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Studies of fruit color development in red seedless table grape Reliance (Vitis hybrid)

Gao, Yu, Ph.D.
The Ohio State University, 1993
STUDIES OF FRUIT COLOR DEVELOPMENT IN
RED SEEDLESS TABLE GRAPE RELIANCE (Vitis hybrid)

Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

by

Yu Gao, B.S., M.S.

* * * *

The Ohio State University

1993

Dissertation Committee:                              Approved by
G.A. Cahoon                                          Advisor
M.L. Evans                                           Department of Horticulture
D.C. Ferree
A.R. Miller
To My Wife, Xiaodong
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VITA

May 8, 1964 ................................................. Born in Taiyuan, Shanxi, P.R. China.

July, 1985 ....................................................... B.S. Shanxi Agricultural University, Shanxi, Taigu, P.R. China.

December, 1985 to March, 1988, ........... Shanxi Exchange Fellow, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio

March, 1988 ................................................. M.S. The Ohio State University, Columbus, Ohio.

April, 1988 to Present ................................. Graduate Research Associate, Ohio Agricultural Research and Development Center, Wooster, Ohio.

Publications


Fields of Studies

Major Field of Study: Horticulture

Studies in Viticulture, Dr. G.A. Cahoon

Studies in Physiology of Fruit Crops, Dr. D.C. Ferree

Studies in Plant Physiology, Dr. M.L. Evans

Studies in Plant Secondary Chemistry, Dr. A.R. Miller
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INTRODUCTION

RELIANCE GRAPES (*Vitis* hybrid):

Reliance grape (*Vitis* hybrid) was released by Dr. James Moore from the University of Arkansas (Moore, 1983). Its fruit is round and red at maturity. Berries are medium in size and have vestigial seeds. Berry skins are very tender and flesh is melting in nature. It has a sweet and pleasing taste rated as outstanding with a delicate labrusca aroma (Moore, 1983). Furthermore, Reliance grapevines are vigorous with exceptional winter hardiness. It is the most promising red seedless cultivar from the table grape cultivar testing program at The Ohio State University (Cahoon, 1983). Reliance grape provides a very interesting system for the study of fruit color development, due to its great color variation at maturity, poor color development when overcropped, and importance in table grape production. In Ohio, Reliance fruit color at maturity, can range from light red, to brilliant red, to deep dark red, to purplish red. Preliminary work conducted in 1989-1990 showed that crop control by cluster thinning in Reliance grapes, increased red color and fruit cluster shading decreased red color (Gao and Cahoon, 1991).
FRUIT COLOR IN PIGMENTED TABLE GRAPES:

Grapes intended for use as fresh fruit, either for food or decorative purposes, are commonly designated as table grapes (Winkler, 1974). They must be attractive and be of high eating quality. Appearance factors include berry size, berry shape, berry color, compactness of cluster, and physical conditions. Eating quality factors include aroma, taste, texture of skin and pulp, and presence or absence of seeds.

Berry color is one of the most important quality attributes of table grapes. Three major color classes are white, red and black. The uniformity and brilliance of the color are more important than its intensity (Winkler, 1974). Grape color is more or less a varietal characteristic, but the color’s shade, uniformity, and brilliance are much influenced by climatic and cultural practices (Winkler, 1974).

PHYSIOLOGICAL PROCESSES DURING GRAPE DEVELOPMENT AND MATURATION:

Grape development and maturation is a very complex process. The physiological and biochemical processes during development and maturation of grape berries were investigated by Hrazdina et al. (1981) in a great detail. Fruit soluble solids (°Brix), pH, sugars, and anthocyanins showed a sharp rise during and immediately after veraison. Veraison is defined as a developmental stage when the
onset of berry color occurs. There is a concomitant rise in the activity of anthocyanin biosynthetic enzymes. The majority of metabolic events in the berries appear to come to a steady state approximately eight weeks after veraison under the climatic conditions of the northeastern United States.

ENVIRONMENTAL FACTORS AND FRUIT COLOR:

Environmental factors such as light and temperature have a significant effect on fruit color. Light has been found to be important for fruit color development in many grape cultivars (Kliewer, 1970a; Kliewer, 1977; Roubelakis-Angelakis and Kliewer, 1986; Crippen and Morrison, 1986a; Dokoozlian and Kliewer, 1992). Low light (500 to 1200 foot-candles) greatly reduced coloration of 'Pinot noir' grapes (Kliewer, 1970a). Anthocyanin content was significantly less in berries that received 15% sun light than 54% or 100% sunlight (Kliewer, 1977). Anthocyanin concentration increased rapidly after a phase of slow accumulation of 24 to 60 hours, depending on the temperature, in the presence of light but not in the dark (Roubelakis-Angelakis and Kliewer, 1986). Cluster shading was shown to reduce total anthocyanin content in the berries of several pigmented grape cultivars (Kakaota et al., 1984; Crippen and Morrison, 1986a; Rojas-Lara and Morrison, 1989; Gao and Cahoon, 1991; Gao and Cahoon, 1992). A significant reduction of anthocyanin concentration in shaded berries in comparison with berries fully exposed to sunlight was reported (Rojas-Lara and Morrison, 1989). Total anthocyanin concentration in
berries was decreased by 95% shading significantly in comparison with 55% shading or full sun (Gao and Cahoon, 1992).

Cool day and night temperatures within certain range have been reported to be favorable for red color development in pigmented grapes. Fruits ripened under cool day (15°C) and cool night (15°C) temperatures had a much greater coloration than fruits ripened at hot day (35°C) and cool night (15°C), hot day (35°C) and warm night (25°C), or cool day (15°C) and warm night (25°C) temperatures (Kliewer, 1977). Low day temperature (68°F) significantly increased the level of anthocyanins in berries over high day temperature (86°F) at both high light and low light intensity (Kliewer, 1970a). Winkler (1974) stated that cool regions or cool seasons produce grapes with more intense red color yet this darker color is not necessarily more brilliant or attractive.

CULTURAL PRACTICES:

Various culture practices such as cluster thinning, partial defoliation, growth regulators, have shown significant effects on fruit color improvement in grapes. However, high level of nitrogen fertilization showed an adverse effect on fruit color development.
Fruit cluster thinning has been shown to improve pigmentation in pigmented grapes (Hepner and Bravdo, 1985; Reynolds, 1989). Cluster thinning increases the ratio of leaf area to fruit cluster weight so that more carbohydrates can be allocated to fruit growth, development, and anthocyanin accumulation. More leaves to support the remaining clusters, more carbohydrate is produced in these leaves and the more carbohydrates are available for fruit growth, development, and coloration. However, excessive cluster thinning might alter source and sink relationship in grapevines so significantly that an excessive shoot growth is promoted and berry pigmentation is inhibited due to a competition for carbohydrates. Maintenance of consistent production of quality wine grapes with excellent color in low vigor De Chaunac vines growing on marginal sites cannot be achieved unless the crop level is reduced to 7 kg fruit per kg of pruning woods (Reynolds, 1989).

Partial defoliation (Koblet, 1987; Koblet, 1988; Marquis et al., 1989), was used in a search for effective vineyard practices to improve fruit color. A more recent study on the effects of partial defoliation on berry skin color showed that anthocyanin content tended to be higher following partial defoliation and tended to be higher the later the partial defoliation, resulting in the highest concentration with the partial defoliation at veraison (Hunter et al. 1991). Furthermore, partial defoliation increased lateral shoot length and number of lateral shoots without significant effect on total cane mass (Hunter and Visser, 1990a). A 33% defoliation prior to pea size stage and 66% defoliation treatment prior to veraison decreased fresh mass per berry
and yield at harvest (Hunter and Visser, 1990b). Partial defoliation increased photosynthetic activity of leaves by improving light conditions within grape canopy (Candolfi and Koblet, 1991). Furthermore, partial defoliation increased sugar levels in berry skin without marked effect on berry composition and volume (Hunter et al., 1991).

Growth regulators such as abscisic acid (ABA) and ethephon (an ethylene releasing compound) were shown to enhance fruit coloration in grapes. ABA at 1000 ppm applied at the beginning of fruit color development (veraison) increased anthocyanin concentration without affecting soluble solids or titratable acidity in Kyoho grapes (Kataoka et al., 1982). Foliar ethephon application (1000 ppm) at or after veraison enhanced anthocyanin formation of red *Vitis vinifera* table grapes (Weaver and Montgomery, 1974) while ethephon application only to clusters was ineffective.

High levels of nitrogen fertilization (2.12 g N/25-liter pot) reduced coloration of berries in comparison with low levels (0 to 1.70 g N/25-liter pot) of nitrogen fertilization (Kliewer, 1977). This reduced fruit coloration was attributed mainly to the diversion of photosynthate from carbohydrate accumulation, which is critical for anthocyanin biosynthesis, to amino acid, protein synthesis and storage (Kliewer, 1977).
ANTHOCYANIN:

Pigments responsible for the attractive red, blue, purple, or black color in grapes, are anthocyanins, a class of water-soluble flavonoid pigments (Ribereau-Gayon, 1982; Van Buren et al.; 1970). The anthocyanins of grapes exist as acylated or free glycosides of cyanidin, peonidin, delphinidin, petunidin, and malvidin, with great variation among cultivars of Vitis species (Ribereau-Gayon, 1982). Anthocyanins in De Chaunc grapes are present in the vacuoles of fruit subepidermal cells in the berry skin (Moskowitz and Hrazdina, 1981). Furthermore, the anthocyanin profile of a grape cultivar can be very complex. High performance liquid chromatography (HPLC) analysis of anthocyanin profile in pigmented grapes has shown that a grape cultivar can have up to 20 different anthocyanins (Wulf and Nagel, 1976.; Hebrero et al., 1989).

ANTHOCYANIN BIOSYNTHETIC PATHWAY:

The core linear pathway of anthocyanin biosynthesis was described by Dooner et al (1991). The first committed step in flavonoid biosynthesis is the condensation of three molecules of malonyl CoA and one of p-coumaroyl CoA by the enzyme chalcone synthase (CHS) to produce a yellow chalcone (Dooner et al, 1991). The second step, the isomerization of the chalcone into a colorless flavanone, proceeds at a low rate, but is accelerated by the enzyme chalcone-flavanone isomerase (CHI).
The flavanone so formed is hydroxylated at the C₃ position by the action of flavanone 3 hydroxylase (F3H) to give a colorless dihydroflavonol that is reduced by dihydroflavonol 4-reductase (DFR) to yield a still colorless leucoanthocyanidin. The compound is then converted into a colored anthocyanidin in either one or two steps, catalyzed by yet undescribed enzymes (Heller and Forkmann, 1988). The last step shown, glycosylation of anthocyanidin to give an anthocyanin, is catalyzed by the enzyme UDPglucose flavonoid 3-oxy-glucosyltransferase (UF3GT) (Dooner et al. 1991).

The enzymology of the last steps in the biosynthetic pathway that are unique to anthocyanins is still unknown (Stafford, 1991) despite significant advances in understanding anthocyanin metabolism. Understanding of biosynthesis of individual anthocyanins in a plant system could provide more leads to how different anthocyanins are formed.

**PROBLEM AND RATIONALE:**

In a practical sense, effective cultural methods are needed for Reliance so that the maximum amount of fruit, with brilliant red color, can be produced consistently year after year. It is necessary to determine the optimum crop level so premium quality fruit with excellent color can be produced. Crop level can be adjusted by dormant or summer pruning, cluster thinning, flower thinning, or berry thinning
(Winkler. 1974). Shoot number can be determined by the numbers of buds retained after dormant pruning. Usually, shoots originating from buds on one year canes produce two to three clusters per shoot. Hence, dormant pruning by itself is not sufficient to control the crop level. Final cluster thinning is necessary to get the crop to the optimum level for the production of grapes of the best quality.

The optimum or critical leaf area to cluster weight ratios will also need to be determined on field grown Reliance vines. This information can help determine the optimum pruning severity and whether partial defoliation can be applied to improve canopy microclimate for better colored with decreasing vine vigor and winter hardiness.

It is also important to determine whether direct sunlight exposure on the fruit cluster is critical for color development in Reliance grapes. If direct light exposure on fruit cluster is critical, such cultural practices as leaf removal around the fruiting zone and/or shoot positioning will be necessary to expose fruit clusters to sunlight for best fruit color.

Detailed studies of individual anthocyanins in a given grape cultivar can lead to a much better understanding of anthocyanin metabolism. Further, only limited information is available on the effects of cultural practices or environmental factors on individual anthocyanins of a given grape cultivar. Understanding changes in
individual anthocyanins of a given cultivar may also provide leads to the mechanism by which synthesis of different anthocyanins is regulated. Studies on changes of individual anthocyanins in ripening Syrah grapes of different clones during ripening (Roggero and Ragonnet, 1986) and effects of cultivars and climate on individual anthocyanins during ripening (Cacho et al., 1992) have provided very enlightening results in the anthocyanin metabolism of whole grapevines.

HYPOTHESIS STATEMENTS:

1. Manipulation of the ratio of fruiting to vegetative tissue through cluster thinning or defoliation affects fruit color, total anthocyanin content, amount of individual anthocyanins, and relative percentages of individual anthocyanins to total anthocyanin content.

2. Fruit cluster light exposure is critical for fruit color development. Hence, shading of fruit clusters without shading leaves affects fruit color, anthocyanin contents, individual anthocyanins, and relative composition of individual anthocyanins.

3. Individual anthocyanins in Reliance grape contribute to fruit red visual color to different degrees.
OBJECTIVES:

This study will be conducted with the following objectives:

1. To separate, purify, and identify the anthocyanins in Reliance grapes. 

The anthocyanin profile of this cultivar needs to be identified first for the
detailed quantitative and qualitative comparisons of individual anthocyanins of
Reliance grapes.

2. To study mechanisms by which manipulation of the ratios of fruiting to
vegetative growth by crop control or defoliation, affects fruit color and the
biosynthesis of anthocyanins.

Crop control through cluster thinning decreases the ratio of fruiting to
vegetative growth ratio whereas defoliation increases the ratio. Total anthocyanin
content and the content of individual anthocyanins will be analyzed to determine how
crop control or defoliation affects the content and relative percentage of individual
anthocyanins in addition to total anthocyanin content.

3. To study the whether exposure of fruit to light is critical for color
development in Reliance grapes through cluster shading.

Total anthocyanin content, the content and percentage of individual
anthocyanins will be analyzed to determine how fruit cluster shading affects individual
anthocyanins in addition to total anthocyanin.

