provide an alternative explanation as a result of the increased expression of LDOX observed post-veraison (Boss et al., 1996a) presumably leading to an increase in LDOX catalytic activity.

The biochemistry of the galloylation of terminal and extension proanthocyanidin subunits also remains unclear. In grapevines, only epicatechin-gallate has been reported. Why only epicatechin is galloylated currently remains an enigma. That galloylation is limited by the stereochemistry of the 2,3-\textit{trans} isomer catechin and that the timing of epigallocatechin accumulation does not coincide with the activity of an unidentified galloyltransferase, are possible explanations.

Thus, there yet remain aspects of proanthocyanidin biochemistry to be elucidated. The number of tannin deficient mutants downstream of flavan-3-ol synthesis identified in Arabidopsis (Shirley et al., 1995; Abrahams et al., 2002) lends credence to this notion. The genes identified by these mutants may involve a number of processes including galloylation, dimerisation and polymerisation, the role, if any, of an epimerase, as well as
trans-membrane transport, or genes involved in the regulation of proanthocyanidin biosynthesis.

Finally, the bulk of this research in grapevines has been on a single cultivar, Shiraz. It is not yet known whether the pattern observed in Shiraz is representative of all of *Vitis vinifera* or indeed the Vitiaceae. While much of what has been reported here (Chapter 3) is consistent with patterns observed in other cultivars by other workers, substantial deviations from what has previously been reported were also noted. Thus it would be of great viticultural and oenological importance to make a broader survey of tannin accumulation in winegrapes.

### 6.2. Flavonol Accumulation in Shiraz Berries

The predominant flavonols in Shiraz and Chardonnay berries are quercetin-3-glucosides with trace amounts of kaempferol-3-glucosides detected in Shiraz flowers, but not in developing berries. Flavonols were present in the skin of ripening grapes, but were not detected in the seeds or flesh. Flavonols were also present in buds, tendrils, inflorescences, anthers and leaves of the grapevine. The flavonol concentration in flowers was high and decreased between flowering and berry-set then remained relatively constant through berry development. Total flavonols per berry were low until pre-veraison then increased during berry development, particularly before veraison in Chardonnay and during ripening in Shiraz.

In addition to determining the pattern of flavonol accumulation during berry development, genes encoding the biosynthetic enzyme flavonol synthase were also cloned. Two cDNA fragments with homology to genes encoding the enzyme flavonol synthase (FLS) were isolated from Shiraz flowers. One of these, *VvFLS1*, was expressed
in leaves, tendrils, pedicels, buds and inflorescences as well as in the developing grapes. Expression was highest between flowering and fruit-set then declined, increasing again during ripening coincident with the increase in flavonols per berry. The second gene, \( VvFLS2 \), was constitutively expressed at a very low level in berries throughout berry development. The pattern of \( VvFLSI \) expression indicates two distinct periods of flavonol synthesis during berry development, the first around flowering and the second during ripening of the berries. The pattern of flavonol accumulation and \( VvFLSI \) expression in developing berries is consistent with the overall pattern of gene expression in the phenylpropanoid pathway (Boss et al., 1996a) and may account for part of the early expression of pathway genes.

In this investigation, flavonols were measured as the combined total of quercetin-3-glucoside and quercetin-3-glucuronide. These compounds not being readily separated by the method used here. This was considered adequate, as the aim was to investigate the accumulation of total flavonols and changes in expression of the \( FLS \) gene. What remains unanswered here is whether there were compositional changes between the glucoside and the glucuronide during berry development. These modifications of flavonols subsequent to the formation of the aglycone by \( FLS \) have not been investigated. While a range of glycosylation products including, flavonol glucosides, glucuronides, galactosides, glucosylgalactosides, glucosylxylosides and glucosylarabinosides have previously been identified in grapevines (Cheynier & Rigaud, 1986), no flavonol glycoside transferase has yet been identified. To date the only flavonol glycosyltransferase gene characterised has been in Petunia (Miller et al., 1999; Miller et al., 2002). Although flavonol glycosyltransferase activity has been assayed in \( Prunus \times yedoensis \) and \( Vigna mungo \).
(Ishikura & Yamamoto, 1990; Ishikura & Mato, 1993) and a putative gene sequence identified in *Arabidopsis*.

Whether such transferases are specific for flavonols or are similar to or the same as those glycosylating other flavonoids is unknown. However, previous research (Ford & Hoj, 1998; Ford *et al.*, 1998) indicates that the enzyme glucosylating anthocyanidins to form anthocyanins, UFGT, has a high level of substrate specificity. Furthermore, UFGT is not expressed before veraison when significant synthesis of flavonol glycosides occurs. Thus, it is likely that there are specific glycosyltransferases for different classes of flavonoids, such as flavonols.

