**AN ABSTRACT OF THE THESIS OF**

**Steven F. Price** for the Degree of **Doctor of Philosophy** in **Plant Physiology** presented on **April 1, 1994**.

**Title**: **Sun Exposure and Flavonols in Grapes.**

Redacted for Privacy

**Abstract approved**: _____
Patrick J. Breen

UV-screening, in natural sunlight, by phenolic compounds, was investigated in two grape (*Vitis vinifera* L.) cultivars, one with (Pinot noir) and one without (Chardonnay) anthocyanins. Berry epidermis exposed to full sun had higher attenuance of radiation between 300 and 400 nm than shaded epidermis from the same cluster. The concentration of flavonol glycosides ($\mu$g mm$^{-2}$ epidermis) in the sun exposed epidermis was twenty times higher in Chardonnay and six times higher in Pinot noir than in shaded epidermal tissue. The primary flavonols present in both cultivars were quercetin glycosides. Sun exposure increased cinamoyl ester concentrations but did not affect anthocyanins. Flavonol glycosides in Pinot noir, accounted for 70% and 35% of the total absorbance in the UV-A and UV-B ranges, respectively.

Wines were made from Pinot noir clusters from three different sun exposure levels: shaded, moderately exposed, and highly exposed. The concentration of quercetin glycosides in the three wines was 4.5, 14.8, and 33.7 mg L$^{-1}$, respectively. Wines from highly and moderately exposed clusters had similar anthocyanin levels, but polymeric anthocyanin content of wines from highly exposed clusters were 40%

higher than the other two treatments. Concentration of catechins and caftaric acid was inversely related to cluster sun exposure.

Digital imaging and analysis was used to quantify and characterize the light exposure patterns of photo-sensitive paper tubes placed in representative cluster positions in two grape canopies. Blue pixel values from the captured video images had a strong negative correlation with the log of irradience from an integrating quantum sensor. Histograms of incident light, developed from imaging software, were able to quantify the spatial distribution of light on individual tubes and were clearly related to the tubes position in the canopy. Curves of average light distribution in each canopy were able to differentiate the typical cluster light environment of the two canopies.

# Sun Exposure and Flavonols in Grapes.

by
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A Thesis

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed April 1, 1994

Commencement June 1994

APPROVED:

Redacted for Privacy

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Date thesis presented: April 1, 1994

Typed by Steven F. Price

## ACKNOWLEDGMENTS

Much of the work presented in this thesis would not have been possible without the help and support of many of my colleagues. Many of the experiments in this thesis were joint projects and the people described below were instrumental in their design, implementation, and analysis. The grape skin attenuance was the pivotal study in this thesis, providing the key to all the work that followed. Barbara Yoder was indispensable in providing both the equipment and advice that helped get it all started on the right track. Barney Watson gave me my first lesson on phenolic chemistry (drawn on a napkin) and has continued to assist me in almost all aspects of my research since. His winemaking assistance and enological advice were extremely important to the work on cluster exposure presented in Chapter 4 and his continued interest in phenolics generally have made the whole project more fun and tied the work more closely to real problems in the wine industry. Marilin Valladao has, with considerable patience, provided an almost endless supply of excellent chromatograms. Her high standards have raised the tone of the whole project and her good humor and kind support have been much appreciated. Marc Schuette has helped with bits and pieces of almost all the work presented in this thesis. His most noteworthy contribution has been his untiring ability to "pop" the skins of berries. I don't know how many thousands he did, but it was well beyond the call of duty. His contributions to the video imaging study were significant and helped that project move effortlessly into and out of the digital realm. Libby Tassie was a partner in developing the sunprint concept. Her six week visit to Oregon was a period of intense

creativity. Her enthusiasm and interest in all things viticultural started many wheels turning. Angelika Romel and Bob Durst provided important help in the initial identification of quercetin and in the development of our chromatographic techniques.

In addition to direct help with the work of research, many people have helped me in ways less easy to document, but still critical to the whole. Porter Lombard has been a tireless supporter. He encouraged me to start my PhD program and then gave me the time and independence to let it happen. Pat Breen, my ever diligent advisor and editor, has shaped the way I think (no, not warped, more like molded) and write. His interest in the process of science was an inspiration and for his efforts, which I'm sure he thought would never cease, I offer a toast of highly sun exposed (without a hyphen) wine. Glen, Kirk, Dave J., Robin, and Derick (the grad group) have been a valuable sounding board for my ideas. Many thanks for the ideas and comments and thanks for putting up with mine.

And last my family: Sal who never had doubts, and my Mom, to whom I can finally reply "I'm done!".

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# SUN EXPOSURE AND FLAVONOLS IN GRAPES

## CHAPTER 1.

### Introduction

Vineyard management techniques can significantly change the sun exposure of grape clusters. Over the last ten years, the viticulture research program at Oregon State University has tested numerous vineyard management strategies. In most of these trials, sun exposure of clusters was effected by changes in vine training. Generally, an increase in sun exposure resulted in increases in anthocyanins in grape and wine. Typically, there was also an increase in total phenolics and this response was often greater than could be explained by the increase in anthocyanins.

In an experiment evaluating downward trained Pinot noir vines, I observed that clusters on the upper surface of the canopy, that developed in full sunlight, occasionally had a lower anthocyanin content than more shaded clusters. This was contrary to the conventional horticultural wisdom that light intensity and anthocyanin content always go hand in hand. Wines made just from these clusters were described as harsh and had very high level of total phenolics. Pinot gris, a cultivar with a lower anthocyanin content than Pinot noir, had an even more pronounced decrease in anthocyanin levels in exposed clusters.

My initial hypothesis was that anthocyanins in clusters in direct sunlight were being degraded by UV light. I tested this hypothesis in the summer of 1991 by covering sun exposed clusters with aluminum foil just prior to veraison (first color

formation). At harvest, foil-covered clusters appeared very similar to uncovered clusters. They appeared to be less red on the sun-exposed than the shaded side of the cluster, a difference that could not have been due to UV degradation of anthocyanins. These results were inconsistant with my original hypothesis.

An alternate hypothesis I had considered was that these responses were the result of a change in phenolic metabolism, possibly related to UV-screening. In October 1991, I used a spectrophotometer to look at light transmission of sun-exposed and shaded grape berry skins. The sun exposed skins had an absorbance peak at 360 nm that was not present in shaded skins. Absorbance spectra of ethanol extracts of the same tissue showed an additional peak in sun-exposed skin at 260 nm. A search of the literature suggested that either flavonols or flavones could be responsible. The first HPLC analysis of the extracts from sun-exposed berry skin clearly identified quercetin, a flavonol, as the compound responsible for the greater absorbance at 260 and 360 nm.

The research presented in this thesis follows from that point. The first chapter is a detailed study of UV-screening in grape berry tissue. The second, evaluates the practical implications of flavonol accumulation in sun-exposed grape tissue on wine composition. The third chapter presents the results of a project to develop an alternative method of quantifying incident light on grape clusters.

Flavonol research is continuing in our laboratory. The work presented here has been the foundation for several new projects in the departments of Horticulture and Food Science and Technology. Most of the new work revolves around the point

where vineyard and wine processing effects interact. I hope that the research presented in this thesis, and the new work arising from it, contributes to understanding the complex path that leads from the vineyard to wine.

# CHAPTER 2.

## Literature Review

Flavonols are a common class of phenolic compounds found in most higher plants. They are a present in many food plants and are the most common phenolic compound in the human diet with an estimated daily consumption of up to 1 g per day (Leighton et al. 1992). Flavonols are a subclass of flavonoids and are closely related structurally and biosynthetically to anthocyanins and much of the information on flavonol biosynthesis has come from anthocyanin studies. Flavonols have become the subject of increased scientific inquiry in the last ten years due to their potentially positive effects on human health, particularly their effects as inhibitors of certain forms of cancer (reviewed in Ho et al. 1992). They have become a marketing issue in the wine industry following the broadcast of a television news program praising the positive health benefits of quercetin and other phenolics in red wine and the subsequent upsurge in red wine sales (Anon. 1993). Despite the recent interest in quercetin in wine, the occurrence, function, and regulation of flavonols in grapes has generally been ignored.

This thesis attempted to address some of these questions and contributes to the available literature on flavonol functions in grapes and environmental effects on flavonol content of grapes and wines.

**Flavonol Structure** All phenolic compounds contain an aromatic ring with one or more hydroxyl groups attached. Plants produce thousands of compounds containing phenolic residues (Goodwin and Mercer, 1983). All of these compounds share a common biosynthetic intermediate, phenylalanine or its precursor shikimic acid. Flavonoids, $C_6$-$C_3$-$C_6$ compounds, in addition to an aromatic ring and $C_3$ side chain from phenylalanine, have an additional $C_6$ ring derived from three acetyl-CoA groups from the polyketide pathway. Many flavonoids have one or more of their hydroxyl groups attached to a sugar by a ß-glycosidic linkage. These sugars can in turn be attached, via acylation, to additional phenolic or other organic acids. The potential for variation in all of these processes results in a tremendous diversity of phenolic compounds both within individual species and across the full range of higher plants. As an example, more than one hundred anthocyanins have been identified in fruits. They vary in the number of hydroxyl groups, the degree of hydroxyl methylation, the nature and number of sugars attached and the placement of the attachment, and the nature and number of acidic compounds covalently linked to the sugars (Mazza and Miniati 1993).

The structural diversity of phenolics in plants is matched by their extensive range of functions. Many of the physiological roles of specific phenolic compounds are unclear, but phenolic compounds are important plant structural components and act as plant protectants by prevention of insect feeding and pathogen attack, and screening of harmful radiation (reviewed in Stafford 1990).

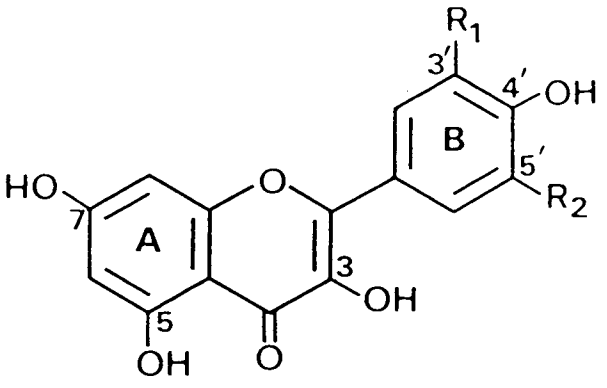


Figure 2.1. General flavonol structure. Kaempferol ($R_1$ = H, $R_2$ = H), quercetin ($R_1$ = OH, $R_2$ = H), myricetin ($R_1$ = OH, $R_2$ = OH), and isorhamentin ($R_1$ = $OCH_3$, $R_2$ = OH). From Macheix et al. (1990).

Flavonols are flavonoids with an unsaturated $C_3$ chain, a double bond between C-2 and C-3, and a hydroxyl attached to C-3 (Fig. 2.1). Hydroxylation and methylation patterns of the B-ring vary between the different aglycones but almost all are hydroxylated in positions 3, 5, and 7 in the A and B' rings. Glycosylation occurs, almost exclusively, at C-3.

**Synthesis.** Flavonols are synthesized in the cytoplasm of plant epidermal cells. In studies with mustard (*Sinapsis alba* L.) cotyledons, Wellman (1974) found that flavonols were synthesized in the upper epidermis, while anthocyanins were synthesized in lower epidermal tissue. Flavonols, anthocyanins, and other phenolic compounds accumulate in the central vacuole. Generally, there is thought to be little

cell to cell transport of flavonols or other phenolic compounds in plants.

Synthesis of flavonols most likely occurs via chalcone, flavanone, and dihydroflavonal precursors (Stafford 1992). Flavonols share these precursors with anthocyanins. The first, and possibly only, unique step in flavonol synthesis occurs with the introduction of a double bond between C-2 and C-3. Flavonol synthase, a soluble deoxygenase requiring $O_2$ , 2-oxoglutarate, $Fe^{2+}$, and ascorbate has been isolated from cell cultures and flower petals of several species, and is capable of converting dihydroquercetin to quercetin (Stafford 1992). The order of glycosylation, methylation, and acylation reactions is not clear for flavonols and is the subject of some debate for flavonoids generally. Stafford (1992) makes a convincing case for tight control of the entire synthesis process by an aggregate of membrane bound proteins all under close genetic regulation, rather than a linear sequence of cytoplasmic enzymes.

**Regulation**. Regulation of synthesis and accumulation of both flavonols and anthocyanins appears to be controlled by several photoreceptors. Three photoreceptors have been implicated in work with cell cultures and seedlings: phytochrome (a red/far-red receptor), a blue/UV receptor (possibly a flavoprotein), and an unknown UV-B receptor (Bruns et al. 1986, reviewed by Ballaré et al. 1992 and Stafford 1990). There are some difficulties in knowing which factors are important under natural sunlight since most studies have used artificial light sources that differ significantly in intensity and spectral distribution from sunlight (Caldwell et al. 1986). In addition to the spatial separation of anthocyanin and flavonol

synthesis mentioned above, there is other evidence that synthesis of flavonols and anthocyanins may be independent. Beggs et al. (1987) found differential accumulation kinetics of anthocyanins and flavonols in mustard and suggested that the two compounds were under separate regulation, and Brödenfeldt (1988) found different accumulation responses of flavonols and anthocyanins to UV treatments in cell culture and seedling studies. Evidence for differences in both temporal and spatial accumulations of anthocyanins and flavonols was found in this thesis (Chapter 3 and Appendix 1).

Multiple gene systems encoding parallel enzymes systems for specific phenolic compounds have recently been found (reviewed by Hahlbrock and Scheel 1989, and Ryder et al. 1987). This work indicates that phenolic compounds with specific physiologic functions could be regulated by distinct sets of enzymes positioned in different regulatory networks. In research on genes controlling anthocyanins in a range of species, Quattrocchio et al. (1993) suggested evolutionary differences in the regulation of flavonol and anthocyanin synthesis, with more advanced species having separate genetic control of each class of compound. The unique response of flavonols to sun exposure, shown in Chapter 3 of this thesis, would seem to indicate that some mechanism exists in grapes to control accumulation of flavonols independently from that of anthocyanins or other phenolic compounds.

**Flavonols and Anthocyanins in Fruit.** The most common flavonols in fruits are glycosides of quercetin, kaempferol, myricetin, and isorhamnin (Macheix et al. 1990).

Although they are structurally very similar to anthocyanins and share biosynthetic precursors, the distribution and relative proportions of the flavonol aglycones in plants usually differs significantly from the distribution of anthocyanin aglycones. Methylated anthocyanins are common in fruit, but with the exception of isorhamnen, methylated flavonols are rare. Flavonol glycosylation patterns in fruit appear to be much more varied than anthocyanin glycosylation. Glucose, galactose, rhamnose, arabinose, xylose, and glucuronic acid are the most common flavonol glycosides (Macheix et al. 1990). Diglycosides, when they occur, are usually 3-diglycosides, with quercetin-3-rutinoside (rutin) being the most common example found in fruit. There are no verified reports in fruit of diglycosides attached to different carbons on the flavonol molecule (Macheix et al. 1990), although in grapes, anthocyanin 3,5-diglucosides are common in American *Vitis* species (Mazza and Miniati 1993). Although acylation of flavonol glycosides with aromatic or other organic acids appears to be rare, acylated anthocyanins are common in fruit of many species, again pointing out the differences in the structural array of anthocyanins and flavonols.

**Functions of Flavonols in Plants.** Stafford (1990) has suggested that the diversity of flavonol structural modifications would seem to indicate a wide range of possible functions. However, most research on flavonols, and phenolics generally, has been limited to phenolics stored in the central vacuole and their role as plant protectants. The primary function of this pool of flavonols is thought to be screening of potentially damaging UV-light (Robberchet et al. 1980, Beggs et al. 1986, Ballaré et al. 1992).

The absorbance spectra of flavonols has two peaks, one at 260 and the other at 360 nm. The peak at 260 nm absorbs UV wavelengths that are the most damaging to cell metabolic structure and DNA (Rundel 1983). Solar radiation reaching the earth's surface is limited below 300 nm, due to absorbance by atmospheric ozone. Changes in atmospheric ozone concentrations caused by pollution may cause a shift in the lower wavelength limits of terrestrial radiation, however, during the evolution of plants, atmospheric ozone was lower than it is today, as were the lower wavelength limits of solar radiation (Caldwell et al. 1983). The flavonol absorbance peak at 360 nm may have more relevance to the current radiation environment of plants. Flavonol absorbance in this wavelength absorbs radiation in a range where there are almost no other absorbing compounds and appears to be responsible for most of the effective screening response observed in grapes in vivo (Chapter 3). These absorbance characteristics and flavonol responses to UV light have led researchers to assume a UV-protective function for flavonols (Markham 1982, Beggs et al. 1987).

Flavones, acylated anthocyanins, and esters of cinamic acids are also present in epidermal vacuoles and have absorbance spectra that could contribute to UV-screening. There has been very little research, however, relating UV-responses to natural sunlight to the chemical composition of epidermal cells, other than the levels of total flavonoids or total phenolics (Robberecht et al. 1980, Caldwell et al. 1983, Flint et al. 1985, Beggs et al. 1986).

Although there is abundant information on the phenolic composition of fruit (reviewed in Macheix et al. 1990, Mazza and Miniati 1993) there is almost no

information on UV-screening in fruit tissue. Most of the research on grape sun exposure has either concentrated on anthocyanins or only measured total phenolics. In almost every case where exposure of grape clusters was increased, there was an increase in levels of anthocyanins and total phenolics (Archer and Strauss 1989, Crippen and Morrison 1986, Freese 1988, Macaulay and Morris 1993, Morrison and Noble 1990, Roubelakis-Angelakis and Kliewer 1986, Smith et al. 1988). Chapter 3 of this thesis is the first study of its type to look at the specific responses of individual phenolic compounds to solar radiation in grapes.