4. To study the relationship between leaf area to cluster weight ratios and
fruit color parameters including fruit red color, total anthocyanin content, and
the content of individual anthocyanins.
CHAPTER I

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF ANTHOCYANINS IN RELIANCE GRAPE (Vitis hybrid)

Abstract

The anthocyanin profile of Reliance grape, a red seedless table grape, was characterized with the joint use of high performance liquid chromatography (HPLC), paper chromatography (PC), thin layer chromatography (TLC), and spectral measurements. Purified anthocyanins were obtained from Reliance grapes by descending preparative PC using Whatman 3MM paper. Reliance anthocyanins were identified by 1) a systematic comparison with authentic anthocyanin and anthocyanidin standards by C18 reverse-phase HPLC, 2) determination of sugar residues using TLC after complete acid hydrolysis, and 3) spectral measurements. The anthocyanin profile of Reliance grape was shown to have seven anthocyanin peaks. The peaks 2, 3, and 5 were identified as delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside. Peaks 1, 4, 6, and 7 were tentatively identified as cyanidin-3,5-diglucoside, petunidin-3-glucoside, malvidin-3-glucoside, and an acylated cyanidin derivative, respectively.
Introduction

Reliance grape, a red seedless table grape cultivar, was released by Dr. James Moore from University of Arkansas (Moore, 1983). It has been one of the most promising red seedless table grape cultivars tested in Ohio from 1970 to present due to its excellent taste, delicate labrusca aroma, medium berry size, moderate disease resistance, and exceptional winter hardiness (Cahoon, 1983). However, its fruit color varies significantly at maturity when Reliance grapevines bear heavy crops. The fruit color development in Reliance needs to be investigated so that an effective cultural procedure to produce premium fresh market grapes can be established.

The pigments responsible for the attractive fruit colors including red, blue, purple, and black in grapes, are anthocyanins (Ribereau-Gayon, 1982). It has been demonstrated that numerous grape cultivars have complex anthocyan profiles (Hebrero et al, 1988; Roggero et al, 1986; Wulf and Nagel, 1976; Hrazdina, 1974). The detailed study of individual anthocyanins in pigmented grapes is critical for the understanding of fruit color development. Reliance grape provides a good system for the study of fruit color development due to its economical importance and great fruit color variation at maturity. There have been no reported studies on the anthocyanin profile of Reliance grapes. Elucidation of the pigment profile in Reliance is essential to the detailed studies of fruit color development and anthocyanin metabolism. This information can also help grape breeders study the heredity of anthocyanins in table
grapes. Furthermore, it can provide a good source of authentic pigments for future anthocyanin research, if Reliance grape has a relatively simple anthocyanin profile. Advances in C18 reverse-phase HPLC have made the separation and identification of complex anthocyanin mixtures from grapes and other red fruits possible (Goiffon et al, 1991; Hebrero et al, 1988; Hong and Wrolstad, 1990; Roggero et al, 1986; Wulf and Nagel, 1976).

The objectives of this study were to isolate, separate, and identify the anthocyanins in the red seedless grape Reliance, with the joint use of C18 reverse-phase HPLC, preparative PC, TLC, and spectral measurements.

Materials and Methods

Samples: Fruit samples used in the study were harvested on August 15, 1992, from Horticulture Unit II at Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio, U.S.A. They were immediately put into plastic bags and stored at -20°C for later analyses.

Extraction of anthocyanins: Frozen Reliance grape berries were thawed in the refrigerator at 4°C for 20-30 minutes to allow ease of berry skin removal. Grape berry skins were removed with tweezers. Berries and berry skins were kept in an ice chilled beaker to retard pigment decomposition. Berry skins were freeze dried and
ground in a coffee mill. Anthocyanins in the grape skins were then extracted with 1% of 12 N HCl in methanol overnight at 4°C in the dark.

Concentration and purification of anthocyanins: Anthocyanin extract was concentrated under vacuum at 30°C using a rotary evaporator (Buchi RE111 Rotavapor, Switzerland). Anthocyanin concentrate was applied to Maxiclean C$_{18}$ cartridges (Alltech Associates, Deerfield, Illinois, U.S.A). These cartridges were washed with distilled deionized water three times to remove sugars that are known to interfere with the separation of anthocyanins in PC, and eluted three times with 0.1% of 12 N HCl in methanol. This anthocyanin extract was concentrated with a rotary evaporator, streaked on Whatmann 3MM chromatography paper, developed sequentially in several solvent systems (Francis, 1982). The sequence of developing solvents was 1% HCl (12 N HCl-water, 3:97), BAW (butanol-glacial acetic acid-water 4:1:5, only upper layer of the mixture was used.), HoAc-HCl (water-glacial acetic acid-12 N HCl 81:15:3), BAW, and BAW. Chromatography papers were air dried after each development. Individual pigment bands were cut out, pooled, redissolved in 0.1% of 12 N HCl in methanol, concentrated, and reapplied to fresh paper. Three developing tanks were used. Four stripes of paper were run simultaneously per tank. This process was repeated at least five times so that sufficient purified anthocyanins were obtained. Purity of anthocyanins was checked with C$_{18}$ reverse-phase HPLC.
Identification of anthocyanins: Identification of anthocyanins from Reliance grape was carried out following a method by Francis (1982) with some modifications.

Positive identification of anthocyanin was first based on a comparison of retention times of purified Reliance anthocyanins with those of authentic anthocyanidin standards. C\textsubscript{18} reverse-phase HPLC was used instead of PC. Second, the anthocyanidins and sugar residues after complete acid hydrolysis were compared with authentic anthocyanidins and sugars. Retention times of Reliance anthocyanidins on C\textsubscript{18} reverse-phase HPLC column, instead of chromatographic mobility off PC, were compared to the retention times of anthocyanidin standards, obtained by complete acid hydrolysis of authentic anthocyanins. Sugar residues were identified by comparing their chromatographic mobility (R\textsubscript{f}x100) with that of authentic sugar standards.

Cellulose TLC was used instead of PC. Third, spectral characteristics in UV-Visible range of Reliance anthocyanins were compared with those of authentic anthocyanin standards. Controlled acid hydrolysis of Reliance anthocyanins was not performed because amount of purified anthocyanins obtained with preparative PC was not enough for such analysis. Purified pigments and authentic anthocyanin standards were concentrated to dryness, redissolved in 0.1 N HCl, and filtered through a 0.2 μm membrane filter for HPLC analyses.

Two anthocyanin components and their respective anthocyanidins from Reliance grapes were also identified independently with PC (Francis, 1982) as a confirmation. Dried anthocyanin components and authentic anthocyanin standards
were dissolved in 1% 12 N HCl in methanol. The dissolved anthocyanins were then spotted on Whatman 1 paper, and developed in BAW and BuHCl (n-butanol-2 N HCl, 1:1, upper layer). Anthocyanidins from Reliance grapes and authentic anthocyanidins were dissolved in 1% HCl in methanol and spotted on three Whatman 1 papers. Each spotted paper was then developed in one of the three different solvent systems including Formic acid (formic acid-12 N HCl-water, 5:2:3), Forestal (glacial acidic acid-12 N HCl-water, 30:3:10), and BAW. Anthocyanins and anthocyanidins from Reliance grapes were identified based on the chromatographic mobility (Rf x 100) of anthocyanin and anthocyanidin standards.

Complete acid hydrolysis: Complete acid hydrolysis of anthocyanins yielded anthocyanidins and sugar residues. Approximately 0.5 to 1 mg of purified pigments dissolved in 1 ml of 0.1% HCl in methanol with two ml of 2 N HCl added, were heated at 100°C in test tubes for 30 minutes. Anthocyanidins were extracted with 2 ml of amyl alcohol after mixtures were cooled to about 20°C, then dried with a rotary evaporator at 30°C under vacuum, redissolved in 0.1 N HCl, and filtered through a 0.2 μm membrane filter for HPLC analyses.

Sugar residue analysis. Determination of sugar residues was carried out following the method by Francis (1982) using cellulose TLC plates (Merk precoated cellulose plates, 0.1 mm in thickness, Darmstadt, Germany) instead of Whatman 1 cellulose PC. The chromatographic mobility (Rf x 100) of sugar residues was compared with
that of authentic sugar standards. The aqueous solution remaining, after removal of the anthocyanidins with amyl alcohol, contained the sugar residues. Successive washings with small portions of 10% di-n-octylmethylamine in chloroform removed acid. A final rinse with chloroform removed traces of amine. Sugar solutions were then dried. Two drops of water were added to the dried sugar residue to dissolve all the sugar. Sugar residues and authentic sugar standards were spotted on Merk cellulose TLC plates. TLC plates were developed in BBPW (n-butanol-benzene-pyridine-water) or Phenol (Phenol-water, 4:1). These plates were then air dried thoroughly. The dry chromatograms were sprayed with aniline-hydrogen phthalate reagent, dried, and heated at 105°C for 2-3 minutes. The sugar spots showed up as brown spots under visible light.

**Spectral data:** UV-visible spectra (250 to 650 nm) of Reliance anthocyanins and their respective anthocyanidins in 0.1% HCl in methanol were recorded with a Gilford Response Research Scanning Spectrophotometer (Ciba-Corning Analytical Corp, Medfield, Massachusetts, U.S.A). One drop of 1% AlCl₃ in ethanol was also added to 0.5 ml of anthocyanins and their respective anthocyanidins for the observation of a possible spectral shift.

**Alkaline hydrolysis:** If the UV-Visible spectrum of an anthocyanin shows a peak around 300-310 nm, alkaline hydrolysis should be performed to identify acyl groups. Approximately 0.5 mg of anthocyanins dissolved in 2 ml of methanol in a screw cap
vial were flushed with nitrogen, then 1 ml of 2 N NaOH was added (Francis, 1982). After again flushing with nitrogen, the mixture was allowed to stand at room temperature in the dark for two hours, acidified with 1 ml of 2 N HCl, and then filtered through a 0.2 μm membrane filter for HPLC analysis.

**High performance liquid chromatography:** HPLC analysis was performed on a Model SP4000 system (Spectra-Physics, San Jose, California, USA) equipped with a 20 μl Rehodyne sample loop. The analytical column was a pH stable RP-18 Spherisorb (E. Merck, Darmstadt, Germany) (150 mm x 4.6 mm I.D.) packed with 5 μm particles by Alltech (Deerfield, Illinois, U.S.A). A SP UV1000 variable wavelength detector and an SP 4600 integrator from Spectra-Physics were used.

The following conditions were used for the analyses of anthocyanins and anthocyanidins: Solvent A—10% formic acid in water; B—100% acetonitrile. These solvents were filtered through 0.20 μm filter and sparged with helium; solvent flow rate 1 ml/min. The solvent program used for anthocyanins was 95% A to 72% A following a linear curve over 20 minutes. The Solvent program for anthocyanidins was 95% A to 91% A over 5 minutes, to 89% A over 10 minutes, to 85% A over 5 minutes, to 75% A over 10 minutes (Hebrero et al., 1989). Detection was carried out at 520 nm for both anthocyanins and anthocyanidins.
**Authentic anthocyanin and anthocyanidin standards:** Cyanidin-3-glucoside, cyanidin-3,5-diglucoside, delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside were generously provided by Dr. Geza Hrazdina from Department of Food Science and Technology, Cornell University, New York State Agricultural Experiment Station, Geneva, New York, U.S.A. Cyanidin-3-glucoside and cyanidin-3,5-diglucoside were also isolated from Heritage raspberry fruits because of its simple anthocyanin profile (Francis, 1972). Delphinidin-3-glucoside was also isolated from mung bean hypocotyles because it was shown to be the major anthocyanin (Nozzolillo, 1972). Authentic anthocyanidin standards including delphinidin, cyanidin, petunidin, and malvidin, were obtained by the complete acid hydrolysis of authentic anthocyanin standards.

**Results and Discussion**

The HPLC chromatogram of crude anthocyanin extract from Reliance fruit skin determined at 520 nm shows seven peaks (Figure 1). It can be assumed that this is also the number of anthocyanins present in Reliance grapes since this is where Reliance anthocyanins have the maximum absorbance (Wulf and Nagel, 1976). The elution order of anthocyanins on C₁₈ reverse-phase column is based on their polarity where retention time of anthocyanins decreases as they become more polar (Goiffon et al., 1991; Hebrero et al 1988 and 1989; Wulf and Nagel, 1976). The polarity of anthocyanidins is directly related to the degree of hydroxylation and/or methylation of
B ring. The anthocyanins become more polar with more hydroxyl groups and less polar with more methyl groups on their B ring. In terms of monoglycosides of anthocyanidins, it can be predicated that their elution order was based on their respective anthocyanidins (Hebrero et al., 1989). In the case of diglycosides or triglycosides of anthocyanidins, it can also be predicted that their elution order was based on the nature of anthocyanidins. However, it should not be assumed that monoglycoside of anthocyanidin always elutes later on reverse-phase HPLC than diglucoside. Grapes are known to include five classes of anthocyanidins such as delphinidin, cyanidin, petunidin, peonidin, and malvidin (Van Buren et al., 1970; Ribereau-Gayon, 1982; Hebrero et al., 1988 and 1989; Wulf and Nagel, 1976). Delphinidin elutes first on C18 reverse-phase column followed in order by cyanidin, petunidin, peonidin, and malvidin on reverse-phase HPLC (Hebrero et al., 1989).

Reliance anthocyanins were first matched with available authentic anthocyanin standards based on their retention times on a C18 reverse-phase column (Figures 2 and 3). The retention times of individual Reliance anthocyanin peaks shown in Figure 1 and authentic anthocyanin standards are listed in Table 1. Peaks 1, 2, 3, 4, and 6 have similar retention times as cyanidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside, respectively. Peaks 5 and 7 could not be identified by retention times of their glycosides due to the lack of appropriate anthocyanin standards. Peak 5 was hypothesized to be peonidin-3-glucoside since peak 5 elutes before malvidin-3-glucoside and after petunidin-3-
Peonidin-3-glucoside is more polar than malvidin-3-glucoside and less polar than petunidin-3-glucoside. Peonidin-3-glucoside was shown to elute after petunidin-3-glucoside and before malvidin-3-glucoside from C₁₈ reverse-phase column with several solvent systems (Wulf and Nagel, 1978; Hebrero et al., 1988 and 1989; and Goiffon et al., 1991). Peak 7 elutes about six minutes after malvidin-3-glucoside. Peak 7 was predicted to be an acylated anthocyanin since only anthocyanidins or acylated anthocyanins are much less polar than monoglycoside of malvidin (Wulf and Nagel, 1978; Hebrero et al., 1989). Furthermore, no free anthocyanidins were found in pigmented grapes in vivo (Ribereau-Gayon, 1982; Stafford, 1990). In addition to the comparison of retention time, Reliance anthocyanin extract was also spiked with each available authentic anthocyanin standards individually. These spiking data (Table 2) further support the match between Reliance anthocyanins and authentic standards based on the increase in peak area of corresponding Reliance anthocyanins (Table 1). Anthocyanin peaks 2 and 3 shown in Figure 1 also had similar chromatographic mobility (Rₓ100) on PC chromatogram as delphinidin-3-glucoside, and cyanidin-3-glucoside, respectively (Table 3).