**6.3. Effect of Shading on Berry Development and Flavonoid Biosynthesis**

To explore the effects of light on berry development and flavonoid biosynthesis, opaque boxes were applied to bunches of Shiraz grapes prior to flowering. The boxes were designed to exclude light while maintaining airflow to minimise changes in temperature and humidity. There was no significant effect of shading on sugar accumulation and in two of the three seasons studied there was no effect on berry weight. Thus, we concluded that berry development was not significantly effected by shading. The chlorophyll concentration was much lower in the shaded fruit, which appeared pale yellow until veraison. Post-veraison, the fruit coloured normally in the shaded bunches and in two of the three seasons, there was no significant change in anthocyanin content. However, in one season, total anthocyanins were substantially lower in the shaded fruit, around half the level in the exposed fruit, and the cause of this difference remains unclear. Despite these differences in total anthocyanins between shaded and exposed fruit, expression of the gene encoding UDP-glucose flavonoid-3-O-glucosyl transferase (UFGT), a key gene in anthocyanin synthesis, was similar between treatments. That gene expression was so
similar while accumulation differed by around 50% suggests a post-translational effect of light on anthocyanin accumulation. This difference in anthocyanin accumulation between shaded and exposed fruit seems difficult to reconcile in both cause and outcome. Two possibilities assert themselves as likely causes, that the difference was caused by the early application of the shading treatment, five weeks pre-flowering as opposed to fruit-set, or by the unusually high temperatures recorded during that season. As for the outcome, early shading or high temperature may cause reduced flux through the biosynthetic pathway, limited by the expression of other genes upstream of UFGT, not examined here, resulting in reduced substrate availability for anthocyanin synthesis. Alternatively, high temperature has been shown to cause anthocyanin degradation in grapes and other plant systems (Buttrose et al., 1971; Pirie, 1977; Markakis, 1982; Crippen & Morrison, 1986a; Yamasaki et al., 1996) and that supplementary anthocyanin biosynthesis in response to degradation is light induced. To explore these possibilities, it would be necessary to test the effect of the timing of shading application as well as the effects of temperature independent of light.

While the effect of shading on total anthocyanins exhibited some seasonal variation, the anthocyanin profile of the shaded fruit was consistently altered. The shaded fruit had a greater proportion of dihydroxylated anthocyanins, the glycosides of cyanidin and peonidin, while the exposed fruit had a higher proportion of the trihydroxylated anthocyanins, the glycosides of delphinidin, petunidin and malvidin. The suggestion implicit in this result is that the enzyme F3'5'H, or the gene encoding F3'5'H, is light sensitive and activity or expression is downregulated in low light conditions. To date this area of flavonoid biochemistry has been little explored other than in Petunia (Holton et al., 1993a). Given the possibility of downregulation of F3'5'H in low light conditions
suggested by changes in grape skin composition, the immediately obvious experiment would be to determine if the reverse trend was observed under high light conditions. Further, if F3'S'H is sensitive to high temperature, the combined effect of shade and temperature could account for the lower colour in the shaded fruit during the hot 2000-2001 season. Another possibility is that the shift in anthocyanin profile with shading results in a pool of anthocyanins that are less thermally stable than those favoured by high light conditions. Given that generally high light and high temperature would be closely linked under normal environmental and growth conditions, this seems a likely scenario. Thus, exploring the temperature effects on anthocyanin biosynthesis and chemistry both in vivo and in vitro would be of considerable interest.

While the glycosylation pattern of anthocyanins was not consistently changed with shading, in the hotter 2000-2001 season when significant differences in total anthocyanins were observed, there were also shifts in composition based on glucoside substitution. Similar changes in composition have been previously reported (Haselgrove *et al*., 2000; Spayd *et al*., 2002) and the indication is that temperature rather than light is the primary influencing factor. The general trend with increasing temperature is an increase in coumaroylated anthocyanin glucosides and a decrease in acetylglucosides and non-acylated glucosides. While this effect is yet equivocal, these observations strongly encourage investigation of the effects of temperature on flavonoid biosynthesis in grapevines independent of the influence light. If this effect on anthocyanin composition based on the glycosylation pattern is indeed effected by temperature it would be of interest to examine the biochemical mechanism by which this occurs, whether by altering enzyme kinetics or regulating gene expression.
In contrast to anthocyanin accumulation, shading appears to have little effect on grape tannin accumulation. Shading had virtually no effect on the levels of condensed tannins in grape seeds, while the level of condensed tannins in the ripe skin of shaded grape berries was not significantly different to that of the exposed fruit. However, there were significant differences early in berry development, particularly in the levels of extension subunits. The post-veraison decrease in apparent extractability of skin tannins resulted in similar tannin levels in the skins at harvest. In addition to this difference, there were also differences in skin tannin composition based on the “B” ring hydroxylation pattern. It is arguable that many of the factors causing similar shifts in anthocyanin composition are applicable to tannin subunits composition in response to light and temperature. Of these factors, the possible light-sensitivity of F3’5’H stands out, however, the changes observed here were only in the order of ten to fifteen percent of extension subunits, five to ten percent of anthocyanins. It is unlikely that such a small shift in tannin composition would represent a quantifiable shift in gene expression.

