Cultivar, juice extraction, ultra violet irradiation and storage influence the stilbene content of muscadine grapes (Vitis rotundifolia Michx.)

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CULTIVAR, JUICE EXTRACTION, ULTRA VIOLET IRRADIATION AND STORAGE INFLUENCE THE STILBENE CONTENT OF MUSCADINE GRAPE (Vitis rotundifolia Michx.)

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Horticulture

by

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B. S., Louisiana State University, 1992
M.S., Louisiana State University, 1996
May 2006
ACKNOWLEDGMENTS

I would like to thank everyone who assisted me in my pursuit of this degree. Specifically, I would like to thank Dr. Charles Johnson, my advisor, for his advise and encouragement through this long process. I would like to thank my committee, Dr. Paul Wilson, Dr. Witoon Prinyawiwatkul, Dr. Jeff Kuehny and Dr. David Himelrick for their support through my studies. I would also like to thank Dr. Steve Stringer of the USDA Small Fruit Crop Laboratory in Poplarville, MS, for providing fruit and for his help with collecting samples.

I would especially like to thank Gloria McClure for her advise, counseling and the countless hours she spent helping me with HPLC analysis. Much of the work done in pursuit of this degree would have been impossible without her assistance.

Most importantly, I would like to thank my wife, Ellen and my children. I have been blessed with a wife with the patience of a saint and four wonderful children, Madeline, Emma, Luke and Vianne. It has been Ellen’s hard work and loving care of our children that has made it possible for me to pursue this degree. Arriving home each day to their love and support has given me the determination to complete my studies.

Finally, I want to thank my parents, Eugene and Rachel LeBlanc. It was their years of sacrifice and determination that made it possible for me and my ten brothers and sisters to get college educations. I will never be able to thank you enough.
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ABSTRACT

Stilbene concentration was analyzed in juice and tissue of muscadine grape (Vitis rotundifolia Michx.) and bunch grape (Vitis labrusca L.) from fresh fruit, after processing and after postharvest treatments. Five filter types were evaluated for percent resveratrol recovery when filtering standard and spiked juice samples in preparation for HPLC analysis. Only two (polycarbonate and Anopore) of the five filter types had more than 90 percent recovery. Polycarbonate was chosen for sample preparation since it was more durable during handling. Eight muscadine grape cultivars and three bunch grape cultivars were evaluated. Skin tissue had approximately 100 times higher stilbene concentration than did the pulp for all cultivars studied. ‘Carlos’ and ‘Magnolia’ muscadine cultivars had the greatest skin stilbene concentration of all the muscadine cultivars evaluated. Except for ‘Sweet Jenny’, bronze cultivars had greater skin stilbene concentration than black skinned cultivars. ‘Miss Blanc’ Vitis labrusca grape had greater skin stilbene concentration than all other cultivars. Stilbene concentration of fresh juice extracted from ‘Noble’ and ‘Carlos’ muscadine grapes was relatively low compared to processed juices. Juices obtained using hot press and freezing methods of juices extraction had significantly higher stilbene concentration than free run or cold pressed juice. Although ‘Carlos’ skin tissue had significantly more stilbenes than ‘Noble’ skins, there were no significant differences between free run, cold press or hot press juices obtained from the two cultivars. Although pectic enzyme treatment significantly increased juice yields, stilbene concentrations were not significantly higher than other juice extraction methods. In contrast to ‘Carlos’ muscadine grape, where high skin stilbene concentration did not result in high juice concentration, Vitis labrusca grape juices had
relatively high stilbene concentration when compared to muscadine juices. UV irradiation
and cold storage had a significant effect on stilbene concentration of muscadine grape tissue.
For ‘Carlos’ muscadine grape, cold storage alone doubled skin stilbene concentration, but UV
irradiation did not significantly change stilbene levels. In contrast, in ‘Noble’ muscadine
grape, UV irradiation increased skin stilbene concentration by 50%, but cold storage alone had
no effect.
CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

In the late part of the 20th century the advancement of knowledge regarding nutrition and disease prevention provided an opportunity for individuals to affect their own health. This expanding body of information helped people understand how the environment and their own behavior affected their body. People now had powerful tools to help maintain and protect their health. The understanding of how our diet affects our well being has dramatically changed the lifestyles and attitudes of the public. People began to make menu and purchasing decisions based on how foods would affect their health. A movement toward healthier lifestyles and healthier diets began. Food processors and marketers had to refocus their efforts from promoting foods for pleasure to promoting foods that fit in to a healthy diet.

Primarily, the focus was on reducing fat and cholesterol in the diet and supplementing vitamins and minerals. Research began to demonstrate the presence of various phytochemicals in fruits and vegetables. These phytochemicals have come to be known as nutraceuticals. The list of nutraceuticals present in fruit and vegetables that are believed to have positive biological properties has been expanding. It has become clear that the presence of vitamins and minerals in fruits and vegetables was only part of the beneficial aspects of consuming them. Food processors and developers have become very interested in exploiting these nutraceuticals for the production of foods that are not only part of a healthy diet but also improve the consumers health in another specific way. These foods have become known as “functional foods”. The primary focus of this work was on examining the concentration of a group of these nutraceuticals called stilbenes in muscadine grapes (Vitis rotundifolia Michx.).
LITERATURE REVIEW

**Resveratrol.** Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenolic compound classified as a stilbene. It is synthesized from p-coumaroyl CoA and malonyl CoA by an enzyme called stilbene synthase. Its biosynthesis is stimulated by stress, including injury, infection or UV irradiation. It has been demonstrated to provide resistance in grapes to fungal diseases (Jeandet et al., 1995). It is synthesized almost exclusively in the skins of *V. vinifera* grapes but is also synthesized in the seeds of muscadine grapes (Jeandet et al., 1995; Ector et al., 1996). It exists in both trans and cis isomeric forms. The cis form is rarely found in

![Chemical structures of trans-Resveratrol and cis-Resveratrol](image1.png)

**Figure 1.1** Chemical structures.
grapes in significant concentration, but it is found in substantial amounts in wines (Jeandet et al., 1995; Mattivi et al., 1995; Lamuela-Raventos et al., 1995). The reaction producing resveratrol in the plant is very similar to another reaction using the same products catalyzed by a similar enzyme, chalcone synthase (Goodwin et al., 2000). Chalcone synthase combines p-coumaroyl CoA and malonyl CoA to form chalcones including naringenin and eriodictyol which go on to form flavonoids which are responsible for the anthocyanins. Anthocyanins are a class of compounds that include the pigments in grapes (Croteau et al., 2000). Developmental studies have demonstrated that resveratrol concentration decreases with veraison, (pigment formation) in the grape (Jeandet et al., 1991; Strigler et al., 2005). It is suggested that the formation of chalcones to produce anthocyanins may come at the expense of resveratrol production by stilbene synthase.

Resveratrol also exists in a glucoside form called piceid (5,4' dihydroxy-3-glucopyranosylstilbene). The two compounds are from a class of secondary metabolites called stilbenes. Studies have shown that piceid can exist in large amounts, sometimes exceeding resveratrol, in both wine and grapes (Matti et al., 1995; Romero-Perez et al., 1996a, 1996b; Romero-Perez et al., 2001). During fermentation there can be complex changes in the concentrations of the four monomers of resveratrol and piceid (Matti et al., 1995; Lamuela-Raventos et al., 1997; Romero-Perez et al., 1999). Many early studies only quantified trans resveratrol and sometimes the cis isomer. With the quantification of all four monomers, a more accurate representation of the beneficial properties of a wine or juice can be obtained.
Health Benefits. During the 1980's, researchers looking into World Health Organization data from the United States and Europe noted an anomaly with regard to diet and mortality from heart disease (NRC, 1989). Data from the United States showed that generally with increased consumption of fatty foods there was a similar increase in the rate of coronary heart disease (CHD). Data from areas in France did not follow this pattern. In some areas where diets were traditionally high in fat, there were not similar elevations in CHD. This phenomenon has become known as “the French Paradox”. In 1992, Renaud and Lorgeril demonstrated that wine consumption was statistically the only factor correlated to the reduction in CHD. Subsequent data suggested that somehow wine consumption resulted in a larger reduction in CHD than did the consumption of beer and spirits. It was proposed that, although alcohol was a factor, there were other components in wine that were providing the protection.

Prior to these investigations, a number of studies established that a phytoalexin called resveratrol (3,5,4’ trihydroxystilbene) was present in grape skins (Langcake and Pryce, 1976; Pool et al., 1981; Jeandet et al., 1991). Initially, these studies focused on resveratrol’s presence as a marker for disease resistance. Some demonstrated that resveratrol metabolism can be stimulated by plant pathogen infection and by exposure to ultraviolet light (Langcake and Price, 1976). Similarly, a group of scientists investigating a traditional Japanese folk remedy demonstrated that resveratrol was the primary active ingredient in a medicine composed of the dried powdered root of the Japanese knotweed (Polygonum cuspidatum Sieb. et Zucc.) (Arichi et al., 1982).
For more than two decades, scientists have been reporting the various ways that resveratrol can positively effect our health (Arichi et al., 1982; Kimura et al., 1985; Kinsella et al., 1993; Jang et al., 1997; Lu and Serrero, 1999; De Santi, et al., 2000a, 2000b; Brakenhielm et al., 2001; El-Mowafy, 2002). By the mid to late nineties, work was underway around the world to both quantify resveratrol in wine and grapes and to verify its mode of action as a protective agent for human health.

In 1985, Kimura et al. reported that resveratrol inhibited platelet aggregation in rats. Jang et al., reported in 1997 that resveratrol acted as an antioxidant and as an anti-mutagen. Resveratrol reduced tumor formation in rats and reduced initiation and promotion of human cancer cells. In a 1999 publication, Cheong et al. reported that resveratrol had anti-allergenic properties. Resveratrol inhibited the release of $\beta$-hexosaminidase from mast cells. $\beta$-hexosaminidase is released along with histamine in response to allergic reactions. Resveratrol has also been reported to inhibit the growth of human breast cancer cells by acting as a estrogen receptor antagonist (Lu and Serrero, 1999). Tedesco et al. (2000) studied the effect of red wine extract and resveratrol singularly on red blood cells. They reported that the red wine extract acted as a strong antioxidant and that resveratrol by itself did not have as strong an effect. They suggested that the effects of red wine may be associated with the combined effect of the components of the wine and not with the individual compounds. Huang et al. (1999), proposed that resveratrol reduced tumor growth by inducing apoptosis (programmed cell death).

Although there is a substantial amount of information about the effect of resveratrol in \textit{in vitro}, it is unclear how and how much of the compound is absorbed in the digestive tract.
Kuhnle et al. (2000) studied the absorption of resveratrol in rat intestines. They reported that only small amounts were absorbed, but larger amounts of a resveratrol glucuronide was absorbed through the intestine. The authors suggested that resveratrol was converted to the glucuronide during absorption and postulated that the resulting molecule could be cleaved back into resveratrol in various organs of the body. The presence of flavanoids in products containing resveratrol may improve its bio-availability. Two studies in 2000 suggested that flavanoids inhibit the sulphation and glucuronidation of resveratrol in the liver and therefore improve the bio-availability of the compound (De Santi et al., 2000a, 2000b).

Kimura and Okuda reported in a 2001 study that resveratrol inhibited tumor growth in mice and inhibited angiogenesis in human umbilical cells which suggests a mechanism for the reduction in tumor growth. Another 2001 study demonstrated that resveratrol suppressed angiogenesis and tumor growth, but also reduced wound healing in bovine and mouse cells. Resveratrol has also been shown to inhibit human squalene monooxygenase, an enzyme that is part of the cholesterol biosynthetic pathway (Laden and Porter, 2001). A 2002 (El-Mowafy, 2002) study reports that resveratrol has vascular relaxation properties. The authors suggest that resveratrol could have significant effects on vascular disorders such as atherosclerosis, chronic hyperlipidemia and diabetes.

Early Studies. In 1976 Langcake and Pryce published a paper demonstrating that resveratrol was produced by “Vitis vinifera ... as a response to infection or injury”. This work was focused exclusively on its effect on disease resistance. For the next 15 years, resveratrol was studied extensively in grapes (Langcake and Pryce, 1977; Creasy and Coffee, 1988; Derecks and Creasy, 1989; Jeandet et al., 1991).
In 1992, Siemann and Creasy published a paper demonstrating that resveratrol (sum of trans and cis) was present in finished wine. They sampled 22 wines and found resveratrol (HPLC with UV detector) concentrations ranging from below detection to 0.7 mg/L. In general, red wines had higher resveratrol levels than white wines although this was not always the case. The authors proposed that since resveratrol is produced almost exclusively in the skins, wines with longer skin contact during vinification (red wines) should have higher levels of resveratrol. Their data also suggested that growing region had an effect on resveratrol concentration. Chardonnays from New York had higher resveratrol concentrations than Chardonnays from California. The authors proposed that since resveratrol production is stimulated by fungal attack, regions with greater fungal pressure would produce grapes with higher resveratrol concentration.

In 1993, Jeandet et al. quantified resveratrol in Burgundy wines using gas chromatography and a mass spectrophotometer. Their results confirmed the findings of Siemann and Creasy. Resveratrol concentrations were higher in red wines than in white and they also found differences based on the growing conditions. Unlike Siemann and Creasy, they were able to quantify the trans and cis isomers of resveratrol, separately. They were surprised to find, since it had not been found in grapes, that the cis isomer was the predominant form in the wines. The authors suggested that exposure to sunlight during processing or reactions occurring during vinification converted the trans isomer to the cis. This study found slightly greater amounts of resveratrol than did Siemann and Creasy. They found levels from not detected to 0.06 mg/L for Chardonnay to 0.4 to 2.0 mg/L for Pinot Noir.
The previous two studies demonstrated conclusively that cis and trans resveratrol was present in finished wines. Although studies had concluded by then that resveratrol had biological activity (Frankel et al., 1993; Shan et al., 1990; Kimura et al., 1985; Kimura et al., 1983), there were still those who argued that the small amounts of resveratrol in wines were unlikely to have a meaningful effect on human health.

A 1994 study by Waterhouse and Lamuela-Raventos demonstrated that grape berries contained not only resveratrol but also contained a 3-beta-glucoside of resveratrol (piceid). This compound could be converted to resveratrol during vinification and could also provide for a greater biological effect from wine if it survives processing and is present in the finished wine. Two studies (Lamuela-Raventos et al., 1995a, 1995b; Romero-Perez et al., 1996a, 1996b) subsequently demonstrated that piceid was present in wine. The 1995 article reports that resveratrol and piceid (stilbenes) were present in wines in proportions that agree with previous studies (Lamuela-Raventos and Waterhouse, 1993; McMurtey et al., 1997; Soleas et al., 1995). They report that Pinot Noir wines generally have the highest levels of stilbenes (9.39 mg/L) followed by Merlot (9.19 mg/L), Grenache (6.37 mg/L), Cabernet Sauvignon (3.23 mg/L) and Tempranillo (3.43mg/L). Romero-Perez et al., in a 1996 study of resveratrol and piceid isomers in Spanish white wines, reports that isomers of both compounds are present in all samples and the levels range from 0.46 to 1.24 mg/L total stilbenes. This is consistent with previous reports of lower levels in white wines.

When considering trans and cis resveratrol and trans and cis piceid, the doses one would receive from a typical serving of wine is significantly greater than when only trans resveratrol was considered alone. Since glycosidase is known to be present in the digestive
tract, it is possible that piceid could be converted to resveratrol and absorbed during digestion (Hackett, 1986). Several studies have demonstrated that piceid is itself biologically active in animal systems (Shan et al., 1990; Kimura et al., 1995).