The complete hydrolysis of anthocyanin extract from Reliance grape reveal the classes of anthocyanidin present. There were five anthocyanidin peaks present (Figure 4). The retention times of authentic anthocyanidins including delphinidin, cyanidin, petunidin, and malvidin, were established first (Figure 4). Anthocyanidin peaks 1, 2, 3, and 5 had similar retention times as delphinidin, cyanidin, petunidin,
and malvidin, respectively. Peak 4 was predicted as peonidin based on its relative retention time as shown in Figures 4 and 5, and published anthocyanidin chromatogram using identical solvent system on C$_{18}$ reverse-phase column (Hebrero et al., 1989). Peonidin was shown to elute later than petunidin and earlier than malvidin on C$_{18}$ reverse-phase column (Hebrero et al., 1989). Retention times of Reliance anthocyanidins and authentic anthocyanidins are shown in Table 4. Spiking of Reliance anthocyanidin mixture with authentic anthocyanidins also supported the identification (Table 5). Based on these information, Reliance anthocyanidins were identified as delphinidin, cyanidin, petunidin, peonidin, and malvidin.

The anthocyanidins of anthocyanin peaks 2, 3, 5, and 7 as shown in Figure 1, after complete hydrolysis, are shown in Figure 5A to 5D. The anthocyanidin profile from Concord, determined at similar time as anthocyanidin data show in Figure 5A to 5D, is shown in Figure 5E. It should be noted that retention times of anthocyanidins in Figure 5 were different from retention times of anthocyanidins in Figure 4 because these runs were made at different time. C$_{18}$ reverse-phase could loose its some active sites at low pH. The pH of the solvent for HPLC analysis had a pH range of 1.5. This low pH was maintained because anthocyanins were all converted to flavylium cation at this low pH. The anthocyanidin profile of Concord grape was known to be delphinidin, cyanidin, petunidin, peonidin, and malvidin (Hrazdina, 1975). The solvent system used for HPLC analysis of anthocyanidins is identical to that used by Hebrero et al (1989). Hebrero et al (1989) showed that the elution order was
delphinidin, cyanidin, petunidin, peonidin, and malvidin. Anthocyanidin peaks 1 to 5 in Figure 5E were identified as delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively. Anthocyanidins of Reliance anthocyanin peaks 2, 3, 5, and 7 matched in retention times to those of Concord grape anthocyanidin peaks 1, 2, 4, and 2 respectively (Figure 5A to 5E). Hence, anthocyanidin peaks 1 to 5 in Figure 6E were also delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively. The anthocyanidins of anthocyanin peaks 2 and 3 shown in Figure 1 had similar retention times as delphinidin and cyanidin, respectively (Figure 5A, 5B, and 5E). Anthocyanidins of anthocyanin peaks 5 and 7 shown in Figure 1 had similar retention times as peonidin and cyanidin, respectively (Figures 5C, 5D, and 5E). Anthocyanidins of anthocyanin peaks 1, 4, and 6 were not obtained because their low concentration did not make the isolation of these pigments possible with preparative PC. Furthermore, analysis of anthocyanidin mixture from Reliance grape supported the identification of anthocyanidins of anthocyanin peaks 2, 3, 5, and 7. In addition, PC analysis showed that anthocyanidins of anthocyanin peaks 2 and 3 were delphinidin and cyanidin, respectively (Table 6). Based on the information in Figures 5 and 6, Table 6, results by Hebrero et al. (1989) and Hrazdina (1975), anthocyanidins of anthocyanin peaks 2, 3, 5, and 7 shown in Figure 1, were identified as delphinidin, cyanidin, peonidin, and cyanidin derivatives, respectively.
The sugar residues of purified anthocyanins peak 2, 3, and 5 shown in Figure 1 were analyzed with cellulose TLC after complete acid hydrolysis (Table 7). The sugar residues of peaks 2, 3, and 5 were identified as glucose.

The spectra of purified Reliance pigments as peaks 2, 3, and 5, shown in Figure 1, are shown in Figure 6. Spectra of these purified anthocyanins in 0.1% of HCl in methanol helped determine whether these pigments were acylated or not based on the possible presence of an extra peak around 300-310 nm. A slight shoulder was observed on the spectra of peaks 2 shown in Figure 1 (Figure 6A). However, this shoulder was not significant enough in comparison to the kinds of shoulders that suggested presence of acyl groups (Harborne, 1958; Hebrero et al., 1989). Furthermore, the relative retention time of peak 2 did not suggest that peak 2 was acylated. No significant shoulders were observed on the spectra of peak 3 or 5 shown in Figure 1 (Figure 6). This indicates that peaks 3 or 5 were not acylated anthocyanins. The absorbance peaks of the spectra in UV-visible range, general shape of the spectra, $E_{\text{UV, max}}/E_{\text{Vis, max}}$, and $E_{440}/E_{\text{Vis, max}}$, also added more information to the positive identification of anthocyanins since the spectrum of each anthocyanin is characteristic. It is well known that delphinidin, or cyanidin, or petunidin derivatives exhibits a blue shift after an addition of AlCl$_3$ in ethanol to the anthocyanin or anthocyanidin where peonidin, or malvidin, or pelargonidin derivatives does not exhibit a blue shift (Harborne, 1958; Wulf and Nagel, 1978). These spectral characteristics of peaks 2, 3, and 5, and their matching anthocyanins are shown in
Table 8. The absorbance maximum in UV (UV<sub>max</sub>) and visible (Vis<sub>max</sub>) range, E<sub>440/460</sub>/E<sub>Vis, max</sub>, and spectral shift of peak 2, were identical to that of delphinidin-3-glucoside (Table 8). The UV<sub>max</sub> and Vis<sub>max</sub> range, E<sub>UV, max</sub>/E<sub>Vis, max</sub>, E<sub>440/460</sub>/E<sub>Vis, max</sub>, and spectral shift of peak 3, were identical to that of cyanidin-3-glucoside (Table 8). The absorbance Vis<sub>max</sub>, E<sub>440/460</sub>/E<sub>Vis, max</sub>, and spectral shift of peak 5, were identical to that of peonidin-3-glucoside (Figure 6C). Possible presence of impurities may have altered the spectrum of peak 5 in UV range (Figure 6C). The spectra of peaks 2, 3, and 5 further supported the positive identification of these anthocyanins. The spectrum of peak 7 was not available because of the difficulty in isolating that pigment with preparative PC. However, anthocyanin containing mainly peak 7 did not show a blue shift. This information further supported that the anthocyanidin of peak 7 was a cyanidin.

Based on the information available on the purified anthocyanins from Reliance grapes, peaks 2, 3, and 5 were positively identified (Table 9) as delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside, respectively. Identification of peaks 2 and 3 shown in Figure 1 were also confirmed by PC (Tables 3, 6, and 7). Peaks 1, and 4 were tentatively identified (Table 9) as cyanidin-3,5-diglucoside, and petunidin-3-glucoside based on their retention times. Peak 7 was tentatively identified as an acylated cyanidin derivative based its relative retention time. Identifications of individual peaks are shown in Table 2. The molecular structures of the postulated Reliance anthocyanins are shown in Figure 7.
Preparative PC was effective in separating the majority of anthocyanins in Reliance. However, difficulties in separating more complex and less concentrated anthocyanins with preparative PC are well known. Recent applications of HPLC and Diode Array Spectroscopy (HPLC-DAS) in anthocyanin identifications proved to be very efficient, effective, and much less time consuming. However, the cost of HPLC-DAS is still quite high for many researchers. A semi-preparative or preparative C18 reverse-phase column could also been used in purifying anthocyanins. However, these columns are still relatively expensive.

The pigments in Reliance grapes are mainly monoglucosides of delphinidin, cyanidin, and peonidin. This characteristic resembles that of *Vitis vinifera* more than *Vitis labrusca* because anthocyanins in *Vitis vinifera* grapes are well known to be only monoglycosides of anthocyanidins (Ribereau-Gayon, 1982; Van Buren et. al, 1970) whereas anthocyanins in *Vitis labrusca* grapes are a mixture of monoglycosides, diglycosides, and triglycosides. This is very significant for the breeding of table grapes since most of the characteristics in Reliance grape such as fruit aroma and taste, vine vigor, and winter hardiness resemble that of *Vitis labrusca*. A systematic study of anthocyanin components among progenies of grape crosses should shed more light on heredity of individual anthocyanin in grapes.

Reliance grape is a good source of cyanidin-3-glucoside, delphinidin-3-glucoside, and peonidin-3-glucoside due to its relatively simple anthocyanin
composition. It also provides a good system for the study of anthocyanin metabolism due to the great fruit color variation at maturity and relatively simple anthocyanin composition. A series of detailed studies on the changes in individual anthocyanins affected by cultural practices or fruit cluster shading in Reliance was conducted. Results are shown in following Chapters II to IV.

In conclusion, the anthocyanin profile of the red seedless table grape cultivar Reliance (*Vitis* hybrid) was identified mainly as delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside with combined use of C18 reverse-phase HPLC, preparative PC, TLC, and spectral measurements. One of the anthocyanin components was tentatively identified as an acylated cyanidin derivative based on the retention times of its glycoside and aglycone in C18 reverse-phase HPLC. Other three anthocyanin components were tentatively identified as cyanidin-3,5-diglucoside, petunidin-3-glucoside, and malvidin-3-glucoside based on the comparison of their retention times with those of authentic anthocyanins on C18 reverse-phase HPLC.
Figure 1. The anthocyanin profile of Reliance grape determined by C_{18} reverse-phase HPLC. Peak identifications were listed in Table 9.
Figure 2. The chromatograms of authentic anthocyanidin standards cyanidin-3,5-diglucoside (A), cyanidin-3-glucoside (B), petunidin-3-glucoside (C), malvidin-3-glucoside (D), and Reliance anthocyanin extract (E).
Absorbance at 520 nm
Figure 3. The chromatograms of authentic anthocyanin standard delphinidin-3-glucoside extracted from hypocotyle of mung bean seedlings (A) and Reliance anthocyanin extract (B).
Table 1. Retention times of Reliance anthocyanin peaks shown in Figure 1 and authentic anthocyanin standards shown in Figures 2 and 3 determined by C18 reverse-phase HPLC.

<table>
<thead>
<tr>
<th>Reliance Anthocyanin Peak Number</th>
<th>Retention Time (min)</th>
<th>Anthocyanin Standards</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.16-4.20</td>
<td>Cy-3,5-GG</td>
<td>4.16</td>
</tr>
<tr>
<td>2</td>
<td>4.60</td>
<td>Dp-3-G</td>
<td>4.60</td>
</tr>
<tr>
<td>3</td>
<td>5.67-5.64</td>
<td>Cy-3-G</td>
<td>5.85</td>
</tr>
<tr>
<td>4</td>
<td>6.86-6.90</td>
<td>Pt-3-G</td>
<td>6.82</td>
</tr>
<tr>
<td>5</td>
<td>8.10-8.17</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>9.17</td>
<td>Mv-3-G</td>
<td>9.16</td>
</tr>
<tr>
<td>7</td>
<td>15.40</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: Cy-3,5-GG=cyanidin-3,5-diglucoside; Dp-3-G=delphinidin-3-glucoside; Cy-3-G=cyanidin-3-glucoside; Pt-3-G=petunidin-3-glucoside; NA=no matching authentic anthocyanin available; Mv-3-G=malvidin-3-glucoside.
Table 2. Peak area of Reliance anthocyanin extract spiked with available authentic anthocyanin standards determined by reverse-phase HPLC.

<table>
<thead>
<tr>
<th>Reliance Anthocyanin Peak Number</th>
<th>Peak Area Before Spiking</th>
<th>Peak Area After Spiking</th>
<th>Anthocyanin Standards Spiked With</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>104538</td>
<td>708480</td>
<td>Cy-3,5-GG</td>
</tr>
<tr>
<td>2</td>
<td>1971964</td>
<td>2793265</td>
<td>Dp-3-G</td>
</tr>
<tr>
<td>3</td>
<td>3473737</td>
<td>7192965</td>
<td>Cy-3-G</td>
</tr>
<tr>
<td>4</td>
<td>83869</td>
<td>3319077</td>
<td>Pt-3-G</td>
</tr>
<tr>
<td>5</td>
<td>206338</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>26305</td>
<td>5342320</td>
<td>Mv-3-G</td>
</tr>
<tr>
<td>7</td>
<td>315641</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviations: Cy-3,5-GG=cyanidin-3,5-diglucoside; Dp-3-G=delphinidin-3-glucoside; Cy-3-G=cyanidin-3-glucoside; Pt-3-G=petunidin-3-glucoside; NA=no matching authentic anthocyanin available; Mv-3-G=malvidin-3-glucoside.
Table 3. Chromatographic mobility (R\textsubscript{x}100) of authentic anthocyanins standards and Reliance anthocyanins peak 2 and 3 as shown in Figure 1 in two solvent systems BAW and Bu-HCl.

<table>
<thead>
<tr>
<th>Source of Anthocyanins</th>
<th>BAW</th>
<th>Bu-HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 2</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Peak 3</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>33</td>
<td>29</td>
</tr>
</tbody>
</table>

Authentic anthocyanin standards and Reliance anthocyanin peaks 2 and 3 shown in Figure 1 were developed on Whatman No.1 chromatography paper with two solvent systems BAW (n-Butanol-glacial acetic acid-water, 4:1:5, upper layer) and Bu-HCl (n-Butanol-2 N HCl, 1:1, upper layer).
Figure 4. Chromatograms of authentic anthocyanidins delphinidin (A), cyanidin (B), petunidin (C), malvidin (D) and Reliance anthocyanidins (E).
Absorbance at 520 nm

Figure 4
Figure 5. The anthocyanidins of purified Reliance anthocyanin peaks 2 (A), 3 (B), 5 (C), and 7 (D) shown in Figure 1 after complete acid hydrolysis and anthocyanidins (E) from Concord grapes.
Table 4. Retention times of Reliance anthocyanidins shown in Figure 2 and authentic anthocyanidin standards determined by reverse-phase HPLC.

<table>
<thead>
<tr>
<th>Reliance Anthocyanidins Peak Number</th>
<th>Retention Time (min)</th>
<th>Authentic Anthocyanidins</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.19</td>
<td>Cyanidin</td>
<td>8.46</td>
</tr>
<tr>
<td>2</td>
<td>11.00</td>
<td>Delphinidin</td>
<td>11.42</td>
</tr>
<tr>
<td>3</td>
<td>14.88</td>
<td>Petunidin</td>
<td>14.88</td>
</tr>
<tr>
<td>4</td>
<td>20.37</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>23.70</td>
<td>Malvidin</td>
<td>23.44</td>
</tr>
</tbody>
</table>

Abbreviations: NA = no matching authentic anthocyanidin available.

Table 5. Peak area of Reliance anthocyanidins shown in Figure 2 spiked with available authentic anthocyanidin standards determined by reverse-phase HPLC.