The flavonol pool in the vacuole may have other functions as well. Phenolic compounds are thought to act as taste aversion compounds and may have a role in plant defenses against pathogens. Flavonols, like many phenolic compounds, can cause the precipitation of proteins, an assumed part of plant protective mechanisms (Stafford 1990). Flavonols, are not effective substrates for polyphenoloxydases although it is likely that they participate in secondary coupled oxidations tied to enzyme mediated formation of ortho quinones (Macheix et al. 1991). Flavonols are present in dark grown tomato seedlings, where they have no conceivable screening function (Price and Ballaré unpublished data), but with exposure to white and UV light the number of flavonols present and their concentration increased greatly.

Other roles for flavonols outside of the vacuole have been suggested. Quercetin is present in spinach chloroplast and in vitro studies show that it suppresses carotenoid photobleaching (Takahama 1984). However, ascorbate appeared to be a more effective antioxidant. Quercetin and kaempferol aglycones have been shown to affect

auxin transport by binding to membrane bound receptor proteins in vitro (Jacobs and Rubery 1993, Jones et al. 1991), but assumptions on the presence of aglycones in the vascular system have yet to be proven.

**Flavonols in Grape.** Flavonols were first isolated and identified in grape leaves in 1837 (cited in Singleton 1969). Glycosides of quercetin, kaempferol, myricetin, and isorhamnetin have been identified in grape berry epidermal tissue (Cheynier and Rigaud 1986). Glycosides are most commonly glucosides, galactosides, and glucuronides, with reports of other diglycosides appearing less frequently (Singleton 1969, Cheynier and Rigaud 1986, Spanos and Wrolstad 1990). Singleton (1969) and Machiex et al. (1990) report that the concentration of flavonol glycosides in grape berries ranges from 8 to 97 $\mu$g $gFW^{-1}$. Flavonol aglycones do not appear to be present in grape berries. None of the studies on flavonol concentrations mention possible environmental interactions and there is an overlying assumption that genetic differences between cultivars are the main factors affecting flavonol levels in fruit. Flavonols are not found in grape berry pulp or seeds, but leaves contain high levels of quercetin that can end up in wine following mechanical harvesting (Somers and Vérette 1988). Grape stems also appear to have significant flavonol levels, and could potentially affect wine flavonol concentrations in whole cluster fermentations (S.F. Price unpublished data).

**Flavonols in Wine**. Fermentation practices that favor a more thorough extraction of skin phenolic compounds will result in higher flavonol levels in wine. As a result, red wines usually have substantially higher flavonols than white, white wines given skin contact before pressing have higher flavonol levels that those without, and longer maceration times and higher temperatures increase the flavonol content of red wines (Macheix et al. 1990, Ramey et al. 1986, Merida et al. 1991). Singleton (1988) reported flavonol content of wine can range from 0 to 30 mg $L^{-1}$. Results from this thesis (Chapter 4) show that wines from the same cultivar and the same vineyard can range from 5 to 35 mg $L^{-1}$ based on the degree of cluster sun exposure and young Pinot noir wines can have flavonol levels up to 50 mg $L^{-1}$ (S. F. Price unpublished data). Both flavonol glycosides and aglycones are found in wine. Presumably, the aglycones are derived from the glycosides due to a slow acid hydrolysis or to glycosidase enzymes present in grapes or yeasts, but this has not been researched in wine. Evidence from a wine maceration trial now in progress suggests that both mechanisms could be important (B.T. Watson and S.F. Price unpublished data). Pre-fermentation maceration treatments that delay alcoholic fermentation have had higher flavonol aglycone concentrations in the wine than treatments with an immediate alcoholic fermentation, suggesting an enzyme involvement, and all the treatments show a gradual reduction of glycosides with time accompanied by increases in aglycones, suggesting a non-enzymatic hydrolysis.

The importance of flavonols in wine quality has generally been discounted or ignored (Singleton 1969, Singleton 1988, Somers and Vérette 1988, Macheix et al.

1990, Ribéreau-Gayon 1974). Part of this may be due to analytical tests commonly used in wine. Analysis of wine phenolics often stops at quantification of anthocyanins and an estimate of total phenols, both simple assays on a spectrophotometer (Singleton 1974, Singleton 1988, Somers and Vérette 1988). Because absorbance at 280 nm is a low point in the flavonol spectrum (Markham 1982), estimates of total phenolics based on wine absorbance at 280 nm (Sommers and Vérette 1988) could seriously underestimate the concentration of flavonols and, in high flavonol wines, would also underestimate total phenolics. Total phenolic estimates, using the Folin Ciocalteu method (Singleton 1974), are less likely to underestimate the contribution of flavonols but still do not separate flavonols from other phenolic compounds. HPLC techniques that monitor phenolics only at 280 nm or other low flavonol absorbing wavelengths (Sommers and Vérette 1988) could also underestimate the flavonol contribution to a wines phenolic profile.

Flavonols in wine could have a wide range of quality implications, potentially affecting wine color, taste, and stability. Flavonols have significant absorbance in the visible wavelength between 400 and 420 nm (Markham 1982). At 30 mg $L^{-1}$, a concentration that could occur in wine, a quercetin solution is visibly yellow. Flavonols may also affect wine color by altering and enhancing absorbance characteristics of anthocyanins. At high concentrations, flavonols and anthocyanins can form stacked structures held together by hydrophobic and hydrogen bonds (Brouillard 1983, Macheix et al. 1990). This "copigmentation" effect also stabilizes anthocyanins in their colored flavillium ion form and reduces the formation of a

colorless psuedobase. The addition of rutin to a colored grape juice resulted in a 1000% intensification in color of malvidin 3,5-diglucoside (Scheffeldt and Hrazdina 1978). In Chapter 3 of this thesis, a shift in the wavelength of maximum absorbance was seen in grape skin absorbance that could be due to copigmentation in the vacuoles and recent spectral analysis of wines from exposed clusters from the experiment described in Chapter 4 showed both a wavelength shift and a absorbance increase in wine that could not be explained by anthocyanin concentration alone.

Many phenolic compounds are both bitter and astringent. The quercetin aglycone in an alcoholic solutions is reportably intensely bitter (Merk Index). However, there is almost no information on the taste effects of flavonols in wine. Quercetin and myricetin, however, were found to be bitter, "winey", aromatic, and harsh in beer at 10 to 20 mg $L^{-1}$ (Dadic and Belleau 1973). Flavonols could also affect wine quality through indirect means by interacting with other wine phenolic compounds. Flavonols could combine with flavan 3-ols and anthocyanins to form polymeric compounds (Macheix et al. 1990). In the exposure trial reported in Chapter 4, grapes with higher flavonols levels made wines with higher amounts of polymeric phenols. This could effect long term color stability by stabilizing anthocyanins in polymeric forms (Sommers 1988, Nagel and Wulf 1979). Singelton and Trousdale (1992) recently demonstrated the importance of anthocyanins in maintaining polymeric phenolic tannins in solution. Anthocyanins are considerably more soluble than flavonols. It is possible in wines with high flavonol levels, that insertion of flavonols into polymeric tannins, in place of anthocyanins, could affect

the solubility of tannins and potentially the average molecular weight of the wine tannin complex in solution. The molecular weight of tannins is thought to be very important for their sensory characteristics, significantly affecting their astringency (Singleton and Trousdale 1992, Macheix et al. 1990).

Phenolic compounds have strong antioxidant capabilities including flavonols. The effects of large increases in flavonol content on a wine's oxidative status is unknown but could be important to a wine's oxidative stability. Some of the unexplained changes in wine chemistry related to cluster sun exposure shown in Chapter 4, particularly the apparent conversion of caftaric acid to caffeic acid could be related to the antioxidant characteristics of flavonols.

Although the wine industry's current interest in "healthful" wines with high quercetin levels has led some researchers to advocate fermentation techniques to maximize flavonol content of wine, there are too many unknowns to be able to predict the quality of a high flavonol wines. The chemistry of flavonols in wine is a subject worthy of further research.

# CHAPTER 3

## Light Attenuance by Phenylpropanoid and Flavonoid Compounds in Grape Berry Epidermal Tissue


### Abstract

UV screening, in response to natural sunlight, by phenylpropanoids and flavonoids in grape (*Vitis vinifera* L.) berry epidermal tissue was investigated in two cultivars, one with (Pinot Noir) and one without (Chardonnay) anthocyanins. Berry epidermis exposed to full sun had substantially higher attenuance between 300 and 400 nm than shaded epidermis on the same cluster with the greatest differences around 360 nm. The concentration of flavonol glycosides in ethanol extracts of sun exposed epidermis was twenty times higher in Chardonnay and six times higher in Pinot noir than that from the shaded position. The primary flavonol present in both cultivars was quercetin glucoside. Sun exposure increased the content of cinamoyl esters in both cultivars by two fold. The anthocyanin content of Pinot Noir epidermis was not affected by sun exposure. A novel method of determining the spectral effects of compounds in complex solutions using HPLC diode array data is described. This method showed that flavonols were the primary compounds absorbing UV in exposed Pinot noir epidermal extracts, accounting for 70 and 35% of the total absorbance in the UV-A and UV-B ranges, respectively. This study suggests that increases in UV-B radiation could have significant effects on fruit composition and food quality.

## Introduction

Light attenuance by phenolic compounds in the epidermis of plants is the primary mechanism protecting sensitive tissue from the damaging UV radiation in sunlight (Robberchet et al. 1980, Beggs et al. 1986, Stafford 1990). Anthocyanins, flavonols, flavones, and cinamoyl esters are thought to be the primary phenylpropanoid and flavonoid compounds involved in UV screening due to their strong absorbance in UV-A (325-400 nm) and UV-B (280-325 nm) wavelengths (Markham 1982, Beggs et al. 1987), their presence in epidermal tissue (Beggs et al. 1986, Macheix et al. 1990), and their accumulation in response to supplemental UV-A and UV-B radiation treatments (Beggs et al. 1986, Bruns et al. 1986, Tevini et al. 1991). Research with cell cultures and developing seedlings has elucidated many of the physiological and genetic processes involved in these responses (reviewed in Beggs et al. 1986, Hahlbrock and Scheel 1989, Stafford 1990). There have been fewer studies evaluating phenolic attenuation of UV radiation under natural conditions.

Robberecht et al. (1980) evaluated leaf epidermal transmittance of arctic-alpine plants along a latitudinal gradient. Species from high UV-B radiation environments (i.e. equatorial or high elevation species) more effectively attenuated UV-B light, most commonly by increases in epidermal absorbance. High altitude, Hawaiian species were also used by Caldwell et al. (1983) to evaluate the role of reflectance and absorbance in UV protection. They suggested that the UV epidermal absorbance of most species was primarily a function of flavonoids in the vacuole. Flint et al. (1985), using field-

grown *Vicia faba* plants with two levels of supplemental UV light, found that UV treatments increased concentrations of flavonoid pigments and decreased UV transmittance through the leaf epidermis. Natural sunlight, modulated with a series of cutoff filters, was used by Beggs et al. (1986) to vary the quality of UV light reaching young seedlings of several important crop species. Ethanol extracts of seedlings had increased UV absorbance with increased seedling exposure to short wavelength solar radiation. The role of flavonoids in all these studies was assumed based on absorbance changes, but the specific compounds responsible for epidermal absorbance were not identified.

Our objectives were to determine the changes in phenolic composition that occur in response to natural sunlight in grape berry epidermal tissue and to determine phenolic effects on light attenuance in vivo and in plant extracts. Shaded and exposed tissues were obtained by utilizing natural variation in sun exposure that exists within grape clusters.

Although there is voluminous information on the phenolic composition of many fruits (Macheix et al. 1990, Mazza and Miniati 1993), there is very little information on UV screening responses in fruit tissue. We were particularly interested in changes that might occur in relative concentrations of different flavonoids in grapes; many of these compounds are primary constituents of wine with significant effects on wine quality (Macheix et al. 1990). Two cultivars were used, one with and one without anthocyanins, to determine the effective degree of UV protection provided by

anthocyanins and whether other phenolic responses were linked to anthocyanin accumulation.

Photodiode array detection coupled with the separation power of HPLC was used to evaluate the relative composition and spectral properties of the phenolic compounds in epidermal extracts. We used these tools to develop several novel approaches to show the contribution of specific flavonoids and phenylpropanoids to attenuance in plant tissue and tissue extracts and their responses to solar radiation.

## Materials and Methods

**Plant Material.** Ten fruit clusters of Pinot Noir and Chardonnay wine grapes were collected at full maturity from sun exposed positions on the top surface of grape canopies. Pinot Noir is a "black" grape with anthocyanins in the epidermal tissue; the pulp is not pigmented. Chardonnay is a yellow or green grape with no anthocyanins. The vines were trained with shoots positioned downward so that the fruit clusters, which are located at the base of shoots, were in the upper part of the canopy, above the foliage, and exposed to direct sunlight for most of the growing season. The sun exposed side of each cluster was marked and harvested clusters stored at 4° C until analysis (1 to 5 days).

Preliminary experiments had determined that there is significant variation in total phenolic content between sun exposed and shaded berries in a single grape cluster. The variation was related to the position of a berry in the cluster, with exterior, sunlit berries having the highest levels and interior, shaded berries the lowest. For the

present experiment, two 9 mm disks of grape epidermis were cut from sun exposed positions on each cluster and two disks from the inward facing surfaces of shaded berries on the same cluster (twenty disks for each exposure and cultivar). Light levels at exposed epidermal surfaces were essentially equal to ambient levels above the grape canopy throughout the growing season, whereas light levels at the surface of interior berries decreased through the season as the berries enlarged and berry to berry shading increased. By harvest, light levels on the shaded exterior surface of the clusters were less than 10% of ambient (data not shown) and the surface of interior berries were undoubtedly much lower than that. Hereafter the two sampling sites are referred to as "exposed" and "shaded". The disks consisted of endo- and hypodermal tissues (Pratt 1972). All pulp was removed from the inner surface and the disks blotted dry with tissue.

**Measuring Epidermal Light Attenuance**. Epidermal light attenuance was recorded on a Shimadzu model 265 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) with an integrating sphere attachment to maximize measurement of scattered light (Yoder and Daley 1990). Each epidermal disk was placed between a black metal template, with a 7 mm hole, and a quartz microscope slide, with the external side of the disk facing the quartz slide and the incident light. Less than 60 sec elapsed between cutting a disk and placing it in the spectrophotometer. Attenuance was recorded between 750 and 190 nm at 0.1 nm intervals on the "slow scan" setting. Slit width was 5 nm. The base line was established with the metal template and quartz slide

in place and each sample was zeroed at 750 nm before scanning. The spectrophotometer was connected to a personal computer, and data were transferred to a spreadsheet program for analysis. Single scans were smoothed over 1 nm before averaging. Spectra presented in Figure 3.1 are the average of twenty disks for each exposure from each cultivar.

**Ethanol Extracts**. After each scan, a 7 mm disk, corresponding to the portion of the disk scanned in the spectrophotometer, was cut from the center of each 9 mm disk and placed in 1 mL of acidulated ethanol (9:1, 95% ethanol:KCl/HCl pH1 buffer). The mixture was heated to 70°C for 5 min then kept at 0°C. After 12 h the disks were removed and discarded. Extracts were stored at -80°C.

One hundred microliters of each extract was added to a pooled sample for each cultivar and exposure treatment. Spectra of the pooled extracts for each treatment were obtained with the Shimadzu spectrophotometer, with a cuvette holder replacing the integrating sphere. Quartz cuvetes with a 10 mm pathlength were used on all the extracts. Extracts were diluted 1:5 with acidulated ethanol prior to reading in the spectrophotometer. Settings were the same as in the skin attenuance runs. Acidulated ethanol was used as a blank.

**Chromatographic Profiles of Phenolic Compounds**. Pooled ethanol extracts of each cultivar and treatment were passed through 0.4 $\mu$m nylon filters and 20 $\mu$L injected into the HPLC. A Hewlett-Packard 1050 series HPLC with a model 1040 series II diode

array detector and HP Chemstation 3D software (Hewlett-Packard Inc, Palo Alto, CA) were used for all chromatographic analysis. The column was a 250 x 4.6 mm Polymer Labs PLRP-S column (Polymer Laboratories Inc, Amherst, MA). A gradient elution was used. Solvent A consisted of water with 1.5% (v/v) phosphoric acid and solvent B was acetonitrile with 1.5% (v/v) phosphoric acid. Gradient conditions were: 0 min, A 95%, B 5%; 85 min, A 78%, B 22%; 88 to 95 min, A 50%, B 50%, 100 min, A 95%, B 5%.

Full spectral scans were taken every 1.6 sec at 4 nm intervals to develop a three dimensional database (retention time x wavelength x absorbance). Peaks were identified by spectral comparison to published spectra (Markham 1982) and by retention time and spectral comparisons to known standards.

The three dimensional data base for each run was used to develop chromatograms at 8 nm intervals from 220 to 600 nm for each pooled ethanol extract. Each chromatogram was integrated and total peak area and peak areas for total anthocyanins, total flavonols, and total hydroxy cinamic acid derivatives were recorded.

## Results

**Epidermal Light Attenuance.** Sun exposure markedly increased epidermal light attenuance in grape berries in both Chardonnay and Pinot Noir (Fig. 3.1A and B). The inset in each figure shows the difference between the two spectra (exposed minus shaded). The greatest differences in the attenuance spectra were in the UV-A range with the maximum difference at 360 nm for both cultivars. Compared to shaded,the

exposed berry epidermis of both cultivars had higher attenuance at all wavelengths except near 675 nm and below 300 nm for Pinot Noir. Attenuance readings were saturated, compromising their reliability, below 350 nm in the exposed Pinot Noir and Chardonnay berries and below 310 nm in shaded Pinot noir.