**Juices.** Waterhouse and Lamuela-Raventos demonstrated in 1994 that both piceid and resveratrol were present in the skins of *V. vinifera* grapes. In 1995, Soleas et al. found trans resveratrol in ten cultivars of grape juice. A 1999 study, by Romero-Perez et al., quantified resveratrol and piceid in a number of red and white Spanish grape juices. They reported that for most juices whether white or red, piceid was the predominant compound. Concentrations of resveratrol and piceid combined averaged 0.49 mg/L for white juices and 4.73 mg/L for red juices. The average concentration for piceid alone was 0.44 mg/L for whites and 4.11 mg/L for reds. The differences between white and red wines can be explained by the differences in skin contact during fermentation. The differences between the red and white juices, which were ten fold, can only be attributed to differences in juice processing (pressing, temperature), since previous work demonstrated that the resveratrol concentration of the skins of white and red grapes were similar (Okuda and Yokotsuka, 1996). The predominance of piceid in the juices and the reports of high resveratrol in wines suggests that there must be a process during fermentation and bottling that converts the piceid to resveratrol in wine. It also indicates that if piceid does have human biological activity, consumption of grape juices could provide some of the benefits that wine consumption has been shown to possess. The authors point out that the average resveratrol concentration of the wine-making juices was twice that of the commercial juices. These differences are unexplained, but may be attributed to varietal differences or the processing techniques used to produce the respective juices.
Factors Affecting Resveratrol. There has been a number of studies conducted on the effects of various factors on the concentration of resveratrol in wine. Several of these were centered on the type and time of fermentation. Jeandet et al., in 1995, studied the effect of maceration (exposure to skins during fermentation) on the resveratrol concentration of wines. Resveratrol concentration of wine increased with exposure to the skins. There was an approximate ten fold increase in resveratrol with skin exposure compared to the same wine made without exposure. Although the white wine prepared with maceration had much higher levels of resveratrol than without, these wines still had less than half the resveratrol of red wines with maceration. These data indicate for the cultivars used in this study (‘Pinot Noir’ (red) and ‘Chardonnay’(white)) that there is still a significant effect of skin color on resveratrol concentration. These differences may be associated with these cultivars and not with the overall color of the grapes. This study also investigated the effect of botrytis infection on resveratrol concentration of wines. Previous studies demonstrated that fungal infection stimulates production of resveratrol in grapes (Langcake and Pryce, 1976), therefore, it would be expected that with highly infected fruit there would be higher resveratrol levels in the wine. The results of this study demonstrated that resveratrol levels decrease with high levels of botrytis infection. It is suggested by the authors that a fungal exo-cellular enzyme may aid in degrading resveratrol after infection. Such an enzyme would lower resveratrol concentration in the grapes and would also be active in the wine must, lowering resveratrol in the finished wine. Although high resveratrol levels were found in wine with no infection, the highest levels of resveratrol were in wines made with grapes with approximately 10% infection. These grapes would have benefitted from a limited fungal attack that would stimulate resveratrol
metabolism, but would lack the volume of fungal enzymes needed to degrade the compound. A 2000 study by Darias-Martin et al. also demonstrated a significant increase in resveratrol with fungal exposure to grape skins.

Another 1995 study investigated the evolution of both isomers of resveratrol and piceid during fermentation (Mattivi et al.). In this study, levels of trans and cis resveratrol and piceid were monitored during fermentation. In the initial must (crushed fruit), cis piceid was the predominant monomer followed by trans piceid and trans resveratrol. Cis resveratrol was not present in the initial must. During the first four days of fermentation, cis piceid declines while all other monomers increase. Trans resveratrol increases almost ten fold in the first four days while cis resveratrol increases from not detected to 3.4 mg/L. Initially, most of the increase of the monomers is attributed to extraction from the skins. But after day four, both trans and cis piceid decrease while trans and cis resveratrol increase. The authors suggest that there is either an acid catalyzed or enzymatic hydrolysis of the glucosides to either of the two isomers of resveratrol. The final wine contained predominantly trans resveratrol followed closely by cis resveratrol and with small amounts of cis piceid and trans piceid. Although hydrolysis is proposed as the primary source of the trans and cis resveratrol, isomerization is also probable since the isomers of resveratrol are less stable than the isomers of piceid. The authors suggest that the final concentration of trans resveratrol is more related to extraction from the grape than from hydrolysis of the glucosides, whereas the concentration of cis resveratrol in the final wine is likely exclusively produced by hydrolysis of the two glucosides.

A 1997 study by Lamuela-Raventos et al. investigated the evolution of the four monomers of resveratrol during fermentation of ‘Merlot’ and ‘Cabernet Sauvignon’ grapes.
The ‘Cabernet Sauvignon’ final wine had a similar distribution of the monomers as the Mattivi study, but there was an overall increase in all monomers during the fermentation. All four of the monomers increased during the entire fermentation for ‘Merlot’ as well, but the final ‘Merlot’ wine had a different distribution of the monomers. For ‘Merlot’, trans piceid was the predominant monomer followed by trans resveratrol, cis piceid and cis resveratrol. For the ‘Merlot’ fermentation, there appeared to be less hydrolysis of the glucosides than in the ‘Cabernet’ fermentations. For ‘Merlot’ there were only slight decreases in the glucosides at the end of the fermentation, but for ‘Cabernet’ trans piceid decreased dramatically during the second half of the fermentation. This corresponded with an increase in both trans and cis resveratrol.

A 2000 study (Baveresco et al.) investigated the effect of cluster stems on resveratrol concentration of simulated wines. Resveratrol was extracted from cluster stems with methanol and in a hydro-alcoholic solution designed to mimic wine. The study only looked for trans and cis resveratrol. Cis resveratrol was not detected. Three amounts of stems and four times of extraction were evaluated. The highest amount of stems (0.9 g/100ml) yielded the greatest amount of resveratrol for both the methanol (0.2 mg/L) and the hydro-alcoholic solution (1.4 mg/L). For the times of exposure (2, 3, 4 and 8 days), the greatest extraction was for the 4 day period for both the methanol extraction (0.2 mg/L) and for the hydro-alcoholic solution (1.2 mg/L). There was a reduction in resveratrol for both extractions from 4 to 8 days. The authors suggested that oxidative degradation or transformation to an unknown compound may have been responsible for the decrease. The authors suggest that the addition of stem components to the must might be used as a method of increasing resveratrol concentration of
wine although they recognized that other undesirable compounds could be extracted from the stems during fermentation.

Jeandet et al. (1991) studied the UV light induced production of resveratrol in grape berries of different developmental stages. The study suggested that the ability of the grape to produce resveratrol after UV elicitation declined with maturity. There was a gradual decline in resveratrol production from initiation to veraison with a rapid decline from veraison to maturity. These data suggest that the metabolic pathways responsible for resveratrol production diminish with maturity of the fruit. The study also investigated the possibility that the diminishing ability to produce resveratrol may be a result of the rise in UV absorbing anthocyanins that develop in the grape skin after veraison. Production of resveratrol was stimulated by UV light and by sucrose solution for both immature and mature grapes. Resveratrol production was reduced dramatically from immature to mature fruit for both elicitation factors. This suggests that the reduction with maturation in the ability to produce resveratrol is not a result of the production of anthocyanins in the maturing fruit.

In 2002, Magee et al. studied the effect of disease control spray program on resveratrol in muscadine berries. Resveratrol levels were determined for berry skins, juice/pulp and seeds separately from both fungicide treated and untreated vines. Resveratrol levels in the untreated vines were higher than the treated vines for all cultivars tested, although only three out of the five was the decrease statistically significant. For the two that were not significant, the overall levels of resveratrol were relatively low. There were no significant effect on the resveratrol values for the juice/pulp or for the seeds. The authors suggested that the fungicide treatment
resulted in less fungal pressure on the berries and therefore lower levels of elicitation of the metabolic pathway producing resveratrol.

Gonzalez-Candela et al. in 2000 studied the effect of transgenic wine yeasts encoding a glycosyl-hydrolase enzyme on the concentration of the monomers of resveratrol and piceid. Wines made with the transgenic yeasts had trans resveratrol levels four times that of the untransformed yeast and cis resveratrol levels ten times that of the untransformed yeasts. Trans piceid was reduced by half for the transformed yeast, but cis piceid was unaffected. The authors suggested that the enzyme encoded in the transformed yeast hydrolyzed the trans piceid into trans resveratrol, but this would only explain a small part of the increase in trans resveratrol and none of the increase in cis resveratrol. The authors speculate that either enzymes produced by the transformed yeast are providing more substrate for the hydrolysis enzyme from cell wall fragments or there are unknown conjugated forms of resveratrol present that have not been described.

**Effect of UV Light on Resveratrol.** A number of studies have demonstrated that UV light can induce the production of resveratrol in grapevine tissues (Langcake and Pryce, 1977; Jendet et al., 1997). In more recent years there have been several additional studies conducted in this area. In 1999, Douillet-Breuil et al. studied changes in the phytoalexin concentration of grape leaf tissue after exposure to UV light. The authors studied four *Vitis* species: three American species (*Vitis rupestris*, *Vitis cineria* and *Vitis labrusca*) and three cultivars of *Vitis vinifera*. All three American species showed a higher capacity for resveratrol synthesis than *V. vinifera*. Although, *V. rupestris* and *V. cineria* had higher resveratrol synthesis capacity than *V. labrusca*. All American species took longer to reach peak resveratrol concentration (30 to 45
hours) than \textit{V. vinifera} (18 to 25 hours). The American species were considered to be more disease resistant than \textit{V. vinifera}. The authors proposed that the results they obtained confirmed the role of resveratrol in defense of the plant against fungal attack.

Adrian et al., in 2000, studied the concentration of various stilbenes in grape berries in response to UV light and level of infection of \textit{Botrytis cinerea}. Three cultivars of \textit{V. vinifera} (‘Gamay’, ‘Pinot Noir’ and ‘Chardonnay’) were studied. Five compounds were quantified: trans piceid, cis piceid, trans resveratrol, \(\varepsilon\)-viniferin (resveratrol dimer) and pterostilbene (3,5 methylated resveratrol). For ‘Gamay’ and ‘Chardonnay’, all compounds were detected in berries that were not UV elicited except for the highly infected berries. For the highly infected berries little or none of the compounds was quantified. This was probably due to the ability of the fungal organism to enzymatically degrade the compounds produced by the plant to defend itself. For the non-UV induced ‘Pinot Noir’, the only compounds detected were trans resveratrol and \(\varepsilon\)-viniferin and only in infected berries and those surrounding the infected berries. For UV elicited berries, neither trans nor cis piceid was detected in the ‘Pinot Noir’ cultivar and only very small amounts of pterostilbene was detected. Pterostilbene was only detected in very small amounts for all berries. Trans piceid, cis piceid, trans resveratrol and \(\varepsilon\)-viniferin were quantified in UV elicited berries of both ‘Chardonnay’ and ‘Gamay’ cultivars. Generally incubation of 48 hours after UV elicitation produced greater concentrations of the compounds than an incubation of only 24 hours. Like the non-induced berries, concentrations of all compounds were lower in the highly infected berries than for lesser infected or non-infected berries. Overall, UV elicitation increased the concentration of all compounds in the berries. The data reported in this study demonstrate that both moderate fungal attack and UV
light can be strong elicitors of production of piceid, resveratrol and \( \epsilon \)-viniferin and also that fungal organisms can eventually overwhelm the protective properties of these compounds.

A study published in 2000, by Cantos et al., studied the effect of cold storage and postharvest UV irradiation on ‘Napoleon’ table grapes. Both piceid and resveratrol were quantified using HPLC and a diode array detector. Cold storage (15 days) alone resulted in approximately 75% increase in piceid and a 300% increase in resveratrol. Cold storage in combination with UV irradiation increased piceid slightly more than cold storage alone, but resulted in a 900% increase for resveratrol. The authors suggested that one 200 g serving of ‘Napoleon’ table grapes after cold storage could provide the same dose (approximately 1 mg) of stilbenes (resveratrol+piceid) as a serving of red wine (200ml). The same grapes after UV irradiation could provide 2 to 3 times the dose of the cold storage grapes alone.

**Muscadine Studies.** The first studies to evaluate resveratrol in muscadine grapes were published in 1996. Two articles were published looking at resveratrol in wines and in muscadine fruit components. Lamikanra et al. investigated the trans and cis resveratrol concentration of 18 different wines. Five commercial muscadine table wines, eight commercial \( V. \) \textit{vinifera} table wines, two commercial \( V. \) \textit{labrusca} wines, a muscadine port and two \( V. \) \textit{vinifera} ports were evaluated in the study. All of the muscadine table wines sampled had greater trans and cis resveratrol concentrations than any other wines sampled. The muscadine table wines varied between 9.2 and 31.9 mg/L cis resveratrol and between 4.9 and 13.4 mg/L trans resveratrol. The \( V. \) \textit{vinifera} table wines ranged between 0.8 to 3.3 mg/L cis resveratrol and between 1.1 and 4.5 mg/L trans resveratrol. The two ‘Concord’ \( V. \) \textit{labrusca} wines had 1.5 and 4.0 mg/L cis resveratrol and 1.1 and 2.7 mg/L trans resveratrol. The muscadine port wine
had 3.3 mg/L cis resveratrol and 3.6 mg/L trans resveratrol while the *V. vinifera* port wines had 0.3 and 0.1 cis resveratrol and had no trans resveratrol detected. The data show that wine made from muscadine grapes consistently had more cis and trans resveratrol than wines made from either *V. labrusca* or *vinifera* grapes. But what was interesting about these data was that for all muscadine wines, cis resveratrol was present in greater proportions than trans resveratrol. For the *V. vinifera* wines, the opposite was true for all but one wine. Since cis resveratrol has been either not found or in very low amounts in grapes in previous studies, it has been suggested that some process during fermentation isomerizes the trans resveratrol into cis in wine. No data regarding the cis and trans resveratrol concentration of fruit or juice were reported in this study, but it would be interesting to know if muscadine grapes have relatively large amounts of cis resveratrol or if the resulting large amounts of cis resveratrol are a result of reactions during fermentation.

The second study published in 1996 investigated the concentration of resveratrol in muscadine berries, juice, pomace, purees, seeds and wine (Ector et al., 1996). Resveratrol was quantified in bronze and dark-skinned berries for the fruit parts and also for products made from the fruit. Each replication of a sample of each berry type was selected from one of 12 cultivars for bronze and one of 10 cultivars for the dark skinned fruit. Samples were not analyzed by cultivar. Bronze and dark skinned samples represent a combination of several cultivars for each type. The data indicated that muscadine berries and seeds have substantial amounts of resveratrol. Concentrations for the berries without seeds range from 2.7 to 11.3 µg/g dwt for bronze and 5.0 to 23.5 µg/g dwt for dark skinned fruit. The data also indicated that muscadine seeds contained a substantial amount of resveratrol. Overall, seeds of both fruit
types contained between 24.5 and 62.2 μg/g dwt resveratrol. This is somewhat surprising since Jeandet et al. (1995) reported only approximately 1.0 μg/g dwt resveratrol in vinifera grape seeds. This represents a substantial difference between the two species. The high seed concentration of resveratrol could be significant during red wine making when the fermenting wine is in contact with the seed. The study also reported the resveratrol concentration of various products from muscadine fruit. Muscadine pomace, the solids left after pressing, contained 22 to 84 μg/g dwt resveratrol for dark skinned fruit and 18 to 72 μg/g dwt resveratrol for bronze fruit. A puree made from the pomace with the seeds removed contained 10 to 83 μg/g dwt resveratrol for dark skinned fruit and 10 to 62 μg/g dwt resveratrol for bronze fruit. Muscadine wine was reported to have from 0.7 to 1.9 mg/L resveratrol for red wines and 0.3 to 0.9 mg/L resveratrol for white wine. These values are higher to or similar to values published in previous work for V. vinifera wines (Pezet et al., 1994; Siemann and Creasy, 1992; Jeandet et al., 1993), but somewhat low compared to other reported values for muscadine wines (Lamikanra et al., 1996). One thing to consider about the wine data is that only trans resveratrol was quantified. In the previous study discussed (Lamikanra et al., 1996), cis resveratrol was found to be the predominant isomer in muscadine wines. Also, trans and cis piceid have been quantified in various wines, sometimes in quantities greater than the resveratrol isomers (Mattivi et al., 1995). If the wines in this study had been quantified for all monomers of resveratrol, the values may have been much higher. For the juices analyzed in this study, resveratrol was found in concentrations ranging from 2.6 to 17 mg/L for dark skinned fruit and from 2.6 to 12.8 mg/L for bronze fruit. The authors were surprised by the relatively large amounts of resveratrol in the juice considering its relative lack of contact with
the skins after crushing. It is apparent that some resveratrol is extracted during the crushing and pressing processes.

A article published in 1997 studied the resveratrol concentration of various wines including some white muscadine wines (McMurtrey, 1997). Wines were analyzed for trans resveratrol only. White wines made from ‘Noble’, ‘Cowart’, ‘Carlos’, ‘Doreen’ and ‘Magnolia’ cultivars of muscadine were analyzed. Resveratrol concentration varied between 0.29 and 1.2 mg/L for the white muscadine wines. These values represent ten fold more trans resveratrol than white vinifera wines, some of which had undetectable levels of resveratrol. The white muscadine wines had levels higher than the white but somewhat lower than red vinifera wines. Of all of the muscadine wines analyzed, ‘Doreen’ produced the wine with the greatest resveratrol concentration (1.2 mg/L) compared to ‘Magnolia’ which had the lowest level of resveratrol (0.3 mg/L).

The objectives of this work are as follows: 1) Detect and quantify the presence of trans and cis resveratrol and trans and cis piceid (stilbenes) in tissue and juice of eight cultivars of muscadine grape and three cultivars of bunch grape. 2) Determine the effect of juice extraction method on stilbene concentration of ‘Noble’ and ‘Carlos’ muscadine and ‘Midsouth’ and ‘Miss Blue’ bunch grape juice. 3) Determine the effect of postharvest UV irradiation on the stilbene content of ‘Noble’ and ‘Carlos’ muscadine grape tissue.