<table>
<thead>
<tr>
<th>Reliance Anthocyanidins Peak Number</th>
<th>Peak Area Before Spiking</th>
<th>Peak Area After Spiking</th>
<th>Anthocyanidin Standards Spiked With</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>465324</td>
<td>715992</td>
<td>Delphinidin</td>
</tr>
<tr>
<td>2</td>
<td>3734268</td>
<td>3900436</td>
<td>Cyanidin</td>
</tr>
<tr>
<td>3</td>
<td>120991</td>
<td>932492</td>
<td>Petunidin</td>
</tr>
<tr>
<td>4</td>
<td>83869</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>33332</td>
<td>126330</td>
<td>Malvidin</td>
</tr>
</tbody>
</table>

Abbreviations: NA = no matching authentic anthocyanin available.
Table 6. The chromatographic mobility of authentic anthocyanidins and anthocyanidins of isolated Reliance anthocyanins after complete acid hydrolysis ($R_f \times 100$)

<table>
<thead>
<tr>
<th>Source of Anthocyanidins</th>
<th>$R_f \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formic Acid</td>
</tr>
<tr>
<td>Peak 2</td>
<td>15</td>
</tr>
<tr>
<td>Peak 3</td>
<td>25</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>16</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>25</td>
</tr>
</tbody>
</table>

Reliance anthocyanidins and authentic anthocyanidins (cyanidin and delphinidin) were developed on Whatman No. 1 chromatography paper with such solvents as formic acid (formic acid-12 N HCl-water, 5:2:3), forestal (Glacial acetic acid-12 N HCl-water, 30:3:10) and BAW (n-Butanol-glacial acetic acid-water, 4:1:5, upper layer).
Table 7. Chromatographic mobility of sugar residue in two solvent systems BBPW and Phenol after complete acid hydrolysis of isolated Reliance anthocyanins peak 2 and 3 shown in Figure 1 ($R_f \times 100$)

<table>
<thead>
<tr>
<th>Source of Sugar Residue</th>
<th>$R_f \times 100$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBPW</td>
</tr>
<tr>
<td>Peak 2</td>
<td>66</td>
</tr>
<tr>
<td>Peak 3</td>
<td>66</td>
</tr>
<tr>
<td>Glucose</td>
<td>66</td>
</tr>
</tbody>
</table>

Sugar residue and sugar standard was developed on Thin Layer Plates (Whatman LK2 Microcrystalline Cellulose TLC Plates) with BBFW (1-butanol-benzene-formic acid-water, 100:19:10:25) and Phenol (Phenol-water, 4:1).
Figure 6. The UV-Visible spectra of purified Reliance anthocyanin peaks 2, 3, and 5 shown in Figure 1 and anthocyanin standards delphinidin-3-glucoside (Dp-3-G) and cyanidin-3-glucoside (Cy-3-G).
Table 8. Spectral characteristics of isolated major anthocyanins in dissolved in 0.01% HCl in methanol.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$E_{\text{UV, max}}/E_{\text{vis, max}}$ %</th>
<th>$E_{440}/E_{\text{vis, max}}$ %</th>
<th>AlCl$_3$ Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>278, 542</td>
<td>60</td>
<td>17</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>282, 530</td>
<td>60</td>
<td>22</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>276, 527</td>
<td>205</td>
<td>28</td>
<td>No</td>
</tr>
<tr>
<td>Delphinidin-3-glucoside</td>
<td>278, 542</td>
<td>50</td>
<td>18</td>
<td>Yes</td>
</tr>
<tr>
<td>Cyanidin-3-glucoside</td>
<td>282, 530</td>
<td>60</td>
<td>24</td>
<td>Yes</td>
</tr>
<tr>
<td>Peonidin-3-glucoside</td>
<td>280, 528</td>
<td>60</td>
<td>24</td>
<td>No</td>
</tr>
</tbody>
</table>

Anthocyanin peaks were shown in Figure 1. $\lambda_{\text{max}}$ in UV and visible range of authentic anthocyanins were reported by Wulf and Nagel (1978). AlCl$_3$ Shift data of authentic anthocyanins were taken from the paper by Wulf and Nagel (1978). $E_{\text{UV, max}}/E_{\text{vis, max}}$ and $E_{440}/E_{\text{vis, max}}$ of authentic anthocyanin standards were reported by Francis (1982).
Table 9. Identification of anthocyanins in Reliance grape based on retention times of their glycoside, anthocyanidin, and sugar residue, and UV-Visible spectra.

<table>
<thead>
<tr>
<th>Peak No</th>
<th>Glycoside</th>
<th>Aglycone</th>
<th>Sugar Residue</th>
<th>Identification</th>
<th>Identification Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cy-3,5-GG</td>
<td>NA</td>
<td>NA</td>
<td>Cy-3,5-GG</td>
<td>RT</td>
</tr>
<tr>
<td>2</td>
<td>Dp-3-G</td>
<td>Delphinidin</td>
<td>Glucose</td>
<td>Dp-3-G</td>
<td>RT, SP, HY</td>
</tr>
<tr>
<td>3</td>
<td>Cy-3-G</td>
<td>Cyanidin</td>
<td>Glucose</td>
<td>Cy-3-G</td>
<td>RT, SP, HY</td>
</tr>
<tr>
<td>4</td>
<td>Pt-3-G</td>
<td>NA</td>
<td>NA</td>
<td>Pt-3-G</td>
<td>RT</td>
</tr>
<tr>
<td>5</td>
<td>NA</td>
<td>Peonidin</td>
<td>Glucose</td>
<td>Pn-3-G</td>
<td>HY</td>
</tr>
<tr>
<td>6</td>
<td>Mv-3-G</td>
<td>NA</td>
<td>NA</td>
<td>Mv-3-G</td>
<td>RT</td>
</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>Cyanidin</td>
<td>Glucose</td>
<td>Acylated Cy</td>
<td>RT*, HY</td>
</tr>
</tbody>
</table>

Abbreviations: Cy-3,5-GG=cyanidin-3,5-diglucoside; Dp-3-G=delphinidin-3-glucoside; Cy-3-G=cyanidin-3-glucoside; Pt-3-G=petunidin-3-glucoside; Mv-3-G=malvidin-3-glucoside; NA=not available; RT = retention time; SP = spectral characteristics; HY = complete acid hydrolysis; RT* = relative retention time.
Figure 7. The molecular structures of Reliance anthocyanins shown in Figure 1. Peak 1, cyanidin-3,6-diglucoside; Peak 2, delphinidin-3-glucoside; Peak 3, cyanidin-3-glucoside; Peak 4, petunidin-3-glucoside; Peak 5, peonidin-3-glucoside; Peak 6, malvidin-3-glucoside; Peak 7, cyanidin-3-(p-coumarin)-glucoside.
References


CHAPTER II

CLUSTER THINNING EFFECTS ON FRUIT QUALITY, FRUIT SKIN COLOR, TOTAL ANTHOCYANIN CONTENT, CONTENT OF INDIVIDUAL ANTHOCYANIN, AND PERCENTAGES OF INDIVIDUAL ANTHOCYANINS IN BERRY SKINS OF RELIANCE GRAPES (Vitis hybrid)

Abstract

Fruit quality, fruit skin color, total anthocyanin content, content of individual anthocyanin, and percentages of individual anthocyanins in the berry skin of Reliance grape were investigated with cluster thinning treatments. Cluster thinning treatments were 60 (control), 40 and 20 clusters per vine, applied when the berries were 2-3 mm in diameter. Fruit cluster thinning decreased vine yield significantly following a quadratic relationship. Juice soluble solids were increased significantly by cluster thinning treatments. Berry weight was increased by 20 clusters per vine in comparison with 60 clusters per vine. Juice pH was not affected although juice TA was decreased by 20 clusters per vine treatment in comparison with 40 or 60 clusters per vine treatments. Twenty clusters per vine treatment produced fruits darker (CIE 1976 L') and less yellow (CIE 1976 b') in color in comparison with 60 clusters per vine. However, fruit red color characterized by CIE 1976 a' was not affected
significantly by clusters thinning treatments. Twenty clusters per vine treatment increased total anthocyanin content over 60 but not 40 clusters per vine. The content of individual anthocyanins including cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative, were increased significantly by 20 clusters per vine in comparison with 40 or 60 clusters per vine. However, the content of delphinidin-3-glucoside or petunidin-3-glucoside were not affected significantly. Twenty clusters per vine treatment increased the percentage of cyanidin-3-glucoside, decreased the percentages of malvidin-3-glucoside or acylated cyanidin derivative in comparison with 40 or 60 clusters per vine. The percentages of delphinidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside, were not affected by fruit cluster thinning treatments.

Introduction

Fruit color is one of the important quality attributes in table grapes. The pigments responsible for the attractive red, blue, purple, and black color, are anthocyanins, a class of water soluble flavonoid pigments. Fruit color development in grapes has been studied extensively due to its importance in both the table grape and wine grape industry. Fruit cluster thinning has been shown to improve pigmentation of pigmented grapes (Weaver et al., 1957; Kliwer and Weaver, 1971; Hepner and Bravdo, 1985; Reynolds, 1989; Gao and Cahoon, 1991; Gao and Cahoon, 1992).
The detailed study of individual anthocyanins in pigmented grape cultivars is essential to the understanding of anthocyanin metabolism and fruit color improvement. Various published studies on fruit coloration in grapes have dealt with changes in total anthocyanin content (Kliewer, 1970a; Kliewer and Torris, 1972; Kliewer, 1977; Weaver and Montgomery, 1974; Pirie and Mullins, 1977; Kataoka et al, 1982; Wicks and Kliewer, 1983; Roubelakis-Angelakis and Kliewer, 1986; Hunter et al, 1991). Anthocyanin profiles of most pigmented grape cultivars are known to be very complex (Van Buren et al, 1970; Ribereau-Gayon, 1982). Cabernet Sauvignon (*Vitis vinifera* L) and Concord (*Vitis labruscana* L) have potentially up to 20 different kinds of anthocyanins (Wulf and Nagel, 1978; Hrazdina, 1975).

Wide utilization of C18 reverse-phase high performance liquid chromatography (HPLC) in simultaneous separation, identification, quantification of anthocyanins in grapes and other colored fruits made the detailed study of anthocyanin metabolism possible (Wulf and Nagel, 1976; Roggero et al, 1986; Hebrero et al., 1989; Hong and Wrolstad, 1990). HPLC studies of the effects of cultivars and climatological factors on changes in individual anthocyanins of Syrah clones during ripening (Roggero et al., 1986; Cacho et al., 1992) produced results significant to our understanding of anthocyanin metabolism in field grown pigmented grapes.

A red seedless table grape cultivar, Reliance (*Vitis* hybrid) was chosen in this study due to its fruit color variation at maturity and economical importance in Ohio
(Cahoon, 1983). C_{18} reverse-phase HPLC analysis of anthocyanins from Reliance grape showed 7 components (Chapter I). With the joint use of HPLC, paper chromatography, thin layer chromatography, and spectral measurement, three components were identified as delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside (Chapter I). Other four anthocyanin components were tentatively identified as cyanidin-3,5-diglucoside, petunidin-3-glucoside, malvidin-3-glucoside, acylated cyanidin derivative. Preliminary studies on fruit color development in Reliance showed that fruit color was enhanced by chelated nutrients, ethephon, or cluster thinning (Gao et al., 1989; Gao and Cahoon, 1991; Gao and Cahoon, 1992).

The objective of this study was to investigate effects of fruit cluster thinning on fruit quality, fruit color, total anthocyanin content, the content of individual anthocyanins, and percentages of individual anthocyanin in the berry skin of Reliance grape.

**Materials and Methods**

**Grapevines and treatments:** Reliance grapevines were planted in 1985 at Horticulture Unit II of the Ohio Agricultural Research and Development Center, The Ohio State university, Wooster, Ohio, U.S.A. Grapevines were trained to the Single Curtain, Cordon System. Row and vine spacings were 3.05 m x 2.44 m. In 1991,
grapevines were pruned to 60 buds per vine. Shoot number was adjusted to 50 per vine one week after full bloom. Cluster thinning was done when berries reached about 2-3 mm in diameter. Treatments were 60 (control), 40, and 20 clusters per vine. The experimental design was a randomized complete block where a whole vine served as an experimental unit. Treatments were replicated eight times.

**Fruit quality and color:** Fruit juice was obtained by pressing 100 berries per sample through a Garden-Way Squeezo strainer. Soluble solids of the fruit juice was measured with an ABBE-3L Refractometer (Baush & Lomb Inc, Rochester, New York). Juice pH was measured with a Beckman pH meter (Model PHI 45, Beckman Instruments Inc, Fullerton, California). Titratable acidity (TA%) was measured by a titration of 5 ml of juice with 0.1 N NaOH. Fruit skin color was measured with a Minolta Chroma Meter (Model CR-100, Minolta Camera Co., Ltd, Higashi-Ku, Osaka, Japan) as CIE (Commission Internationale de l'Eclairage translated as the International Commission of Illumination) 1976 L*, a*, and b*. CIE L* represents black to white color as CIE L* values increase from negative to positive. CIE a* represents green to red color as CIE a* values increase from negative to positive. CIE b* represents blue to yellow color as CIE b* values increase from negative to positive. Fruit skin readings were taken from the east-facing side of each fruit cluster. Fruit skin color readings were taken from top, middle, and bottom portion of each treated cluster. The mean of these three readings was used.
Sample preparation for anthocyanin analysis: Fruit cluster samples were collected on August 18, 1991, and immediately placed in 4°C storage. They were weighed and berry number per fruit cluster counted. Clusters were then frozen and stored at -20°C for future analyses. Berry skins were removed and collected by first thawing the frozen berries in a refrigerator at 4°C for 20 minutes. The berry skin was then peeled with tweezers, and kept in an ice chilled beaker. Finally, these berry skins were freeze dried and ground with a coffee mill (Oster Model 663-06, Sunbeam corporation, Milwaukee, Wisconsin, U.S.A).

Anthocyanin extraction and concentration: One gram of ground berry skin was placed in 100 ml of 1% 12 N HCl in methanol. The anthocyanin extraction was carried out overnight in a refrigerator at 4°C. These anthocyanin extracts were then filtered through Whatman No.1 filter paper in a Buchner funnel. Twenty ml of deionized distilled water was added to each anthocyanin extract. The anthocyanin extracts were concentrated with a rotary evaporator under vacuum at 30°C. Each anthocyanin concentrate was transferred to a 25 ml volumetric flask and then brought to volume with deionized distilled water. Five ml of each anthocyanin concentrate was then passed through 0.2 μm syringe membrane filters which were equilibrated with 1 ml of respective anthocyanin concentrate to avoid anthocyanin dilution by syringe membrane filters. Each anthocyanin filtrate was stored at room temperature for less than 20 minutes in a screw capped sample vial before HPLC analysis.
HPLC analyses: HPLC analyses were performed on a Model SP4000 pump (Spectra-Physics, San Jose, California, USA) equipped with a 20 μl Rheodyne sample loop. The analytical column was pH stable RP-18 Spherisorb (Merck, Darmstadt, Germany) (150 mm x 4.6 mm I.D.) packed with 5 μm particles by Alltech (Deerfield, Illinois, USA). A Spectra-Physics UV1000 variable wavelength detector and Spectra-Physics Model 4600 integrator were used.