**Light Absorbance by Ethanol Extracts.** Absorbance spectra for the pooled ethanol extracts of Chardonnay and Pinot Noir are shown in Figures 3.2A and B. In comparison to the shaded extract, the exposed Chardonnay had greater absorbance at all wavelengths below 420 nm. Absorbance of the exposed Pinot Noir extract was higher than that of the shaded extract at all wavelengths. The shaded extract of Chardonnay had a distinct peak at 280 nm, the absorbance maxima of many phenolic compounds (Fig. 3.2A). The extracts of shaded Pinot Noir had an absorbance peak at 280 nm and also at 540 nm, where anthocyanins absorb strongly. Extracts of exposed epidermis of both cultivars showed additional peaks at 260 nm and 360 nm, absorbance maxima for flavonols (Markham 1982). The spectral differences (Fig 3.2A and B, insets) strongly suggested the presence of flavonols in the extracts, with distinct peaks at 260 nm and 360 nm for both cultivars. The difference spectrum of the Chardonnay extract clearly resembles published spectra for flavonol glycosides (Markham 1982) and in fact is almost identical to the spectrum of quercetin glucoside, the main flavonol glycoside peak identified in the ethanol extracts (Fig. 3.5B).

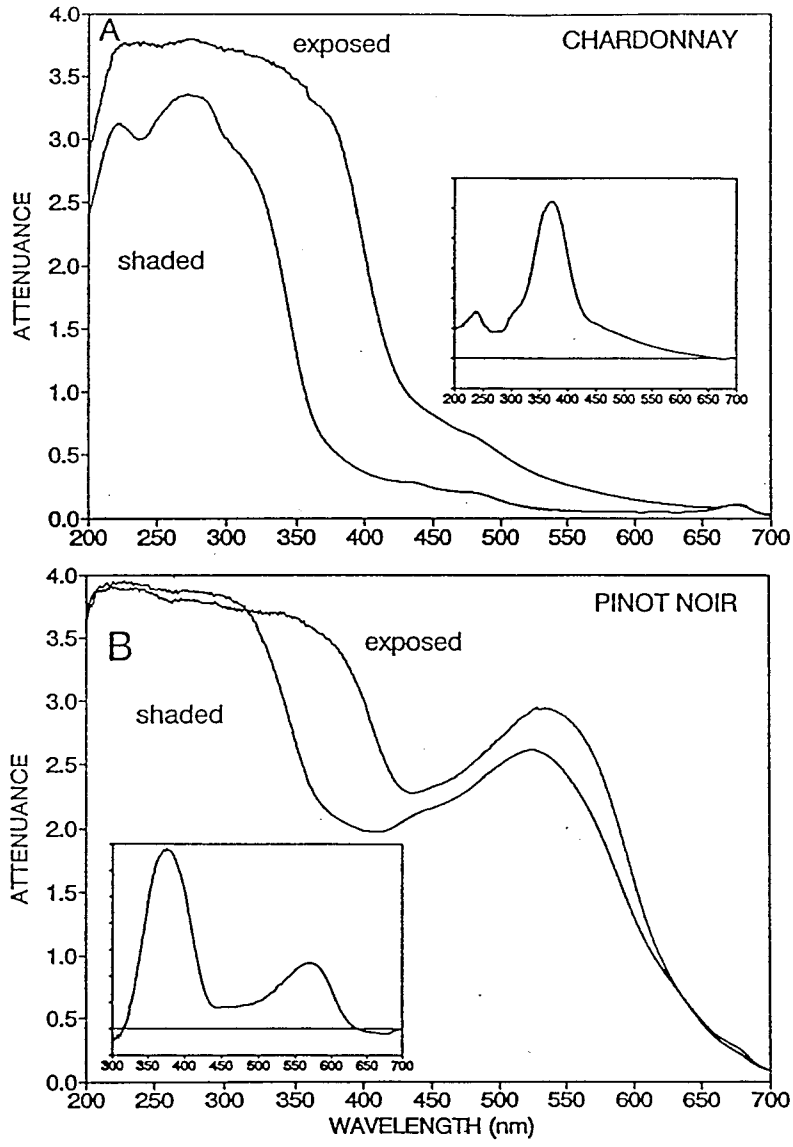


Figure 3.1. Light attenuance by sun exposed and shaded grape epidermal tissue of two cultivars recorded on spectrophotometer with an integrating sphere attachment: A, Chardonnay; B, Pinot Noir. Attenuance was recorded between 750 and 190 nm at 0.1 nm intervals. Single scans were smoothed over 1 nm before averaging. Spectra presented are the average of twenty disks from each exposure and cultivar. Insets are the difference spectra of exposed minus shaded spectra.

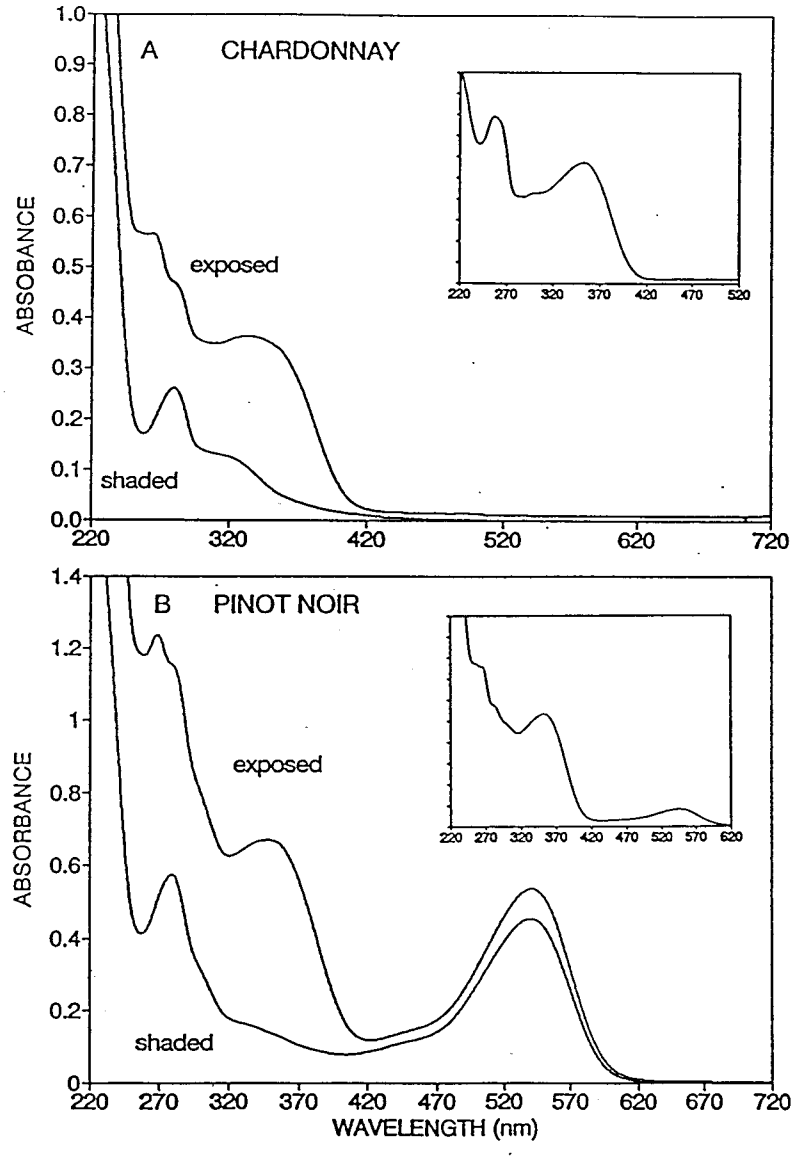


Figure 3.2. Absorbance spectra of sun exposed and shaded grape epidermal extracts from two cultivars: A, Chardonnay; B, Pinot Noir. Absorbance was recorded at 0.1 nm intervals. Single scans were smoothed over 1 nm before averaging. Each spectra is the average of twenty epidermal disk extracts from each exposure and cultivar. Insets are the difference spectra of exposed minus shaded spectra.

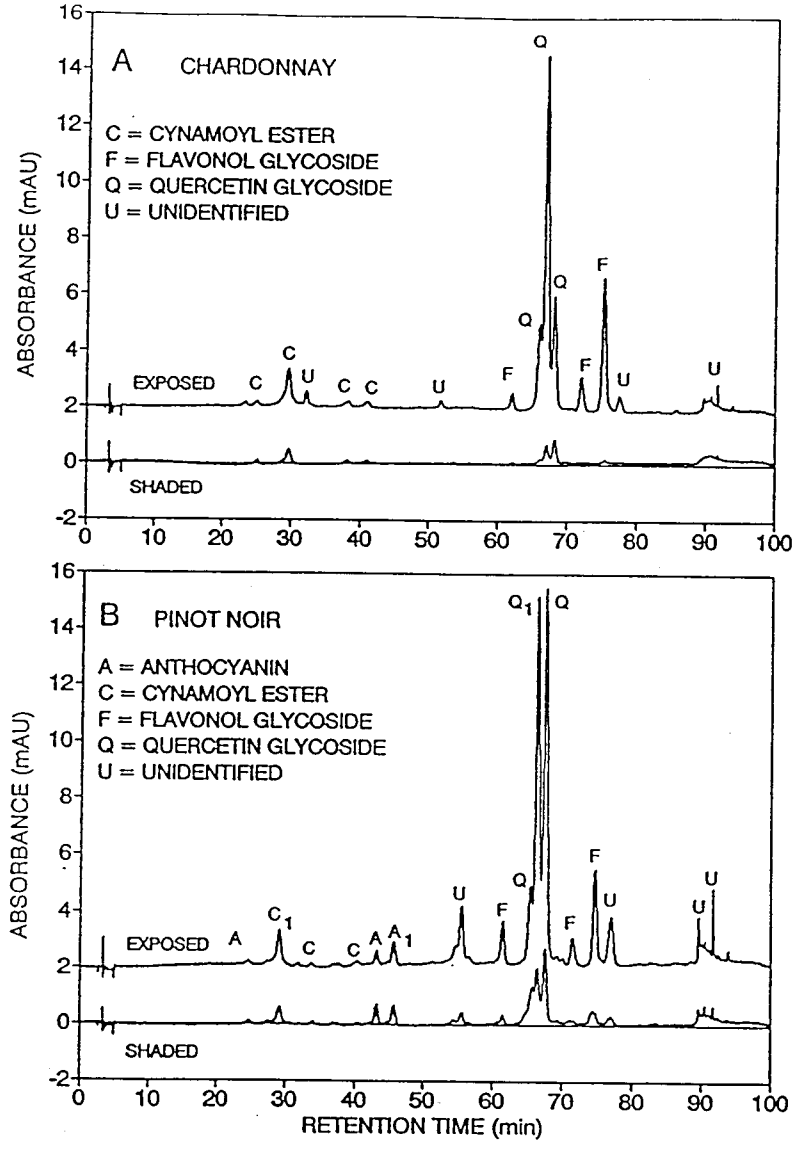


Figure 3.3. HPLC chromatograms at 360 nm of sun exposed and shaded grape epidermal extracts from two cultivars: A, Chardonnay; B, Pinot Noir. Extracts are pooled from 20 individual extracts from each cultivar and exposure treatment. Peaks were identified by comparison to published spectra and spectral and retention time comparisons to known standards. The spectra of peaks $C_1$, $A_1$, and $Q_1$ from both exposed and shaded extracts of 'Pinot Noir' are presented in Figure 3.4.

**HPLC Chromatograms and Spectral Data**. Chromatograms at 360 nm of extracts of exposed and shaded epidermis of Pinot Noir and Chardonnay are shown in Figures 3.3A and B. This wavelength was selected to show the responses of flavonols and cinamoyl esters. Extracts of exposed epidermis in both cultivars had higher concentrations of cinamoyl esters and flavonols than shaded tissue. Peak areas of cinamoyol esters at 360 nm were approximately two-fold higher in exposed than shaded epidermal extracts. Total flavonol peak areas at 360 nm (F and Q in Fig. 3.3A and B) for the exposed extracts were about 20 times higher than the shaded extracts in Chardonnay and 6 times higher in Pinot Noir. Shaded Pinot Noir extracts had higher flavonol levels than shaded Chardonnay. Quercetin glycosides were the main flavonols present in both cultivars, accounting for more than 80% of the total flavonol peak area. No flavonol aglycones were found in the extracts. As expected, five anthocyanin peaks were identified in Pinot Noir extracts (Mazza and Miniati 1993). There were no significant differences between their peak areas in the chromatograms of exposed and shaded extracts at 520 nm, the absorbance maxima for anthocyanins (data not shown).

Spectra of the most prominent anthocyanin, cinamoyl ester, and quercetin glycoside peaks from chromatograms of the exposed and shaded Pinot Noir extracts were used to define the spectral characteristics of each compound class as well as absorbance differences between exposure treatments (Fig. 3.4). Spectra were not normalized to emphasize the effects of concentration differences in the extracts.

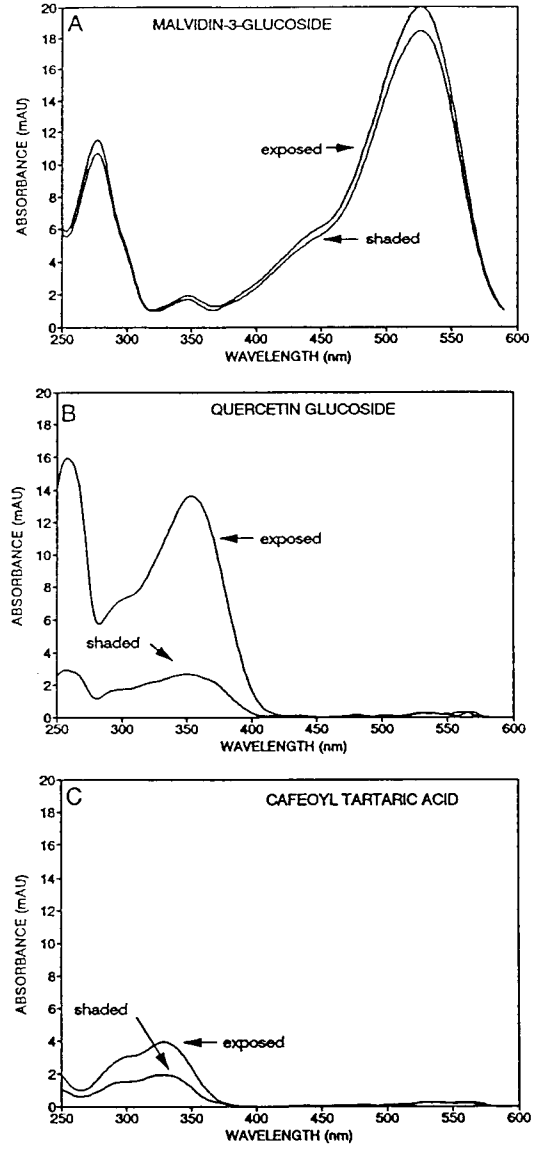


Figure 3.4. Spectra of malvidin 3 glucoside (A), quercetin glucoside (B), and cafeoyl tartaric acid (C) peaks from sun exposed and shaded Pinot noir epidermal extracts. Respectively peaks $C_1$, $A_1$, and $Q_1$ in Figure 3.3.

Malvidin-3-glucoside (peak $A_1$ in Fig. 3.3B) was the primary anthocyanin present in Pinot Noir. There were no significant differences in the spectral characteristics of anthocyanin peaks from exposed and shaded epidermal extracts (Fig. 3.4A).

The most prominent flavonol glycoside in Chardonnay was quercetin glucoside (Fig. 3.3A). In Pinot noir there were two quercetin glycosides one of which was the glucoside (peak $Q_1$ in Fig. 3.3B). Its spectra had substantially higher absorbance in extracts of sun exposed tissue of Pinot Noir than shaded tissue. From 300 to 400 nm, absorbance of the quercetin glucoside exceeded absorbance of malvidin in extracts of exposed epidermis (Fig. 3.4A and B). The most common cinamoyl ester in grapes is cafeoyl tartaric acid (Macheix et al. 1990) (peak $C_1$ in Fig. 3.4C). The absorbance spectrum of this peak was 2 fold higher in extracts of exposed compared to shaded tissue (Fig. 1.4C). In extracts from exposed epidermal disks, however, the absorbance of cafeoyl tartaric acid was substantially lower than that of quercetin glucoside (Fig. 3.4B).

To determine the effects of the anthocyanins, flavonols, and cinamoyl esters on absorbance at different wavelengths, chromatograms were generated at 10 nm intervals across the full wavelength range of the three dimensional database for the Pinot noir extracts (200 nm to 600 nm). Peaks in each chromatogram were identified by spectral characteristics and retention times and then grouped into appropriate classes. Integrated peak areas from these chromatograms were used to show total peak area at each wavelength and the peak area contributed by anthocyanins, flavonols, and

cinamoyl esters. This information was used to develop the graphs in Figure 3.5 and the information in Table 3.1.

Total peak area from the chromatograms of Pinot Noir extracts, plotted against wavelength, resulted in a curve very similar to the absorbance spectra of the ethanol extracts determined with the spectrophotometer (compare Fig. 3.5A and 3.2B). The greatest differences between exposed and shaded treatments were at 260 nm and 360 nm, as was shown in Figure 3.2B, again showing the close relationship of the peak area plots to the absorbance spectra of the extracts.

The chromatographic peak area contributed by the three main phenolic classes in Pinot Noir is shown in Figure 3.5B (exposed) and 3.5C (shaded). Anthocyanins accounted for almost all of the peak area in the visible wavelengths in extracts from both exposed and shaded epidermis. In the shaded epidermal extract, peak area in the UV-A range is the result of absorbance by anthocyanins, flavonols and cinamoyl esters; below 320 nm, anthocyanins were the major phenolic class affecting peak area. However, due to the higher flavonol concentration in the exposed extracts, flavonols accounted for most of the UV-A peak area and more than half of the total at 260 nm.