LITERATURE CITED


INTRODUCTION

Resveratrol and piceid are naturally occurring compounds found in grape tissue (Figure 2.1). There has been considerable interest in these compounds over the last decade because of their reported health benefits. Resveratrol has been reported to have numerous health benefits, including cardiovascular protective, anti-cancer and anti-inflammatory properties (Arichi et al., 1982; Kinsella et al., 1993; Jang et al., 1997; Lu and Serrero, 1999; De Santi et al., 2000; Burns et al., 2000; Brakenhielm et al., 2001; Kimura et al., 2001; El-Mowafy, 2002).

Figure 2.1 Chemical structures.
There is no consensus in the literature on the best method to quantify the four compounds collectively known as stilbenes (cis and trans piceid and cis and trans resveratrol). Over the years, stilbenes have been quantified a number of ways. The earliest work used high performance liquid chromatography (HPLC) fitted with a ultraviolet light(UV) detector (Siemann and Creasy, 1992; Waterhouse and Lamuela-Raventos, 1994; Jeandet et al., 1995). Others have used gas chromatography (GC) combined with a mass spectrometer (Jeandet et al., 1993), liquid chromatography with GC (Wang et al., 2002) and HPLC fitted with a flurometer (Pezet et al., 1994). Of all the methods reviewed in the literature to quantify stilbenes, HPLC fitted with a UV detector appeared to be the most reliable for analyzing stilbenes.

There have been diverse HPLC techniques reported in the literature about stilbene analysis. These techniques used high performance liquid chromatography principally as a means to separate stilbenes from other components in a sample matrix to quantify them individually. The instrument used to detect and quantify the compound of interest is chosen based on the chemical attributes of the compound. In our work, all of the stilbenes studied have absorbance in the UV spectral region. For this reason, a UV detector was used as the instrument to detect and quantify stilbenes in our samples. In other research, a flurometer has been used for detection of stilbenes (Jeandet et al., 1997). In our work, initial samples were analyzed using both UV and fluorescence detectors. After comparisons of the resulting chromatograms, it became evident that the UV detector would provide the most useful detection of the four stilbenes.

Another facet of stilbene analysis is the procedure used to prepare the sample matrix for analysis. Before samples are analyzed, regardless of instrumentation, the sample matrix is
usually “processed” to remove compounds that would interfere with detection. There are several examples of wine and fruit tissue sample preparation detailed in the literature (Siemann and Creasy, 1992; Jeandet et al., 1993; Waterhouse and Lamuela-Raventos, 1994). These protocols used techniques to purify or concentrate the stilbenes. Purification or concentration of the stilbenes require a significant investment of time and materials. This investment often limits the amount of samples that can be analyzed.

As interest regarding resveratrol and its monomers became more common, faster and less costly methods to quantify the compounds were needed. Several investigators began quantifying these compounds by direct injection of the sample on to the HPLC columns (Lamuela-Ravento et al., 1995; Romero-Perez et al., 1996). Direct injection procedures skipped many of the elaborate sample preparation steps and it allowed a greater number of samples to be processed while decreasing the time and cost of sample preparation. However, the sample matrix often contained compounds that responded similarly to those of interest, making isolation of the compounds of interest more difficult. Because of unknown interfering compounds, we developed an HPLC method that would separate, detect and quantify the presence of the stilbene compounds.

Among the literature where HPLC has been used, there have been a variety of procedures reported with respect to mobile phase, flow rate, column temperature and column type. As with any HPLC analysis, it is necessary to develop a method that will allow maximum separation of compounds while minimizing processing time and consumption of mobile phase. Since we elected to directly inject the sample matrix, the composition of the
mobile phase, the flow rate and the column temperature were manipulated to find the best combination for detection of resveratrol and its monomers.

**Method Development.** Sample analysis was completed using an HPLC system, equipped with a Waters 600 pump, a Waters 717 Plus autosampler and a Waters 2487 dual wave length UV detector (Waters Corporation, Milford, Mass.). The injection volume was twenty µL of each sample and standard. Samples were analyzed at 285 nm and 306 nm for the cis isomers and the trans isomers respectively (Carreri et al., 2003). Sample peaks and retention times were compared to standard peaks and retention times for quantification.

The HPLC method ultimately used to analyze samples was developed after evaluating various columns, column temperatures, mobile phases and pump flow rates. All of the evaluations were conducted using trans resveratrol (Sigma, St. Louis, MO) and trans piceid standards (Apin Chemicals, Abingdon, Oxon, England), selected juice and tissue extractions and extractions mixed or spiked with standards.

We began our investigation by replicating the conditions reported by Lamuela-Raventos, et al. (1995a) (Table 2.1). Initial conditions were a linear gradient of 5.5% glacial acetic acid in HPLC grade water (A) and 80% acetonitrile (B) for 30 minutes at a 1.5 ml/min flow rate using a Nucleosil C18 column (250mm X 4.5 mm, 5 µm particle size, Supelco Inc., Bellefonte, Penn.). Under the conditions described above, the system pressure approached column limits. In an effort to reduce the system pressure, we increased the column temperature from ambient to 40 °C with no significant reduction in system pressure. The gradient was modified by decreasing the initial organic phase and increasing the aqueous phase. System pressure remained at column limits. Although pressure remained near system limits, standard
<table>
<thead>
<tr>
<th>Column</th>
<th>Conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Gradient, 5.5% glacial acetic acid in water: acetonitrile, 1.5 ml/min, ambient column temp.</td>
<td>Good peak separation and base line but high system pressure.</td>
</tr>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Same as above but with 40 °C column</td>
<td>No change in system pressure.</td>
</tr>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Same as above but with decreased organic phase.</td>
<td>No change in system pressure.</td>
</tr>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Isocratic, 30:70, 5% formic acid in water: acetonitrile, 0.5 ml/min, 40 °C column</td>
<td>Poor peak separation. Flat baselines in standards but poor in samples. Pressure reduced but still high.</td>
</tr>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Same as above but with 36:64 ratio.</td>
<td>No change.</td>
</tr>
<tr>
<td>Nucleosil C18, 250 X 4.5 mm, 5 µm.</td>
<td>Isocratic, 69.3:22:8:0.7, water: acetonitrile: propanol: formic acid, 0.5 ml/ min, 40 °C column</td>
<td>Pressure exceeded system limits.</td>
</tr>
<tr>
<td>Column</td>
<td>Conditions</td>
<td>Results</td>
</tr>
<tr>
<td>--------------------------------------</td>
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</tr>
<tr>
<td>Xterra RP18, 150 X 4.6 mm, 5 µm.</td>
<td>Gradient, 5.5% glacial acetic acid in water: acetonitrile, 1.5 ml/min, ambient column temp.</td>
<td>Pressure lower than with Nucleosil and with shorter retention times. Peaks well separated with flat baselines in standards. Samples had unknown compounds eluting near trans piceid.</td>
</tr>
<tr>
<td>Xterra RP18, 150 X 4.6 mm, 5 µm.</td>
<td>Isocratic, 0.5 % formic acid in methanol. 0.5 ml/min, 40 °C column.</td>
<td>Unknown compound interfering with detection apparently liberated from column. Discontinued use of Xterra.</td>
</tr>
<tr>
<td>Nova-Pak C18, 150 X 3.9mm, 5 µm.</td>
<td>Gradient, 30:70, 0.5% formic acid in water: acetonitrile, 0.5 ml/min, 40 °C column.</td>
<td>Pressure within limits, peaks separated but with irregular baselines.</td>
</tr>
<tr>
<td>Nova-Pak C18, 150 X 3.9mm, 5 µm.</td>
<td>Isocratic, 69.3:22:8:0.7, water: acetonitrile: propanol: formic acid, 0.2 ml/ min, 30 °C column.</td>
<td>System pressure was low, peaks were well separated but broad, trans piceid was integrated but surrounding baseline was irregular.</td>
</tr>
<tr>
<td>Sunfire C18, 250 X 3.0 mm, 5 µm.</td>
<td>Isocratic, 69.3:22:8:0.7, water: acetonitrile: propanol: formic acid, 0.2 ml/ min, 30 °C column.</td>
<td>Good peak separation, flat baselines, narrower peaks, trans piceid separated from surrounding peaks.</td>
</tr>
</tbody>
</table>
chromatograms revealed that individual peaks were easily distinguishable from one another and that the base line between peaks was flat.

Using the Supelco column, we next attempted an isocratic method using 30% formic acid in water (5%) and 70% acetonitrile with a flow rate of 0.5 ml/min and a column temperature of 40 °C. While the system pressure was reduced compared to the gradient conditions, it remained at levels approaching system limits. These parameters produced flat baselines for the standards, but samples baselines were irregular. Detection of trans piceid in spiked samples was unsatisfactory. Other compounds in the sample matrix appeared to be co-eluting with trans piceid. The mobile phase was modified to 64% formic acid/water and 36% acetonitrile with little change in either system pressure or peak separation. Careri et al. in 2003 reported successful stilbene detection using isocratic conditions and a mobile phase of 69.3:22:8:0.7/ water: acetonitrile: propanol: formic acid by volume. We were unable to completely test this method as the system pressure exceeded the set column limits and the pump’s protective mechanism disengaged the pump.

Because of the continuing pressure challenges with the Nucleosil column, we experimented with a Xterra RP 18 column (150 mm X 4.6 mm, 5 µm particle size, Waters, Milford, Mass.). This column was tested using many of the previously described conditions. Our evaluation of this column began with the gradient originally used with the Nucleosil column. The system pressure was lower than the Nucleosil. Since the Xterra column was significantly shorter than the Nucleosil, the retention times were reduced, thereby decreasing the time of sample analysis. Peaks were well separated and baselines were flat for the standard
solutions. In spiked samples, trans piceid was detected, but eluting near other compounds. After trans piceid had eluted, base lines were flat in spiked samples.

We also assessed an isocratic mobile phase (McMurtry, 1997; Wang et al., 2002) utilizing 0.5% formic acid and methanol using the X-Terra column. An unknown compound appeared on chromatograms. The compound was present on chromatograms for all injections even when injections of mobile phase were made. The unknown compound interfered with detection and was apparently being liberated from the column material. This unknown peak was apparently caused by incompatibility with the mobile phase. Therefore, further attempts to utilize this column were abandoned.

The third column evaluated was a Nova-Pak C 18 column (150 mm X 3.9mm, 4 µm particle size, Waters, Milford, Mass.). Specifications for this column indicated it could be used with an acetified mobile phase. Initially, we used a 30% formic acid in water (0.5%) and 70% acetonitrile mobile phase using a gradient. Flow rate was 0.5 ml/min therefore system pressure was well within limits. Spiked sample peaks were separated, but baselines were irregular. An isocratic method using 69.3:22:8:0.7/ water: acetonitril: propanol: formic acid was tested. We modified the protocol by maintaining the column at 30 °C to prevent any fluctuations in room temperature from influencing detection and quantification over time. Except for trans piceid, peaks were well separated, although broad. Trans piceid was separated enough to be integrated by the system software, but the surrounding base line was irregular due to co-eluting compounds. The system pressure was within system limits. Different column temperatures and different flow rates were assessed. The best detection and quantification was achieved with a flow rate of 0.2 ml/min at 30 °C. Spiked juice and tissue samples were tested using these
conditions. All stilbenes of interest were detected in spiked samples; however, peak separation of isomers was not ideal and baselines were irregular.

In an attempt to improve peak separation and quality, an additional column was tested. A Sunfire C18 column (250 mm X 3 mm, 5 µm particle size, Waters, Milford, Mass.) was evaluated. A report by Careri et al. in 2003 reported using a Luna column of similar characteristics that demonstrated good results. Our initial assessment of this column began with the method last used with the Waters Novapak column. The mobile phase used was 69.3:22:8:0.7/ water: acetonitrile: propanol: formic acid, with a flow rate of 0.2 ml/min and a column temperature of 30 °C. These conditions resulted in improved separation of trans piceid from surrounding compounds. Peaks were narrower and baselines were smoother. Since the Sunfire column was longer than the Novapak column, retention times were greater. Though sample analysis required a greater amount of time, the improved peak separation and shape resulted in improved detection and quantification. We were satisfied with the analytical method we had prepared as well as with the Sunfire column; therefore we proceeded with sample analysis.

**Standard Preparation and Compound Quantification.** Trans resveratrol was obtained from Sigma (St. Louis, MO) and trans piceid was obtained from Apin Chemicals (Abingdon, Oxon, UK). The trans piceid standard was of unknown concentration; therefore, the trans piceid chromatogram was used as the means to identify the trans piceid peaks in the samples. To verify the identity of trans piceid, a standard solution was incubated with β-D-glucosidase (Sigma, St. Louis, MO). The chromatogram obtained after enzyme incubation
Figure 2.2 Chromatograms comparing the effect of glucosidase enzyme on trans piceid standard solution. A. Trans piceid standard without glucosidase incubation. B. Trans piceid standard after glucosidase incubation. C. Trans resveratrol standard. AU = Absorbance Units.
revealed the disappearance of the trans piceid peak and the appearance of a peak whose retention time corresponded with the standard trans resveratrol peak (Figure 2.2).

No standards are available for cis isomers of resveratrol and piceid. To identify the peaks for the cis compounds in the samples, standards of both trans resveratrol and trans piceid were exposed to direct unobstructed sunlight in the window sill for approximately 15 minutes. This resulted in the conversion of approximately 85% of the trans compounds to their cis isomers (Figure 2.3). Peaks for cis isomers were verified by comparison to retention times reported in literature. Conversion of trans isomers to cis was confirmed by HPLC analysis of the standard before and after exposure to sunlight.

Trans resveratrol was the only compound of interest for which there was a standard of known concentration. Both trans resveratrol and trans piceid were quantified by comparing sample peak area with the standard peak area for trans resveratrol since the two compounds have identical UV absorbance spectra (Romero-Perez et al., 2001; Cantos et al, 2000; Adrian et al., 2000). Both cis resveratrol and cis piceid were quantified by comparing the sample peak area with the standard peak area for cis resveratrol. The concentration of the cis resveratrol peak was determined by calculating the amount of trans resveratrol that disappeared from the sunlight exposed trans standard.

Filter Study. A 1995 study by Lamuela-Raventos et al. reported that many commonly used HPLC preparative filter membranes can retain trans resveratrol during the filtration process. The authors evaluated nylon, PVDF, polysulfone and aluminum oxide filter membranes. They reported that nylon, PVDF and polysulfone retained greater than 60% of the trans resveratrol. In contrast, an aluminum oxide membrane, or “Anopore” membrane, did not
Figure 2.3 Chromatograms of trans piceid and trans resveratrol before and after exposure to direct sunlight. A. Trans piceid and trans resveratrol standard before sunlight exposure. B. Trans piceid and trans resveratrol standard after sunlight exposure. AU = Absorbance Units.
retain the trans resveratrol compound. Though Anopore does not bind the compound the nature of the membrane presents challenges. The membrane is a thin fragile disc that is difficult to insert into the membrane filter holder without fracturing the material. Once the membrane disc is loaded into the holder, great care must be taken when assembling the filter holder. Applying too much pressure during assembly often results in rupturing the disc. For these reasons, a study was initiated to evaluate the performance of a number of membrane filters that are commonly used for HPLC sample preparation.

Five membrane filters were selected for evaluation, Anopore (aluminum oxide, Whatman, Maidstone, England), GHP (hydrophillic polypropylene, Pall, Ann Arbor, MI), nylon (Nylaflo, Pall, Ann Arbor, MI), polycarbonate (PC) (Whatman, Maidstone, England) and mixed esters of cellulose (Membra-Fil) (Whatman, Maidstone, England). All filters were 25 mm in diameter. Anapore, GHP, nylon and PC membranes had a pore size of 0.2 µm. Membra-fil membranes had a pore size of 0.45 µm. For performance comparison, a standard solution of trans resveratrol was prepared using 1:1 ethanol and water (v/v) with a final concentration of 10mg/L. In addition, a muscadine juice sample was spiked with the trans resveratrol standard (10mg/L). The standard and spiked sample were filtered through each of the five membranes in triplicate. An unfiltered standard was also prepared.

Both filtered and unfiltered samples were analyzed by HPLC/UV under the same conditions. The analytical equipment consisted of a Waters 600 pump, a Waters 717 Plus autosampler, a Waters 2487 dual wave length UV detector. The column was a Waters Sunfire 3 x 250 mm C18 (5 µm particle size) with a 20 mm pre-column of the same material. Column temperature was maintained at 30°C. Twenty µL of each sample was injected and eluted using
an isocratic method with a mobile phase of 70:22:8 ratio of 1% formic acid in water: acetonitrile: propanol at a flow rate of 0.2 ml/min. Samples were analyzed by a UV detector at 285 nm and 306 nm for the cis isomers and the trans isomers, respectively (Careri et al., 2003). Data were analyzed using PROC MIXED and means separated using Tukey’s studentized range test, $\alpha = 0.05$ (SAS, Carey, NC).