A more noticeable shift in tannin composition with shading was in the proportion of galloylated epicatechin extension subunits. While extension subunits were almost 40% lower in the shaded fruit (at 24°Brix), the proportion of galloylated extension subunits doubled. Whether this difference is discernible through molecular techniques remains to be seen, and awaits the cloning and characterisation of a galloylating enzyme.

Of the flavonoids studied here, the greatest, consistent effect of shading was on flavonol biosynthesis. When shading was applied before flowering, shaded fruit had much lower levels of flavonols throughout berry development and at harvest the level of flavonols were less than 10% of that in exposed fruit. A gene encoding flavonol synthase (VvFLSI) was expressed at flowering and during ripening in exposed grapes but its expression was
greatly reduced in shaded fruit. Flavonol accumulation and \( VvFLSI \) expression were also greatly reduced in shaded leaves and developing inflorescences. Furthermore, when this shading treatment was removed and tissues sampled three days later, flavonol levels and \( VvFLSI \) expression increased in response to exposure to light. In the newly exposed inflorescence the increase from the shaded tissue was around 14-fold, while the increase in the newly exposed leaf was more than 120-fold that in the shaded leaf. This clearly demonstrates that flavonol accumulation and \( VvFLSI \) expression in grapevines are light induced.

What is still unclear is whether increased exposure of the fruit would result in a further increase in flavonol accumulation and \( VvFLSI \) expression, is there an upper limit to the flavonol level in a tissue? It is also uncertain whether changes in total flavonols have any impact on flavonol composition. It would be interesting to see if there was a shift from one glycosidic form to the other with changing levels of light exposure. Indeed, in terms of light exposure it would be interesting to compare not only light quantity, but also light quality, by excluding selected wavelengths.

6.4. Implications for Winemaking

The outcomes of this research indicate that shading had little effect on berry development and ripening and while total anthocyanins and tannins were not greatly effected by shading there were consistent changes in composition. Shading did however significantly decrease flavonol synthesis. While much of this is of considerable interest from a physiological and biochemical perspective, the greater immediate importance is how this new knowledge might impact upon the final wine and upon winemaking techniques.
Changes in grape berry composition indubitably impact upon wine composition and therefore wine quality, however many of these effects have not been thoroughly explored or quantified. One of the shortcomings of this, and much other, research into grape berry composition is that the analysis terminates with the fruit at harvest, rather than making the logical progression into winemaking.

The role of flavonols as a sunscreen in grape skin is consistent with the observed changes in flavonol accumulation due to shading and exposure. It has also been demonstrated that flavonols have a significant copigment effect with malvidin-3-glucoside (Lambert, 2002). However, the role of flavonols as copigments either in the berry or in the wine has not been firmly established. Research has shown flavonols and anthocyanins to co-occur in the vacuole of skin cells (Hrazdina & Moskowitz, 1980; Moskowitz & Hrazdina, 1981), although the concentrations were considered insufficient for a copigmentation effect. Further, the levels present in the grapes, and must, were also considered inadequate for any appreciable copigmentation effect in the wine (Lambert, 2002). Of more importance is the observation that greatest copigmentation effect results from the self-association of malvidin-3-glucoside (Hoshino et al., 1982; Houbiers, et al., 1998; Lambert, 2002). Thus, while it would be of great interest to make wine from shaded, normally exposed and highly exposed fruit to examine the effects of changes in flavonol content on wine colour, it would be equally interesting to observe the effects in wine from treatments that incline the anthocyanin composition of the fruit towards malvidin-3-glucoside. Based on the work presented here and other recent research (Downey, unpublished) these conditions would be high light and low temperature.

