

## ABSTRACT

LIU, ALISON ANN. Comparative Compositional and Biological Properties of Muscadine and Cabernet franc Grape Skins. (Under the direction of Leon C. Boyd).

Numerous studies have documented the health benefits of moderate consumption of wines, and especially red wines. Much of this activity has been attributed to the extraction of phytochemicals from the skin and seeds during on-skin fermentation procedures. This study focused on the extraction and biological properties of phytochemicals from freeze dried skins of muscadine and Cabernet franc. The Cabernet franc grapes are a good source of resveratrol whereas muscadine grapes are an excellent source of ellagic acid with the resveratrol content varying considerably from batch to batch. Both grapes contain a complex mixture of phytochemicals, many of which have been shown to have potential health benefits *in vitro*. The skins of two muscadine cultivars, Carlos and Noble, and the skins of Cabernet franc grapes obtained during two harvests were freeze-dried, extracted with 80:20 methanol-water, and analyzed for antioxidant capacity followed by high pressure liquid chromatography (HPLC) and HPLC coupled with time of flight mass spectrometry (HPLC-TOF-MS) analyses for phenolic compounds. The anticarcinogenic activity of extracts was determined against LNCaP prostate carcinoma cells with the XTT proliferation assay and the caspase-3 assay used to determine apoptosis.

The 2006 Cabernet franc extract contained 70.3  $\mu\text{g/g}$  resveratrol and no ellagic acid, whereas muscadine extracts contained 4.2-12.2  $\mu\text{g}$  resveratrol and 117-269  $\mu\text{g}$  ellagic acid per g dried sample. Neither resveratrol nor ellagic acid was detected in the 2007 Cabernet franc extract. The antioxidant capacity was measured using the oxygen radical absorbance capacity

(ORAC) assay. ORAC values of grape skin extracts ranged from 110-354  $\mu\text{mol Trolox}$  equivalents per g dried sample with Carlos being the lowest, followed by Cabernet franc, and Noble being the highest. At concentrations of 5 mg/mL, all skin extracts induced apoptosis in LNCaP cells as the increases in caspase-3 and other closely related proteases were significantly different from the controls. LNCaP proliferation was also inhibited at doses of 5 mg/mL for all extracts. This study shows that even though the phytochemical composition of muscadine and Cabernet franc differ considerably, as by products of the winemaking industry, they both may have economic and potential health benefits as dietary supplements.

Comparative Compositional and Biological Properties of  
Muscadine and Cabernet franc Grape Skins

by  
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A thesis submitted to the Graduate Faculty of  
North Carolina State University  
In partial fulfillment of the  
Requirements for the degree of  
Master of Science

Food Science

Raleigh, North Carolina

2009

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## **BIOGRAPHY**

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## ACKNOWLEDGEMENTS

I would like to thank all those who have helped me and guided me through my graduate studies here at NC State. I would especially like to thank my advisor Dr. Leon Boyd and my committee members Dr. Lisa Dean and Dr. Jonathan Allen. Thank you to Rong Reynolds for teaching me the lab procedures and helping me with troubleshooting. Thank you to Dr. Keith Harris and each of you in the Harris group – Sara Cohen, Teri Davis, Erica Story, Caroline Summers, and Ruth Watkins – for taking the time to help me with my cell culture work and also thank you for graciously sharing your lab space. I greatly appreciate everyone's generosity and kindness throughout my research.

I would also like to thank my family and friends for their continual support.

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## CHAPTER 1. LITERATURE REVIEW

### *Introduction*

Nutraceuticals, functional foods, and phytochemicals are topics of extreme interest in the United States today as the health and nutrition relationship has become more and more evident, much research is being done on the potential of foods for medicinal purposes. Foods we consume everyday are being analyzed for antioxidants, anticarcinogenic properties, and other characteristics that may help prevent, treat, or cure diseases such as cardiovascular disease or lung cancer. Red wine is known to contain the phenolic compound resveratrol which may have cardioprotective effects. Cabernet franc grapes are a good source of resveratrol, along with many other phenolic compounds that may potentially be beneficial to our health. Muscadine grapes, which are native to the southeastern United States, have a growing reputation for possessing advantageous attributes. However, the primary component of the muscadine is not resveratrol, but a compound called ellagic acid. Both grape varieties have positive aspects, but are very different in composition. It is of interest to compare the compositional properties between Cabernet franc grapes and muscadine grapes to determine the major differences between the two varieties.