The results of the standard solutions indicated that recovery rates of Anopore, PC and Mebra-fil were above 90% (Figure 2.4) compared to the unfiltered standard. The GHP and nylon membranes had 82% and 20% recovery, respectively. The recovery rates for the spiked

![Bar chart showing percent recovery of trans resveratrol after micro-filtration through different filter media. Anapore = aluminum oxide, GHP = hydrophilic polypropylene, PC = Polycarbonate, MF = mixed esters of cellulose. Bars with the same letter are not significantly different at $\alpha=0.05$.](image)

Figure 2.4 Percent recovery of trans resveratrol after micro-filtration through different filter media. Anapore = aluminum oxide, GHP = hydrophilic polypropylene, PC = Polycarbonate, MF = mixed esters of cellulose. Bars with the same letter are not significantly different at $\alpha=0.05$.  


samples were 90% for the Anopore and PC membranes. Membra-fil membranes had a recovery rate of 60% while GHP and nylon had recovery rates of 13% and 15%, respectively. These results are consistent with the findings of Lamuela-Raventos et al. (1995) that the filter membrane chosen for sample preparation can influence the recovery rates of trans resveratrol. In this study, recovery rates above 90% were achieved when standard solutions were filtered with Anopore, PC and Membra-fil membranes. However, the results from the spiked samples demonstrated the influence of the sample matrix on the recovery of the compounds of interest. Recovery rates dropped from 90% to 60% for Membra-fil and 82% to 13% for GHP. The sample matrix did not greatly influence the nylon filters as they remained low at 15%. These data suggests that of the five membranes evaluated, only Anopore and PC are suitable for sample preparation of matrices where trans resveratrol is the compound of interest.

Anopore and PC membranes are marketed primarily for sample preparation for microscopy. Both membranes have very stable and uniform pore size. They are ideal for filtering out particles that must be examined under a microscope. Both membranes are fragile and are subject to fracturing during handling. It was our experience that the PC membranes were more durable than the Anopore membranes during filter holder assembly and during sample matrix filtering. The cost of the the Anopore membranes was almost 3 times that of the PC membranes. For these reasons coupled with the fact that there was little difference in their recovery rates, the PC membranes were used for sample preparation in our work.

LITERATURE CITED


INTRODUCTION

Resveratrol and piceid belong to a class of compounds called stilbenes. Stilbenes are non-flavanoid phenolics which are secondary metabolites in plants of the genus *Vitis*. Both of these compounds exist in both the trans and cis form (Figure 3.1). Trans resveratrol has been the subject of numerous works detailing its presence in grapes and wine as well as its potential

![chemical structures](image)

*trans*-Resveratrol

* cis-Resveratrol

*trans*-Resveratrol-3-O-glucoside (*trans*-Piceid)

* cis-Resveratrol-3-O-glucoside (*cis*-Piceid)

Figure 3.1 Chemical structures.
health benefits to humans. From the late 1970’s to the 1990’s, several studies reported the presence of resveratrol in grape stems, leaves, wine and berries (Langcake and Price, 1977; Creasy and Coffee, 1988; Jeandet et al., 1991; Frankel et al., 1993; Jeandet et al., 1995). Subsequent work also quantified a related compound, piceid, in grape tissue (Lamuela-Raventos et al., 1995; Mattivi et al., 1995; Jeandet et al., 1997).

Much of the interest in resveratrol and piceid is based on numerous reports about their potential health benefits. Research published in 1992 suggested that increased wine consumption may result in lower coronary heart disease (CHD) rates (Reneaud and Lorgeril). Subsequent work identified resveratrol as a possible active compound in red wine responsible for reductions in CHD (Arichi et al., 1982; Jeandet et al., 1991). Since then many studies have been initiated to explain how resveratrol may be beneficial to human health. Resveratrol has been reported to inhibit platelet aggregation in rats (Kimura et al., 1985). Jang et al. (1997) noted that resveratrol acted as an antioxidant and anti-mutagen. It has also been reported to have anti-allergenic and anti-carcinogenic properties as well (Cheong et al., 1999; Lu and Serrero, 1999). The health benefits of piceid have also been studied. Piceid is a glycosylated version of resveratrol. It has been reported that since glucosidase is present in the digestive tract, piceid may be converted into resveratrol during digestion (Hackett, 1986; Day et al., 1997). Studies have also demonstrated that in rats, piceid can be absorbed and converted into resveratrol in the digestive tract (Henry et al., 2005). Kimura et al. (1995) and Shan et al., (1990) reported that piceid is biologically active as well.

Although resveratrol has been studied in most commercially significant Vitis species, including muscadine (Vitis rotundifolia Michx.), work quantifying piceid in fruit and wine has
been primarily done only with *Vitis vinifera* grapes (Romero-Perez, 1999; Cantos et al., 2000, 2001, 2002, 2003; Adrian et al., 2000; Careri et al., 2003). Work that detected piceid in *V. vinifera* grapes suggests that piceid concentration can often be greater than resveratrol (Mattivi et al., 1995; Romero-Perez et al., 1996; Romero-Perez et al., 2001). There has been little research to quantify stilbenes in grape tissue and juice outside of the typical wine and table grape species.

Previous work regarding the resveratrol concentration of muscadine grape tissue, juice and wine suggest that muscadine grapes generally have higher resveratrol concentration than other *Vitis* species (Lamikanra et al., 1996; Ector et al., 1996; McMurtrey, 1997;). The limited work with muscadine grapes has not included analyzing for piceid (Magee et al., 2002; Pastrana-Bonilla et al., 2003).

To establish a better understanding of the overall stilbene concentration of muscadine grapes, a study was undertaken to quantify trans and cis resveratrol and trans and cis piceid in skins, pulp, seeds and juice of eight cultivars of muscadine grape and three cultivars of *Vitis labrusca* grapes.

MATERIALS AND METHODS

During August 2002, 2003 and 2004, muscadine grape berries (*Vitis rotundifolia* Michx.) were collected from the USDA Small Fruit Crop Laboratory in Poplarville, MS. Three replications were collected for each cultivar. Each replication was taken from an individual vine. Fruit was refrigerated until samples were processed. Muscadine cultivars sampled included ‘Fry’, ‘Hunt’, ‘Magnolia’, ‘Watergate’, ‘Carlos’, ‘Noble’, ‘Sweet Jenny’ and ‘Albermarle’. In addition to the eight cultivars of muscadine grape, ‘Midsouth’, ‘Miss Blanc’
and ‘Stover’ bunch grapes (*Vitis labrusca*) were also sampled. The bunch grape samples were collected in July from the Hill Farm on the Louisiana State University campus in Baton Rouge, LA. Three replications of each *Vitis labrusca* cultivar were collected. Each replication was taken from a separate arm of a single vine for each cultivar.

Each of the samples was divided into two groups. The first group was crushed and juice was extracted using a hydraulic rack and frame press. A juice sample was collected from the resulting juice. The second group of fruit was divided into skins, pulp and seed and placed in individual plastic bags. Each of the tissue and juice samples were frozen at -40 °C. The frozen tissue samples were lyophilized and re-frozen at -40 °C until further sample preparation. Juice samples were centrifuged to remove particulate matter and an aliquot of the supernatant was filtered through a 0.2 μm Nucleopore Track-Etch membrane (polycarbonate, Whatman, Maidstone, England) in preparation for HPLC analysis and placed into amber vials to protect from light induced isomerization or degradation.

One gram of freeze dried tissue was weighed and placed in centrifuge tubes along with 12.5 ml of 80% ethanol. Solutions were incubated for 30 minutes at 60 °C with agitation every 5 minutes (Romero-Perez et al., 2001). After heating, tubes were removed and centrifuged for 20 minutes. Supernatant was then decanted into a graduated cylinder and brought to a volume of 10 ml. An aliquot was filtered through 0.2 μm Nucleopore Track-Etch membrane (polycarbonate, Whatman, Maidstone, England) in preparation for HPLC analysis.

Tissue samples were analyzed using HPLC with a UV detector. The analytical equipment consisted of a Waters 600 pump, a Waters 717 Plus autosampler and a Waters 2487 dual wave length UV detector (Milford, Mass.). Stilbenes were separated using a Waters
Sunfire 3 x 250 mm C18 (5 μm particle size, Milford, Mass.) with a twenty mm pre-column of the same material. Column temperature was maintained at 30°C. Twenty μL of each sample was eluted using an isocratic method with a mobile phase of 70:22:8 ratio by volume of: 1% formic acid in water: acetonitrile: propanol, at a flow rate of 0.2 ml/min. Samples were analyzed by the UV detector at 285 nm and 306 nm for the cis isomers and the trans isomers, respectively (Careri et al., 2003).

Sample peak areas were compared to standards of known concentration for quantification. Trans resveratrol was purchased from Sigma Chemicals (St. Louis, Mo.) and trans piceid was purchased from Apin Chemicals (Abingdon, England). Since the trans piceid standard was of unknown concentration, its chromatogram was used as the means to identify the trans piceid peaks in the samples. To verify the identity of trans piceid, the standard was subjected to incubation with β-D-glucosidase (Sigma Chemicals, St. Louis, Mo.). The HPLC chromatogram obtained after the enzyme incubation revealed the disappearance of the trans piceid peak and the appearance of a peak whose retention time corresponded to the standard trans resveratrol peak. To identify peaks for the cis compounds, standards of both trans resveratrol and trans piceid were exposed to direct sunlight for approximately 15 minutes. This resulted in the conversion of approximately 85% of the trans compounds to their cis isomers as determined by analysis of the peak areas before and after sunlight exposure.

Trans resveratrol was the only compound for which there was a standard of known concentration. Since both piceid and resveratrol have identical uv absorbance spectra, both trans resveratrol and trans piceid were quantified using the standard peak for trans resveratrol (Romero-Perez et al., 2001; Cantos et al, 2000; Adrian et al., 2000). Due to the instability of
both cis resveratrol and cis piceid, no standards of these compounds are available for purchase. Cis resveratrol and cis piceid were quantified using the standard peak for cis resveratrol. The concentration of the cis resveratrol peak was determined by calculating the amount of trans resveratrol that had disappeared from the sunlight exposed trans resveratrol standard. Data were analyzed using PROC MIXED and means separated using Tukey’s studentized range test, \( \alpha = 0.05 \) (SAS, Carey, NC).

RESULTS AND DISCUSSION

Although muscadine grape tissue was collected for both 2003 and 2004, only two of the eight muscadine cultivars were sampled for both years. For this reason, data for each year were analyzed separately (Tables 3.1 and 3.2).

In 2003, tissue samples of ‘Noble’ (black) and ‘Sweet Jenny’ (bronze) muscadine cultivars and two \( Vitis labrusca \) bunch grape cultivars were collected (Table 3.1). ‘Miss Blanc’ (white) and ‘Midsouth’ (purple) bunch grapes were grown on the LSU Campus in Baton Rouge, Louisiana. These grapes were analyzed as a comparison with the muscadine grapes that are more commonly grown in the southeast. \( Vitis labrusca \) grapes are more similar to \( Vitis vinifera \) grapes than are muscadine. Previous work suggested that muscadine grapes had greater resveratrol concentration than vinifera or labrusca grapes (Ector et al., 1996). The data collected here suggest that \( Vitis labrusca \) grapes have comparable or even greater levels of stilbenes than do some muscadine cultivars. For 2003, ‘Miss Blanc’ had greater total stilbenes than both ‘Noble’ and ‘Sweet Jenny’. ‘Midsouth’ had levels comparable to those of ‘Noble’. While it is significant that these bunch grapes had high levels of stilbenes compared to the two muscadine cultivars, it is important to note that the only data available for these cultivars was
Table 3.1  Stilbenes (μg/g dwt) in muscadine and bunch grape tissue (2003).

<table>
<thead>
<tr>
<th>Skin</th>
<th>Piceid</th>
<th>Cis Piceid</th>
<th>Resv.</th>
<th>Cis Resv.</th>
<th>Total&lt;sup&gt;Y&lt;/sup&gt;</th>
<th>Piceid</th>
<th>Cis Piceid</th>
<th>Resv.</th>
<th>Total</th>
<th>Cis Piceid</th>
<th>Resv.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweet Jenny (br)</td>
<td>124.8 b</td>
<td>21.2 b</td>
<td>4.43 b</td>
<td>nd b</td>
<td>150.4 c</td>
<td>1.5 b</td>
<td>0.2 ns</td>
<td>nd ns</td>
<td>1.7 b</td>
<td>94.07n</td>
<td>3.6 ns</td>
</tr>
<tr>
<td>Noble (bl)</td>
<td>88.2 b</td>
<td>144.3 a</td>
<td>113.2 a</td>
<td>5.4 a</td>
<td>351.0 b</td>
<td>0.2 b</td>
<td>1.3 ns</td>
<td>0.2 ns</td>
<td>1.7 b</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Midsouth&lt;sup&gt;x&lt;/sup&gt; (p)</td>
<td>322.6 a</td>
<td>7.1 b</td>
<td>12.3 b</td>
<td>nd b</td>
<td>341.9 b</td>
<td>2.8 b</td>
<td>1.3 ns</td>
<td>nd ns</td>
<td>4.1 b</td>
<td>nd ns</td>
<td>1.1 ns</td>
</tr>
<tr>
<td>Miss Blanc&lt;sup&gt;x&lt;/sup&gt; (w)</td>
<td>320.1 a</td>
<td>110.9 a</td>
<td>85.7 a</td>
<td>2.5 ab</td>
<td>519.4 a</td>
<td>29.4 a</td>
<td>0.5 ns</td>
<td>0.7 ns</td>
<td>30.7 a</td>
<td>nd ns</td>
<td>8.1 ns</td>
</tr>
</tbody>
</table>

Means (n=3) within a column, followed by the same letter, are not significantly different at P ≤ 0.05.  ns = Not significant at P ≤ 0.05.  br = bronze.  bl = black.  p = purple.  w = white.  <sup>2</sup>Trans piceid not quantified and Cis resveratrol not detected in seed tissue.  <sup>Y</sup>Total = Piceid+Cis piceid+Resv.+Cis resv.  <sup>2</sup>Bunch grape cultivars.
collected during a year where stilbene levels were significantly higher than recorded during the subsequent year for muscadine tissue. The fruit were also harvested in a different location than the muscadine grapes. More work comparing the two species would be helpful in determining the relevance of this limited data.

Since only two of the eight cultivars (‘Noble’ and ‘Sweet Jenny’) were sampled in both 2003 and 2004 for tissue, data for each year was reported and discussed separately. When comparing the data between years for those two cultivars (Figure 3.2), it is obvious that year of harvest can have a large effect on levels of all of the stilbenes detected here. All of the compounds analyzed for skin tissue were significantly greater in 2003 than in 2004 for ‘Noble’. For ‘Sweet Jenny’, only trans piceid and total skin stilbenes was significantly greater in 2003.

Figure 3.2 Effect of harvest year on the stilbene concentration of muscadine skin tissue. Columns with the same letters are not significantly different at $P \leq 0.05$. ns = not significant at $P \leq 0.05$. Total = Piceid+Cis Pceid+Resv.+Cis Resv.
Trans resveratrol was significantly greater for skins in 2004 than in 2003. There were no significant differences between the years for pulp tissue and only cis piceid was significantly different for seed tissue. The data above demonstrate that most if not all of the stilbene metabolism occurs in the skins and seed. Stilbene concentration in pulp remained statistically unchanged over the two years, while there was significant differences in the skin. This suggests that the effects of cultural conditions, environment and harvest time have a much greater effect on skin stilbene concentration than on pulp concentration.

In addition to the differences between the overall stilbene levels, there were some differences in the relationship between the cultivars as well. In 2003 (Table 3.2), total stilbene levels for ‘Sweet Jenny’ were approximately 43% of those for ‘Noble’. In 2004, total stilbene levels for ‘Sweet Jenny’ were approximately 66% of those for ‘Noble’. The difference between the cultivars decreased between 2003 and 2004. Differences between years for total stilbene levels for ‘Noble’ were greater than those for ‘Sweet Jenny’. This may indicate that under ideal conditions for stilbene production, cultivars may have differing capabilities to metabolize the compounds.

Many published works have reported that variations in climate and environment from year to year can have substantial effects on stilbene concentration of fruit, juice and wine (Lamuela-Raventos et al., 1995; Romero-Perez et al., 1996; McMurtrey, 1997; Romero-Perez et al., 1999). Resveratrol is considered to be a phytoalexin, meaning that it is produced by the fruit in response to fungal pathogen pressure (Siemann and Creasy, 1992; Magee and Smith, 2002). Different environments from one year to the next might produce different fungal pressures on the developing fruit. This may explain the wide variations in stilbenes between
one year to the next. Other work has suggested that developmental stage can have a substantial
effect on stilbene concentration (Striegler et al., 2005). Early work on vinifera grapes reported
that trans resveratrol levels increased up to veraison (color formation), but decreased with
maturity after that point (Jeandet et al., 1991). More recent work on muscadine reported that
total phenolics increased with ripening (Lee and Talcott, 2004). Although that work was not
specific to piceid and resveratrol and did not specify the stage of development. Based on the
work with vinifera grapes, minor differences in maturity after veraison can significantly affect
stilbene concentration. Muscadine grapes typically ripen at different stages within the cluster,
resulting in various stages of maturity being harvested together. This may result in changes in
stilbene concentration from one harvest to another.