When expressed as a percentage of the total chromatogram peak area, flavonols in extracts from exposed epidermis accounted for 35% of the peak area in the UV-B range and 70% of the total in the UV-A range, whereas in the comparable values from extracts of shaded epidermis were only 9% and 49% (Table 3.1). Although total peak area of cinamates was higher in extracts from exposed epidermis than shaded (Fig. 3.5 B and C), their percentage of the total was lower due to the larger increase in flavonol

peak area (Table 3.1). Since the anthocyanin peak area was essentially the same in extracts from both treatments the percentage of total anthocyanin peak area in the UV-A and UV-B range was markedly lower in the exposed treatment.

Chardonnay epidermal extracts did not have a high enough concentration of flavonols or cinamoyl esters to evaluate using this technique. Chromatographic peaks were generally too small to be integrated at any wavelengths between 200 and 600 nm except near 280 and 360 nm. Efforts to concentrate the extracts in a rotory evaporator, in a stream of nitrogen gas, or in a centrifical evaporator, all resulted in the appearance of flavonol aglycone artifacts, presumably due to acid hydrolysis of the glycoside in the concentrated solutions.

**Table 3.1**. Percent of total peak area on chromatograms of an exposed and shaded Pinot noir grape epidermal extract. Three dimensional diode array data from an HPLC run of each extract was used to construct chromatograms at 10 nm intervals between 220 and 600 nm. Peak areas of each compound class were summed and compared to total peak area to determine percentage of total absorbance in each wavelength range.

| | | Wavelength range | | |
|---|---|---|---|---|
| Compound class | Exposure treatment | UV-B (280-325nm) | UV-A (325-400nm) | Visible (400-600nm) |
| | | Percent of total peak area | | |
| Anthocyanins | exposed | 17.6 | 6.1 | 94.9 |
| | shaded | 44.8 | 27.6 | 100.0 |
| Flavonols | exposed | 34.5 | 70.1 | 2.6 |
| | shaded | 8.5 | 49.1 | 0.0 |
| Cinamates | exposed | 14.4 | 8.3 | 0.0 |
| | shaded | 16.0 | 14.6 | 0.0 |

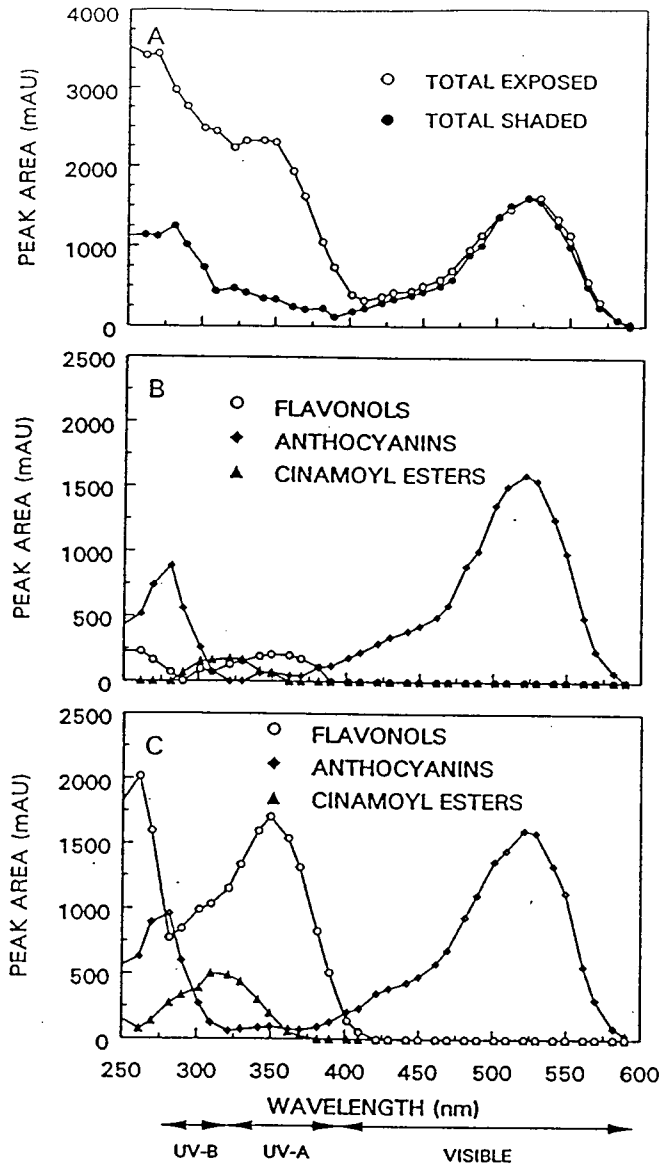


Figure 3.5. Plots of peak areas (mAU s) taken from HPLC chromatograms of sun exposed and shaded Pinot Noir epidermal extracts. Three dimensional diode array data from an HPLC run of each extract was used to construct chromatograms at 10 nm intervals with an 8 nm bandwidth between 220 and 600 nm. A, total peak area of exposed and shaded extracts. B, summed peak area for each compound class of the shaded epidermal extract. C, summed peak area for each compound class for the sun exposed epidermal extract.

## Discussion

Berries of Pinot noir and Chardonnay grapes exposed to prolonged levels of direct solar irradiance during their development and maturation showed higher accumulation of phenolics in epidermal tissues than shaded fruit. This response was observed in two distinct classes of phenolic compounds. In both cultivars, the accumulation of flavonols was most dramatic followed by cinamoyl esters. Anthocyanin content in Pinot noir epidermis was not affected by exposure treatment. High light levels are usually thought to result in an increase in anthocyanin levels in fruit tissue (Macheix et al. 1990), however, Dokoozlian (1990) recently reported that maximum accumulation of anthocyanins in Pinot noir grape berries occurs at fairly low fluence rates (less than 18% ambient PPFD). This is much lower than the fluence levels present on fully sun exposed grape skin used as the exposed treatment in this study but could be similar to the light levels of the shaded berries early in their development.

The apparent independence between the accumulation of flavonols and other phenolic compounds is significant for its relevance to an understanding of the mechanisms regulating phenylpropanoid and flavonoid metabolism as well as for its possible effects on wine quality. Although the role of phenolic compounds in UV protection has been clearly demonstrated, in many studies it was not clear whether the response was the result of a general increase in phenolic metabolism or a compound specific response (Robberecht et al. 1980, Caldwell et al. 1983, Flint et al. 1985, Beggs

et al. 1986). The question is further complicated by the presence of at least three photoreceptors implicated in phenylpropanoid and flavonol synthesis: the phytochrome R/FR system, a blue/UV-A photoreceptor, and a putative UV-B receptor, and a background of constitutive phenylpropanoids formed in response to endogenous factors (Stafford 1990, Ballare et al. 1992).

Biosynthesis pathway models usually show flavonols and anthocyanins sharing a common path and substrate studies in vitro show that dihydroflavonols are precursors for both classes of compounds (Markham 1982, Stafford 1990). However, Beggs et al. (1987) found that accumulation kinetics of anthocyanins and flavonols in mustard were quite different and suggested that they were under separate regulation. Differential accumulation responses of anthocyanins and flavonols have been observed in cell culture and seedling studies with artificial light (Brödenfeldt 1988). Spatial separation between anthocyanin synthesis and flavonols has been observed in mustard seedlings where flavonol synthesis and accumulation was concentrated in the outer epidermis and anthocyanins in the inner epidermis (Wellmann 1974).

Recent genetic studies have found multiple gene systems encoding parallel enzymes for specific phenylpropanoid products (reviewed in Hahlbrock and Scheel 1989). Ryder et al. (1987) identified a set of up to 9 chalcone synthase genes and multiple isoforms of chalcone synthase in bean. They suggested that the multiple genes allow similar enzymes to be positioned in different regulatory networks. The presence of separate, parallel synthesis pathways of anthocyanins and flavonols could explain

accumulations of flavonols in response to environmental signals or stresses without an accompanying increases in other flavonoids, as we have observed here.

The diode array detector was particularly useful in evaluating the spectral characteristics of the epidermal extracts. The resulting three dimensional databases allowed quantitative comparisons of spectra of specific chromatogram peaks in exposed and shaded tissue extracts (Fig. 3.4). In addition, each chromatographic database could be reprocessed to provide additional information on the impact of specific compounds (e.g. flavonols) on the spectral characteristics of the extracts (Fig. 3.5 and Table 3.1). While it is difficult to determine the effects of specific compounds on the spectra of a complex solution, total chomatographic peak area can be easily partitioned between the peak area of integrated individual peaks or all the peaks of a defined group. These data can be graphically presented as peak areas vs wavelength (Fig. 3.5) or can be summarized in tabular form (Table 3.1). Although the data are expressed as peak areas (mAU s) they are directly proportional to absorbance. This technique is a unique application of diode array technology and could have further application for the evaluation of spectral effects of compounds in other physiological studies or for determining the effects of compounds on the optical (i.e. visible) characteristics of solutions.

Flavonols that accumulated in sun exposed epidermal tissue of grape berries appeared to be responsible for almost all of the increases in attenuance associated with exposure. This was apparent in absorbance spectra and HPLC chromatographs of the ethanol extracts. The twenty fold increase in flavonol concentration in Chardonnay and

the six fold increase in Pinot noir were clearly responsible for the observed higher attenuance in the exposed epidermal disks. The attenuance data showed significant increases in UV attenuance for both cultivars, demonstrating that flavonols can create a functional, effective protection against UV-radiation in vivo.

Although anthocyanins have been suggested as having a role in UV-radiation protection (Caldwell 1981, Tevini et al. 1991) they did not accumulate in response to increased solar irradience or significantly block UV-radiation between 300 nm and 400 nm in this study. As a result, the contribution of anthocyanins to total absorbance in the exposed treatments, was low in both UV-B and UV-A wavelengths (Table 3.1). Their low contribution to UV absorbance is due in part to the spectral characteristics of the anthocyanins present in Pinot Noir (Fig. 3.5A). None of the five anthocyanin glucosides present in Pinot Noir are acylated with organic acids or phenolic compounds (Mazza and Miniati 1993). Acylation significantly increases the absorbance spectra of anthocyanins in the 300 to 320 nm range (Hong and Wrolsted 1990).

The accumulation of flavonols in sun exposed berries had not been previously reported in grape. This information may have significant economic importance as the phenolic composition of grapes has pronounced effects on the quality of wine. Flavonols effect bitterness and astringency of wine (Macheix et al. 1990) and some work now in progress in our group suggests that flavonols may interact with anthocyanins to form polymeric compounds in wine. Furthermore, quercetin, the major flavonol found in this study, has been the subject of increased interest due to its role

as an anti-oxidant in human nutrition and its possible effects on high density lipoproteins and human heart disease (Ho et al. 1992).

Most studies on the effects of increased levels of UV-B radiation in crop plants have been concerned with possible impacts on plant productivity. This study suggests that the accumulation of UV screening compounds in crop plants may also result in food quality differences as well.

# CHAPTER 4

## Quercetin in Grapes and Wine


### Abstract

Anthocyanin and flavonol content of disks of sun-exposed Pinot noir berry skin were compared to disks from shaded berries. Anthocyanin content was not affected by sun exposure but quercetin glycoside concentration of sun-exposed disks was 1.46 $\mu$g $mm^{-2}$ for the sun-exposed disks compared to 0.14 $\mu$g $mm^{-2}$ for the shaded. Wines were made from Pinot noir clusters, from a single vineyard block, from three different sun exposure levels: Shaded, moderately exposed and highly exposed. The concentration of quercetin glycosides in wine was 4.5, 14.8, and 33.7 mg $L^{-1}$ in the shaded, moderate and highly exposed treatments respectively. The level of quercetin aglycone also increased with sun exposure. Cluster sun exposure appears to be the primary factor determining quercetin levels in grapes and wine. Wines from highly and moderately exposed cluster positions had higher total anthocyanin levels than those from shaded clusters, but wines from highly exposed clusters had 40% greater polymeric anthocyanins than the other two treatments. Caftaric acid, catechin, and epicatechin concentrations in wine were inversely related to cluster sun exposure. The low levels of caftaric acid in wines from sun-exposed clusters appeared to be related to hydrolysis of the tartaric ester, with wines from highly sun-exposed clusters having 50% more caffeic acid than moderate and 130% more than shaded. Caffeic acid was not present

in fruit samples. It is possible that the increase in polymeric anthocyanins and the low levels of catechin in wines from sun-exposed clusters is directly related to quercetin levels. High wine quercetin levels may increase the rate of polymerization with potential stability and quality implications.

## Introduction

Flavonols are a class of flavonoid compounds found in most higher plants, most often as glycosides sequestered in the vacuoles of epidermal tissue (Stafford 1990). The flavonol quercetin was first identified in grape leaves in 1873 (cited in Singleton 1969) and flavonol glycosides of quercetin , myricetin, and kaempferol have been found in grape berry epidermal tissue (Cheynier and Rigaud 1986). Quercetin levels in grape skins reportedly range from 0.0081 to 0.0975 mg $gFW^{-1}$ (Macheix et al. 1990). There are apparently no flavonols in the pulp or seeds (Singleton 1969). Flavonol glycosides and aglycones are found in grape wine from trace amounts up to 30 mg $L^{-1}$ in some red wines (Singleton 1988). The concentration of flavonols in wine can be affected by processing variables, with factors that increase skin extraction resulting in higher levels (Ramey et al. 1986, Merida et al. 1991). Quercetin in wine has been the subject of increased interest due to potential effects on human health. The health aspects of quercetin and other phenolics in foods have been reviewed in Ho et al. (1991). Despite reports of significant variation in grape and wine flavonol concentrations (Leighton 1990) there have been no studies on factors that influence flavonol levels in grape berry tissue.

Flavonols, anthocyanins, flavones, and cinamoyl esters are thought to be the primary phenylpropanoid and flavonoid compounds involved in UV screening, due to their strong absorbance in UV-A (325-400 nm) and UV-B (280-325 nm) wavelengths (Markham 1982, Beggs et al. 1987), their presence in epidermal tissue (Beggs et al. 1986, Macheix et al. 1990), and their accumulation in response to supplemental UV-A

and UV-B radiation treatments (Beggs et al. 1986, Bruns et al. 1986, Tevini et al. 1991, reviewed in Ballaré). Research with cell cultures and developing seedlings has elucidated many of the physiological and genetic processes involved in these responses (reviewed in Beggs et al. 1986, Hahlbrock and Scheel 1989, Stafford 1990).

In 1991, our group began a research program to determine the effects of sun exposure on grape phenolics. Previous work on sun exposure and grape phenolics had looked only at the anthocyanins and levels of total phenolic accumulation (Crippen and Morrison 1986, Roubelakis-Angelakis and Kliewer 1986, Freese 1988, Smith et al. 1988, Archer and Strauss 1989, Morrison and Noble 1990, and Macauly and Morris 1993). We were particularly interested in changes that might occur in specific phenolic compounds and how they might relate to UV screening responses in grape berry skins. We recently reported that Pinot noir and Chardonnay berry skin exposed to full sun had substantially higher attenuance between 300 and 400 nm than shaded berry epidermis in the same cluster. The greatest differences were near 360 nm (Price et al. 1992). Quercetin glycosides appeared to be responsible for most of the differences in UV attenuance in both skin and ethanol extracts of skin from sun-exposed and shaded berries. Flavonols in extracts of sun-exposed Pinot noir skin accounted for 70 and 35% of the total absorbance in the UV-A and UV-B range respectively, compared to only 49 and 9% in extracts of shaded skin from the same clusters. Anthocyanins did not appear to respond to sun exposure and were ineffective as screening compounds between 300 and 400 nm (Chapter 3).

The magnitude of the flavonol response to sun exposure seemed large enough to have potential effects on wine composition and quality. The objectives of the research presented in this paper were to further characterize and quantify flavonol accumulation in sun-exposed berry skin of Pinot noir and to determine if sun exposure of clusters affects quercetin levels in the resulting wine.

Two studies were conducted, both utilizing the natural variation in sun exposure that occurs in a commercial vineyard environment. The first, a continuation of the work presented in Price et al. (1992), evaluated phenolic accumulation in grape skins from exposed and shaded positions within sun-exposed clusters. The second, utilized the variation in cluster exposure found in a commercial vineyard block. Skin extracts, must, and wine were analyzed from whole clusters selected on the basis of their sun exposure.

## Materials and Methods

**Skin Disk Study**. Ten fruit clusters of Pinot noir were collected at full maturity (about 23° Brix) from sun exposed canopy positions in a commercial Willamette Valley vineyard. The vines were trained to a single wire hanging trellis, with shoots positioned downward, so that the fruit clusters were in the upper part of the canopy, exposed to direct sunlight for most of the growing season. Using a cork borer, two 7 mm diameter disks of grape skin were cut from sun-exposed positions on each cluster and two disks from the inward facing surfaces of shaded berries on the same cluster (twenty disks for each exposure) (sampling details are described in Chapter 3). Levels

of solar radiation at exposed epidermal surfaces were essentially equal to ambient levels above the grape canopy throughout the growing season, whereas the intensity of solar radiation reaching the surface of interior berries decreased through the season as the berries enlarged and berry to berry shading increased. The disks consisted of endo- and hypodermal tissues (Pratt 1972) and all pulp was wiped from the inner surface with tissue. Skin disks were extracted in 1 mL of acidulated ethanol (9:1, 95% ethanol:KCl/HCl pH1 buffer) heated to 70°C for 5 min then kept at 0°C. After 12 h the disks were removed and discarded. Extracts were stored at -80°C.