The following conditions were used for the analyses of anthocyanins: Solvent A—10% formic acid in water; solvent B—high purity acetonitrile. These solvents were filtered through 0.20 μm membrane filters and sparged with helium; solvent flow rate 1 ml/min. The solvent program used for anthocyanins was 95% A initially, decreased from 95% A to 72% A in twenty minutes following a linear curve. Detection was carried out at 520 nm.

Determination of anthocyanin content: In order to convert the peak areas into pigment concentration per gram of dry berry skin, a solution of cyanidin-3-glucoside in 0.1 N HCL was prepared to established a standard curve. The solution was filtered through a 0.2 μm membrane filter. Its absorbance was read at 520 nm. The concentration (g/100 ml) of this solution was calculated based on the extinction coefficient of cyanidin-3-glucoside in 0.1 N HCl. A series of dilutions was then made. Twenty μl of diluted samples was injected into HPLC under identical analytical condition as for the Reliance samples.
A standard curve was established between the concentration of diluted cyanidin-3-glucoside solutions and their peak areas. The concentration of cyanidin-3-glucoside was calculated based on the following equation where the correlation coefficient was 0.998:

\[ \text{mg/100 ml} = \text{peak area} \times 0.00000391 \]

Since one gram of dried berry skin was dissolved in a final volume of 25 ml, the concentration of cyanidin-3-glucoside was calculated as:

\[ \text{mg/gram dried berry skin} = \frac{\text{peak area} \times 0.00000391 \times 25 \text{ml}}{100 \text{ml}} \]

The content of each individual anthocyanin was expressed as cyanidin-3-glucoside equivalent based on their respective peak area since cyanidin-3-glucoside was present at the highest concentration in Reliance anthocyanin profile (Chapter I). The percentage of each individual anthocyanin was calculated as:

\[ \text{Percentage} = \frac{\text{the content of individual anthocyanin} \times 100}{\text{Total anthocyanin content}} \]

Total anthocyanin content was calculated as the sum of the contents of all the individual anthocyanins.
Results and Discussion

Cluster thinning treatments decreased yield significantly (Table 10). Soluble solids were increased significantly by fruit cluster thinning treatments following a quadratic relationship (Table 10). Juice pH was not affected by cluster thinning treatments even juice TA was decreased by 20 clusters per vine in comparison with 60 clusters per vine. Berry weight was increased by 20 clusters per vine treatment in comparison with 40 or 60 clusters per vine. Cluster weight was not increased significantly by cluster thinning treatments. This minimal difference in cluster weight could have been partially due to a variation in berry number per cluster before cluster thinning treatments were applied. Berry numbers for 20, 40, and 60 clusters per vine treatments were 131, 140, and 133, respectively. This minimal difference in cluster weight could also have been partially due to a drought during the final stage of berry enlargement in 1991. Van Zyl and Webber (1977) found veraison to be the most sensitive berry growth stage, followed by the period just after flowering. Hardie and Considine (1976) found the greatest yield loss, caused by reduced fruit set, when water stress was imposed during a 3-week period after flowering. Severe water stress after veraison resulted in fruit failing to mature (Hardie and Considine, 1976). Grimes and Williams (1990) reported that Thompson seedless grape yields followed declining water availability essentially linearly from 40 to 120% of the expected nonstressed crop evapotranspiration. Sixty clusters per vine (control) applied in this study was slightly above the maximum yield (50 clusters per
Cluster thinning did not significantly increase fruit red color characterized as CIE 1976 $a^*$. Twenty clusters per vine treatment produced darker (CIE 1976 $L^*$) and less yellow (CIE 1976 $b^*$) clusters in comparison with 60 clusters per vine treatment (Table 10). Fruit skin color reading readings were taken from east-facing side of fruit cluster. It is possible that fruit red color under 60 clusters per vine could have been overestimated by this approach. Furthermore, differences in fruit color could have been decreased to a certain degree by the dry and hot weather. Total anthocyanin content in freeze dried berry skin from a whole cluster was analyzed so that pigment of a whole fruit cluster can be used to indicate fruit color.

As measured with HPLC, total anthocyanin content of the berry skin was increased by cluster thinning treatments following a linear relationship (Table 11). Twenty clusters per vine increased total anthocyanin content in comparison with 60 cluster per vine but not 40 clusters per vine. Kliewer and Weaver (1971) showed that 18.7 clusters per vine treatment (pruned and thinned) increased the percentage of coloration by 57% in comparison with 31.8 cluster per vine treatment, and by 85% in comparison with 120 clusters per vine treatment. Low crop levels in several red wine grape cultivars (Alicante Bouschet, Carignane, Perlite Sirah, Pinot Pernand, and Zinfandale), were shown to produce better colored fruits than high crop levels.
(Weaver et al., 1957). The content of cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were increased significantly by 20 clusters per vine in comparison with 40 or 60 clusters per vine. However, the content of delphinidin-3-glucoside, or petunidin-3-glucoside were not affected significantly by cluster thinning treatments. It appears that cluster thinning affected individual anthocyanins in Reliance grape differently. Cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were affected by fruit cluster thinning treatments where delphinidin-3-glucoside and petunidin-3-glucoside were not. One question concerning cluster thinning on fruit anthocyanins is whether cluster thinning affects individual anthocyanins by advancing fruit ripening. A study by Cacho et al. (1992) reported that all the individual anthocyanin in such grape cultivars as Tempranillo, Moristel, and Garnacha, increase steadily as total anthocyanin content increases from veraison to full maturity. The ratios of individual anthocyanins help to determine whether the effect of cluster thinning was solely due to the promotion of fruit ripening.

The percentages of several individual anthocyanin components, in relation to total anthocyanin content, were also affected by cluster thinning treatments (Table 12). The percentage of cyanidin-3-glucoside was increased significantly by cluster thinning treatments following a linear relationship. Twenty clusters per vine treatment increased the percentage of cyanidin-3-glucoside in comparison with 40 or 60 clusters per vine. The percentages of malvidin-3-glucoside and acylated cyanidin
derivative were decreased by 20 clusters per vine treatment in comparison with 40 or 60 clusters per vine treatments. However, the percentages of delphinidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside were not affected by cluster thinning treatments. Cluster thinning had an opposite effect on the percentage of cyanidin-3-glucoside as for the ratio of acylated cyanidin derivative. The same relationship exists between the ratios of cyanidin-3-glucoside and malvidin-3-glucoside. The effect of cluster thinning treatments on the percentage of individual anthocyanins does not appear to be only related berry ripening. Roggero et al. (1986) reports that anthocyanin composition in Syrah grape is quickly set after veraison and remains nearly stable until the grapes mature except for cyanidin derivative, which is the precursor of other pigments. Based on a calculation of ratios of individual anthocyanins presented by Cacho et al. (1992), it seems that percentages of anthocyanins remain stable after veraison. Cluster thinning treatments seem to shift the balance among cyanidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative without significant affecting the percentages of other anthocyanins. The effects of cluster thinning seem to have a control on anthocyanin metabolism beside promoting fruit ripening.

Twenty clusters per vine treatment in this study produced fruit clusters with the most anthocyanin in berry skin or in turn the best colored fruit. Is this crop level economical for commercial grape growers? Twenty clusters per vine would be 5.6 kg based on a cluster weight of 0.28 kg/cluster. A 3.03 m x 2.44 m (8 feet x 10
feet) row and vine spacing will have 545 vines per 0.405 hectare (one acre). Based on $1.32/kg ($0.60/lb) at wholesale level, the gross income of 0.405 hectare (one acre) is about $4646. For an established vineyard, a grape grower’s input is around $1500-2000. It seems that a Reliance grape grower can expect $2646-$3146 per 0.405 hectare (acre) if grapevines were cluster thinned to 20 clusters per vine. Price and Baldwin (1980) reported a net return of $508.80 per 0.405 hectare (one acre), an estimated gross return of $1825 per 0.405 hectare (one acre), and an estimated total variable cost of $1316 per 0.405 hectare (one acre), for irrigated table grape vineyards. Lutz et al. (1986) reported a net investment cost of $8124.74 per 0.405 hectare (one acre) for first the four years in establishing a 30-acre Concord grape vineyard. After fourth year, the per 0.405 hectare (one acre) cost associated with maintaining a 30-acre Concord grape vineyard was $2609 (Lutz et al., 1986). These two studies provide a good picture for vineyard economics in Ohio. What about 40 clusters per vine for Reliance grapes? Let us assume that 15 clusters can be sold at $1.32/Kg ($0.60), 15 cluster at $1.10/Kg ($0.50/lb), 10 clusters for juice at $0.66/Kg ($0.27/lb). Based on similar calculations, a gross income is about $6545 per 0.405 hectare. Based on previous calculation, grape grower’s input is $1500-$2000, a Reliance grower can expect about $4545-$5045 per 0.405 hectare. However, an excessive crop level could severely inhibit growth, limit carbohydrate storage, and result in vine losses. A very small crop could lead to too much vegetative growth and hence result in vigorous shoots competing for photosynthate with fruit clusters. Twenty clusters per vine did not seem to result in excessive shoot growth in a dry
year. However, twenty clusters in a wet year could be too little and result in excessive shoot growth. Several more growing seasons and a comprehensive economical analysis are really needed before a definite recommendation can be made to commercial Reliance grape growers.

This study is the first of its kind in attempting to investigate the individual anthocyanins of berry skin in a given grape cultivar affected by cultural practices in addition to total anthocyanin content. Such crop levels as 20, 30, 40, 50, 60, 70, and 80 cluster per vine should be used to investigate metabolism of individual anthocyanins from veraison to maturity. The optimum ratios of yield to vegetative growth measured as prunings should also established for Reliance. The number of leaves to mature a fruit cluster also needs to be determined. Much more research is needed in order to completely understand anthocyanin metabolism in pigmented grapes before manipulation of fruit color can be achieved at will.
Table 10. Effects of cluster thinning on grape yield, quality, and fruit skin color (CIE 1976 L*, a*, and b*).^2

<table>
<thead>
<tr>
<th>Treatment Cluster No/Vine</th>
<th>Yield (kg/vine)</th>
<th>Soluble Solids (°Brix)</th>
<th>pH</th>
<th>TA (%)</th>
<th>Berry Weight (g)</th>
<th>Cluster Weight (g)</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>12.7a</td>
<td>18.5c</td>
<td>3.25a</td>
<td>0.50a</td>
<td>2.0b</td>
<td>269.3a</td>
<td>32.6a</td>
<td>6.2a</td>
<td>4.5a</td>
</tr>
<tr>
<td>40</td>
<td>8.9b</td>
<td>20.0b</td>
<td>3.35a</td>
<td>0.49ab</td>
<td>2.1b</td>
<td>294.4a</td>
<td>31.3ab</td>
<td>7.0a</td>
<td>3.2b</td>
</tr>
<tr>
<td>20</td>
<td>5.4c</td>
<td>21.6a</td>
<td>3.29a</td>
<td>0.48b</td>
<td>2.2a</td>
<td>277.2a</td>
<td>30.0b</td>
<td>7.2a</td>
<td>2.5b</td>
</tr>
<tr>
<td>LSD</td>
<td>1.7</td>
<td>0.7</td>
<td>0.23</td>
<td>0.02</td>
<td>0.1</td>
<td>39.7</td>
<td>1.7</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Linear Contrast</td>
<td>**</td>
<td>**</td>
<td>NS **</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td>Quadratic Contrast</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

^2Mean separation by least significant difference (LSD) at 0.05 level.

*.*, **, NS Significant at 0.05 or 0.01 level, nonsignificant, respectively.

Abbreviations: TA, titratable acidity; L* represents light color to dark color; a* represents red color to green color; b* represents yellow color to blue color.
Table 11. Effects of cluster thinning on individual anthocyanins and total anthocyanin content in fruit berry skin.

<table>
<thead>
<tr>
<th>Treatments Cluster No/Vine</th>
<th>Dp-3-g (mg/g)</th>
<th>Cy-3-g (mg/g)</th>
<th>Pt-3-g (mg/g)</th>
<th>Pn-3-g (mg/g)</th>
<th>Mv-3-g (mg/g)</th>
<th>Acylated Cy derivative (mg/g)</th>
<th>Total Anthocyanin Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0.59a</td>
<td>2.64b</td>
<td>0.06a</td>
<td>0.15a</td>
<td>0.03b</td>
<td>0.50b</td>
<td>3.83b</td>
</tr>
<tr>
<td>40</td>
<td>0.61a</td>
<td>3.29ab</td>
<td>0.06a</td>
<td>0.17a</td>
<td>0.03b</td>
<td>0.58b</td>
<td>4.60ab</td>
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<td>0.76a</td>
<td>4.35a</td>
<td>0.08a</td>
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<td>0.01</td>
<td>0.12</td>
<td>1.65</td>
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<td>NS</td>
<td>**</td>
<td>NS</td>
<td>*</td>
<td>NS</td>
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<td>**</td>
</tr>
<tr>
<td>Quadratic Contrast</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Mean separation by least significant difference (LSD) 0.05 level.
**NSSignificant at 0.01, 0.05 level, and nonsignificant at 0.05 level.

Abbreviations: Dp-3-g, delphinidin-3-glucoside; Cy-3-g, cyanidin-3-glucoside; Pt-3-g, petunidin-3-glucoside; Pn-3-g, peonidin-3-glucoside; Mv-3-g, malvidin-3-glucoside; Acylated Cy derivative, acylated cyanidin glycoside. Total anthocyanin is a sum of the content of individual anthocyanins.
Table 12. Effects of cluster thinning on the percentages of individual anthocyanins.2

<table>
<thead>
<tr>
<th>Treatment Cluster No/Vine</th>
<th>Dp-3-g %</th>
<th>Cy-3-g %</th>
<th>Pt-3-g %</th>
<th>Pn-3-g %</th>
<th>Mv-3-g %</th>
<th>Acylated Cy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>12.5a</td>
<td>67.3b</td>
<td>1.4a</td>
<td>4.4a</td>
<td>0.8a</td>
<td>13.6a</td>
</tr>
<tr>
<td>40</td>
<td>12.0a</td>
<td>68.6b</td>
<td>1.3a</td>
<td>4.4a</td>
<td>0.6b</td>
<td>13.2a</td>
</tr>
<tr>
<td>20</td>
<td>12.2a</td>
<td>71.4a</td>
<td>1.3a</td>
<td>3.7a</td>
<td>0.6b</td>
<td>10.8b</td>
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<tr>
<td>LSD</td>
<td>3.6</td>
<td>4.1</td>
<td>0.4</td>
<td>0.9</td>
<td>0.2</td>
<td>2.85</td>
</tr>
<tr>
<td>Linear Contrast</td>
<td>NS</td>
<td>*</td>
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<td>NS</td>
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<td>*</td>
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<td>Quadratic Contrast</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Mean separation by least significant difference (LSD) at 0.05 level.
* NS Significant at 0.05 level or nonsignificant.