Skin, pulp and seed tissue of all cultvars of muscadine grape were analyzed for
concentrations of trans piceid, cis piceid, trans resveratrol and cis resveratrol (Table 3.2). All
four compounds were able to be analyzed in skin and pulp. In contrast, seed tissue samples
contained interfering compounds that co-eluted with and had similar UV spectra as trans
piceid. Since the baseline around the trans piceid peak was irregular, we were unable to
consistently quantify the compound. For that reason, no data on trans piceid or total stilbenes
are presented for seed tissue.

For 2004 measurements, total stilbene concentration (trans piceid, cis piceid, trans
resveratrol and cis resveratrol) in skin tissue varied significantly between cultivars. ‘Carlos’
and ‘Magnolia’ had the greatest skin total stilbene concentration and was significantly greater
than all other cultivars except ‘Watergate’. Sweet Jenny had the lowest total stilbene
Table 3.2 Stilbenes (µg/g dwt) in muscadine grape tissue (2004).

<table>
<thead>
<tr>
<th></th>
<th>Skin</th>
<th></th>
<th></th>
<th></th>
<th>Pulp</th>
<th>Seed&lt;sup&gt;z&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans Piceid</td>
<td>Cis Piceid</td>
<td>Trans Resv.</td>
<td>Cis Resv.</td>
<td>Total&lt;sup&gt;y&lt;/sup&gt;</td>
<td>Trans Piceid</td>
<td>Cis Piceid</td>
<td>Trans Resv.</td>
<td>Cis Resv.</td>
<td>Total&lt;sup&gt;y&lt;/sup&gt;</td>
<td>Cis Piceid</td>
<td>Trans Resv.</td>
</tr>
<tr>
<td>Carlos (br)</td>
<td>147.7 ab</td>
<td>42.4 ab</td>
<td>4.4 b</td>
<td>0.4 ns</td>
<td>194.9 a</td>
<td>3.5 ns</td>
<td>2.4 ab</td>
<td>0.2 ns</td>
<td>6.1 ns</td>
<td>20.6 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Magnolia (br)</td>
<td>167.1 a</td>
<td>11.6 d</td>
<td>3.6 b</td>
<td>0.2 ns</td>
<td>182.5 a</td>
<td>1.8 ns</td>
<td>0.3 b</td>
<td>0.1 ns</td>
<td>2.2 ns</td>
<td>30.8 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Watergate (br)</td>
<td>141.4 a</td>
<td>16.4 cd</td>
<td>10.9 ab</td>
<td>nd ns</td>
<td>168.7 ab</td>
<td>0.3 ns</td>
<td>0.2 b</td>
<td>nd ns</td>
<td>0.5 ns</td>
<td>34.9 ns</td>
<td>nd ns</td>
<td>2.3 ns</td>
</tr>
<tr>
<td>Fry (br)</td>
<td>77.8 b</td>
<td>33.5 bcd</td>
<td>7.0 ab</td>
<td>0.1 ns</td>
<td>118.4 bc</td>
<td>1.7 ns</td>
<td>0.8 b</td>
<td>0.1 ns</td>
<td>2.5 ns</td>
<td>39.1 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Hunt (bl)</td>
<td>44.0 bc</td>
<td>63.4 a</td>
<td>10.4 ab</td>
<td>nd ns</td>
<td>117.8 bc</td>
<td>0.3 ns</td>
<td>0.5 b</td>
<td>nd ns</td>
<td>0.8 ns</td>
<td>52.7 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Noble (bl)</td>
<td>44.6 bc</td>
<td>40.3 abc</td>
<td>22.8 a</td>
<td>nd ns</td>
<td>107.7 c</td>
<td>0.3 ns</td>
<td>1.2 b</td>
<td>0.3 ns</td>
<td>1.8 ns</td>
<td>52.1 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Albermarle (bl)</td>
<td>60.9 b</td>
<td>32.5 bcd</td>
<td>9.5 ab</td>
<td>0.4 ns</td>
<td>103.2 c</td>
<td>nd ns</td>
<td>6.2 a</td>
<td>nd ns</td>
<td>6.2 ns</td>
<td>19.7 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
<tr>
<td>Sweet Jenny (br)</td>
<td>33.0 bc</td>
<td>23.3 bcd</td>
<td>15.3 ab</td>
<td>nd ns</td>
<td>71.6 c</td>
<td>0.3 ns</td>
<td>1.7 ab</td>
<td>0.2 ns</td>
<td>2.2 ns</td>
<td>74.2 ns</td>
<td>nd ns</td>
<td>nd ns</td>
</tr>
</tbody>
</table>

Means (n = 3) within a column followed by the same letter are not significantly different at $P \leq 0.05$. ns = No significant differences at $P \leq 0.05$. nd = not detected. br = bronze. bl = black. <sup>z</sup>Trans piceid not quantified and and Cis resveratrol not detected in seed tissue. <sup>y</sup>Total = Piceid+Cis piceid+Resv.+Cis resv.
concentration (Table 3.2). Total stilbenes for ‘Sweet Jenny’ skin was significantly lower than the three cultivars with the highest total concentration.

In contrast to previous reports regarding resveratrol, (Jeandet et al., 1995; Ector et al., 1996; Romero-Perez et al., 1999), two white or “bronze” muscadine cultivars (‘Carlos’ and ‘Magnolia’) had greater total stilbene concentrations than did all three dark skinned cultivars (‘Hunt’, ‘Noble’ and ‘Albermarle’). Only one bronze skin cultivar (‘Sweet Jenny’) had total skin levels lower than the dark skinned cultivars.

Trans piceid, cis piceid and trans resveratrol were detected in skins of all cultivars. Cis resveratrol was only found in the skins of ‘Carlos’, ‘Magnolia’, ‘Fry’ and ‘Albermarle’. When comparing trans resveratrol, two dark skinned cultivars (‘Noble’ and ‘Hunt’) had higher concentrations than the two bronze cultivars (‘Carlos’ and ‘Magnolia’) that had the highest total stilbene concentration. This agrees with studies that reported higher trans resveratrol levels in dark skinned cultivars (Jeandet et al., 1995; Ector et al., 1996).

In 2004, for seven of the eight cultivars, trans piceid was present in the greatest concentration in skin tissue. ‘Hunt’ was the only cultivar whose trans piceid concentration was not higher than other stilbenes; however, ‘Hunt’s cis piceid concentration was the greatest of the four compounds.

Though not significant, total stilbenes in pulp tissue was greatest in ‘Albermarle’ and least in ‘Watergate’ (Figure 3.2). Differences in trans resveratrol concentrations between cultivars were not significant. Trans resveratrol was only detected in five of the eight cultivars. Cis resveratrol was not detected in any of the cultivars. Though differences in pulp trans piceid concentrations were not significant, it was detected in greatest concentration in
‘Carlos’. Cis piceid was detected in greatest concentration in ‘Albermarle’. Only three of the eight cultivars had higher pulp trans piceid levels than cis piceid. The other cultivars had greater cis piceid levels. The only differences in pulp tissue data that was significant was for cis piced, where ‘Albermarle’ had significantly greater levels than for all other cultivars except ‘Carlos’ and ‘Sweet Jenny’. For pulp tissue, trans resveratrol was only detected in five of the eight cultivars.

Only cis piceid, trans resveratrol and cis resveratrol data are reported here for seed tissue. Though there were no significant differences between cultivars, concentrations were one hundred times that of pulp tissue and about the same range as skin tissue. No cis resveratrol was detected in seed tissue. ‘Watergate’ was the only cultivar for which trans resveratrol was detected in seed tissue. Cis piceid concentrations were highest in ‘Sweet Jenny’ and lowest in ‘Albermarle’, although the differences were not significant. There was only limited success in quantifying trans piceid in seed tissue. Trans piceid was only detected and quantified for a small number of samples due to interfering compounds. For those samples, concentrations varied widely (30 to 140 µg/g dwt). Additional work with extraction techniques and HPLC methods may help provide trans piceid data in seed tissue so that a clear picture of total stilbenes can be assessed.

For all cultivars, total stilbenes were higher in skins than in pulp tissue. Total levels in pulp tissue ranged from 0.3% to 6% and averaged 2.5% of the total levels for skin tissue. These data agree with previous work that the majority of stilbenes are metabolized in the skins of grape berries rather than in the pulp or juice (Lamuela-Raventos et al., 1997; Careri et al., 2003).
Since trans piceid data were not collected for seed tissue, total values were not determined. Values for cis piceid were generally higher for seed than for pulp, but varied in comparison to the skin tissue (Table 3.2). Previous work (Ector et al., 1996) reported high levels (approximately 44 µg/g dwt) of trans resveratrol in seed tissue. This was not the case with this work. Only small levels (2.3 µg/g dwt) were found in seed tissue and for only one cultivar (‘Watergate’). Perhaps, the extraction technique that was used here, which was optimized for softer tissues such as skins and pulp (Romero-Perez et al., 2001), is not suitable to extract stilbenes from the rather woody tissue of muscadine seeds.

The stilbene values reported in literature for *V. vinifera* grapes vary between 22 and 1026 µg/g dwt for stilbenes and between 1 and 40 µg/g dwt for trans resveratrol (Romero-Perez et al., 2001; Careri et al., 2003; Cantos et al., 2003). The concentrations of stilbenes in muscadine skins (71 to 195 µg/g dwt) are somewhat larger than the values reported for most *V. vinifera* cultivars (22 to 89 µg/g dwt) by Romero-Perez et al. (2001). Of the seven cultivars evaluated in that publication, two (‘Xarello’ and ‘Merlot’) had values (389 and 1026 µg/g dwt, respectively) much greater than the remaining cultivars. The significant variations in stilbene concentration between cultivars found here is comparable to the variations found by Romero-Perez et al. The stilbene concentrations found in the *V. labrusca* grape skins (341 to 519 µg/g dwt) in this research are also comparable to the concentrations found in the *V. vinifera* cultivars reported by Romero-Perez et al. (2001).

The values listed above from literature demonstrate that there are wide variations in the stilbene concentration of *V. vinifera* wine and table grapes. The literature reports on muscadine grape stilbene concentration are also inconsistent. In 1996, Ector et al. reported
high levels of trans resveratrol (20 to 230 µg/g dwt) in muscadine skins and pulps. The most recent publication quantifying trans resveratrol in muscadine tissue reported very low concentrations (not detected to 1 µg/g fw) in muscadine grapes (Pastrana-Bonilla et al., 2003). The trans resveratrol values reported here (3.6 to 22.8 µg/g dwt) for skins is significantly less than those reported by Ector et al. (1996), but are substantially larger than those reported by Pastrana-Bonilla et al. (2003). It is possible that cultural, environmental and genetic differences may explain the large differences in the three reports. The differences may also be explained by the different extraction and quantification techniques used.

As a comparison to the cultivars analyzed in this research, ‘Thompson’s Seedless’ and ‘Flame Seedless’ vinifera table grapes were purchased from a local grocery and analyzed for their tissue stilbene concentration. Grapes of both cultivars were separated into skins and pulp. ‘Thompson Seedless’ had total stilbene concentration of 14.2 µg/g dwt for pulp and 86.7 µg/g dwt for skins. ‘Flame Seedless’ had total stilbene concentrations of 12.3 µg/g dwt for pulp and 170 µg/g dwt for skins. Both vinifera grapes had total stilbene concentrations in skins that fit within the range of values found for muscadine cultivars in this research. Total stilbene concentrations in the pulp tissue of the vinifera grapes was higher than any reported in this work for muscadine grapes. Of the two V. labrusca grapes analyzed here, ‘Miss Blanc’ had greater skin and pulp total stilbene concentrations than the two V. vinifera cultivars. It is important to note that the data for the vinifera grapes represent only a single sample from an individual harvest year. Care must be taken when comparing the values to replicated and multi-year data. Multiyear and replicated samples from the market place would be helpful in
comparing commercial produce to muscadine and \textit{V. labrusca} grapes. Data collected from such sampling would also be useful for consumers.

During 2002, 2003 and 2004, juice of eight muscadine cultivars and one bunch grape cultivar was extracted and sampled for stilbene concentration (Table 3.3). The muscadine grapes were harvested from McNeil, Mississippi, while \textit{V. labrusca} cultivar (‘Stover’) was harvested on the LSU campus in Baton Rouge, LA. Compared to the stilbene levels of the tissue reported above, levels of stilbenes in muscadine juice were relatively low (0.12 to 0.77 mg/L) (Table 3.3). Only one muscadine cultivar (‘Magnolia’) had juice a concentration (0.77 mg/L) significantly greater than the other cultivars. The juice samples analyzed here were extracted from fresh fruit using a hydraulic rack and frame press. The samples were extracted with the intent of determining the stilbene level of the juice in the fresh fruit. No effort was made to improve stilbene extraction.

The stilbene levels in the muscadine juice do not appear to correspond with the levels in muscadine skins. For skin tissue, ‘Carlos’ and ‘Magnolia’ were significantly higher than five of the other cultivars. But for juice levels, ‘Magnolia’ was the only cultivar that was significantly different. ‘Hunt’ had one of the lowest pulp total stilbene levels and had the lowest total stilbene level in juice. These data suggest that there is little extraction of stilbenes from the skin during cold pressing.

Although the stilbene levels were low for the muscadine cultivars, the bunch grape cultivar sampled had significantly greater total stilbene levels than all of the muscadine cultivars. ‘Stover’ juice had a total stilbene concentration (3.39 mg/L) more than three times greater than the highest muscadine juice. This result is surprising given the suggestions by
other published work that muscadine grapes and juice had greater stilbene levels than bunch grape cultivars (Ector et al., 1996).

The total stilbene levels reported here for muscadine juice are comparable to those reported by Romero-Perez et al. (1999) for white vinifera grape juices (0 to 1.44 mg/L). The same publication also reported red grape juices from 0.7 to 11.5 mg/L. Although muscadine

### Table 3.3 Stilbenes (mg/L) in fresh muscadine and bunch grape juice.

|                        | Trans Piceid | Cis Piceid | Total
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlos (br)</td>
<td>0.26 de</td>
<td>0.09 ns</td>
<td>0.35 c</td>
</tr>
<tr>
<td>Magnolia (br)</td>
<td>0.7 c</td>
<td>0.07 ns</td>
<td>0.77 b</td>
</tr>
<tr>
<td>Watergate (br)</td>
<td>0.28 de</td>
<td>0.11 ns</td>
<td>0.39 c</td>
</tr>
<tr>
<td>Fry (br)</td>
<td>0.15 de</td>
<td>0.13 ns</td>
<td>0.28 c</td>
</tr>
<tr>
<td>Hunt (br)</td>
<td>0.05 e</td>
<td>0.07 ns</td>
<td>0.12 c</td>
</tr>
<tr>
<td>Noble (bl)</td>
<td>0.18 de</td>
<td>0.18 ns</td>
<td>0.36 c</td>
</tr>
<tr>
<td>Albermarle (bl)</td>
<td>0.17 de</td>
<td>0.13 ns</td>
<td>0.29 c</td>
</tr>
<tr>
<td>Sweet Jenny (br)</td>
<td>0.22 de</td>
<td>0.10 ns</td>
<td>0.31 c</td>
</tr>
<tr>
<td>Stover(y) (w)</td>
<td>3.32 a</td>
<td>0.07 ns</td>
<td>3.39 a</td>
</tr>
</tbody>
</table>

Means (n = 3) within a column followed by the same letter are not significantly different at \( P \leq 0.05 \). ns = Not significant at \( P \leq 0.05 \). br = bronze. bl = black. w = white. \( ^\gamma \)Total = Trans Piceid + Cis Piceid. No resveratrol or cis resveratrol detected. \( ^\gamma \) = bunch grape cultivar.
grapes have large quantities of stilbenes in their skins, their juices do not have comparable levels. Bunch grape skins are thinner and less fleshy than muscadine grapes. The total stilbene concentration for ‘Stover’ *V. labrusca* grape juice was more comparable to the red grape juices reported above. Stilbenes may be more readily extracted from *Vitis labrusca* skins than those of muscadine grapes.