Extracts were analyzed for total phenols by the method of Singleton (1988). Anthocyanins were determined by absorbance at 520 nm at pH 1 using an extinction coefficient of 38,000 (Singleton 1982). Quercetin glycoside concentration was determined on a Hewlett-Packard 1050 series HPLC with a model 1040 series II diode array detector and HP Chemstation 3D software (Hewlett-Packard Inc, Palo Alto, CA) with a 250 x 4.6 mm polystyrene/divinylbenzene reversed phase column (PLRP-S 100Å 5$\mu$m, Polymer Labs, Amherst, MA). An isocratic solvent system was used with 1.5% phosphoric acid, 19.7% acetonitrile, and 78.8% distilled, deionized water. The flow rate was 1 mL $min^{-1}$. Two main quercetin glycoside peaks eluted at 11.5 and 12.2 minutes. Peak areas at 360 nm were compared to known amounts of quercetrin standard (Sigma). Extracts were not concentrated to avoid artifactual flavonol aglycone peaks in the chromatograms.

A complete chromatographic analysis of each treatment was run on a pooled sample of each treatment (50 $\mu$L from each extract). The same HPLC, detector, and

column were used with a gradient elution. Solvent A was distilled, deionized water with 1.5% (v/v) phosphoric acid. Solvent B was acetonitrile with 1.5% (v/v) phosphoric acid. Gradient conditions were: 0 min, A 95%, B 5%; 85 min, A 78%, B 22%; 88 to 95 min, A 50%, B 50%; 100 min, A 95%, B 5%. To maintain consistent retention times and stable baselines, a blank was run at the start of each day, the column temperature was held at 25°C, and the column was reequilibrated to the starting solvent conditions for 55 min between runs. Ethanol extracts were diluted 50% with distilled, deionized water prior to injection to reduce the effects of the solvent front. Full spectral scans were taken every 1.6 sec at 4 nm intervals to develop a three dimensional database (retention time x wavelength x absorbance). Peaks were identified by spectral comparison to published spectra and retention times (Markham 1982, Macheix et al. 1990, Mazza and Miniati 1993, Hong and Wrolstad 1990) and by retention time and spectral comparisons to known standards (rutin, quercetrin, quercetin, kaempferol, myricetin, gallic acid, caffeic acid, catechin, epicatechin from Sigma; isoquercetin from Roth; caftaric acid kindly provided by V. Singleton).

**Whole Cluster Study.** Clusters were harvested from a 1 ha section of a commercial block of Pinot noir grapes on September 16, 1992. The vineyard was 8 years old and was cane pruned and trained to a single wire hanging trellis system. Clusters were selected in three categories based on their position in the canopy and exposure to sunlight at harvest. "Exposed" clusters were on the top of the canopy in positions with little or no leaf shading. "Moderately exposed" clusters were in more protected canopy

positions but most received at least some direct sun exposure during part of the day. "Shaded" clusters were in interior canopy positions with no direct sun exposure and very little exposure to the sky. Most clusters in the block fit the "moderate" exposure conditions but often all three categories were found on the same vine. Five replicates of 200 clusters each were collected for each category of cluster exposure in a completely random fashion. No more than three clusters of any one category were harvested from any one vine. Twenty five clusters of each lot were frozen at -20°C for skin extract analysis and the remainder was made into wine.

The 25 cluster sample was frozen overnight at -80°C to facilitate separating the berries from the rachis and pedicils. Loose, frozen berries were poured through a seed sampling device (model H-3985, Humbolt Mfg. Co., Norridge, IL) to randomly divide the sample. One eighth of each sample (approximately 300 berries) was counted and weighed then thawed and the skins separated from the pulp and seeds. Skins were homogenized in a blender with 300 mL of acidulated ethanol for 3 min then heated in a water bath to 70°C. Extracts were filtered through Whatman #1 paper and brought to 500 mL with acidulated ethanol. Extracts were stored at -20°C and quercetin quantified as described above.

Clusters for wine (approximately 22 kg per lot) were crushed and de-stemmed with 35 mg $L^{-1}$ $SO_2$ added after crushing. Must was inoculated with 0.13 g $L^{-1}$ Wadensvil 27 yeast (Lalvin). Wines were fermented for 12 days on the skins and were punched down twice daily. Fermentation temperatures ranged from 20° to 30°C. Wines were pressed at 0° Brix and inoculated with 0.053 g $L^{-1}$ OSU *Leuconostoc oenos*

(Lalvin). After completion of malolactic fermentation, new wines were racked and 25 mg $L^{-1}$ $SO_2$ added. Wines were cold stabilized at 5°C for 30 days, and filtered once with Seitz 200 filter pads prior to bottling at 12 months of age.

The must was sampled after crushing, before the addition of $SO_2$. Wines were sampled at pressing and at 4 months, after cold stabilization.

Must was analyzed for °Brix, titratable acidity, and pH by the usual methods and malic acid content was determined enzymatically. New wine, sampled at pressing, was analyzed for total phenols and anthocyanins as described for the disk extracts. At four months, wines were analyzed by HPLC as described for the pooled skin disk extracts. Wines were not diluted and were passed through a 0.4 $\mu$m filter prior to injection. Individual wines from each replicate were run as well as a pooled sample of each exposure. Quercetin glycoside content was quantified by peak area comparison to known amounts of a quercetrin standard.

## Results and Discussion

**Skin Disk Study.** Analysis of the ethanol extracts of exposed and shaded skin disks is shown in Table 4.1. Skin exposure greatly increased total phenolic levels but had no statistically significant effect on anthocyanins. The concentration of quercetin glycosides was ten fold higher in sun-exposed than in shaded skin. The level of

Table 4.1. Total phenolic, anthocyanin, and quercetin glycoside levels in ethanol extracts of 20 skin disks from exposed and shaded positions on sun exposed clusters. Concentrations are expressed per unit skin area.

| | Skin Disk Position | |
|---|---|---|
| | **Shaded** | **Exposed** |
| Total Phenols ($\mu$g gallic acid mm$^{-2}$) | 2.53 | 4.70 ***[1] |
| Anthocyanins ($\mu$g mm$^{-2}$) | 3.09 | 3.43 ns |
| Quercetin ($\mu$g mm$^{-2}$) | 0.14 | 1.46 *** |

[1] *** and ns = two tailed T-test significant at p = 0.001 and not significant respectively.

quercetin in shaded skin was low compared to anthocyanins or total phenolics, however in sun-exposed skin they were 40% of the anthocyanin levels and obviously accounted for much of the difference in total phenolics between sun-exposed and shaded skin. Sun exposure had very little effect on the amount of the five anthocyanin glucosides present in Pinot noir (Fig. 4.1). The level of malvidin (peak 5) in the sun-exposed extract was slightly higher than shaded levels but peonidin (peak 4) was slightly higher in the extract of shaded skin. The total 520 nm peak areas were 935 and 976 mAU sec for the shaded and sun-exposed samples respectively, a difference of less than 5%.

The higher flavonol levels in the sun-exposed skin are clearly visible in the 360 nm chromatograms, the wavelength of maximum absorbance for flavonols (Fig. 4.2). Eight flavonol glycosides were identified on the basis of spectral characteristics, and

all of them were higher in sun-exposed skin. The two most prominent compounds were

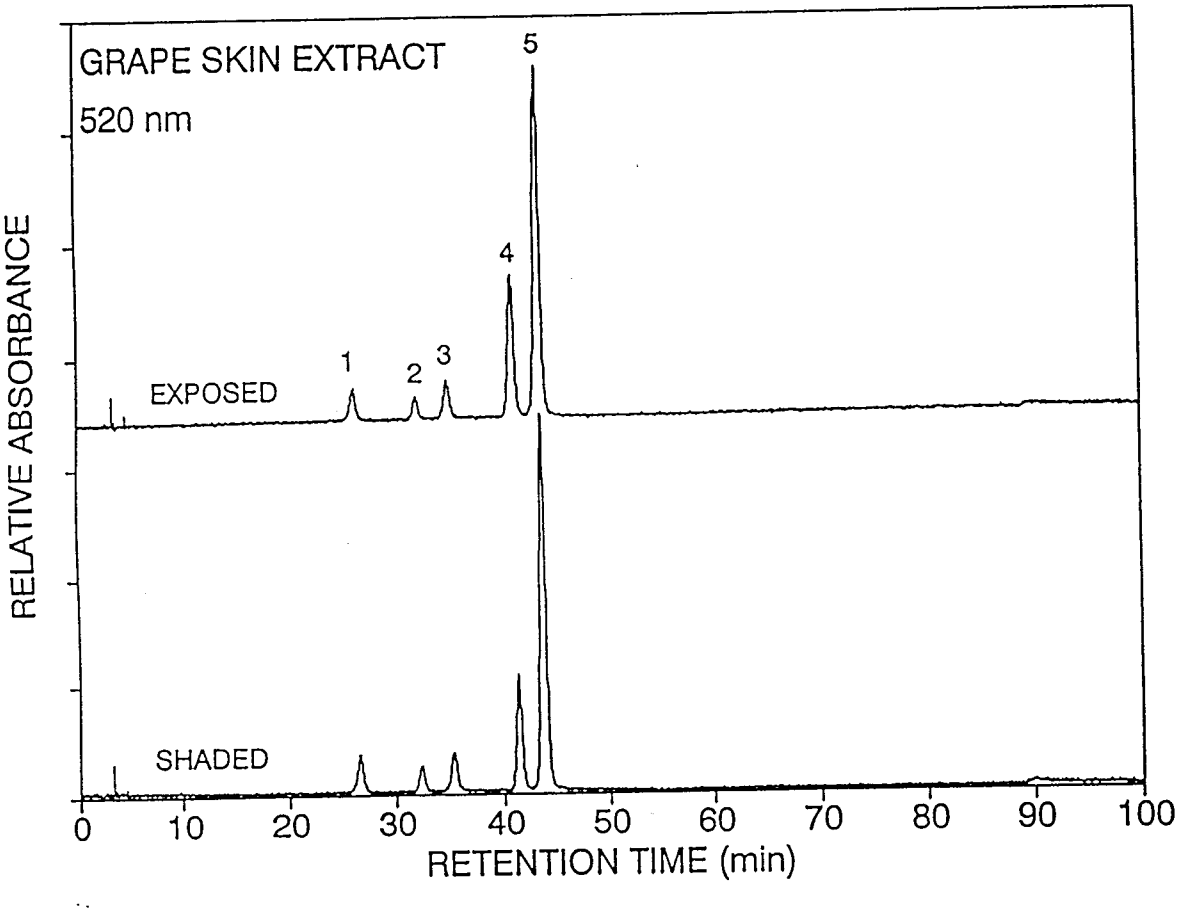


Figure 4.1. HPLC chromatograms, at 520 nm, of grape skin disk extracts from sun-exposed and shaded positions from sun-exposed Pinot noir clusters. All peaks shown are anthocyanin glucosides. Peak 1 = delphinidin, 2 = cyanidin, 3 = petunidin, 4 = peonidin, and 5 = malvidin.

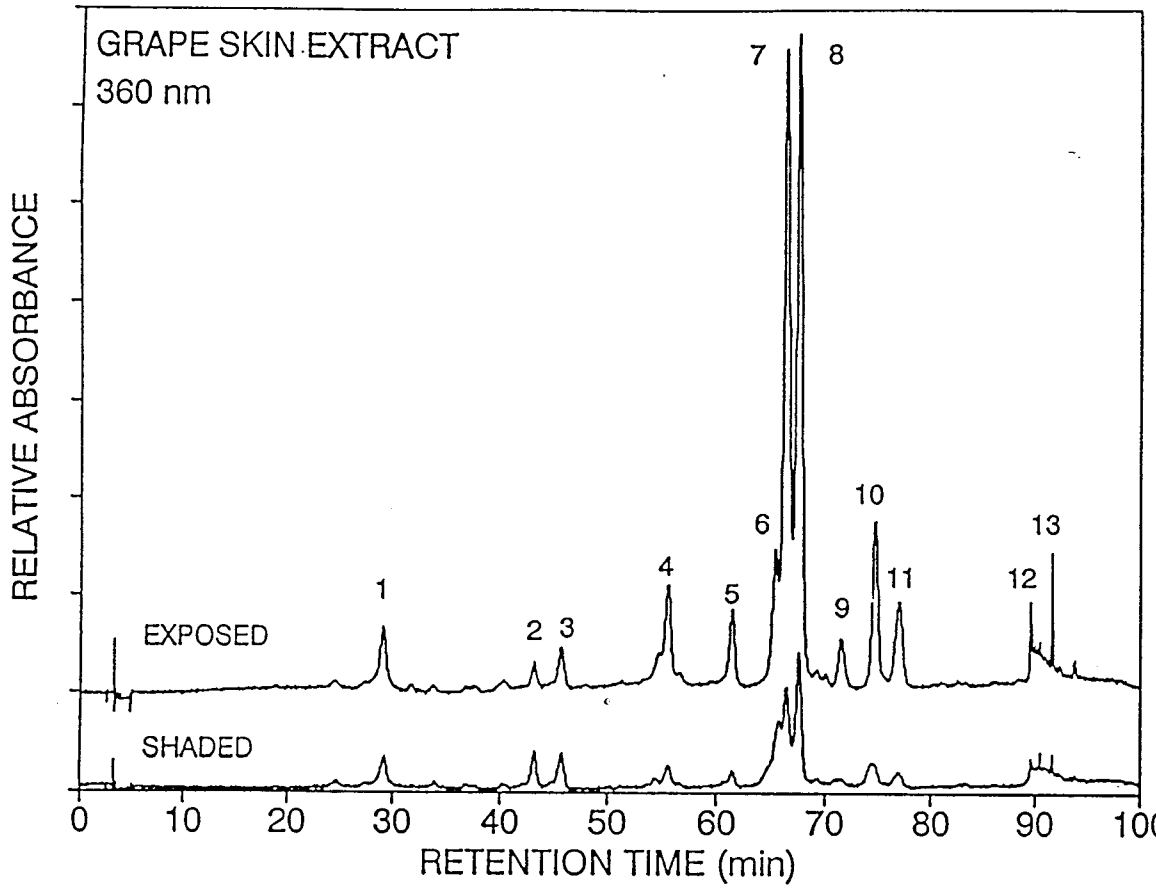


Figure 4.2. HPLC chromatograms, at 360 nm, of grape skin disk extracts from sun-exposed and shaded positions from sun-exposed Pinot noir clusters. Peak 1 = caftaric acid, 2 = peonidin glucoside, 3 = malvidin glucoside, 4 and 5 = unidentified flavonols, 6 = unidentified quercetin glycoside, 7 = quercetin glucoside, 8 = unidentified quercetin glycoside, 9, 10, and 11 = unidentified flavonol glycosides, 12 = unknown, 13 = resveratrol.

quercetin glycosides (peaks 7 and 8, Fig. 4.2). Peak 7 has been identified as quercetin glucoside; peak 8 is an unknown glycoside, possibly a glucuronide (Cheynier and Rigaud 1986). No flavonol aglycones were detected in the skin extracts. Caftaric acid (peak 1) and resveratrol (peak 13) also responded to sun exposure, with peak areas in the sun exposed skin extract at least twice that of the shaded. Total peak area at 360 nm in the exposed samples was 890 mAU sec compared to 92 mAU sec in the shaded.

Flavonols have been found to accumulate in sun-exposed tissue of Chardonnay (Chapter 3). In Pinot gris and Gewurztraminer an increase in sun exposure resulted in an increase in flavonols and a decrease in anthocyanin levels in skin disks (Price et al. 1992). Pre-veraison cluster exposure was also found to result in flavonol accumulation in Pinot gris skins in an experiment using aluminum foil covers to regulate the timing of cluster sun exposure (Appendix 1).

The accumulation of flavonols is thought to be a screening response, intended to protect the plant from UV radiation damage. It is possible that the reduction in sunburn accompanying early season cluster exposure from leaf removal or shoot positioning is the consequence of an early accumulation of flavonols.

**Whole Cluster Study**. There were distinct morphological differences between clusters from the different exposure categories (Table 4.2). Exposed and shaded clusters were smaller than those from moderate exposures, shaded clusters had fewer berries than more exposed clusters, and berry weight decreased as cluster exposure increased. The lower weight of shaded clusters was primarily due to fewer berries, whereas the smaller

size of exposed clusters resulted from smaller berries. The smaller average berry weight of exposed clusters was similar to the results Crippen and Morrison (1986) found in a similar cluster exposure study with Cabernet Sauvignon.

Table 4.2. Pinot noir cluster and berry measurements from three cluster exposure levels. All clusters are from the same vineyard and were selected on the basis of their sun exposure.

| | Cluster Exposure | | |
|---|---|---|---|
| | **Exposed** | **Moderate** | **Shaded** |
| Cluster weight (g) | 120.7 a[1] | 133.7 b | 115.2 a |
| Berries per cluster | 128.0 b | 124.1 b | 97.8 a |
| Berry weight (g) | 0.94 a | 1.08 b | 1.18 c |

[1] Means separated by Duncan's multiple range test ($p = 0.05$).

The °Brix of the must was significantly higher for exposed than less exposed clusters (Table 4.3). Titratable acidity was lowest in exposed clusters and highest in shaded clusters. Most of the differences in acidity could be attributed to differences in malic acid levels. Sun exposure did not seem to affect pH.