While the indication is that high light and cool temperatures are likely to favour the accumulation of malvidin-3-glucoside, these conditions cannot practicably be achieved in
many of the world's winegrowing regions. Thus it is also important to determine the combinations of light and temperature that are most favourable to wine colour, as well as mouthfeel and other quality parameters. One of the aspects of wine colour that has not been thoroughly investigated is the relative extractability and stability of different classes of anthocyanins during winemaking, nor indeed of other classes of flavonoids, such as flavonols and tannins. If viticultural management practices are aimed at increasing grape colour and the shift in the anthocyanin profile that increases grape colour results in less extractable or less stable anthocyanins, increased grape colour may translate into decreased wine colour. Thus, it becomes essential to explore the relationship between the anthocyanin profile of the fruit and the colour intensity and stability of the final wine.

6.5. Summary

As a result of these investigations we can now revise our schematic representation of grape berry development (Figure 2.1) to include flavonols and tannins. While this was not a new field of investigation, much of the published literature, for whatever reason, had dealt only with individual aspects of flavonoid biosynthesis in *Vitis vinifera*. Seeds, but not skin or vice versa, anthocyanins, but not tannins, etc. and included a diverse array of varieties, such that while it was possible to get a general impression of flavonoid biosynthesis in grapevines, no definitive study of all flavonoids in the same cultivar existed. In addition, the extant literature included many contradictions arising from analytical, seasonal and varietal differences, which made comparing and contrasting these data problematic.

Here we have addressed many of these issues and have presented a body of work that clearly shows the pattern of flavonoid accumulation in *Vitis vinifera* cv. Shiraz over several seasons. Thus, the revised schematic (Figure 6.2) now includes flavonol and
tannin accumulation on a whole berry basis, augmented by detailed compositional analysis both spatially and temporally (Chapter 3; Chapter 4).

The second area of investigation presented in this research was the effect of shading on both berry development and flavonoid biosynthesis in the grape. While berry development was largely unaffected by shading, there were observed differences in the flavonoid accumulation and flavonoid gene expression between shaded and normally exposed fruit. Generally, these changes were limited to flavonoids in the grape skin. Consistently flavonol biosynthesis was reduced in the shaded fruit, while changes in total

Figure 6.2. Revised schematic representation of grape berry development including the products of this research, the accumulation of flavonols and tannins in the berry
anthocyanin and tannin accumulation adhered to no discernible pattern. However, closer examination of the data presented here showed that consistent, albeit subtle, changes in anthocyanin and tannin composition did occur in response to shading. These changes had not previously been reported.

While the effect of shading has been closely examined here, there arises an interesting counterpoint, whether the effect of increasing light would result in a reversal of the changes observed. Furthermore, as discussed in Chapter 5, it remains uncertain whether or not changes attributed to bunch exposure are effects of changes in the light environment or in fact, temperature effects. While much of the data here is unequivocal, given that the shading treatment did not have a significant temperature effect, the effect of temperature independent of and interacting with light should be further investigated.

This research represents a comprehensive analysis of these aspects of flavonoid biosynthesis in grapevines, however many aspects of flavonoid biogenesis remain to be elucidated. While this research has closely examined the cultivar Shiraz and to a lesser extent Chardonnay, the patterns observed here may not be representative of the whole *Vitis* genus or Vitaceae family. Indeed, earlier investigations of other cultivars, such as Pinot noir, are not entirely consistent with the observations presented here. Given that broad and fundamental differences in flavonoid composition between cultivars have been extensively documented elsewhere, such inconsistency is to be expected. Indeed, this variability between cultivars forces us to ask whether the trends observed here represent a general pattern or an anomaly in the Vitaceae? Is there genetic variability that could be exploited to improve fruit quality by changed flavonoid biosynthesis/composition? Thus, it is necessary to expand these investigations to other *Vitis* cultivars to determine whether the phenomena observed in Shiraz, spatially, temporally and with respect to light
exclusion, is universal or specific. Description of a very general trend, or extrapolating a general trend from isolated examples establishes an expectation of a single management practice applicable to wide array species or cultivars. However, it is becoming increasingly apparent that this is not appropriate and that targeted practices will be required for individual cultivar, particularly with wineries tending more towards payment based on quality parameters, such as colour. Thus, the industry will be driven to take a more targeted approach to viticultural management, which will inevitably highlight many of the shortcomings of our current knowledge. Indeed, it will become increasingly apparent that much of the extant body of viticultural research needs to be revisited using modern analytical techniques.
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Z


CALVIN AND HOBBES

IF THERE ARE NO QUESTIONS, WE'LL MOVE ON TO THE NEXT CHAPTER.

I HAVE A QUESTION.

CERTAINLY, CALVIN. WHAT IS IT?

WHAT'S THE POINT OF HUMAN EXISTENCE?

I MEANT ANY QUESTIONS ABOUT THE SUBJECT AT HAND.

FRANKLY, I'D LIKE TO HAVE THE ISSUE RESOLVED BEFORE I EXPEND ANY MORE ENERGY ON THIS.

OH.