As mentioned above, climate can have significant effect on stilbene metabolism. Previous work has relied on comparing published data for one species and data collected from another location and another year. The data presented here compare two species that, although are from different sites, have been grown during the same season and similar climates. It is possible that the same cultivar of grape grown in the humid south might have greater levels of stilbenes than it would if grown in a drier, cooler climate. Data from a 1994 (Waterhouse and Lamuela-Raventos) study report that another cultivar of bunch grape (‘Concord’) of the same species (*Vitis labruscana*) as ‘Stover’ had low levels of trans resveratrol and no trans piceid in skin tissue. This study was conducted in California. A sample of a commercially produced ‘Concord’ grape juice obtained at a grocery store contained relatively high levels of stilbenes (20.5 mg/L). Although the source of the commercial juice was unknown and consisted of only one sample, it suggests that climate can have a significant effect on stilbene concentration.

**CONCLUSIONS**

Muscadine grapes have significant levels of trans piceid and resveratrol in their tissue. ‘Carlos’ and ‘Magnolia’ had significantly greater levels of stilbenes in skin tissue than most other cultivars. Genetic variation between cultivars may help determine the metabolic capability of the fruit to produce stilbenes. These data reinforce the fact that stilbenes are
produced almost entirely in the skins of muscadine grapes as has been reported for bunch grapes. These data represent the first report of trans piceid in muscadine grape tissue and juice. Although stilbene levels are high in the skins of the fruit, obtaining any health benefits from eating fresh muscadine fruit would require the consumption of the relatively thick skins.

Much of the of literature regarding stilbenes in grapes has focused on the isomers of resveratrol. In this work, though, the trans piceid isomers appear to be the most significant of the two stilbenes. In almost all cases, isomers of trans piceid are present in greater amounts than those of resveratrol. In muscadine juice, neither trans resveratrol nor cis resveratrol was detected in samples at all. Although there are wide variations in stilbene concentration between the cultivars, trans piceid and cis piceid are consistently present in the greatest concentration.

The relatively large levels of stilbenes in ‘Midsouth’ and ‘Miss Blue’ tissue and ‘Stover’ juice suggest that *Vitis labrusca* grapes can also be a significant source of dietary stilbenes. More work directly comparing muscadine and bunch grape cultivars would be helpful in determining the effect of climate and genetic differences on stilbene production.

LITERATURE CITED


CHAPTER 4. THE EFFECT OF JUICE EXTRACTION METHOD ON THE STILBENE CONTENT OF MUSCADINE JUICE

INTRODUCTION

Resveratrol is a secondary metabolite classified as a stilbene found in grape leaves, stems and berries and can be present in both a trans and cis form (Ector et al., 1996; Bavaresco et al., 2000; Pastana-Bonilla et al., 2003). Much of the work regarding resveratrol has focused on its presence in wine (Jeandet et al., 1995; Adrian et al., 2000; Careri et al., 2003). Interest in resveratrol is due to its association with numerous health benefits, including cardiovascular protective, anti-cancer and anti-inflammatory properties (Arichi et al., 1982; Kimura et al., 1985; Kinsella et al., 1993; Jang et al., 1997; Lu and Serrero, 1999; De Santi et al., 2000; Burns et al., 2000; Brakenhielm et al., 2001; El-Mowafy, 2002). Piceid (5,4' dihydroxy-3-glucopyranosylstilbene) is a glucoside of resveratrol that is often present in quantities greater than resveratrol (Romero-Perez et al., 1999). Since resveratrol may be present in different forms and in order to get a complete picture of the health benefits of a muscadine product, it is necessary to determine the concentration of resveratrol, piceid and their isomers.

Although there have been numerous works investigating resveratrol and piceid concentration of wines, there have been relatively few on the stilbene concentration of grape juices (Ector et al., 1996; Romero-Perez et al., 1999; and Wang et al., 2002). Only very few of these works focus on the stilbene concentration in muscadine grapes. Although muscadine grapes are of less commercial significance than bunch grapes (*Vitis vinifera* and *labrusca*), they are significant crop to the southeastern United States. Muscadine grapes area a native species in the southeastern United States. Most cultivars of muscadines are not affected by Pierces disease bacterium (*Xylella fastidiosa*), which limits the successfull production of most cultivars
of bunch grapes. Information on the stilbene concentration of muscadine grape would help increase consumer awareness of the potential health benefits of this fruit.

Another important area that has not received much attention from researchers is the affect of processing on stilbene concentration of muscadine juice. Over the years, research has been conducted to determine the optimum conditions necessary to produce a high quality muscadine juice (Flora, 1979; Sistrunk and Morris, 1982;). Grape juice can be extracted using either a hot press or a cold press method. The primary difference is whether the crushed grapes are heated before pressing (Morris and Brady, 2004). Hot pressed juice generally has greater juice yields, higher titratable acidity and greater color extraction than cold press juice of the same cultivar (Sistrunk and Morris, 1982; Morris and Brady, 2004). Since hot pressed juices usually also have higher anthocyanin and total phenol levels than cold pressed juices, it would be useful to determine how the process will effect stilbene levels in juice (Sistrunk and Morris, 1982). Both hot and cold press processes generally use pectic enzymes to improve juice extraction (Morris and Brady, 2004). The enzymes help extract more juice by breaking down pectins that otherwise would trap juice in the tissue. Since adding pectolytic enzyme improves juice yield, it may also affect the extraction of stilbenes from the fruit tissue.

This experiment was initiated to: 1) Determine the effect of extraction temperature, addition of pectic enzymes and freezing on the stilbene concentration of juice of two muscadine cultivars. 2) The effect of extraction temperature, addition of pectic enzymes and freezing on stilbene concentration of two bunch grape cultivars.
MATERIALS AND METHODS

During August of 2002, 2003 and 2004, fruit from ‘Noble’ and ‘Carlos’ cultivars of muscadine grapes (*Vitis rotundifolia*) was collected from the USDA Small Fruit Crops Laboratory in Poplarville, MS. ‘Carlos’ is a bronze skinned grape that is often used for juice and wine production. ‘Noble’ is a black skinned grape that is also commonly used for juice and wine production. Three replicates were harvested for each cultivar. Each replication was collected from a separate vine. After harvest, samples were refrigerated during transport. In addition to the two cultivars of muscadine grapes, two cultivars of bunch grapes (*Vitis labrusca*, ‘Midsouth’ and ‘Miss Blue’) were also sampled in summer 2003 from the Hill Farm on the LSU campus in Baton Rouge, LA. ‘Midsouth’ is a purple bunch grape and ‘Miss Blanc’ is a white bunch grape. For *V. labrusca* grapes, three replicates were taken for each cultivar. One replicate represents one arm of a single vine.

For 2002 and 2003 harvest years, fruit from each cultivar were separated into three parts for further processing containing approximately 2 kg each. For the 2004 harvest year, collected fruit was separated into four equal parts containing approximately 2 kg (Figure 4.1). All samples were crushed using a manually operated crusher/de-stemmer.

Free Run: After crushing the first sample of grapes, a 20 ml sample was collected from the juice that ran freely from the crushed fruit.

Cold Pressed: After collecting the free run sample, the remaining fruit were pressed using a hydraulic rack and frame press. A 20 ml sample was collected from the resulting juice.
Figure 4.1 Juice extraction methods.
Hot Pressed: The next group of grapes were crushed and quickly heated in a steam kettle to 60 °C and immediately pressed. A 20 ml sample was collected from the resulting juice.

Frozen: The next group of grapes were placed in a sealed plastic bag and frozen overnight at -20 °C. After freezing, the grapes were thawed and crushed. A 20 ml sample was collected from the resulting juice.

Enzyme: In 2004, the last group of grapes were crushed and placed in a two liter container. Pectic enzyme (pectinase, LD Carlson, Kent, OH) was stirred into the crushed grapes at a concentration of 100 ppm by weight. The sample was placed in cold storage at 45°F overnight. After enzyme treatment, the crushed fruit were pressed. A 20 ml sample was collected from the resulting juice.

Percent soluble solids was measured using a digital refractometer (Bellingham and Stanley, model RFM 80, England) for all juice samples. During the 2004 harvest year, juice yield data were collected for cold press, hot press, frozen and enzyme treatments. Percent yield was calculated as follows: (weight of juice recovered/ weight of crushed fruit) X 100. All juice samples were frozen at -40 °C until processing for HPLC analysis. All juice samples were thawed and centrifuged to remove particulate matter and a sample of the supernatant was filtered through 0.2 µm Nucleopore Track-Etch membrane (polycarbonate) (Whatman, Banbury, Oxon, UK) in preparation for HPLC analysis. After microfiltration, samples were placed in amber autosampler vials to protect them from light induced isomerization.

Sample analysis was done using HPLC with a UV detector. The analytical equipment consisted of a Waters 600 pump, a Waters 717 Plus autosampler, a Waters 2487 dual wave
length UV detector. The column was a Waters Sunfire 3 x 250 mm C18 (5 µm particle size) with a 20 mm pre-column of the same material. Column temperature was maintained at 30°C. Twenty µL of each sample was injected and eluted using an isocratic method with a mobile phase of 69.3:22:8:0.7/ water: acetonitrile: propanol: formic acid by volume, at a flow rate of 0.2 ml/min. Samples were analyzed by a UV detector at 285 nm and 306 nm for the cis isomers and the trans isomers of resveratrol and piceid respectively (Careri et al., 2003). Sample peaks and retention times were compared to standard peaks and retention times for quantification.

Trans resveratrol was obtained from Sigma (St. Louis, MO) and trans piceid was obtained from Apin Chemicals (Abingdon, Oxon, UK). The trans piceid standard was of unknown concentration; therefore, the trans piceid chromatogram was used only to identify the trans piceid peaks in the samples. To verify the identity of trans piceid, a standard solution was incubated with β-D-glucosidase (Sigma, St. Louis, MO). The chromatogram obtained after enzyme incubation revealed the disappearance of the trans piceid peak and the appearance of a peak whose retention time corresponded with the standard trans resveratrol peak.

No standards are available for cis isomers of resveratrol and piceid. To identify the peaks for the cis compounds in the samples, standards of both trans resveratrol and trans piceid were exposed to direct sunlight for approximately 15 minutes. This resulted in the conversion of approximately 85% of the trans compounds to their cis isomers. Conversion of trans isomers to cis was confirmed by HPLC analysis of the standard before and after exposure to sunlight.

Trans resveratrol was the only compound of interest for which there was a standard of known concentration. Both trans resveratrol and trans piceid were quantified using the
standard peak for trans resveratrol since the two compounds have identical UV absorbance spectra (Romero-Perez et al., 2001; Cantos et al, 2000; Adrian et al., 2000). Both cis resveratrol and cis piceid were quantified using the standard peak for cis resveratrol. The concentration of the cis resveratrol peak was determined by calculating the amount of trans resveratrol that disappeared from the sunlight exposed trans standard. Data were analyzed using PROC MIXED, PROC CORR and means separated using Tukey’s studentized range test, $\alpha = 0.05$ (SAS, Carey, NC).

RESULTS AND DISCUSSION

Previous work has demonstrated that different processing methods can result in juices with varying qualities (Flora, 1979; Sistrunk and Morris, 1982). The results of this research suggest that processing can affect the stilbene concentration as well. Since the predominant source of stilbenes in grapes is skin tissue (Chapter 3), it is expected that methods designed to extract more juice from the skins may also extract more stilbenes. Hot pressed juice of ‘Noble’ had significantly greater trans piceid concentration than that of free run, cold press or frozen treatments (Table 4.1). For ‘Carlos’, hot press and frozen treatments were significantly greater ($p \leq 0.05$) when compared to other treatments. Hot press and frozen juice of ‘Noble’ had significantly greater concentration of cis piceid when compared to juice from free run or cold press. There were no significant differences between treatments for cis piceid in ‘Carlos’ juice. Trans resveratrol was only detected in hot press juice for ‘Carlos’ and ‘Noble’ and at levels at or near the detection limits. For both ‘Carlos’ and ‘Noble’, total stilbenes were greater for hot press and frozen treatments than for free run and cold press treatments.
<table>
<thead>
<tr>
<th>Method</th>
<th>Noble (bl)</th>
<th>Carlos (br)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans Piceid</td>
<td>Cis Piceid</td>
</tr>
<tr>
<td>Free Run</td>
<td>0.21 b</td>
<td>0.22 b</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Cold Press</td>
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<td>0.19 b</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Hot Press</td>
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<td>0.88 a</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Frozen</td>
<td>0.43 b</td>
<td>0.66 b</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A</td>
</tr>
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</tr>
<tr>
<td></td>
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<td>ns</td>
</tr>
</tbody>
</table>

Means (n = 3) within a column followed by the same lower case letter are not significantly different at \( P \leq 0.05 \). Uppercase letters below means compares between cultivars within rows. ns = No significant differences at \( P \leq 0.05 \). bl = black. br = bronze. ^Percent juice yield = wt of juice/wt of crushed grapes. ^Yield data not collected for free run treatment. ^Enzyme treatment stilbene data from one year only and is not statistically compared to multi-year data.
Previous data showed that hot pressed juice had darker color, higher total phenols and greater acid extraction in muscadine grapes (Sistrunk and Morris, 1982; Morris and Brady, 2004; Lee and Talcott, 2004). It is possible that stilbene extraction parallels the increased extraction of color, phenols and acids from muscadine skins. In industry, hot pressing is the predominant method used to extract dark grape juices. One of the primary reasons to use this process is to increase the color extraction from the grape tissue (McLellan and Acree, 1993). Anthocyanins are responsible for the color of dark muscadine and bunch grape juices. The mechanism that produces resveratrol and its monomers, is very similar to the metabolic process that produces anthocyanins. Since much of the initial pathway for the production of anthocyanins and stilbenes is shared (Fritzemeier and Kindl, 1981; Melchior and Kindl, 1990), the site of metabolism for the two compounds may also be similarly located in the anatomy of the muscadine fruit.

Freezing (-10 °C) fruit resulted in greater juice stilbene concentration than for cold or free run. Freezing causes the rupture of most cell membranes and cell walls. Breakdown of the membrane structure may allow stilbenes to become dissolved in the juice. One possible side effect of freezing may be that other compounds may be extracted as well that may not contribute positively to the sensory characteristics of the juice. Research evaluating the sensory characteristics of juices obtained after freezing would be required to determine if freezing fruit would be a practical method of increasing stilbenes in muscadine juice.

Pectic enzymes were also used to increase the amount of juice extracted from fruit. This treatment was only applied to fruit collected in 2004, therefore comparisons are restricted to other treatments for that harvest year. Stilbene levels in enzyme samples were very similar
to those of the frozen samples. Frozen and enzyme total stilbene levels were greater for ‘Noble’ than free run and cold press but less than hot press levels. For ‘Carlos’, hot press, enzyme and frozen total stilbene levels were all greater than for free run and cold press juice. Pectic enzymes break down the natural pectins that are present in the juice tissue of the grape. By adding pectic enzymes before pressing, juice that would otherwise be trapped in the tissue can be released. Although the action of the pectic enzymes would most affect the pulp, much of the additional stilbenes that are released likely comes from within the skin tissue where the metabolic pathways for stilbene synthesis are likely present.

Use of pectic enzyme is common both in white and dark grape juice extraction as well as in wine production. It results in substantially larger juice yields (Table 4.1). In this case, pectic enzyme treatment significantly increased ‘Carlos’ juice yield from 41.5 % (fresh press) to 61.1 % and ‘Noble’ juice yield from 40.8% (fresh press) to 56.2%. Pectic enzyme treatment resulted in the greatest increase in juice yield of all the treatments.

Although enzyme treatment produced the greatest increase in juice yield, it did not result in the greatest stilbene concentration. There were no significant correlation between juice yield and the concentration of any of the individual or total stilbenes for either cultivar. Hot press treatment also increased juice yield over cold press but the increase was not as great as the enzyme treatment. The hot press treatment had the greatest total stilbene levels. One reason for this disparity may be that much of the increase in juice yield resulting from the pectic enzyme treatment likely comes from the pulp. As reported in Chapter 3, the muscadine fruit pulp has the lowest stilbene concentration. The additional juice extracted from the pulp may be diluting the final stilbene concentration of the juice in the enzyme treatment as
compared to the hot press treatment. The hot press treatment is likely more effective in extracting stilbenes from the skin than is the pectic enzyme treatment.

It is important to note that most commercial dark grape juices are extracted using both pectic enzymes and heat (Morris and Brady, 2004). Several commercially prepared grape juices were analyzed for stilbene concentration as a comparison to the juices studied in our work. ‘Concord’ (purple) and ‘Niagara’ (white) grape juices were purchased at a local grocery store. ‘Concord’ and the ‘Niagara’ juices had total stilbenes concentrations of 20.5 mg/L and 2.1 mg/L, respectively. The stilbene concentration for the ‘Concord’ juice is considerably greater than the highest concentration for muscadine juices quantified in our research (2.05 mg/L). Several factors may contribute to the differences in concentration. Commercial dark grape juices are extracted using both enzyme treatment and heat. White juices use only an enzyme treatment since color extraction from the skins is not necessary or desired. As was reported in chapter 3, there can be significant varietal differences in stilbene concentration for muscadine grapes, as is the case for bunch grapes. Since the values reported above for the commercial juices only represent a single sample, care must be taken when comparing the stilbene concentrations with the muscadine juice samples analyzed in this work. Differences such as processing method, grape cultivar and climatic conditions of the vines can have significant influence on stilbene concentration of grapes.