As expected, the concentration of anthocyanins in new wines was greatly affected by cluster exposure. Wines from exposed clusters had a 60% higher anthocyanin concentration than those from shaded clusters, and 14% more than wines from the moderately exposed clusters (Table 4.3). Most of the difference in wine

anthocyanins between the exposed and moderate exposure treatments was probably caused by the difference in berry weights (also a 14% difference) and a concomitant

Table 4.3. Analysis of Pinot noir musts and new wines from three cluster exposure levels. Must was sampled at crushing and new wine after pressing.

| | Cluster Exposure | | |
|---|---|---|---|
| | **Exposed** | **Moderate** | **Shaded** |
| **Must** | | | |
| °Brix | 25.3 b[1] | 24.5 a | 24.1 a |
| Titratable Acidity (g $L^{-1}$) | 6.37 a | 7.19 b | 8.63 c |
| pH | 3.22 | 3.19 | 3.18 |
| Malic Acid (g $L^{-1}$) | 2.42 a | 3.11 b | 3.95 c |
| **New Wine** | | | |
| Anthocyanins (mg $L^{-1}$) | 384 c | 336 b | 239 a |
| Total Phenolics (mg $L^{-1}$ gallic acid) | 2361 | 1995 | 2072 |

[1] Means separated by Duncan's multiple range test ($p = 0.05$).

change in the juice to skin ratio. The low anthocyanin levels in wine from shaded clusters was partly due to large berries but most likely was also due to reduced synthesis and accumulation of anthocyanins in the shaded skin. Dookuslian (1990) recently found that maximum anthocyanin synthesis in greenhouse-grown Pinot noir occurred at relatively low light levels (less than 19% ambient). In the present study,

it would appear that light was not limiting anthocyanin accumulation in either the exposed or moderate-exposed clusters but may have been in the shaded clusters. Light intensity in shaded clusters from interior canopy positions is well below 19% of ambient. (Chapter 3, Smart 1987). Despite the large differences in anthocyanin content cluster exposure did not have a significant effect on the levels of total phenols in new wine.

Quercetin glycoside levels in skin extracts from whole clusters showed a dramatic response to sun exposure (Fig. 4.3). The quercetin concentration in skin extracts from exposed clusters was 83% higher than moderately exposed and more than 385% higher than that from shaded clusters. Although large, the difference between the shaded and exposed clusters was less than the difference between the exposed and unexposed skin disks described above (Table 4.1). The whole cluster samples represent an average of berry skin exposures found within the clusters. Based on the skin disk study, it seems likely that most of the quercetin in whole clusters originated from the exterior, outward facing berry surfaces of the clusters.

Quercetin concentrations in four-month old wines paralleled the levels in the cluster samples (Fig. 4.3). Quercetin glycoside content of wine was related to cluster exposure, with wine from sun-exposed clusters having the highest concentrations and those from shaded clusters the lowest. The quercetin concentration of all the replicate wines ranged from a low of 0.5 mg $L^{-1}$ in one of the wines from shaded clusters to 35.2 mg $L^{-1}$ in a wine from sun-exposed clusters. This range, from clusters in a single vineyard block, made with similar wine making practices, is equal to the range of

quercetin levels reported for all red wines by Singleton (1988). Regression analysis, comparing grape and wine quercetin glycoside levels in all the replicates, showed an $R^2 = 0.932$.

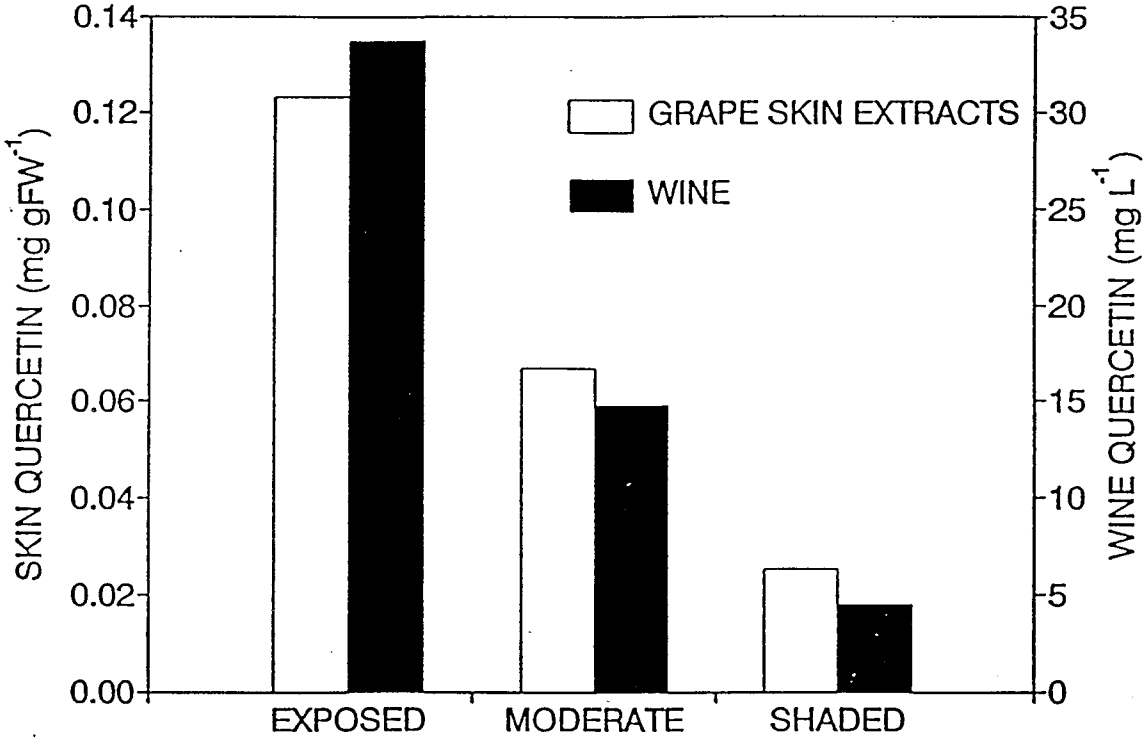


Figure 4.3. Quercetin concentrations of Pinot noir grape skins and wine from clusters from three different sun exposures. Differences in quercetin concentrations between cluster sun exposures in grape skins and wines were significant at $p = 0.01$.

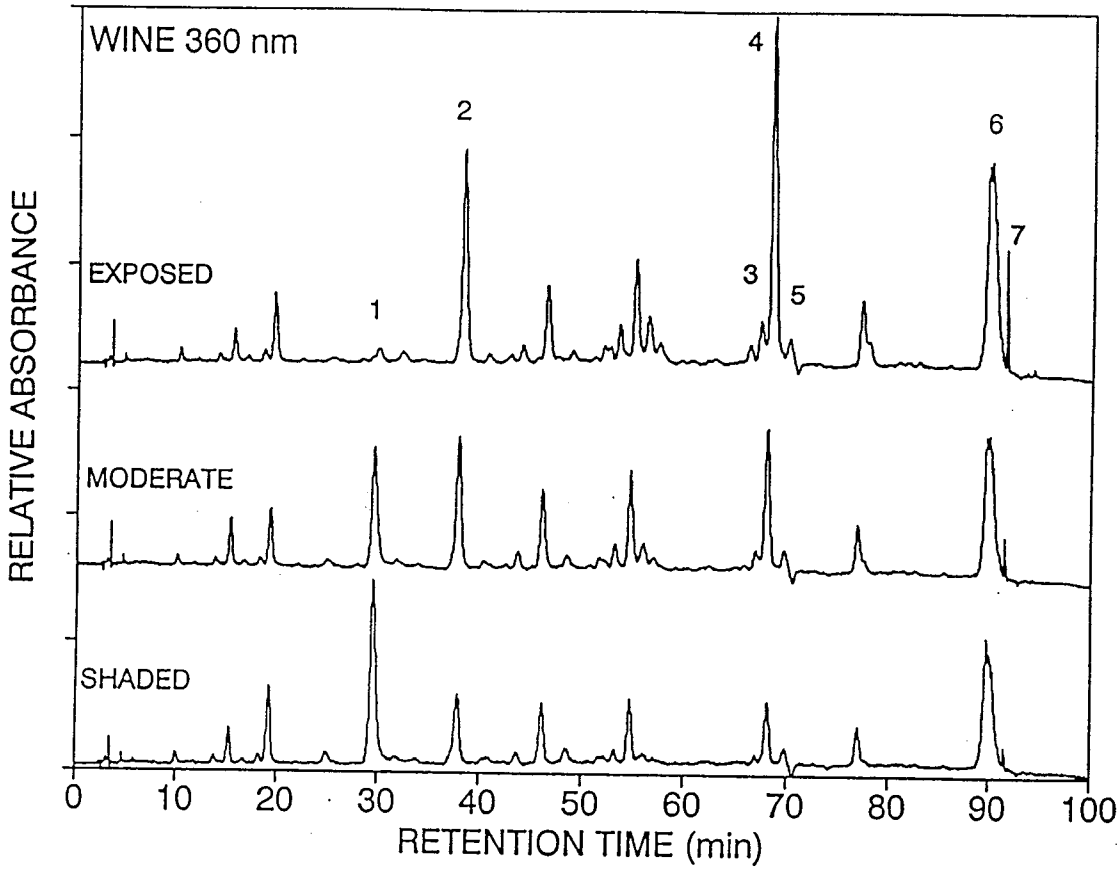


Figure 4.4. HPLC chromatograms, at 360 nm, of Pinot noir wines from three different cluster sun exposures. Peak 1 = caftaric acid, 2 = caffeic acid, 3 = quercetin glucoside, 4 = unidentified quercetin glycoside, 5 = unidentified quercetin glycoside, 6 = polymeric compounds, 7 = quercetin aglycone, 8 = keampferol aglycone.

The difference in quercetin content of wines from different cluster exposures is clearly apparent on the wine chromatograms at 360 nm (Fig. 4.4, peak 4). In addition, there was a distinct change in the flavonol profile from grape skins to wine (compare Figures 4.2 and 4.4). Wine chromatograms show only one prominent flavonol glycoside instead of two as well as the quercetin and kaempferol aglycones (Fig. 4.4 peaks 7 and 8). The myricetin aglycone was not detected using this method, presumably due to its co-elution with the large polymeric peak (Fig. 4.4, peak 6). The content of the quercetin aglycone in wine, like the glycoside, was strongly influenced by cluster exposure (Table 4.4).

The concentration of several other phenolic compounds in wine also appeared to be affected by grape cluster exposure. Caftaric acid and caffeic acid (Fig. 4.4, peaks 1 and 2, and Table 4.4) showed opposite, and probably related, responses. Caftaric acid was only detected in trace amounts in wines from sun-exposed clusters but it was the highest peak at 360 nm in wines from shaded clusters. In grape skins, however, caftaric acid was higher in exposed skin extracts than shaded (Fig. 4.2, peak 1). The higher levels of caftaric acid in wines from shaded clusters is possibly due to different rates of caftaric acid degradation in wines from the different treatments. Apparently, the tartaric acid moiety was hydrolyzed more rapidly in wines from exposed than shaded clusters, as evidenced by the higher concentration of caffeic acid in wines from exposed clusters. Caffeic acid was not detectable in grape skin extracts.

Catechin concentrations in wine were also influenced by cluster exposure, with the highest catechin levels in wines from shaded clusters and the lowest levels in those

Table 4.4. Analysis of chromatograms of Pinot noir wines made from three cluster exposure levels. Monomeric anthocyanins are the sum of peaks 1 through 5 in Figure 3. Polymeric anthocyanins are peaks 10 in Figure 3. Caftaric acid, caffeic acid, and quercetin aglycone are peaks 1, 2, and 7 respectively in Figure 4.4. Catechin and polymeric phenols are from 280 nm chromatograms (chromatograms not shown).

| | Cluster Exposure | | |
|---|---|---|---|
| **Peak Area (mAU sec)** | **Exposed** | **Moderate** | **Shaded** |
| Total (520 nm) | 3494 b[1] | 3394 b | 2264 a |
| Monomeric anthocyanins (520 nm) | 2560 b | 2704 b | 1701 a |
| Polymeric anthocyanins (520 nm) | 730 b | 517 a | 404 a |
| Caftaric acid (360 nm) | 0.4 a | 166.6 b | 259.4 c |
| Caffeic acid (360 nm) | 316 c | 203 b | 132 c |
| Quercetin aglycone (360 nm) | 26.4 b | 15.2 ab | 8.2 a |
| Catechin (280 nm) | 838 a | 1183 b | 1875 c |
| Polymeric phenolics (280 nm) | 6906 b | 5483 a | 4691 a |

[1] Means separated by Duncan's multiple range test ($p = 0.05$).

from exposed clusters (Table 4.4, chromatogram not shown). Catechin is an important wine constituent, particularly due to its involvement in polymerization reactions and as a substrate for polyphenoloxydase and browning reactions (Macheix et al. 1991). It is

generally thought to come from grape seeds and skins (Oszmianski et al. 1986). However we were unable to detect catechin in chromatograms of grapes skin extracts. Peak areas of epicatechin and two other unidentified compounds with similar spectral characteristics (possibly galocatechins) were also highest in wines from shaded clusters and lowest in wines from exposed clusters (data not shown). It is possible that most of the catechin present in these wines originated in the grape seeds. Seed catechin levels were not measured in this study, so it is not clear whether the higher catechin levels in wines from shaded clusters were caused by higher seed catechin levels in the shaded clusters or from changes in catechin concentrations that occurred during wine fermentation, maceration, and aging, as was apparently the case with caftaric acid. The second possibility seems most likely, particularly in light of the differences in polymeric compounds present in the wines (see below).

Anthocyanin profiles in wine, like the flavonol profiles, differed from those of the grape skin extracts (compare Fig. 4.1 and 4.5). In addition to the usual five anthocyanin glucosides there were at least four oligomeric anthocyanin peaks in wine and a large polymeric peak (Fig. 4.5). None of the oligo- or polymeric compounds have been positively identified. Our designation of peak 10 as "polymeric" is based on a higher 280nm:520nm ratio of the peak's spectra than that of peaks 1 through 5 or 6 through 9, the longer retention time on the reversed phase column, and the broad nature of the peak, suggesting a heterogenous chemical composition (Kantz and Singleton 1990).

Cluster exposure affected both total anthocyanin content (peak area) as well as the relative concentrations of the different anthocyanin groups (Table 4.4). Total anthocyanins were higher in the wines from exposed and moderately exposed clusters than from shaded clusters, as were total monomeric anthocyanins. Polymeric anthocyanins, however, were 40% higher in wines from exposed clusters than from moderate exposures and 80% higher than those from shaded clusters. Wine polymeric phenols (the same peak as peak 10 in Fig. 4.5 but measured at 280 nm) showed a similar response to cluster exposure.

It is possible, although by no means proven, that the higher quercetin levels in wines from sun-exposed clusters are related to the lower levels of catechin and caftaric acid found in these same wines. The formation of polymers involving flavonols, anthocyanins, and flavan-3-ols via oxidative polymerizations could explain the higher levels of polymeric phenols and anthocyanins as well as the lower catechin levels seen in wines from exposed clusters in this study. Flavonols are not good substrates for polyphenyloxydase (Macheix et al. 1990), but they could participate in subsequent coupled oxidations. Oxidation of caftaric acid has been shown to be affected by other phenolic compounds present in musts (Cheynier et al. 1989, Cheynier et al. 1988, Cheynier et al. 1990), however, these reactions are not usually accompanied by the appearance of caffeic acid, as was seen in this experiment. Appearance of free hydroxy cinamates in wine is thought to be related to enzymatic hydrolysis of the hydroxycinamoyl esters (Somers and Vérette 1988). Free caffeic acid in wine has also been reported under conditions of anaerobiosis (Macheix et al. 1990) but wines in these

experiments were made using standard fermentation techniques. Flavonol chemistry in wine has not been studied in depth, and these and other possible chemical interactions seem worthy of further study.

There has been very little research on the affects of quercetin on wine quality. Flavonols have been shown to have both bitter and astringent properties in alcohol solutions and in beer (Dadic and Belleau 1973), and preliminary tastings of the quercetin aglycone by our research group suggest that it could have detectable effects in wines. Flavonol effects on other chemical reactions such as oxidations and polymerizations also have the potential to have marked affects on wine quality. In an informal public tasting of the wines from this trial, the wine from the moderately exposed clusters was invariably preferred over the highly exposed, with the general impression being that the wines from exposed clusters were more harsh. However, it was not possible in this study to know if flavonols were responsible for this impression.

Flavonols effects on wine color have generally been discounted (Somers and Vérette 1988), however, a quercetin solution of 30 mg $L^{-1}$, equivalent to the concentration in a wine from exposed grapes, is visibly yellow with significant absorbance between 400 and 420 nm. In addition, flavonols can act as co-pigments with the potential to alter, enhance, and stabilize anthocyanins (Scheffeldt and Hrazdina 1979). If they are involved with polymeric color formation, as discussed above, they could affect long term color stability (Ribéreau-Gayon 1974) . Flavonol hazes have been reported in white wines (Somers and Ziemelis 1985), but the flavonol source was thought to be grape leaves from mechanical harvesting.

The HPLC techniques used in this study are worthy of some discussion. The choice of a polymeric column and acetonitrile as the organic solvent, and the addition of phosphoric acid to both the aqueous and organic solvent was based on our need for limited spectral interference from the mobile phase (Hong and Wrolstad 1990). Methanol and acetic acid, which are commonly used to separate phenolic compounds, both have significantly higher UV absorbance than acetonitrile, and silica based C-18 columns do not hold up well to the low pH of the solvents used in our separations. The long, slow gradient (5 to 22% acetonitrile over 85 min) was necessary to achieve separation of the flavonol glycosides. The method is an improvement on the method of Cheynier et al. (1986), in that it clearly separated six distinct flavonol glycosides and two aglycones and did not require pre-injection fractionations. The method of Cheynier does, however, provide additional information on glycosylation of flavonols, particularly the presence of glucurinides. It is possible in red grape cultivars with acylated anthocyanins that some co-elution of flavonols and anthocyanins could occur. We have used the method mostly on Pinot noir and Pinot gris, neither of which have any acylated pigments. The HPLC method worked well for both grape extracts and wine, although the ethanol content of extracts should not exceed 50% to avoid problems with the solvent front causing peak distortions. Our early attempts to transfer ethanol extracts to aqueous solutions via evaporation resulted in some hydrolysis of flavonol glycosides and the formation of insoluble precipitates of unknown origin. In addition to separating flavonols, chromatograms at 520, 320, and 280 nm allowed quantification

of anthocyanins, hydroxycinamates and their esters, and flavn-3-ols, and provided an estimate of polymeric phenolics.