Abbreviations: Dp-3-g, delphinidin-3-glucoside; Cy-3-g, cyanidin-3-glucoside; Pt-3-g, petunidin-3-glucoside; Pn-3-g, peonidin-3-glucoside; Mv-3-g, malvidin-3-glucoside; Acylated Cy, acylated cyanidin derivative. The ratio of individual anthocyanins was calculated as a percentage of total anthocyanin.
References


CHAPTER III

CLUSTER SHADING EFFECTS ON FRUIT QUALITY, FRUIT SKIN COLOR, TOTAL ANTHOCYANIN CONTENT, CONTENT OF INDIVIDUAL ANTHOCYANINS, AND PERCENTAGES OF INDIVIDUAL ANTHOCYANINS

Abstract

The effects of 55% and 95% cluster shading on fruit quality, fruit visual color, total anthocyanin content, the content of individual anthocyanins, and percentages of individual anthocyanins in the berry skins, were examined in detail in 1991. This experiment was conducted on five year old field grown grapevines of a red seedless grape cultivar Reliance. Juice soluble solids was decreased significantly by 95% shading in comparison to 55% shading or the full sun control. There were no significant differences in fruit soluble solids between 55% shading and full sun control. Juice pH was not affected significantly by cluster shading. However, juice TA was increased by significantly by 55% shading in comparison to full sun or 95% shading. Cluster weight was not affected significantly by cluster shading. Berry weight was decreased by cluster shading treatments following a linear relationship. Ninety five percent shading produced fruit clusters that were lighter in color (CIE 1976 L*), less red (CIE 1976 a*), and more yellow (CIE 1976 b*) in comparison to
full sun control or 55% shading. Total anthocyanin content in the berry skin measured with HPLC was decreased by 95% shading in comparison to full sun or 55% shading. No differences in fruit total anthocyanin content were found between 55% shading and full sun control. Fruit individual anthocyanins including delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were also decreased by 95% shading in comparison with full sun control or 55% shading. There were no significant differences in the content of individual anthocyanins between 55% shading and full sun. The percentages of most individual anthocyanins in the berry skin were also affected significantly by 95% cluster shading treatment. Ninety five percent shade decreased the percentage of delphinidin-3-glucoside in comparison with 55% but not full sun control. The percentage of cyanidin-3-glucoside was decreased significantly by 95% shading in comparison with 55% shading or full sun. The percentages of peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were increased by 95% shading in comparison with 55% shading or full sun. Cluster shading treatments had no effect on the percentage of petunidin-3-glucoside in comparison with full sun. It seems that light is more critical to the biosynthesis of cyanidin-3-glucoside. The biosynthesis of peonidin-3-glucoside, malvidin-3-glucoside, or acylated cyanidin derivative seem to be much less light dependent than cyanidin-3-glucoside. The percentage of petunidin-3-glucoside was not affected by cluster shading.
Introduction

Sunlight has been found to be very critical for fruit color development in many grape cultivars (Kliewer, 1970a; Kliewer, 1977; Roubelakis-Angelakis and Kliewer, 1986, Crippen and Morrison, 1986; Dokoozlian and Kliewer, 1992). Cluster shading was shown to reduce total anthocyanin content in pigmented grape cultivars (Kakaota et al., 1984; Crippen and Morrison, 1986; Rojas-Lara and Morrison, 1989; Gao and Cahoon, 1991; Gao and Cahoon, 1992). Pigments responsible for the attractive red, blue, purple, and black color in grapes, are anthocyanins, a class of water soluble flavonoid pigments (Ribereau-Gayon, 1982; Van Buren et al.; 1970). Anthocyanins in a grape cultivar are present in the vacuole of fruit subepidermal cells in the berry skin (Moskowitz and Hrazdina, 1981). Furthermore, the anthocyanin profile in a grape cultivar can be very complex. HPLC analyses of grape anthocyanin profiles have shown that a grape cultivar may have up to 20 different anthocyanins (Wulf and Nagel, 1976.; Hebrero et al., 1989). Detailed studies of individual anthocyanins in a given grape cultivar provided significant insight to the understanding of anthocyanin metabolism in grapes (Cacho et al., 1992; Roggero and Ragonnet, 1986; Wicks, 1982).

Reliance (Vitis hybrid) is a red seedless grape with excellent taste, delicate aroma, early ripening, moderate disease resistance, and exceptional winter hardiness (Moore, 1983). It was used in this study due to its economic importance as a red
seedless table grape cultivar in Ohio (Cahoon, 1983), a great fruit color variation at maturity, and the relatively simple anthocyanin profile (Chapter I).

It was very important to determine the importance of exposure of fruit clusters to sunlight to red color development or production of brilliant red colored fruit in Reliance grapes. In a practical sense, this information can help justify the necessity of such cultural practices as selective leaf removal around fruit zone or shoot positioning to expose fruit clusters to sunlight for the best fruit pigmentation. Two levels of shading (95 and 55%) treatments with full sun (control) were used to test the effect of sunlight on fruit quality, visual color, total anthocyanin content, content of individual anthocyanins, and percentages of individual anthocyanins.

Detailed studies of individual anthocyanins in Reliance grape can lead to a better understanding of the mechanism in which anthocyanin biosynthesis in Reliance grape is regulated. Fruit cluster shading treatments can be used to test how the synthesis of different anthocyanins in Reliance grape is regulated by such environmental factors as light. C18 reverse-phase HPLC analysis of the anthocyanins from Reliance grape showed 7 components (Chapter I). With the joint use of HPLC, paper chromatography, thin layer chromatography, and spectral measurement, three components were identified as delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside (Chapter I). Other four anthocyanin components were tentatively identified as cyanidin-3,5-diglucoside, petunidin-3-glucoside, malvidin-3-
glucoside, and an acylated cyanidin derivative. Simultaneous separation and quantification of the anthocyanins from Reliance grape were achieved within 20 minutes with HPLC (Chapter I).

The objective of this study was to investigate the effects of cluster shading on fruit color development in Reliance grape. Fruit quality, visual color, content of total anthocyanin and individual anthocyanins, and the relative percentages of individual anthocyanins in the berry skin of the Reliance grapes, were studied to gain a better understanding of the controlling mechanism in fruit color development.

**Materials and Methods**

**Grapevines and treatments:** Reliance grapevines in the experiment were planted in 1985 at Horticulture Unit II of the Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio, U.S.A. Grapevines were trained to the Single Curtain Cordon System. Row and vine spacings were 3.05 m x 2.44 m. Vines were pruned to 60 buds per vine. Shoot number was then adjusted to 50 per vine one week after full bloom. Cluster shading treatments applied were full sun (control), 55% shading, and 95% shading when berries reached about 2-3 mm in diameter. Clusters were shaded with shading cages constructed of 55% or 95% polypropylene shade cloth (Pak Unlimited, Norcross, Georgia, U.S.A). Two clusters were selected per treatment on each experimental vine. Each vine served as a
replicate. The experimental design was a complete randomized block with 12 replicates.

**Temperature around fruit clusters:** Temperature readings were measured with thermocouples that were placed beside control and treated clusters on August 5, 1991, two weeks before harvest. A 21X Micrologger (Campbell Scientific Inc, Logan, Utah, U.S.A) was used to continuously record air temperature readings beside fruit clusters over a period of two weeks.

**Fruit quality and visual color:** Refer to Chapter II for the measurements of fruit quality and berry skin color.

**Sample preparation for anthocyanin analysis:** Refer to Chapter II for sample preparation in anthocyanin analysis.

**Anthocyanin extraction and concentration:** Refer to Chapter II for anthocyanin extraction and concentration.

**HPLC analyses:** Refer to Chapter II for HPLC analyses.
Results and Discussion

Temperature readings were taken to verify that differences created by the shading treatments were not from a secondary warming effect created by the shade cloth. The air temperature of one daily cycle beside the grape clusters was shown because trends were similar over the two week period. Air temperature readings beside control and shaded fruit clusters on August 8, 1991 are shown in Figure 8. Air temperature beside 95% shaded clusters was lower than that of control from one AM to 6 AM (6:00 hour), higher from 6 AM (6:00 hour) to 10 PM (22:00 hour), similar or equal from 10 PM (22:00 hour) to midnight (24:00 hour). The air temperature of 55% shaded clusters was similar to that of control except at noon when 55% shade provided a cooling effect. In studies with potted Carbernet Sauvignon grapes, pigmentation was enhanced by low day temperature of 20°C in comparison with high day temperature of 30°C (Buttrose et al., 1971). Kliwer and Torres (1972) showed that fruits ripened at cool night temperature (15°C) had much greater coloration that at warm night temperatures (25°C). Kliwer (1970a) showed that 10°C decrease in day temperatures under high light improved fruit coloration by about 40%. Weaver and McCune (1960) found 1.1°C difference between control clusters and clusters enclosed in brown bags. Waver and McCune (1960) stated "It is clear that bagged clusters warm up more slowly, but cool off more slowly. There was a shift in temperature within the bags as compared to control clusters. However, temperature difference was very small and of questionable significance." The
maximum temperature differences in this study between 95% shaded and full sun control were observed to be 2.8°C. Differences in air temperature beside control and shaded fruit clusters were much smaller than 10°C. However, the possible effects of this temperature differences of fruit color could not be ruled out completely.

Fruit soluble solids was decreased significantly by 95% shading in comparison to 55% shading or the full sun control (Table 13). There were no significant differences in fruit soluble solids between 55% shading and full sun control. Kliewer (1977) found that 54% full sun had no significant effect while 15% full sun reduced soluble solids in comparison to 100% full sun control in Emperor grapes. Cluster weight was not affected significantly by cluster shading. Juice pH was not affected significantly by cluster shading. However, juice TA was increased significantly by 55% shading in comparison to full sun or 95% shading. Berry weight was decreased by cluster shading treatments following a linear relationship. Ninety five percent shading produced fruit clusters that were lighter in color, less red, and more yellow in comparison to full sun or 55% shading.

Total anthocyanin content in the berry skin measured with HPLC was decreased by 95% shading in comparison to full sun or 55% shading (Table 14). No difference in fruit total anthocyanin content was found between 55% shading and full sun control. Kliewer (1977) found similar results with Emperor grapes. Kliewer (1977) showed that 15% full sun (85% shading) decreased fruit anthocyanin content
whereas 54% full sun (46% shading) did not in comparison to 100% full sun control.

The content of individual anthocyanins including delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were also decreased by 95% shading in comparison with full sun control or 55% shading (Table 14). There were no significant differences in individual anthocyanins between 55% shading and full sun. The results demonstrated that cluster shading not only decreased total anthocyanin content but also the concentration of individual anthocyanins. Wicks et al. (1982) studied the influences of light and ethephon (an ethylene releasing compound) on anthocyanins determined by thin layer chromatography in Tokay, Emperor, Cardinal, and Ribier grapes. Wicks et al. (1982) found that cyanidin-peonidin pathway was induced by light, but light requirement can be overcome by an appropriate application of ethephon. Biosynthesis of anthocyanins from Reliance grapes was not completely stopped by 95% shading. This agrees with the results obtained by Kliewer (1977) with Emperor grapes where clusters under 3% or 0.8% full sun had around 34% of the anthocyanin in clusters under full sun.

The percentages of most individual anthocyanins in the berry skin were also affected significantly by 95% cluster shading treatment (Table 15). Ninety five percent shading decreased the percentage of delphinidin-3-glucoside in comparison with 55% but not full sun control. The percentage of cyanidin-3-glucoside was
decreased significantly by 95% shading in comparison with 55% shading or full sun. The percentages of peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative were increased by 95% shading in comparison with 55% shading or full sun. Cluster shading treatments had no effect on the percentage of petunidin-3-glucoside in comparison with full sun control. It seems that light is very critical to the biosynthesis of cyanidin-3-glucoside. The biosynthesis of peonidin-3-glucoside, malvidin-3-glucoside, or acylated cyanidin derivative seem to be much less light dependent than cyanidin-3-glucoside in Reliance grape. Wicks et al. (1982) found that cyanidin-3-glucoside, the only anthocyanin in Tokay grape, appeared only in the presence of light with or without ethephon. Results from Tokay agree with the finding that cyanidin-3-glucose in Reliance grape was more light dependent than other anthocyanins. The percentage of petunidin-3-glucoside was not affected by cluster shading.

In a practical sense, results from this cluster shading study can help Reliance grape growers determine whether shoot positioning or selective leaf removal around fruit zone to expose fruit cluster to more sunlight is necessary to improve fruit color. Smart et al. (1982) found that basal leaf illuminance was improved early in the season by canopy division and low shoot density; after flowering, the major effect was of shoot positioning. Morris et al. (1985) found that shoot positioning in Niagara grapes produced darker (more red color) fruit clusters than without shoot positioning. Shaulis and May (1971) found that shoot crowding reduced yield while control of the
shoot crowding with canopy dividing increased yield in Sultana grapes. Reliance clusters reaches the full color potential if light levels were above 45% full sunlight. However, when fruit clusters receive only 5% full sunlight (95% shading), cultural means such as selective leaf removal or shoot positioning are needed to exposure fruit cluster to the sun light in order for the Reliance fruit to reach its characteristic red color. Partial defoliation at veraison was shown to have the highest anthocyanin content in Cabernet Sauvignon grapes in comparison with partial defoliation at bud break, berry set, or pea size (Hunter et al., 1991).