Soluble solids were measured in juice samples to determine if there were any relationships between sugar and stilbene extraction. Soluble solids varied between approximately 13 and 16 °Brix. There were no significant differences between treatments or cultivars for soluble solids in the juice samples (Appendix 1). There were no significant
correlations between soluble solids and any of the individual or total stilbene concentrations. This would suggest that soluble solid extraction from the tissue is not directly related to stilbene extraction from the tissue of muscadine grapes.

In 2003, data were taken from ‘Midsouth’ and ‘Miss Blue’ *V. labrusca* grape cultivars as well as ‘Carlos’ and ‘Noble’ muscadine grapes in order to compare the stilbene concentration and the effects of processing between the two grape species (Table 4.2). The relationship between processing treatments was similar for ‘Noble’ and ‘Carlos’. For this one year of data, there were no statistical differences between the treatments for total stilbenes. Except for ‘Noble’ hot press, trans piceid and cis piceid were the only stilbenes detected in the juice samples (Appendix 2). In ‘Noble’ hot press, trans resveratrol was detected only at a very low concentration. Cis resveratrol was not detected in any juice sample. Much of the previous literature quantifying stilbenes in grapes and grape juice only analyzed for trans resveratrol (Ector et al., 1996; McMurtry, 1997; Pastrana-Bonilla et al., 2003; Careri et al., 2003). If that were the case here, there would be little to report on the trans resveratrol concentration of muscadine and bunch grape juice.

‘Midsouth’ and ‘Miss Blue’ juice had similar relationships between the processing treatments. Hot press juice had the greatest total stilbene levels and was significantly greater than free run, cold press and frozen juices (Table 4.2). Free run total stilbene levels were relatively high for ‘Miss Blue’. Although the concentrations were not significantly greater than cold press and frozen, they were significantly greater than free run concentrations for ‘Noble’, ‘Carlos’ and ‘Midsouth’. There were no significant differences in total stilbenes between the cultivars for either cold press or frozen juice treatments. The most striking differences in
these data were the total stilbene levels for the hot press treatments. Both ‘Midsouth’ and ‘Miss Blanc’ bunch grape juice had total stilbene levels significantly (p < 0.05) greater than ‘Carlos’ and ‘Noble’ muscadine grape juice. Total stilbene levels for the two bunch grape cultivars had levels more than five times greater than the muscadine grape cultivars. It is

<table>
<thead>
<tr>
<th></th>
<th>Carlos (br)</th>
<th>Noble (bl)</th>
<th>Midsouth^z (p)</th>
<th>Miss Blanc^z</th>
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</tbody>
</table>

Means (n = 3) within a column followed by the same letter are not significantly different at P ≤0.05. Capital letters below means compares between cultivars within rows. ns = No significant differences at α = 0.05. br = bronze. bl = black. p = purple. w = white. ^zBunch grape cultivar. ^γTotal Stilbenes = Trans Piceid+Cis Piceid+Trans Resveratrol+Cis Resveratrol. ^xNo data collected.
surprising that these two bunch grape cultivars that are relatively thin skinned and somewhat more susceptible to disease than muscadine grapes would have such high stilbene levels. Resveratrol and its monomers have been reported to be phytoalexins. Phytoalexins are produced by the plant to defend against disease attack. Muscadine’s resistance to disease has been used as an explanation for previous reports of high levels of resveratrol in the species. It is possible that the presence of large amounts of stilbenes in the skins of the *V. labrusca* grapes may be partially responsible for their resistance or tolerance to Pierce’s disease.

It has been reported in *V. vinifera* grapes that the levels of resveratrol reach their highest just before veraison (Jeandet et al., 1991). The levels are reported to decline with maturity from that point on. If *V. labrusca* grapes have similar developmental characteristics, this may help explain the relatively large concentration of stilbenes in the *V. labrusca* grape juices. Soluble solids data collected on the juices studied show that the two *V. labrusca* cultivars had soluble solids concentrations significantly lower than the muscadine juices. There were no significant correlations between soluble solids and the concentration of any of the individual or total stilbenes. The relatively low soluble solids of the *V. labrusca* juice may reflect on the maturity of the fruit at harvest. The *V. labrusca* grapes were harvested in early July. It is possible that the fruit were harvested before peak maturity (soluble solids). If this were the case, it is possible that the stilbene levels were higher in the labrusca grapes because of their relative immaturity. ‘Midsouth’ is a purple grape and was fully colored at the time of harvest. ‘Miss Blanc’ is a white grape and was fully expanded and appeared to be fully mature. It is possible that the grapes could have matured longer and resulted in higher soluble solids and lower stilbenes if they follow the same pattern for resveratrol levels in vinifera grapes. Future
studies should consider comparing stilbene levels in muscadine and labrusca grapes at different developmental stages to answer some of these questions.

It appears that the majority of the stilbenes present in the juice appear to have been extracted from the skins since the majority of the stilbenes in the fruit is located in the skins. Since the skins of the bunch grapes are generally thinner than those of muscadine skins, the relative concentration of stilbenes must be somewhat great. Stilbenes may be more easily extracted from the thin skins of the bunch grapes than from the thick fleshy skins of the muscadine grapes. It appears that ‘Miss Blue’ and ‘Midsouth’ would produce a juice with greater stilbene concentration than ‘Carlos’ and ‘Noble’ muscadine grapes.

CONCLUSIONS

The presence of stilbenes such as resveratrol and piceid in fruit juices would provide an additional health benefit to consumers. If methods can be developed to increase the levels of these potential disease preventing compounds, a more desirable and marketable product can be produced. It appears from the data presented here that both hot press and enzyme treatments produced juices with greater stilbene concentration than free run or cold pressed juice. Additional work investigating a combination of hot pressing and enzyme treatment might improve the stilbene concentration further. Sensory evaluation of the juices obtained from the various treatments would also be helpful to determine if the products would be acceptable to consumers.

Also, more work directly comparing Vitis labrusca and Vitis rotundifolia grapes for their stilbene concentrations might help clarify differences between the two species. Work comparing the relative stilbene concentration of both muscadine and labrusca grapes grown at
different climates might help determine if the production of stilbenes is a result of climatic condition or genetic programing.

LITERATURE CITED


CHAPTER 5. THE EFFECT OF POSTHARVEST ULTRA VIOLET IRRADIATION ON THE STILBENE CONTENT OF MUSCADINE GRAPE

INTRODUCTION

One of the recommended ways to reduce obesity, prevent disease and improve overall health is to increase daily consumption of fruits and vegetables. The nutritional benefits of fruits and vegetables include dietary fiber, vitamins and minerals. In recent years, an additional benefit of fruit and vegetable consumption that has been reported, is the presence of phytochemicals that may help prevent certain diseases. These phytochemicals have come to be known as nutraceuticals. This term refers to naturally occurring compounds that have potential disease fighting properties.

Muscadine grapes (Vitis rotundifolia) are a popular native fruit crop of the southeast U.S. grown in back yards as well as in commercial vineyards. There have been numerous publications detailing the nutritional and nutraceutical value of muscadine grapes (Ector et al., 1996; Basiouny and Himelrick, 2001; Lee and Talcott, 2004; Pastrana-Bonilla et al., 2003; Striegler et al., 2005; Hartle et al., 2005). One important nutraceutical previously found in muscadine grapes is resveratrol. Resveratrol has been reported to have numerous health benefits, including cardiovascular protective, anti-cancer and anti-inflammatory properties (Arichi et al., 1982; Kimura et al., 1985; Kinsella et al., 1993; Jang et al., 1997; Lu and Serrero, 1999; De Santi et al., 2000; Burns et al., 2000; Brakenhielm et al., 2001; El-Mowafy, 2002). Resveratrol has been reported in grape (Vitis vinifera and V. labrusca) tissue, juice and wine (Siemann and Creasy, 1992; Romero-Perez et al., 1999; Cantos et al., 2000). Resveratrol has been reported in muscadine tissue, juice and wine (Ector et al., 1996, McMurtry, 1997; Pastrana-Bonilla et al., 2003). Resveratrol exists in different forms in the plant. It is often
present as both the trans and cis isomer as well as a glycosylated form called piceid. Piceid also exists in both the trans and cis forms. These four compounds are part of a class of chemicals labeled stilbenes. All of the previous work quantifying resveratrol in muscadine has focused on the trans isomer only. Although cis resveratrol is rare in grape tissue and juice, trans and cis piceid are often present at levels that exceed that of trans resveratrol (Romero-Perez et al., 1999). Piceid is essentially a glycosylated resveratrol molecule. Since glucosidase is known to be present in the digestive tract, it is possible that the sugar moiety could be cleaved from the piceid molecule, leaving trans resveratrol to be absorbed during digestion (Hackett, 1986; Day et al., 1998). Several studies have demonstrated that piceid is biologically active in animal systems (Kimura et al., 1995, Shan et al., 1990). Any investigation into the nutriceutical value of muscadine fruit would be incomplete without including the contribution of piceid.

Stilbenes (trans and cis resveratrol and piceid) are metabolized in the plant by the enzyme called stilbene synthase (STS). It is synthesized from p-coumaroyl CoA and malonyl CoA (Fritzemeier and Kindl, 1981; Melchior and Kindl, 1990). The metabolic pathway leading to stilbenes is very similar to the pathway that produces flavanoids. Flavanoids are precursors of anthocyanin pigments. UV light is one of the many stresses that can stimulate the metabolism of stilbenes (Douillet-Breuil et al., 1999). The ability of ultraviolet light to stimulate this process has been shown to peak immediately before veraison (color formation) and decline with maturity (Jeandet et al., 1991). It has been proposed that this may be the result of anthocyanin metabolism competing for the same precursors as resveratrol metabolism (Jeandet et al., 1995). Work published in 2001 suggests that this may not be the case. Versari
et al. (2001) investigated stilbene synthase (STS) gene expression and demonstrated that UV light was able to stimulate STS expression in the immature fruit but was unable to significantly stimulate it in mature fruit. This indicates that the expression of the genes necessary for stilbene production are ramped down as the fruit approaches full ripe stage.

Several authors have investigated the potential of UV light to increase stilbene concentration in fresh fruit. Langcake and Price reported in 1977 that UV irradiation induced trans resveratrol production in tissue of *Vitis vinifera* grapes. Adrian et al. reported in 2000 that UV light increased trans piceid, cis piceid and trans resveratrol levels in berries 48 hours after exposure for two of the three *V. vinifera* cultivars studied. In 2000, Cantos et al. studied the effect of UV light on stilbenes in ‘Napolean’ table grapes. They found that grapes held under cold storage alone had a two fold increase in trans piceid and a three fold increase for trans resveratrol. UV irradiation increased trans resveratrol ten fold after cold storage. In 2001, Versari et al. was able to increase trans resveratrol concentration from undetectable to 39.8 µg/g FW for vinifera grapes at veraison but only from 1.5 to 4.8 µg/g FW at maturity. Cantos et al. in 2002 studied UV irradiation on seven different *V. vinifera* table grape cultivars. Both separately and in combination, UV light and cold storage significantly increased trans resveratrol in all cultivars. UV treatment and cold storage increased trans piceid levels in two of the four red cultivars and all three white cultivars. Peak stilbene concentration after treatment varied between three and five days when stored at 22° C. In a 2003 study, Cantos et al. demonstrated that UV exposure of grapes more than doubled the ultimate trans resveratrol concentration of the wine.
The works described above indicate that increased stilbene levels can be induced using UV irradiation for *V. vinifera* grapes. Increasing stilbene concentration of muscadine grapes could make this already nutritional fruit more appealing to health conscious consumers. UV treated muscadine fruit may produce juices and wines with greater stilbene levels and potentially higher nutraceutical value. A study was initiated to determine the effect of UV irradiation and cold storage on the stilbene levels of ‘Carlos’ and ‘Noble’ muscadine grapes.

**MATERIALS AND METHODS**

Fruit of ‘Noble’ and ‘Carlos’ cultivars of muscadine grapes (*Vitis rotundifolia*) were collected from vines in the research vineyard at the USDA Small Fruit Crops Laboratory in Poplarville, MS. Three replicates were harvested for each cultivar. One vine represents one replication. After harvest samples were stored on ice during transport and refrigerated at 1 °C until processing. Berries were carefully separated from the stems and placed in clear plastic bags. Berries for the fresh treatment were immediately processed and frozen. Berries for the cold storage only treatment were placed immediately into a cooler. Berries were placed in plastic bags which permitted transmittance of UV light. Berries for the UV treatment were exposed to UV irradiation using a Fotodyne Foto/UV 300 UV (313nm, 4X15 watt) lamp. The berries were exposed to UV light for 30 minutes and bags were rotated and exposed for 30 additional minutes in order to expose the entire berry. After UV exposure, samples were placed with the unexposed samples in a cooler at 10° C for 5 days. After cold storage, all samples were firm and in good condition.

After treatment, berries were divided into skins, pulp and seed. Each of the tissue samples were frozen at -40° C. The frozen tissue samples were lyophylized and re-frozen at -
40°C until further processing. One gram of freeze dried tissue was weighed and placed in centrifuge tubes along with 12.5 ml of 80% ethanol. Solutions were incubated for 30 minutes at 60°C with agitation every 5 minutes. (Romero-Perez et al., 2001). After heating, tubes were centrifuged for 20 minutes. The supernatant was decanted into a graduated cylinder and brought to 10 ml volume. An aliquot of each solution was filtered through 0.2 μm Nucleopore Track-Etch membrane (polycarbonate) (Whatman, Maidstone, England) in preparation for HPLC analysis.

Tissue samples were analyzed using HPLC with a UV detector. The analytical equipment consisted of a Waters 600 pump, a Waters 717 Plus autosampler, a Waters 2487 dual wave length UV detector (Milford, MA). Stilbenes were separated using a Waters Sunfire 3 x 250 mm C18 (5 μm particle size) with a 20 mm pre-column of the same material. Column temperature was maintained at 30 °C. 20 μL of each sample was eluted using an isocratic method with a mobile phase of 69.3:22:8:0.7/water: acetonitrile: propanol: formic acid by volume at a flow rate of 0.2 ml/min. Samples were analyzed by the UV detector at 285 nm and 306 nm for the cis isomers and the trans isomers respectively (Careri et al., 2003).

Sample peak areas were compared to those of known standards for quantification. Trans resveratrol was purchased from Sigma Chemicals (St. Louis, MO) and trans piceid was purchased from Apin Chemicals (Abingdon, Oxon, UK). Since the trans piceid standard was of unknown concentration, its chromatogram was only used to identify the trans piceid peaks in the samples. To verify the identity of trans piceid, the standard was subjected to incubation with β-D-glucosidase (Sigma Chemicals). The HPLC chromatogram obtained after the enzyme incubation revealed the disappearance of the trans piceid peak and the appearance of a
peak whose retention time corresponded to the standard trans resveratrol peak. To identify peaks for the cis compounds, standards of both trans resveratrol and trans piceid were exposed to direct sunlight for approximately 15 minutes. This resulted in the conversion of approximately 85% of the trans compounds to their cis isomers as determined by analysis of the peak areas before and after sunlight exposure.

Trans resveratrol was the only compound for which there was a standard of known concentration. Since both piceid and resveratrol have identical uv absorbance spectra, both trans resveratrol and trans piceid were quantified using the standard peak for trans resveratrol (Romero-Perez et al., 2001; Cantos et al., 2000; Adrian et al., 2000). Due to the instability of both cis resveratrol and cis piceid, no standards of these compounds are available for purchase. Cis resveratrol and cis piceid were quantified using the standard peak for cis resveratrol. The concentration of the cis resveratrol peak was determined by calculating the amount of trans resveratrol that had disappeared from the sunlight exposed trans standard. Data were analyzed using PROC CORR, PROC MIXED and means separated using Tukey’s studentized range test, \( \alpha = 0.05 \) (SAS, Carey, NC).