## Conclusions

This is the first study that has shown that the sun exposure of grape clusters can influence the flavonol content of both grapes and wine. It is important, both for adding to the understanding of the causes of flavonol variation in wine and because it points the way to controlling flavonol levels in wine through vineyard practices. The sun exposure of grape clusters is easily subject to viticultural manipulation by a range of techniques including leaf removal, hedging, canopy division, and shoot positioning, and spacing as well as through controls on vine vigor using irrigation, fertilization, or the use of cover crops. The variation in quercetin levels within clusters is also important as it suggests that cluster morphology as well as cluster exposure could potentially affect wine flavonol content.

The current interest in quercetin as a healthful component of wine has led to an interest in increasing wine quercetin content. Increasing cluster exposure will probably have that effect, but this tool needs to be balanced with the understanding that very little is known about the quality effects of quercetin in wine.

# CHAPTER 5

## Measurement of incident light on grape clusters using photo-sensitive paper and image analysis.


### Abstract

Digital imaging and analysis was used to quantify and characterize the light exposure patterns of photo-sensitive paper tubes placed in representative cluster postions in two grape (*Vitis vinifera* L.) canopies: a minimally pruned and a vertically trained canopy. Blue pixel values of the captured video images had a strong negative correlation with the log of irradience from an integrating quantum sensor ($R^2$=0.9308). Histograms of incident light distribution on individual paper tubes were developed using imaging software. Histograms were able to quantify the spatial distribution of light on individual tubes and were clearly related to the tube's exposure in the canopy. Average population curves of pixel light distribution of twenty tubes in each canopy were able to differentiate the typical cluster light environment in the two canopies. Tubes in the minimally pruned canopy had a larger proportion of their surface exposed to irradiences greater than 50 $\mu$mol $s^{-1}$ $m^{-2}$ and 65% higher average irradience than the vertical canopy. Image analysis of photo-sensitive paper appears to be a workable method to record variation in spatial and temporal light in plant canopies.

## Introduction

The light environment of grape clusters can have pronounced effects on grape physiological processes and fruit and wine composition. We have been investigating the effects of sunlight on the phenolic composition of grape skin and have found that sun exposure greatly increases flavonol content (Price et al. 1992). Only grape skin directly exposed to full sunlight appeared to show this response. As a result, significant variation can exist in flavonol content between shaded and sun exposed sides of the same cluster, between clusters with different exposures on the same vine, and between typical clusters from different canopy systems. As one of the first steps in developing a model to explain the variation in flavonol content of grapes, we needed a method to quantify and characterize the incident solar radiation on the cluster surface, both for individual clusters and for representative clusters in canopy management experiments.

Several approaches have been used to describe canopy effects on the light microclimate of grapes. The most common are descriptions of whole vine structural parameters affecting the light environment of clusters, such as leaf area indices, leaf layer number, and number of shoots or pruning weights per unit length of canopy (Smart 1985). Point quadrat analysis has been used to describe several parameters relating to cluster exposure, including canopy gaps, leaf layer number, and percent fruit exposure (Smart 1982, Reynolds and Wardle 1989a). Fisheye photography has been used to describe light transmission through canopies (Reynolds and Wardle 1989a) and measures of sunflecks used to indicate light penetration into a canopy (Smart 1988). While all of these methods can be used to estimate changes in the canopy light

environment on a whole plant level none of them describe the light environment of an individual cluster.

Light sensors of various types have been used to describe canopy effects on light quantity and quality on a whole plant level and also to measure incident light on clusters (Reynolds and Wardle 1989b). Smart (1988) used a spot quantum meter to describe the photosynthetic photon flux density incident on individual clusters. He found intensive sampling was required under sunny conditions due to variation due in sunflecks and the sensor angle. In order to get acceptible readings it was neccessary to avoid sun flecks or take readings on cloudy days. Because of these same factors we were unable to obtain repeatable light readings for individual clusters using a spot quantum sensor in a preliminary study.

Long term placement of multiple sensors in plant canopies can eliminate problems with sunfleck variation (Reifsnyder et al. 1971), but problems with sensor placement and the geometry of both the sensor and incident light can still be significant when these methods are used estimate light levels on plant surfaces. Gutschick et al. (1985) used large arrays of non-filtered photodiodes to measure light incident on individual leaves. Multiple sensors were attached directly to the leaves and histograms of irradience were used to describe variation of incident light on leaves with different angles and canopy positions. This system provides significantly more information than single sensor measurements, but equipment requirements are substantial.

In response to these problems we developed a system for describing the cluster light environment using photo-sensitive paper and computer image analysis. Photo-

sensitive ozilid papers have been used to quantify the light environment in numerous ecological studies (Emmingham and Waring 1973, Friend 1969). They were primarily used as a simple integrator in which stacks of paper were exposed to the sun and the number of layers that were colored were correlated to light meter readings. In our study, computer image analysis was used to describe the patterns of light exposure on individual sun exposed papers. This technique eliminates short term temporal variation in average readings. In addition, the captured images provide detailed information on the spatial distribution of light incident on individual clusters. The objective of this study was to determine if photo-sensitive paper and image analysis could describe differences in incident light on individual clusters and if this information could then be used to describe the "average" cluster light environment in canopy management studies.

## Materials and Methods

**Grape canopies.** Two twenty vine sections of 'Cabernet Sauvignon' grape canopies from a minimal pruning experiment were selected on the basis of apparent differences in cluster exposure: a vertical canopy with shoots positioned upward and a minimally pruned canopy with no shoot positioning (Clingleffer 1984). The vertical canopy was cane pruned annually. The minimally pruned canopy had no dormant pruning for three years resulting in short shoots with clusters located toward the outside of the canopy. Reduced shoot growth and exterior cluster positions are typical vine responses to minimal pruning (Clingleffer 1984). The vertically trained vines were vigorous, with

long shoots and clusters generally located in the lower interior of the canopy. The row orientation in the plot was north-south.

**Photo-sensitive paper.** Sheets of photo-sensitive paper were from Sunprint Kit (Lawrence Hall of Science, University of California, Berkeley, CA 94720), an educational science toy. When exposed to sunlight the paper becomes blue.

**Calibration.** Twenty nine 50 x 50 mm sheets of photo-sensitive paper were exposed to varying light intensities under full sun, cloud cover, and in various plant canopies for 5 min. Light exposure levels for calibration were deliberately selected to obtain a complete range from full sun to deep shade. Light levels were recorded on an integrating quantum sensor (LICOR Li-188B, LICOR Inc., Licoln, NE 68504). The paper and sensor were placed adjacent to each other on a rigid board to insure that the angle of light interception was the same for both. Exposed papers were developed in running water for 2 min and air dried for 4 days before image capture and analysis.

**Cluster Exposure Analysis.** Forty 100 x 100 mm sheets of photo-sensitive paper were taped into tube shapes in a darkroom under a safe light with a wratten series OA safe light filter and kept in a dark box until placed in a canopy. Twenty paper tubes were placed in each canopy type at selected cluster positions. Each cluster in the canopy was assigned a number and a random number generator was used to select 20 cluster positions for analysis. Paper tubes were placed near the selected clusters in a similar

light environment and orientation (grape clusters are roughly cylindrical in shape). The paper tubes were held in place with an alligator clip on a flexible wire. Tubes were put out in pairs, one in each canopy, at 1 min intervals during the course of the experiment. The experiment was conducted under clear skies at mid-day in early September. After a 5 min exposure tubes were placed in a dark bag and later opened, flattened and developed and dried as described above.

**Image capture and analysis.** The images of the exposed sheets were captured with a video camera on a light stand with two 60 watt incandescent bulbs. Lighting conditions and camera settings were kept constant for exposed sheets from the canopies and the calibration set. Video output was to an AT&T Truevision (TARGA-24) image capture, digitizing, and display adapter with 512 x 512 pixel resolution and 256 levels of intensity for each of the red, green, and blue pixel components (AT&T Electronic Photography and Imaging Center, Indianapolis, IN). The digital data from the captured image were analyzed using an in-house program that gives minimum, maximum, and average pixel intensity values for red, green, and blue for the entire image. The program also generated a histogram of the distribution of pixel intensity in the captured image for red, green, and blue. Data were transferred to a spreadsheet program for analysis.

Average values for blue pixel intensity for the entire image of each sheet in the calibration set and light readings from the quantum sensor were used to develop an equation to convert blue pixel intensity values to irradience in $\mu$mol $s^{-1}$ $m^{-2}$ (Fig 5.1).

Data for analysis of average light distribution for each canopy was smoothed prior to statistical analysis using a running mean of four values. Individual cluster histograms are shown without smoothing. Canopy numerical data was compared using a two-tailed paired T-test.

## Results and Discussion

**Photo-sensitive Paper.** The Sunprint paper used in this study is one of several products available for making permanent shadow images. The advantage of this type of paper is their ease of development. Exposed papers are fixed and developed in water. The ozilid paper used in earlier studies must be developed in a chamber with ammonia gas.

**Calibration.** The blue pixel value in the captured images had a strong negative correlation with the log of the incident irradience ($r = -0.965$) (Fig 5.1). A similar log relationship between paper sensitivity and light was found in other studies of photo-sensitive paper (Emmingham and Waring 1973, Friend 1969). Red, green, total intensity, and various mathematical combinations of blue, red, green, and total pixel intensity gave significantly poorer correlations (data not shown).

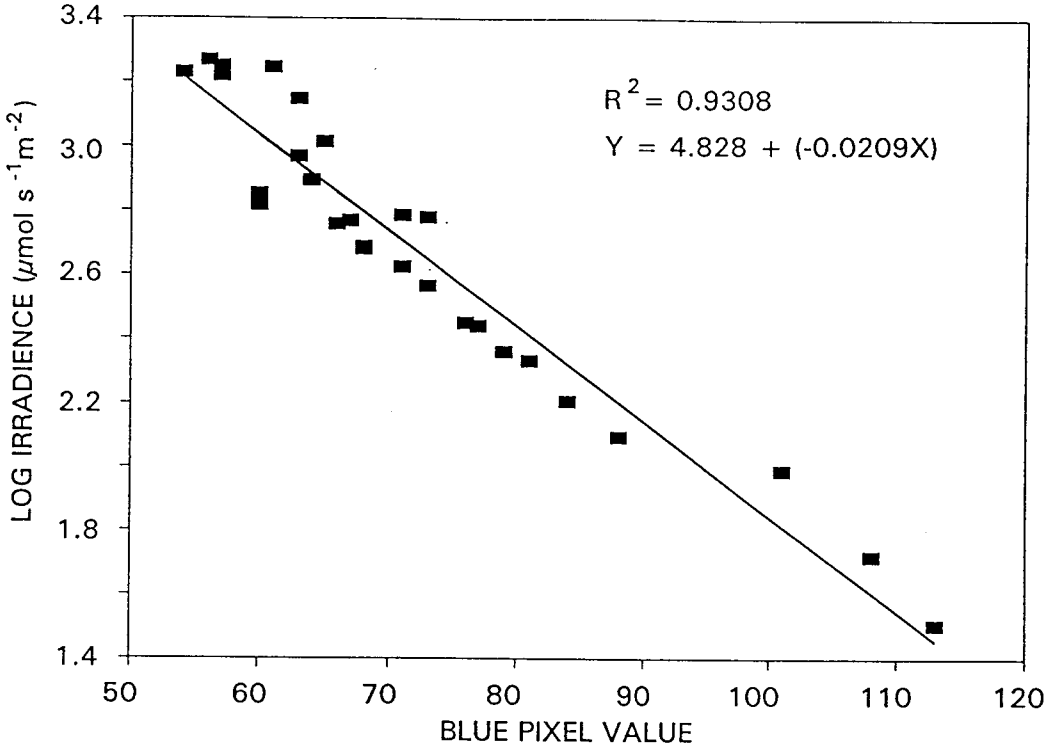


Figure 5.1. Calibration curve for Sunprint photo-sensitive paper. Light levels were recorded with a cosine corrected quantum meter.

To find a full range of light levels for this calibration we used a variety of different plant canopies. In retrospect, it may have been better to use only grape canopies for the shaded readings in the calibration set. Canopies of plant species have been reported to have differing effects on light quality and on the calibration curve for ozalid paper, particularly in low, diffuse light (Federer and Tanner 1966). The narrow spectral response range of photo-chemical methods of measuring light levels have been a subject of criticism (Pearcy 1989). However, the calibration curve in Figure 5.1 appeared linear over the wide range of light conditions used in the calibration set. The wavelength response range for the photo-sensitive paper used in this study was not available from the manufacturer but ozilid papers used in other studies were most responsive in the violet and ultraviolet wavelengths (Emingham and Waring 1973). Tests of Sunprint paper in this study with a Wratten #12 filter suggest that paper was not affected by wavelengths greater than 500nm (data not shown). Sensitivity to shorter wavelengths may be an advantage for our work with flavonols since blue and UV light have been shown to increase flavonol synthesis in plants (Stafford 1990, Brodenfeldt and Mohr 1988). Where a specific spectral response is desired, calibration with a spectral radiometer might be more appropriate.

The lighting conditions during video image capture were particularly important. The best resolution across the full range of exposure conditions was achieved when the light levels during the video capture were adjusted to maximize the visible differences in the darkest papers (most sun exposed). It was essential that the light set up for video

capture was identical for the calibration set and the papers exposed in the grape canopies.

**Single cluster analysis**. A unique aspect of this method is that the variation in incident light on individual clusters can be described in significant detail. A selection of exposed papers from the canopy experiment is shown in Figure 5.2. Exposed papers from both canopies developed a range of patterns. Papers from shaded cluster positions appear white whereas those from highly exposed positions have a dark band from the most exposed side of the tube. The effects of sunflecks are readily apparent in many of the papers as dark spots. Three exposed papers from very different cluster positions are presented in Figure 5.3 to illustrate the information available from individual exposures. Paper A was in one of the most exposed positions near the top of the minimally pruned canopy. It has a dark, sun exposed band that faced the sky and a white area that faced the interior of the canopy. Paper B was from a moderately exposed position from the upright trellis. It was exposed to indirect sky light with one prominent dark spot from a sunfleck. Paper C was from a shaded position in the upright trellis with no direct sunlight and very little indirect light.

The distinct distribution pattern of blue pixels in the three exposures is illustrated in the histograms (Fig 5.4A, B, and C). The letter designation for each histogram corresponds to the photo in Figure 5.3. The x axis of each histogram is in log scale to keep the histogram bin spacings equal (blue pixel value is related to the log of irradience) and to more clearly differentiate the light distribution at low light levels

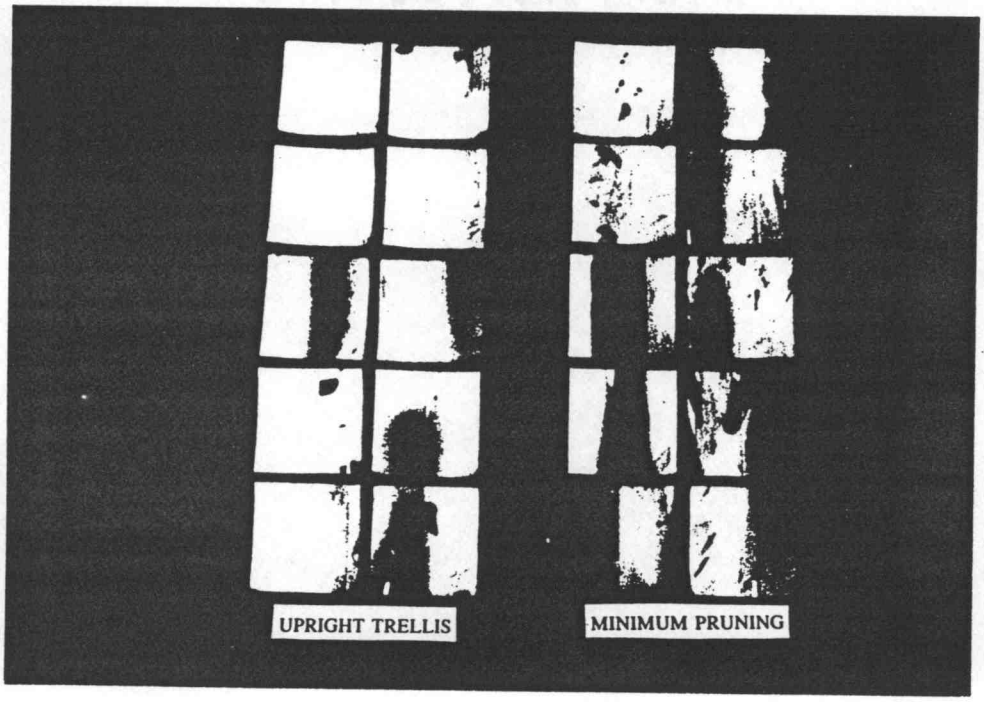


Figure 5.2. Photo-sensitive papers from two grape canopies: an upright vertical canopy with clusters at the base of the canopy and a minimally pruned canopy with clusters on the periphery of the canopy. Papers were rolled into a cylindrical shape and placed in random cluster locations. Darker areas are more sun exposed. Dark spots are from sun flecks.