Reliance grape, with its relatively simple anthocyanin profile, made the study of individual anthocyanin a little easier. Advances in HPLC makes the simultaneous quantitative analyses of individual anthocyanins in a given grape cultivar possible. In the future studies, lights of different wavelength with same intensity, should be used to determine how the light quality affects individual anthocyanin components and possible enzymes involved in anthocyanin biosynthesis of a given pigmented grape cultivar, i.e Reliance. Light of different intensity with same temperature should also be studied in relation to fruit color development. With a better understanding of fruit color development, fruit color can be improved more effectively by the manipulation of light intensity or light quality around fruiting zone and grape leaf canopy, i.e supplemental lighting, light reflectors, shoot positioning, and partial defoliation.
Figure 8. The air temperature readings beside fruit clusters under full sun (control), 55% or 95% shade, were taken on August 5, 1991.
Table 13. Effects of cluster shading on cluster weight, fruit quality and skin color (CIE 1976 L*, a*, and b*).z

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble Solids °Brix</th>
<th>pH</th>
<th>TA (%)</th>
<th>Cluster Weight (g)</th>
<th>Berry Weight (g)</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Sun</td>
<td>20.7a</td>
<td>3.37a</td>
<td>0.47a</td>
<td>276.7a</td>
<td>2.2a</td>
<td>27.5b</td>
<td>9.1a</td>
<td>1.9b</td>
</tr>
<tr>
<td>55% Shade</td>
<td>20.3a</td>
<td>3.17a</td>
<td>0.51b</td>
<td>279.2a</td>
<td>2.1b</td>
<td>29.1b</td>
<td>7.8a</td>
<td>1.2b</td>
</tr>
<tr>
<td>95% Shade</td>
<td>19.1b</td>
<td>3.30a</td>
<td>0.48a</td>
<td>264.7a</td>
<td>1.9c</td>
<td>34.5a</td>
<td>4.9b</td>
<td>5.8a</td>
</tr>
<tr>
<td>LSD</td>
<td>0.74</td>
<td>0.23</td>
<td>0.02</td>
<td>39.7</td>
<td>0.1</td>
<td>1.7</td>
<td>1.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Linear Contrast ** NS NS NS ** ** **

Quadratic Contrast * NS NS NS NS ** NS **

*Mean separation by least significant difference (LSD) at 0.05 level.
NS, NS, NS Nonsignificant at 0.05 level or significant at 0.05 or 0.01 level, respectively.
Abbreviations: TA: titratable acidity; L* represents light to dark color; a*, red color to green color; b*, yellow to blue color.
Table 14. Effects of fruit cluster shading on content of total anthocyanin and individual anthocyanins in the berry skin.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dp-3-g (mg/g)</th>
<th>Cy-3-g (mg/g)</th>
<th>Pt-3-g (mg/g)</th>
<th>Pn-3-g (mg/g)</th>
<th>Mv-3-g (mg/g)</th>
<th>Acylated Cy (mg/g)</th>
<th>Total Anthocyanin Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Sun (Control)</td>
<td>0.98a</td>
<td>5.31a</td>
<td>0.11a</td>
<td>0.25a</td>
<td>0.04a</td>
<td>0.70a</td>
<td>7.21a</td>
</tr>
<tr>
<td>55% Shade</td>
<td>0.97a</td>
<td>4.69a</td>
<td>0.11a</td>
<td>0.25a</td>
<td>0.04a</td>
<td>0.61a</td>
<td>6.51a</td>
</tr>
<tr>
<td>95% Shade</td>
<td>0.20b</td>
<td>0.99b</td>
<td>0.02b</td>
<td>0.07b</td>
<td>0.01b</td>
<td>0.22b</td>
<td>1.46b</td>
</tr>
<tr>
<td>LSD</td>
<td>0.36</td>
<td>1.54</td>
<td>0.05</td>
<td>0.09</td>
<td>0.09</td>
<td>0.13</td>
<td>2.05</td>
</tr>
<tr>
<td>Linear Contrast</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Quadratic Contrast</td>
<td>**</td>
<td>**</td>
<td>*</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>

*Mean separation by least significant difference (LSD) at 0.05 level.
**Significant at 0.05 or 0.01 level, nonsignificant at 0.05 level, respectively.
*Abbreviations: Dp-3-g, delphinidin-3-glucoside; Cy-3-g, cyanidin-3-glucoside; Pt-3-g, petunidin-3-glucoside; Pn-3-g, peonidin-3-glucoside; Mv-3-g, malvidin-3-glucoside; Acylated Cy, acylated cyanidin derivative.
Table 15. Effects of fruit cluster shading on the percentages of individual anthocyanins in berry skin.\(^z\)

<table>
<thead>
<tr>
<th>Treatment Cluster shading</th>
<th>Dp-3-g %</th>
<th>Cy-3-g %</th>
<th>Pt-3-g %</th>
<th>Pn-3-g %</th>
<th>Mv-3-g %</th>
<th>Acylated Cy %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full Sun (Control)</td>
<td>13.5ab</td>
<td>73.5a</td>
<td>1.3a</td>
<td>3.3b</td>
<td>0.5b</td>
<td>7.9b</td>
</tr>
<tr>
<td>55% Shade</td>
<td>15.5a</td>
<td>71.2a</td>
<td>1.6a</td>
<td>3.8b</td>
<td>0.6b</td>
<td>7.4b</td>
</tr>
<tr>
<td>95% Shade</td>
<td>11.3b</td>
<td>66.9b</td>
<td>1.6a</td>
<td>5.4a</td>
<td>0.8a</td>
<td>14.2a</td>
</tr>
<tr>
<td>LSD</td>
<td>3.0</td>
<td>4.3</td>
<td>0.5</td>
<td>1.0</td>
<td>0.2</td>
<td>2.9</td>
</tr>
<tr>
<td>Linear Contrast</td>
<td>NS</td>
<td>**</td>
<td>NS</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Quadratic Contrast</td>
<td>**</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
</tr>
</tbody>
</table>

\(^z\) Mean separation by least significant difference (LSD) at 0.05 level.

**NS** Significant at 0.05 or 0.01 level, nonsignificant at 0.05 level respectively.

Abbreviations: Dp-3-g, delphinidin-3-glucoside; Cy-3-g, cyanidin-3-glucoside; Pt-3-g, petunidin-3-glucoside; Pn-3-g, peonidin-3-glucoside; Mv-3-g, malvidin-3-glucoside; Acylated Cy, acylated cyanidin derivative.
References


CHAPTER IV

EFFECTS OF LEAF AREA TO CLUSTER WEIGHT RATIOS ON FRUIT RED COLOR, TOTAL ANTHOCYANIN CONTENT, AND THE CONTENT OF INDIVIDUAL ANTHOCYANINS IN RELIANCE GRAPES (Vitis hybrid)

Abstract

Fruit color parameters including fruit red color characterized as CIE 1976 a*, total anthocyanin content, and the content of individual anthocyanins in response to leaf area to cluster weight ratios (LACWRs, cm²/g), were investigated using four-year-old Reliance grapevines (Vitis hybrid) trained to the Single Curtain Cordon System. Non-linear parametric models were used to describe the relationship between LACWRs and fruit color parameters. The critical LACWR levels required for the maximum fruit red color (CIE 1976 a*), total anthocyanin content, delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, malvidin-3-glucoside, and acylated cyanidin derivative, were 7.85, 8.12, 7.86, 8.20, 8.52, 8.52, 9.25, and 8.03, respectively. Fruit red color characterized by CIE 1976 a* increased following the linear equation, \( Y = -11.764 + 2.4782X \), when LACWRs were \( \leq 7.85 \), and stayed at 7.69 when LACWRs increased from 7.85 to 12.80. Total anthocyanin content in the berry skin (mg/g of dried berry skin) increased
following the linear equation, $\ln(Y+0.00201) = -5.0488 + 0.7205X$, when LACWRs were $\leq 8.12$, and stayed at 2.23 mg/g when LACWRs increased from 8.12 to 12.80. The content of delphinidin-3-glucoside increased following the linear equation, $\ln(Y+0.001325) = -7.8247 + 0.8420X$, when LACWRs were $\leq 7.86$, and stayed at 0.30 mg/g when LACWRs increased from 7.86 to 12.80. The content of cyanidin-3-glucoside increased following the linear equation, $\ln(Y+0.00155) = -5.3000 + 0.6933X$, when LACWRs were $\leq 8.20$, and stayed at 1.47 mg/g when LACWRs increased from 8.20 to 12.0. The content of petunidin-3-glucoside increased following the linear curve, $\ln(Y+0.002904) = -6.9390 + 0.4176X$, when LACWRs were $\leq 8.52$, and stayed at 0.03 mg/g when LACWRs increased from 8.52 to 12.80. The content of peonidin-3-glucoside increased following the linear curve, $\ln(Y+0.001795) = -7.3490 + 0.6013X$, when LACWRs were $\leq 8.52$, and stayed at 0.11 mg/g when LACWRs increased from 8.52 to 12.8. The content of malvidin-3-glucoside increased following the linear curve, $\ln(Y+0.001322) = -7.5932 + 0.3597X$, when LACWRs were $\leq 9.25$, and stayed 0.01 when LACWRs increased from 9.25 to 12.8. The content of acylated cyanidin derivative increased following the linear curve, $\ln(Y+0.001895) = -6.0721 + 0.5792$, when LACWRs were $\leq 8.03$, and stayed at 0.24 mg/g when LACWRs increased from 8.03 to 12.8.
Introduction

Fruit color is one of the most important quality attributes in table and wine grapes. Partial defoliation (Koblet, 1987; Koblet, 1988; Marquis et al., 1989), was used in a search for effective vineyard practices in improving fruit color. A more recent study on the effects of partial defoliation on berry skin color showed that anthocyanin content tended to be higher following partial defoliation and tended to be higher the later partial defoliation was applied (Hunter et al. 1991). Furthermore, partial defoliation affected growth (Hunter and Visser, 1990a and 1990b), microclimate (Smart 1980; Smart et al., 1985), photosynthetic activity (Candolfi and Koblet, 1991), and fruit quality (Hunter et al, 1991), beside altering leaf area to fruit weight ratio.

In order to gain a better understanding of defoliation effects on grapevines, it was essential to separate changes in leaf area to fruit weight ratio (LACWR) from microclimate, i.e., light. This study is the first attempt to use single shoots with single clusters on field grown vines as experimental units and whole vines as replicates. These shoots were isolated in terms of photosynthate translocation by girdling the canes above each respective shoot. Different LACWRs were achieved by first adjusting the cluster number to one per shoot, then adjusting leaf numbers to selected levels. The shoots surrounding treated shoots and clusters were manipulated to minimize shading. This approach is also superior to controlled greenhouse
experiments because artificial temperature and light conditions can be avoided.

It is well known that pigments responsible for the attractive red, blue, and black color in grape, are anthocyanins, a class of water soluble flavonoid pigments (Ribereau-Gayon, 1982; Van Buren et al.; 1970). Furthermore, the anthocyanin profile in a grape cultivar can be very complex (Hebrero et al., 1989; Ribereau-Gayon, 1982; Wulf and Nagel, 1976; Van Buren et al., 1970;). High performance liquid chromatography (HPLC) analyses of grape anthocyanin profiles revealed that a grape cultivar could have up to 20 different anthocyanins (Hebrero et al., 1989; Wulf and Nagel, 1976). Detailed studies of individual anthocyanins in a given grape cultivar can lead to a much better understanding of anthocyanin metabolism in pigmented grapes cultivars. The detailed studies on individual anthocyanins affected by climate and cultivars (Cacho et al., 1992) or clones of grape cultivars (Roggero and Ragonnet, 1986) have provided very enlightening results on anthocyanin metabolism of field grown grapes.

Reliance grape was used in the study due to its economic importance in Ohio (Cahoon, 1983), great fruit color variation at maturity, and relatively simple anthocyanin profile (Chapter 1). Results from the studies of fruit color development in Reliance grapes showed that cluster thinning increased pigment concentration where shading or defoliation decreased pigment concentration (Chapter II; Chapter III; Gao and Cahoon, 1991; Gao and Cahoon, 1992).
The objective of this study was to investigate the relationship between LACWRs and red fruit color development in Reliance grapes. The LACWRs were adjusted without altering light conditions. Changes of fruit red color, total anthocyanin content, and the content of individual anthocyanins, in relation to LACWRs, were investigated to determine the relationship among LACWRs and red fruit color parameters including fruit red color, total anthocyanin content, and the content of individual anthocyanins.

**Materials and Methods**

**Grapevines and treatments:** Reliance grapevines in the experiment were planted in 1987 at the Overlook Research Branch, The Ohio Agricultural Research and Development Center (OARDC), The Ohio State University, Carroll, Ohio, U.S.A. Grapevines were trained to the Single Curtain Cordon System. Row and vine spacings were 3.05 m x 2.44 m. Grapevines were pruned to 8 five-bud canes per vine. The remaining canes were spur pruned. In the spring, from these 8 five-bud canes per vine, 5 canes of similar vigor were chosen and randomized. Two weeks after full bloom, two shoots with at least 15 leaves and a similar cluster size, were selected from each five-bud cane. The remaining three shoots on these 5-bud canes were removed. Treated shoots were thinned to one cluster per shoot. Each cane was girdled between the first and second shoots (nodes). Leaf number per shoot was adjusted to 15, 12, 9, 6, and 3. Treated shoots and their clusters were all well
exposed to sun by positioning and tying the surrounding shoots to minimize the
shading effect. The leaf area to cluster weight ratio was calculated based on the final
total leaf area to cluster weight at harvest. Shoot number and cluster number of each
vine were all adjusted to 40 when berries were about 2-3 mm in diameter since
spurred canes produced a few shoots and clusters. The experimental design was a
randomized complete block with 10 single vine replicates. **LACWR** was calculated
as total leaf area (cm²) divided by total cluster weight (g).

**Fruit red color:** Refer to Chapter II fruit red color measurement.

**Sample preparation for anthocyanin analysis:** Fruit cluster samples were collected
on August 18, 1991, brought to OARDC, The Ohio State University, Wooster, Ohio,
and stored at 4°C immediately upon arrival. Refer to Chapter II for
sample preparation.

**Anthocyanin extraction and concentration:** Refer to Chapter II for anthocyanin
extraction and concentration.

**HPLC analyses:** Refer to Chapter II for HPLC analyses.

**Determination of anthocyanin content:** Refer to Chapter II for determination of
anthocyanin content.
Statistical analysis: Regression models for fruit red color, total anthocyanin, and the content of individual anthocyanins in relation to LACWRs were calculated based on non-linear parametric procedures (SAS, 1986). Data transformation was performed on the total anthocyanin content and the content of individual anthocyanins. Half of the minimum individual anthocyanin content was added to the total and individual content so that zeros in data set did not cause any problem in natural logarithmic transformation.

Results

Fruit red color: Fruit red color characterized by CIE 1976 a* increased following the linear equation, $Y = -11.764 + 2.4782X$, when LACWR increased from 0.02 to 7.85 (Figure 10). Fruit red color reached its maximum when LACWR was 7.85. Then fruit red color stayed at 7.69 when LACWR increased from 7.85 to 12.80.

Total anthocyanin content: Total anthocyanin content in the berry skin (mg/g of dried berry skin) increased following the linear curve equation, $\ln(Y + 0.00201) = -5.0488 + 0.7205X$, when LACWR increased from 0.02 to 8.12 (Figure 11). Total anthocyanin content in the berry skin reached its maximum when LACWR was 8.12. Then total anthocyanin content in the berry skin stayed 2.23 mg/g when LACWR increased from 8.12 to 12.80.
Delphinidin-3-glucoside: In addition to the changes in fruit visual color and total anthocyanin content, individual anthocyanins showed similar trends. The content of delphinidin-3-glucoside increased following the linear equation, \( \ln(Y+0.001325) = -7.8247 + 0.8420X \), when LACWR increased from 0.02 to 7.86 (Figure 12). The content of delphinidin-3-glucoside reached its maximum when LACWR was 7.86. The content of delphinidin-3-glucoside stayed at 0.30 mg/g when LACWR increased from 7.86 to 12.80.

Cyanidin-3-glucoside: The content of cyanidin-3-glucoside increased following the linear equation, \( \ln(Y+0.00155) = -5.3000 + 0.6933X \), when LACWR increased from 0.02 to 8.20 (Figure 13). The content of cyanidin-3-glucoside reached its maximum when LACWR was 8.20. The content cyanidin-3-glucoside stayed at 1.47 mg/g when leaf area to LACWR increased from 8.20 to 12.0.

Petunidin-3-glucoside: The content of petunidin-3-glucoside increased following the linear curve, \( \ln(Y+0.002904) = -6.9390 + 0.4176X \), when LACWR increased from 0.02 to 8.52 (Figure 14). The content of petunidin-3-glucoside reached its maximum 0.03 mg/g when LACWR was 8.52. Then it stayed at 0.03 when LACWR increased from 8.52 to 12.80.