RESULTS AND DISCUSSION

Previous work has established that UV irradiation can stimulate stilbene metabolism in grape tissue. Our work is the first to test the hypotheses that ultra-violet irradiation stimulates stilbene metabolism in muscadine grapes. For ‘Carlos’ grape skins, cold storage alone significantly increased trans resveratrol and total stilbenes (Table 5.1). This agrees with previous work where cold storage increased trans resveratrol by more than 2 fold in \( V. \) vinifera grapes (Cantos et al., 2000). Unlike previous work, UV treatment did not further increase trans
resveratrol concentration from cold storage alone in ‘Carlos’ skins. There were no significant differences between treatments for levels of trans piceid, cis piceid or cis resveratrol in ‘Carlos’ skins. In previous studies of UV irradiation of grapes, it was reported that stilbene concentrations change with time after exposure (Cantos et al., 2000; Adrian et al., 2000). In our experiment, the grapes were sampled after five days of cold storage. It is possible that the effect of UV treatment on stilbene levels may have peaked either earlier or later than five days. In this study, the fruit was stored at 10°C. This temperature was selected as a compromise between the need to preserve the fruit from decomposition and the need to allow physiological

<table>
<thead>
<tr>
<th>Table 5.1 Effect of UV irradiation and cold storage (10°C) on stilbene concentration (µg/g dwt) of muscadine skin tissue.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlos (br) Fresh</td>
</tr>
<tr>
<td>CS/UT</td>
</tr>
<tr>
<td>CS/UV</td>
</tr>
<tr>
<td>Noble (bl) Fresh</td>
</tr>
<tr>
<td>CS/UT</td>
</tr>
<tr>
<td>CS/UV</td>
</tr>
</tbody>
</table>

Means within a column and within a cultivar followed by the same letter are not significantly different at $P \leq 0.05$. ns = Not significant at $P \leq 0.05$. Fresh = immediately after harvest, CS/UT = cold storage no UV treatment, CS/UV = cold storage with UV treatment. br = bronze. bl = black. $^2$Total = Trans Piceid+Cis Piceid+Trans Resveratrol+ Cin Resveratrol.
activity to occur. Since the grapes were mature, storage longer than 5-7 days at this temperature might have allowed for decomposition to begin.

Unlike ‘Carlos’, UV treatment did significantly increase trans resveratrol for ‘Noble’ skins over both cold storage and fresh treatments. Cold storage slightly increased trans piceid and trans resveratrol levels and decreased cis piceid levels, although not significantly. This is in contrast to ‘Carlos’ skins, where only cold storage appears to have affected stilbene levels. Cis resveratrol was not detected for ‘Noble’ skins in either the fresh or the cold storage

<table>
<thead>
<tr>
<th></th>
<th>Pulp</th>
<th>Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans Piceid</td>
<td>Cis Piceid</td>
</tr>
<tr>
<td>Carlos (br)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>0.3 b</td>
<td>0.7 ns</td>
</tr>
<tr>
<td>CS/UT</td>
<td>3.2 a</td>
<td>nd ns</td>
</tr>
<tr>
<td>CS/UV</td>
<td>2.5 a</td>
<td>nd ns</td>
</tr>
<tr>
<td>Noble (bl)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh</td>
<td>0.3 a</td>
<td>1.2 ns</td>
</tr>
<tr>
<td>CS/UT</td>
<td>1.0 a</td>
<td>0.5 ns</td>
</tr>
<tr>
<td>CS/UV</td>
<td>0.9 a</td>
<td>1.6 ns</td>
</tr>
</tbody>
</table>

Means within a column and within a cultivar followed by the same letter are not significantly different at $P \leq 0.05$. ns = Not significant at $P \leq 0.05$. Fresh = immediately after harvest, CS/UT = cold storage no UV treatment, CS/UV = cold storage with UV treatment. Trans piceid not quantified and and Cis resveratrol not detected in seed tissue. $^\gamma$Total = Trans Piceid+Cis Piceid+Trans Resveratrol+ Cis Resveratrol.
Cis resveratrol was detected in UV irradiated grape skins. The appearance of cis resveratrol is possibly due to UV induced isomerization from trans to the cis form. Since sunlight can convert most trans resveratrol in standard solutions to cis resveratrol, it is not surprising that the same might happen in the fruit with exposure to UV irradiation.

Cold storage alone significantly increased both trans piceid and total stilbenes in ‘Carlos’ pulp over fresh grape pulp. This is in contrast to ‘Carlos’ skins where cold storage had no effect on trans piceid levels. Because piceid is more water soluble than resveratrol, perhaps the compound was more able to move from the skins into the pulp of the berries. This might also explain the lack of increase in trans piceid in the skins with cold storage. Trans resveratrol levels increased slightly with cold storage but were not significantly greater. Since it is believed that resveratrol is metabolized almost exclusively in the skins, it may be possible that only small amounts were able to move from the skins to the pulp. As was the case with ‘Carlos’ skins, UV treatment did not significantly affect stilbene levels in pulp over cold storage alone.

Although the differences were not statistically significant, UV treated pulp had slightly higher concentrations of cis piceid, trans resveratrol and total stilbenes than fresh and cold stored alone. This agrees with the ‘Noble’ skin data. Pulp trans piceid levels slightly increased for both cold storage alone and UV treatment over fresh pulp, although this was not significant. This corresponds with a similar reaction in ‘Noble’ skins.

There were slight and statistically insignificant increases in cis piceid and trans resveratrol for ‘Carlos’ cold storage and UV treated seed over fresh seed as was the case with
‘Carlos’ skin and pulp. There were no significant changes in cis piceid in ‘Noble’ seed. Trans resveratrol was only detected in UV treated seed.

The two muscadine cultivars studied here had substantially different reactions to UV exposure and cold storage. The fact that the two cultivars differ in their skin colors may have much to do with their different reactions. ‘Carlos’ is a bronze cultivar, while ‘Noble’ is a black cultivar. Early work with trans resveratrol in wine revealed greater levels in red wine than in white. This led many to suppose that dark skinned grapes had higher trans resveratrol levels than did white skin grapes. Subsequent research has both supported and contradicted this theory. It is obviously not the case with either muscadine or V. labrusca grapes. Tissue data presented in Chapter 3 demonstrates that ‘Carlos’ and one other bronze cultivar has significantly greater total stilbene levels than all of the dark skinned cultivars studied. It also showed that a white skinned ‘Miss Blanc’ V. labrusca grape had significantly greater total stilbene levels than did a dark skinned cultivar (‘Midsouth’). Although it is clear that the metabolic pathways responsible for metabolism of stilbenes is present in both dark and light skinned muscadines, it is similarly clear that those pathways react differently to environmental stress.

Both cultivars were able to synthesize trans resveratrol in response to stress. ‘Carlos’ appeared to respond well to lower temperatures while ‘Noble’ had a greater response with UV light. The great similarity between the metabolic pathways of stilbenes and flavanoids (anthocyanin precursors), may help explain the different reactions of the two cultivars. Previous work has shown that in V. vinifera grapes, STS gene expression declines from color formation to fruit maturity. The ability for this metabolic pathway to be promoted by
environmental factors is similarly reduced (Versari et al., 2001). The STS pathway is clearly
still active in both cultivars since they both responded to stress by the increased production of
trans resveratrol. What is unclear is why the two cultivars reacted differently. As a dark
skinned fruit, ‘Noble’ may have a more active chalcone synthase pathway, since it requires the
production of anthocyanins. There may be a mechanism that promotes the anthocyanin
production pathway as a protective reaction to UV light. Since the STS pathway is so similar,
it may be promoted by the same mechanism.

As a light skinned muscadine, ‘Carlos’ does not rely on anthocyanins to protect against
UV irradiation. They do contain other pigments that color the fruit skin. There may be
mechanisms that promotes the production of these pigments. In response to UV light, these
mechanisms may promote pathways that are not as closely related to stilbene metabolism.

CONCLUSIONS

‘Carlos’ and ‘Noble’ muscadine grapes contain substantial amounts of stilbenes in their
skins. Both cultivars were stimulated by postharvest environmental stress to produce
significantly greater levels of total stilbenes. ‘Carlos’ muscadine grapes can be induced to
produce stilbenes by cold storage, while ‘Noble’ can similarly be induced by exposure to UV
light. There is a potential that postharvest treatment of muscadine grapes may help yield fresh
fruit, juices and wines with greater nutraceutical value to consumers.

This work also demonstrated that ‘Carlos’ and ‘Noble’ have differing mechanisms that
promote the metabolism of stilbenes. More work investigating differing temperature, time and
UV wavelengths may help understand the different reaction of the two cultivars to the
treatments given here.
LITERATURE CITED


The stilbene concentration of muscadine grapes varies widely among muscadine cultivars. Eight cultivars of muscadine grapes were evaluated for their tissue and juice concentration of trans piceid, cis piceid, trans resveratrol and cis resveratrol. These four compounds are part of a class of phytochemicals called stilbenes. Among the cultivars evaluated, ‘Carlos’ and ‘Magnolia’ had significantly greater total stilbene levels in skin tissue than all other cultivars except ‘Watergate’. The three cultivars with the greatest skin total stilbene concentration (‘Carlos’, ‘Magnolia’, ‘Watergate’) were all bronze skinned cultivars. They had significantly greater levels than all of the dark skinned cultivars evaluated here (‘Hunt’, ‘Noble’, ‘Albermarle’). Previous work suggested that dark skinned grapes had generally higher resveratrol levels than light skinned grapes. Since both piceid and resveratrol were analyzed here, a better picture of the stilbene concentration of the grapes was available. Although the three highest skin total stilbene levels were in bronze cultivars, one other bronze cultivar (‘Sweet Jenny’) had the lowest total levels of all cultivars. There were no significant differences between the total stilbene levels in pulp and seed tissue.

Stilbene concentration of the skins of muscadine grapes does not appear to be directly related to the levels found in fresh pressed juice. Only one of the top three cultivars for stilbene levels in skins had juice stilbene levels significantly greater than the remaining muscadine cultivars. ‘Magnolia’ juice had a total stilbene level (0.77 mg/L) greater than all other muscadine juices studied. Although ‘Magnolia’ had the greatest level among muscadine grapes, ‘Stover’, a labrusca grape had a juice stilbene level (3.39 mg/L) significantly higher than ‘Magnolia’.
Tissue data collected during 2003 also compared *V. labrusca* grapes with muscadine grapes for total stilbene levels. These data found that ‘Miss Blanc’ labrusca grape skins (519.4 µg/g dwt) and pulp (30.7 µg/g dwt) had significantly greater total stilbene levels than ‘Sweet Jenny’ (150.4 µg/g dwt and 1.7 µg/g dwt) and ‘Noble’ (351 µg/g dwt and 1.7 µg/g dwt) muscadine grapes.

Processing method significantly affected both juice yield and stilbene concentration of ‘Carlos’ and ‘Noble’ muscadine juice. Juice obtained from grapes treated with pectolytic enzyme had significantly greater juice yield than cold press and frozen grapes for both cultivars. Although pectolytic enzyme treatment resulted in the greatest juice yield for both ‘Noble’ and ‘Carlos’ grapes, hot pressed juice had the greatest total stilbene concentration for both cultivars. Hot pressed juice for both cultivars had total stilbene levels that were significantly greater than both free run and cold pressed juice. Hot pressed total stilbene levels were greater than but not significantly greater than frozen grapes for both cultivars.

During 2003, data were collected comparing the effect of processing on ‘Carlos’ and ‘Noble’ muscadine grape juice and ‘Miss Blanc’ and ‘Midsouth’ labrusca grape juice. Hot pressed juice for the labrusca grapes had significantly greater total stilbene levels than the two muscadine cultivars. This agrees with the findings for the skin tissue presented above. Although ‘Carlos’ has significantly greater stilbenes in skin tissue than ‘Noble’, it had slightly lower stilbene levels in its juice compared to ‘Noble’. The high levels of stilbenes present in ‘Carlos’ skins are apparently more difficult to remove during juice extraction than those present ‘Noble’ and the labrusca grape skins.
The effect of postharvest UV irradiation and cold storage on ‘Carlos’ and ‘Noble’ muscadine grapes was also evaluated. Cold storage alone but not UV treatment significantly increased total stilbene levels for ‘Carlos’ grape skins and pulps. There was an opposite reaction for ‘Noble’ grape skins. UV treatment but not cold storage alone significantly increased total stilbene levels in ‘Noble’ skins. There were no significant changes in ‘Noble’ pulp with the different treatments. There were no significant differences between treatments for either cultivar for seed total stilbene levels. It appears that different mechanisms were stimulated in the two cultivars to increase stilbene metabolism by UV light and cold storage.

The research described here suggests significant differences between muscadine cultivars with respect to their stilbene concentration and response to environmental stress. ‘Carlos’, ‘Magnolia’ and ‘Watergate’ appear to have the greatest tissue stilbene concentration of the cultivars evaluated here. Of the eight muscadine cultivars studied here, the bronze skinned fruit generally have greater skin stilbene levels than the dark skinned fruit with the exception of ‘Sweet Jenny’. Of the eight muscadine cultivars, ‘Magnolia had the greatest fresh press juice stilbene concentration. Hot press extraction resulted in the highest stilbene concentration in juice while enzyme treatment resulted in the greatest juice yield.

In both fruit tissue and juice, V. labrusca cultivars studied here had greater stilbene levels than the muscadine cultivars studied here. Additional work is needed to confirm this finding for multiple crop years and climates.

Muscadine grapes are a popular backyard fruit crop for the southeastern U.S. As a native plant of the region, many people have fond memories of encountering the grapes both in the wild and in cultivation. For this reason, muscadine grapes will most likely remain popular
with home gardeners for many years to come. Although muscadine grapes are well know and popular in the southeast, their commercial uses both in the southeast and elsewhere have been somewhat limited. As with any regional crop, muscadine grapes need to be promoted to develop a healthy and growing market base. In order to achieve this goal, current and relevant information must be available to promote the various qualities and benefits of muscadine grapes. The work presented should help demonstrate and document some of the nutraceutical value of muscadine grapes.
REFERENCES


## APPENDIX 1. EFFECT OF JUICE EXTRACTION METHOD ON SOLUBLE SOLIDS (°Brix) OF MUSCADINE JUICE (2004)

<table>
<thead>
<tr>
<th>Method</th>
<th>Carlos (br)</th>
<th>Noble (bl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Run</td>
<td>15.1</td>
<td>15.2</td>
</tr>
<tr>
<td>Cold Press</td>
<td>13.5</td>
<td>14.9</td>
</tr>
<tr>
<td>Hot Press</td>
<td>14.9</td>
<td>16.2</td>
</tr>
<tr>
<td>Frozen</td>
<td>15.3</td>
<td>16.8</td>
</tr>
<tr>
<td>Enzyme</td>
<td>14.9</td>
<td>14.6</td>
</tr>
</tbody>
</table>

There were no significant differences at $\alpha = 0.05$. br = bronze. bl = black.
APPENDIX 2. EFFECT OF JUICE EXTRACTION METHOD ON THE STILBENE CONCENTRATION (mg/L) OF MUSCADINE AND BUNCH GRAPE JUICE (2003)

<table>
<thead>
<tr>
<th>Method</th>
<th>Noble (bl)</th>
<th>Carlos (br)</th>
<th>Midsouth (p)</th>
<th>Miss Blanc (w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trans Piceid</td>
<td>Cis Piceid</td>
<td>Trans Resv.</td>
<td>Cis Piceid</td>
</tr>
<tr>
<td>Free Run</td>
<td>0.28 ns B</td>
<td>0.19 b ns</td>
<td>nd b --</td>
<td>0.13 ns B</td>
</tr>
<tr>
<td>Cold Press</td>
<td>0.22 ns ns</td>
<td>0.12 b ns</td>
<td>nd b --</td>
<td>0.16 ns ns</td>
</tr>
<tr>
<td>Hot Press</td>
<td>1.00 ns B A</td>
<td>1.43 a B AB</td>
<td>0.19 a --</td>
<td>1.46 ns B A</td>
</tr>
<tr>
<td>Frozen</td>
<td>0.39 ns ns</td>
<td>1.05 ab ns</td>
<td>nd b --</td>
<td>1.69 na ns</td>
</tr>
</tbody>
</table>

Means (n = 3) within a column followed by the same lower case letter are not significantly different at \( P \leq 0.05 \). Uppercase letters below means compares between cultivars within rows. ns = Not significant at \( P \leq 0.05 \). bl = black. br = bronze. p = purple. w = white.
VITA

Mark Rene’ LeBlanc was born in Baton Rouge, Louisiana, on February 3, 1970, to Eugene and Rachael LeBlanc, the youngest of eleven children. He attended St. Gabriel Elementary and Sunshine High School in Sunshine, Louisiana. He enrolled in the Louisiana State University Department of Horticulture in Baton Rouge, Louisiana, in 1988.

In 1992, Mark received a Bachelor of Science in horticulture. That same year he began his pursuit of a Master of Science degree in turfgrass science at Louisiana State University. In 1995 he accepted a Research Associate position in ornamentals at Louisiana State University Department of Horticulture. In 1996 he received his Master of Science in horticulture.

In 1997 Mark began his pursuit of a doctoral degree in horticulture at Louisiana State University. In December of 1998, he accepted a full time position at the Louisiana Department of Agriculture and Forestry as Assistant Director of the Louisiana Horticulture Commission. He is currently a candidate for the degree of Doctor of Philosophy in horticulture.

Mark is married to Ellen and they have four children, Madeline, Emma, Luke and Vianne.