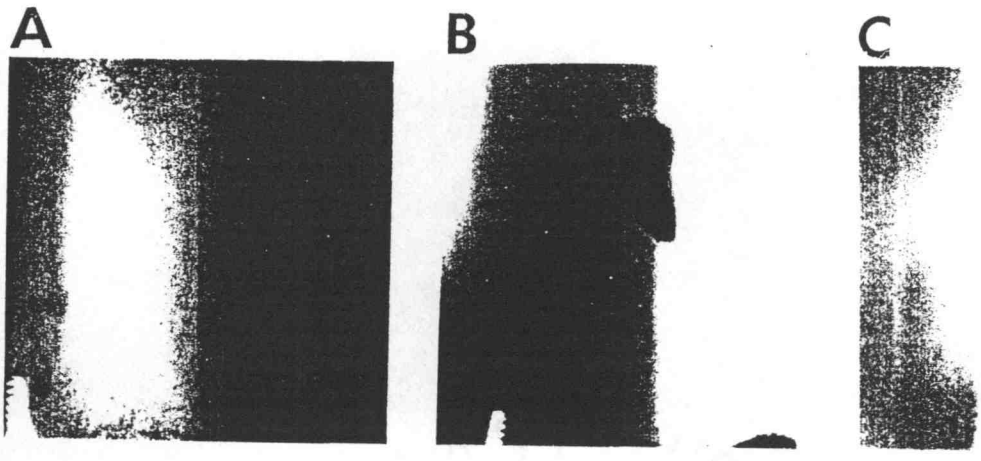


Figure 5.3. Photo-sensitive papers representing three cluster light exposure situations. A: a well exposed cluster on the top of a canopy, B: a moderately exposed cluster with sunflecks, and C: a shaded cluster. Faint leaf shadows are visible in the dark section of A and dark spots resulting from sunflecks are clearly visible in B.

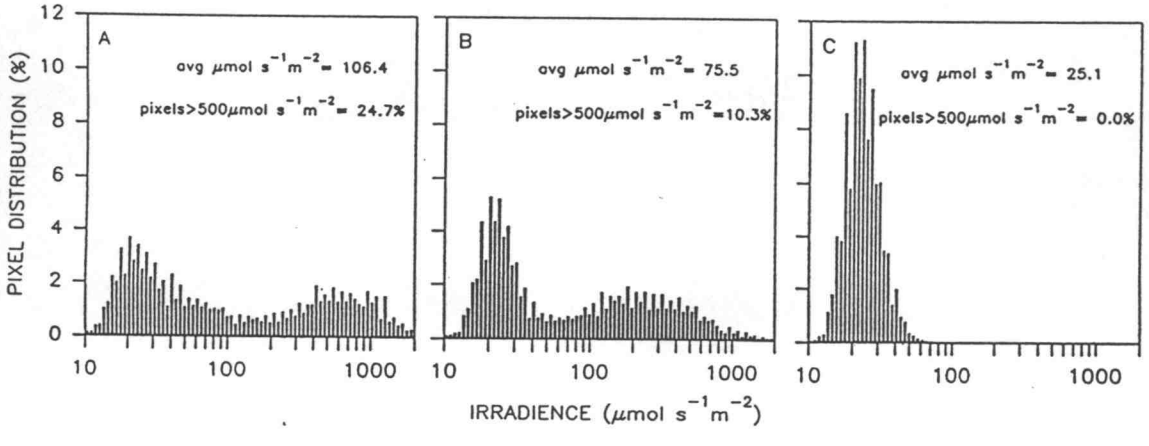


Figure 5.4. Histograms of the distribution of pixel light values in three photo-sensitive paper tubes from three cluster light exposure situations. A: a well exposed cluster on the top of a canopy, B: a moderately exposed cluster with sunflecks, and C: a shaded cluster. The height of each point is the percent of the total pixels at that light level. The area of each curve is the same and is equal to 100%.

(less than 100 $\mu$mol $s^{-1}$ $m^{-2}$). The height of each bar in the histogram is equal to the percent of pixels found at that light intensity level, and the total area of each histogram is the same and equal to 100%. Average light levels for the entire image are shown at the top of each histogram as well as the percent of pixels with a light value greater than 500 $\mu$mol $s^{-1}$ $m^{-2}$.

Paper C, from the shaded position, clearly was exposed to the lowest overall light level and received no light greater than 100 $\mu$mol $s^{-1}$ $m^{-2}$. The other two papers are similar but paper A, from the top of the minimally pruned canopy, was exposed to more direct sunlight (greater than 500 $\mu$mol $s^{-1}$ $m^{-2}$) than paper B from the upright canopy. Paper A was also exposed to the highest average light levels.

**Canopy Differences.** A population curve of pixel distribution was assembled from histograms of the twenty paper tubes exposed in each canopy (Fig 5.5). The standard errors at the top of the figure are for the smoothed data. Tubes from the minimal pruning canopy had a greater proportion of their surface area exposed to light levels above 50 $\mu$mol $s^{-1}$ $m^{-2}$ (Fig 5.5). Average irradience incident on typical cluster positions in the minimally pruned canopy was 65% higher than the level of the upright canopy (Table 5.1). Tubes from the minimally pruned canopy had significantly greater percentage of their area exposed to light values greater than 50 and 100 $\mu$mol $s^{-1}$ $m^{-2}$ than the upright canopy but not at levels greater than 500 $\mu$mol $s^{-1}$ $m^{-2}$ ($p=0.077$)(Table 5.1).

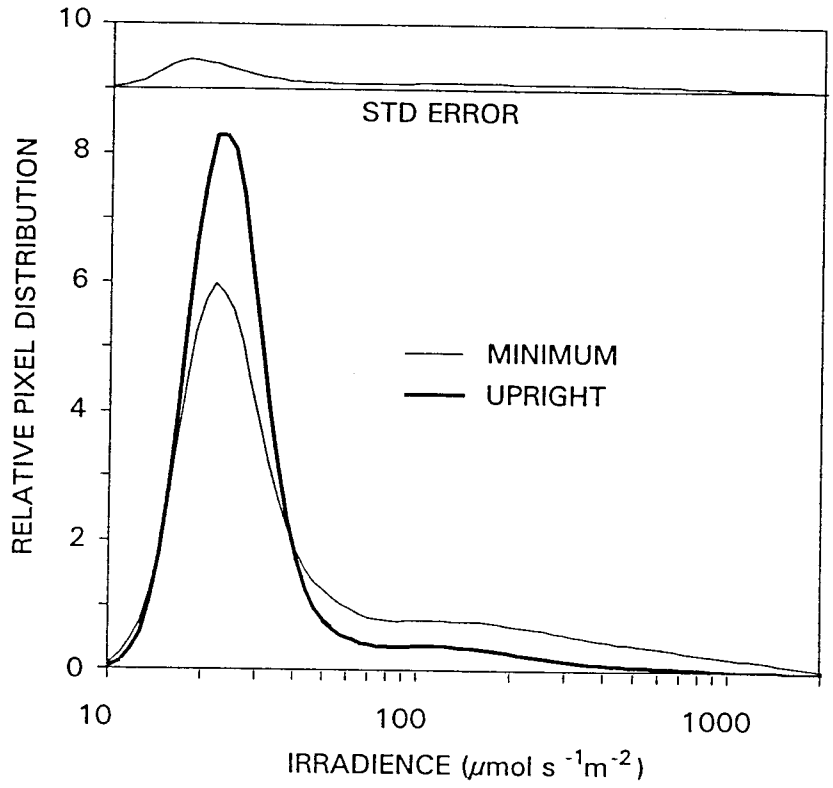


Figure 5.5. Population distribution of pixel light values in photo-sensitive paper tubes from typical cluster positions from two grape canopies: an upright vertical canopy with clusters at the base of the canopy and a minimally pruned canopy with clusters on the periphery of the canopy. The area of each curve is the same. Data was smoothed with a four number running mean. The width of the standard error curve corresponds to the standard error at each point on the plot.

These results agree with visual observation of the two canopies. The canopies used for this study were deliberately chosen for their apparent large differences in cluster exposure. The timing of the study (mid-day) was also chosen to maximize cluster sun exposure differences. Clusters in an upright vertical canopy with a north-south row orientation receive almost no direct sunlight near solar noon because of shading from the upper part of the canopy. The differences would have been less pronounced in early morning or late afternoon. Placing tubes in the same cluster positions morning, noon, and afternoon, as well as at different times during the season could be used to describe season long effects on cluster sun exposure. Larger tubes of photo-sensitive paper could be placed over a cluster rather than near the cluster, as was done in this study.

Table 5.1. Average irradience and distribution of irradience in photo-sensitive paper tubes from typical cluster positions from two grape canopies: an upright vertical canopy with clusters at the base of the canopy and a minimally pruned canopy with clusters on the periphery of the canopy. P values were derived from a two-tailed paired T-test.

| | Canopy | | |
|---|---|---|---|
| | Upright | Minimal | P Value |
| Average irradience ($\mu$mol $s^{-1}$ $m^{-2}$) | 26.7 | 44.2 | 0.025 |
| Pixel distribution of irradience (%) | | | |
| >50 $\mu$mol $s^{-1}$ $m^{-2}$ | 11.1 | 27.7 | 0.011 |
| >100 $\mu$mol $s^{-1}$ $m^{-2}$ | 6.2 | 18.3 | 0.018 |
| >500 $\mu$mol $s^{-1}$ $m^{-2}$ | 0.8 | 4.1 | 0.077 |

## Conclusion

Equipment for digital analysis of video images is becoming more available to researchers in the plant sciences. The power of this procedure, coupled with the detailed images recorded on photosensitive paper make this an attractive technique for analysis of light environments. The cylinders used in this study were a useful approximation of the light absorbing surface of a grape cluster. The information on the spatial distribution of light would have been difficult to obtain from other light recording instruments. We are continuing to evaluate the system as a method of predicting flavonol responses in grape skins, but it would appear that the general concept could be easily applied to a wide range of light studies, particularly where the form of the paper can be easily fitted to the measured surface, such as in studies on leaves. The problem of the spectral response of photo-sensitive papers may be a significant concern, particularly in photosynthesis studies, but careful calibration may eliminate much of that problem.

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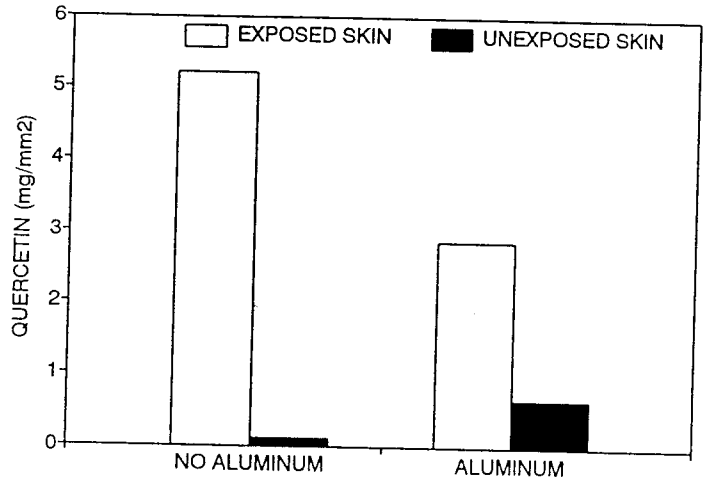
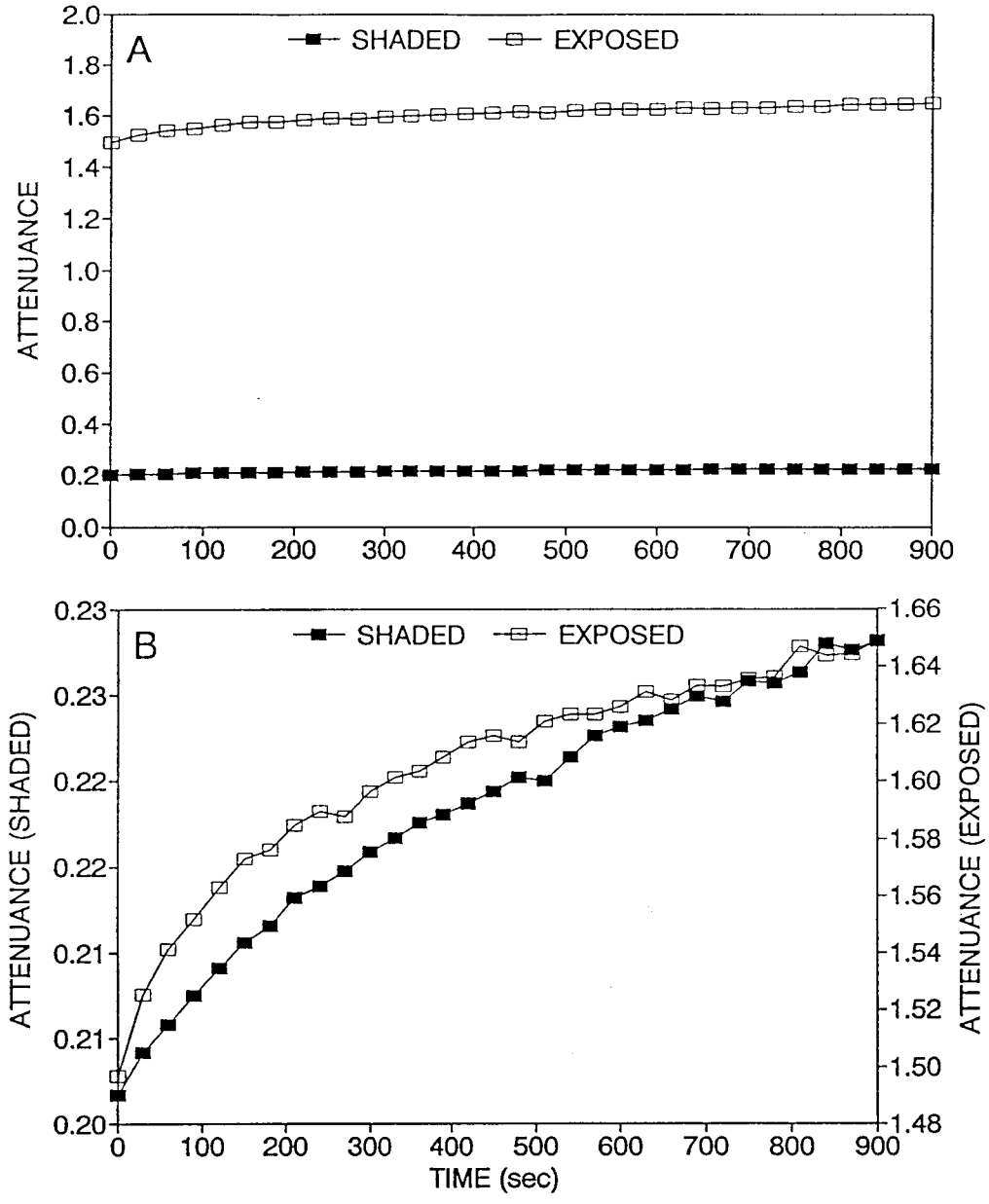
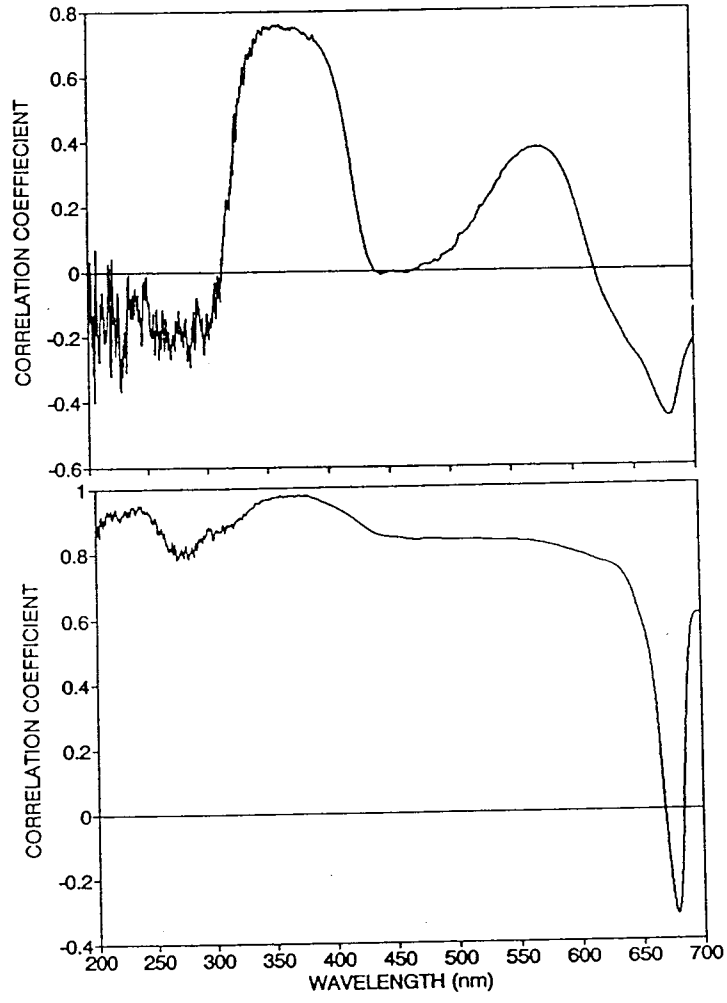
# APPENDICES




Appendix 1. Quercetin concentration in disks of sun-exposed and unexposed Pinot gris berry epidermal tissue. Sun-exposed clusters were either covered with aluminum foil or not, just prior to veraison. Disks were taken at harvest maturity from the exposed and unexposed (shaded) sides of the cluster. Differences between exposed skin with and without aluminum covers and differences between exposed and unexposed skin were highly significant ($p = 0.01$). Differences between unexposed disks with and with aluminum covering are not significant at $p = 0.05$.




Appendix 2. Browning kinetics of sun-exposed and shaded Chardonnay grape epidermal tissue in a spectrophotometer. A is each plot in the same scale and B is each plot in full scale.




Appendix 3. Plots of the correlation coefficient between quercetin concentration and in vivo epidermal absorbance from 200 to 700 nm for Pinot noir (upper) and Chardonnay (lower). The strongest correlation in both plots is at 360 nm, the absorbance maximum for quercetin. The positive correlation in Pinot noir at 575 nm suggests that quercetin may be affecting in vivo absorbance of anthocyanins via a co-pigmentation effect. The negative correlation at 675 nm with both cultivars may be due to a reduction in chlorophyl content in highly exposed, high quercetin tissue.