Peonidin-3-glucoside: The content of peonidin-3-glucoside increased following the linear curve, \( \ln(Y+0.001795) = -7.3490 + 0.6013X \), when LACWR increased from
0.02 to 8.52 (Figure 14). The content of peonidin-3-glucoside reached its maximum 0.11 mg/g when LACWR was 8.52. Then it stayed at 0.11 mg/g when LACWR increased from 8.52 to 12.8.

**Malvidin-3-glucoside:** The content of malvidin-3-glucoside increased following the linear curve, \( \ln(Y+0.001322)=-7.5932+0.3597X \), when LACWR increased from 0.02 to 9.25 (Figure 15). The content of malvidin-3-glucoside reached its maximum 0.01 mg/g when LACWR reached 9.25. Then it stayed at 0.01 mg/g when LACWR increased from 9.25 to 12.8.

**Acylated cyanidin derivative:** The content of acylated cyanidin derivative increased following the linear curve, \( \ln(Y+0.001895)=-6.0721+0.5792 \), when LACWR increased from 0.02 to 8.03 (Figure 17). The content of acylated cyanidin derivative reached its maximum 0.24 mg/g when LACWR was 8.03. Then it stayed at 0.24 mg/g when LACWR increased from 8.03 to 12.8.

**Discussion**

The critical LACWR level required for maximum fruit red color (CIE 1976 a*) was determined to be 7.85 cm²/g. Average Reliance leaf area in this study was 202.3 cm² where average cluster wight was 286.5 g per cluster. Based on these data, a LACWR of 7.85 cm²/g correspond to 11 leaves per cluster. Cross and Webster
(1935) found that 10 or more leaves were required for the best fruit color development in Concord grapes. LACWRs required to mature *Vitis vinifera* grapes were reported to be from 7 to 15 cm$^2$/g (Kliwer, 1970b; Kliwer and Antcliff, 1970; Kliwer and Weaver, 1971; May et al., 1969). Kaps and Cahoon (1992) found that LACWR of 8 to 10 cm$^2$/g was required to mature French-American hybrid grapes from container-grown Seyval blanc grapevines.

Anthocyanins in De Chaunc grapes (*Vitis* hybrid) were shown to be in the vacuoles of subepidermal cells (Moskowitz and Hrazdina, 1981) and vacuolar pH was determined to be 2.7. If vacuolar pH of epidermal cells in Reliance grape was similar to that of De Chaunac grapes, it can be predicted that colors of individual anthocyanins in Reliance would be similar to that which appeared on paper chromatogram under acidic conditions. On paper chromatograms under acidic conditions, Harborne (1984) showed that delphinidin-3-glucoside was mauve colored; cyanidin-3-glucoside, red colored; petunidin-3-glucoside, mauve colored; peonidin-3-glucoside, red colored; malvidin-3-glucoside, mauve colored; and acylated cyanidin derivative, red colored. Although the pH in the vacuoles of subepidermal cells from Reliance grape skins was not determined, it can be assumed that pH in Reliance would be similar to that of De Chaunac. Juice pH is not a good indicator for predicting fruit color since anthocyanins in Reliance grapes are only present in berry skins. Based on the relative composition of Reliance anthocyanins and their predicted color at physiological pH, it can be predicated that Reliance fruit will be
red with purplish tint. Reliance is indeed a dark and purplish red colored grape.

Based on critical LACWR levels for fruit red color and evolution of individual anthocyanins, the importance of individual anthocyanins to fruit color in decreasing order will be delphinidin-3-glucoside (7.86), acylated cyanidin derivative (8.03), cyanidin-3-glucoside (8.20), petunidin-3-glucoside (8.52), peonidin-3-glucoside (8.52), and malvidin-3-glucoside (9.25).

The method used in the experiment to create differences in LACWR was unique because treated shoots with single clusters were isolated in terms of carbohydrate translocation with cane girdling above the respective shoots. This approach permitted an accurate study of the relationship between LACWR and fruit color on mature vines in the field. Possible compounding effects, i.e. carbohydrate translocation among shoots on each vine (Stoev and Ivantochev, 1977), were minimized. The results obtained from greenhouse studies may not be directly representative of that from field grown vines. For example, a leaf from Reliance vines grown in a greenhouse can grow to be four to five times larger than a leaf from field grown Reliance vines (Gao and Cahoon, personal observation). Furthermore, leaf area to cluster weight ratio determined in the field could be larger than that determined in greenhouse since shaded and nonproductive leaves could have been added to the total leaf area in the field study. Shoots around the treated clusters were manipulated so that all clusters received full sun light. Furthermore, single vines served as replicates so that differences in vine vigor had minimal effects.
Fruit Red Color (CIE a*)

Figure 9. The relationship between the leaf area (cm²) to cluster weight (g) ratio and fruit red visual color CIE 1976 a*.
Total Anthocyanin Content

\[
\ln(Y+0.00201) = -5.0488 + 0.7205X \quad \text{When } X \leq 8.12
\]

\[
\ln(Y+0.00201) = 0.8015 \quad \text{When } X > 8.12
\]

Figure 10. The relationship between the leaf area (cm\(^2\)) to cluster weight (g) ratio and total anthocyanin content in berry skin (mg/g of dried berry skin).
Delphinidin-3-Glucoside

Figure 11. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of delphinidin-3-glucoside in berry skin (mg/g of dried skin).
Figure 12. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of cyanidin-3-glucoside (mg/g of dried berry skin).
Figure 13. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of petunidin-3-glucoside (mg/g dried berry skin).
Peonidin-3-Glucoside

Figure 14. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of peonidin-3-glucoside in berry skin (mg/g of dried skin).
Figure 15. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of malvidin-3-glucoside fruit (mg/g of dried berry skin).
Leaf Area to Cluster Weight Ratio

Figure 16. The relationship between the leaf area (cm²) to cluster weight (g) ratio and the content of acylated cyanidin derivative (mg/g dried berry skin).
References


GENERAL DISCUSSION

Fruit color in Reliance grapes was significantly improved by controlling crop level with cluster thinning to 20 clusters per vine (Chapter I), and increasing leaf area to cluster weight ratio (Chapter III). Fruit color failed to reach its full potential when fruit clusters were shaded to 95% of full sun (Chapter II). In order to produce well colored Reliance grapes of premium quality, the following three cultural practices would be suggested.

First of all, a good dormant pruning is an absolute necessity to limit the number of cane buds per vine. Morris et al. (1984) showed that fruit color in Concord was decreased by leaving 70 buds for the first 0.454 kg (one pound) of pruning weight, and 10 more buds for each additional 0.454 kg (one pound) of pruning in comparison to 30 or 50 for the first 0.454 kg (one pound) of pruning weight, and 10 more buds for each additional 0.454 kg (one pound). In Niagara grapes, Mooris et al. (1985) showed that fruit anthocyanin content was decreased about 15% by leaving 50 or 70 buds for the first 0.454 kg (one pound) of pruning weight, and 10 more buds for each additional 0.454 kg (one pound) of pruning weight in comparison to 30 buds for first the 0.454 kg (one pound) of pruning weight, and 10
more buds for each additional 0.454 kg (one pound) of pruning weight. Thus dormant pruning reduces the number of clusters per vine and would be expected to improve color in a manner similar to cluster thinning.

Dormant pruning \((30+10)\) of Reliance grapevines based on leaving 30 buds for the first 0.454 kg of pruning weight, and 10 more buds for each additional 0.454 kg (one pound) of pruning weight in combination with cluster thinning to one cluster per shoot (Cahoon, 1983) has produced Reliance fruits with adequate maturity characterized by soluble solids. With this pruning system, Reliance grapes trained to the Geneva Double Curtain system, from third to eighth year, had an average soluble solids of 17.8%. The same dormant pruning system was used on Reliance grapevines trained to the Single Curtain Cordon System. Dormant pruning alone was not enough to ensure good color in Reliance grapes since the pruning system was developed for Concord grapes (Shaulis, 1966). However, in some years, about 50% to 60% poorly colored fruits were observed in Reliance (Gao and Cahoon, personal observation; Gao and Cahoon, 1989, 1991, and 1992). Hence, more severe cluster thinning is necessary in addition to dormant pruning and one cluster per shoot in Reliance.

Second, an optimum crop level needs to be established with fruit cluster thinning. Twenty clusters per vine in Wooster, Ohio in 1991, increased fruit total anthocyanin content by 36% in comparison to 60 clusters per vine, and by 23% in comparison to 40 clusters per vine (Chapter I). Reynolds (1989) showed that total
fruit weight below 7 kg per kg of pruning weight was necessary to maintain vine size, yield, and acceptable composition of low-vigor De Chaunac vines. The fruit weight per kg of pruning was not determined because a pruning study was not conducted. However, data from Chapter IV indicate that eleven leaves are needed to produce a well colored 0.28 kg Reliance cluster. Based on this number, a preliminary pruning scheme can be determined. Multi-year cluster thinning and dormant pruning studies are needed before an accurate recommendation can be made for Reliance growers. However, based on the cost estimate provided in Chapter II, Reliance grape growers would be able to profitably produce Reliance grapes with either 20 or 40 clusters per vine. The crop level of 60 clusters per vine in Reliance grapes, trained to the Single Curtain Cordon System, resulted in some vine loss due to winter injury (Gao and Cahoon, personal observation).

Third, improvement of light conditions around the fruiting zone and leaf canopy can be an effective tool to improve fruit color. Fruit anthocyanin content in Reliance grape was decreased by 80% (Chapter III) with 95% cluster shading in comparison with full sun control. If Reliance fruit clusters receive between 5% to 45% of full sunlight, shoot positioning or leaf removal can improve fruit color by ensuring that the sunlight level around the fruiting zone is at least 45% of full sunlight. Cultural practices such as cane girdling, foliar application of ethephon, chelated magnesium, or shoot positioning, were only effective in increasing fruit color with moderate Reliance crop levels and were not effective at high crop levels (Gao et
al., 1989; Gao and Cahoon, unpublished results).

The leaf area to cluster weight ratio required for the maximum fruit color in Reliance grape was determined to be 7.85 cm² per gram of fruit (Chapter IV). No fruit red color improvement was found when the leaf area to cluster weight ratio increased from 7.85 to 12.8 cm² per gram of fruit. The critical leaf area to cluster ratio for anthocyanin formation was 8.12 cm² per gram of fruit (Chapter IV). The average cluster weight of Reliance cluster is about 280 gram. The average area of Reliance leaves from field grown-vines is about 202 cm². Based on these three numbers, it can be calculated that it takes about 11 leaves to mature a Reliance cluster with the best color. In apples, Wertheim (1987) found that fruit size in Jonagold increases with increasing number of leaves per fruit up to 220 leaves, while anthocyanin formation increases significantly only up to 45 leaves per fruit. The relation between leaf area to fruit weight ratio and fruit color in Reliance and Jonagold apple is very similar.

The relationship between cluster weight and cluster thinning was discussed in Chapter II. Insignificant differences in cluster weight could have been due to dry weather and variation in berry number. However, the excessive crop thinning could result in the promotion of shoot growth and alter the source and sink relationship so much that fruits would not be able to compete for photosynthate with young shoots. *Fruit trees and vines have a way of maintaining the physiological equilibrium*. The
balance of fruiting to vegetative growth has to be maintained. An excellent example of physiological or functional equilibrium in apples is 'June drop' and 'preharvest drop' of fruits (Luckwell, 1949 and 1953), natural ways of crop adjustment.

This study is the first of its kind to investigate the individual anthocyanins of berry skin with C18 reverse-phase HPLC in a red seedless grape cultivar as affected by various cultural practices or environmental factors under controlled studies. Total anthocyanin and individual anthocyanins in Reliance were affected by cluster thinning, cluster shading, and leaf area to cluster weight ratio. Furthermore, the percentages of some anthocyanins were increased and others were decreased. As discussed in Chapter IV, fruit color can be predicted to certain degrees based on the color of separated anthocyanins present on paper chromatogram under acidic conditions. The grape cultivar Tokay, which has only cyanidin-3-glucoside in its berry skin (Akiyoski et al., 1963), is a red-colored grape. Concord, which has mainly delphinidin, malvidin, and peonidin glycosides (Hrazdina, 1975), is a blue-colored grape. Heritage raspberry, which has mainly cyanidin-3-glucoside and cyanidin-3,5-diglucoside (Francis, 1972), is a deeply red-colored raspberry cultivar. Results from Chapters II and III suggest that the percentages of individual anthocyanins in a grape can be affected quantitatively by cultural means and environmental factors. Further, the color of anthocyanins can be affected by not only by the nature of anthocyanins, but also by medium pH, copigmentation, and metal chelation (Brouillard, 1982; Osawa, 1982; Markakis, 1982).
Studies with stability of different anthocyanins in wines showed that acylated anthocyanins were more stable than non-acylated anthocyanins (Van Buren et al., 1968); Anthocyanidin diglucosides are more stable to decolorization than the corresponding monoglucosides, but the latter were less prone to browning (Robinson et al., 1965). Malvidin and peonidin were most stable where delphinidin was least stable (Robinson et al., 1965). Among diglucosides, malvidin-3,5-diglucoside was the most stable followed by peonidin, petunidin, cyanidin, and delphinidin-3,5-diglucosides (Hrazdina et al., 1970). However, it is not known whether the stability of anthocyanins in vivo would resemble that in wine or solution. If it does, it would be beneficial for grape breeders to breed grapes with more 'stable' anthocyanins so that the 'perfectly colored grape cultivar' can be obtained.

C18 reverse-phase HPLC permits the separation and quantification of individual anthocyanins in grapes or wines (Roggero et al., 1986; Hebrero et al., 1988 and 1989; Wulf and Nagel, 1976; Cacho et al. 1992), avoiding some of the problems inherent to the spectrophotometric techniques. Bakker et al. (1986) found that HPLC method offers a true measurement of the anthocyanin content in red wines while the bisulfite bleaching method gives artificially higher results, attributed to the partial bleaching of polymeric pigments by bisulfite. Rivas-Gonzalo et al., 1992 found that after passing wine anthocyanin through a Polyclar AT column to remove polymeric anthocyanins, the anthocyanin content determined with HPLC, pH shift methods and bisulfite bleaching, were very close. When studying fruit anthocyanin biosynthesis in
grapes or wines, C\textsubscript{18} reverse-phase HPLC is the method of choice.

In future studies, finer divisions in cluster number per vine, i.e. 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 should be employed, to determine the maximum crop load with the best color possible. The relationship between vine size and fruiting level would also need to be taken into consideration. In terms of shading studies, lights of specific wavelength with the same intensity should be used to determine how the light quality affects fruit color development. The individual anthocyanins and enzymes involved in the biosynthesis of a given pigmented grape cultivar, i.e. Reliance, need to be studied in greater detail. With a better understanding of fruit color development, we can improve fruit color more effectively by the manipulation of light intensity or light quality, cluster thinning, or other cultural means. Much more research is needed in order to completely understand anthocyanin metabolism in grape before manipulation of fruit color can be fully achieved at will.
References


LIST OF REFERENCES