In the United States, heart disease is the leading cause of death with cancer as the second leading cause. In 2008, over half a million Americans were expected to die from cancer (ACS 2008). Both Cabernet franc grapes and muscadine grapes have potential in reducing risk factors associated with cardiovascular disease and cancer. With further

research on their mechanisms, extracts from these grapes could be used therapeutically to decrease the risks of these diseases, among others. These extracts have the potential to provide powerful and natural health benefits. It is of great interest to determine which compounds are responsible for the anticarcinogenic properties of these two types of grapes.

Grape marc, or pomace, is the leftover waste comprised of skins and seeds produced from winemaking. Some of the grape marc is added to cattle feed due to its high fiber content. Some grape marc is recycled into compost and used on the vineyard, but high concentrations of phenolic compounds can inhibit germination (Bonilla and others 1999). Some grape marc is mined to produce tartaric acid, although this applies only to large wineries that produce enough pomace, but most winemaking waste ends up in the landfill (Crowe 2005, Stanley 1997). Recently, pomace (including Cabernet franc) was shown to contain particular polyphenols that interfere with the ability of bacteria to contribute to tooth decay (University of Rochester Medical Center 2008). With the potential health benefits of grape skins, the byproducts of winemaking can be extracted and turned into a useful supplement or treatment for diseases.

## ***Background***

### **Cabernet franc grapes**

Centuries old, the Cabernet franc grape originated in France. The grapes are predominantly grown in the Bordeaux and Loire Valley regions. The grape produces a bright, pale red wine that is most often used for blending with Cabernet sauvignon and

Merlot due to its acidity and distinct aroma profile. The unique aroma includes green pepper, tobacco, raspberry, and violet. The Cabernet franc grapes are small, have thin skins, and are blue-black in color. This bunch grape grows on an upright vine with dark green and five lobed leaves. Cabernet franc vines grow best in cool climates and can be found all over the world including France, Canada, Hungary, Italy, and Australia. In the United States, vineyards locations include California, Washington, Michigan, New York, and Virginia (Vinismo 2009).

### **Muscadine grapes**

Indigenous to the southeastern United States, the muscadine grape has unique physical and health characteristics setting the fruit apart from ordinary table grapes. Muscadine grapes, *Vitis rotundifolia*, were first described by explorer Sir Walter Raleigh on the coast of North Carolina in 1584. Today, over three hundred cultivars can be found proliferating as far west as Texas and as far north as New York.

When comparing the muscadine grape to grapes of the group *Vitis vinifera* such as Cabernet Franc, the growth attributes differ greatly. While typical grapes grow in large, tight bunches, muscadine grapes grow in small, loose clusters. Also in contrast to *Vitis vinifera*, the vines bear one to one and a half inch round grapes with thick, tough skin that is often discarded when eaten. Oblong shaped seeds can be found inside the grapes that have colors ranging from bronze, black, and red to purple.

The muscadine has several qualities that make the grape a popular crop for farmers. Muscadine grapes are naturally resistant to Pierce's disease, which is caused by

the pathogen *Xylella fastidiosa*. Pierce's disease affects only grapes and can wipe out an entire vineyard rapidly (Leach and others 2004). The bacterium infects the vine and forms a gel in the tissue of the vine. Water is then prevented from being drawn up the vine causing the leaf to die, which eventually leads to the death of the entire vine. There is no known cure, making the resistance a valuable quality of the muscadine. Muscadines are also one of the most resistant species to the pest *Daktulosphaira vitifoliae*, or more commonly, phylloxera. The tiny insect is native to eastern North America and attacks the grapevine at the roots. They are also resistant to fungal diseases like black rot and microscopic worms called nematodes (Wood and others 2006).

Once started, muscadine grapevines have longevity and grow vigorously. If treated well, the vineyard can produce a yield of eight to twelve tons per acre and mature grapevines can yield up to eighteen tons per acre. A well-managed vineyard can last for more than thirty years (Poling and others 2003). Muscadine grapes produce less juice per ton than typical bunch grapes, at 135 to 140 gallons compared to 165 gallons or greater. Despite the lower yield, the potential health benefits from the other parts of the grape may overcome this initial seeming disadvantage.

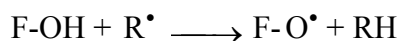
Not only are muscadines used for winemaking, but the versatile grapes are also used to make juice, jellies, jams, preserves, syrups, and dessert toppings. Muscadines have a distinct flavor and aroma and potential health benefits and the grapes have become increasingly popular in the past ten to fifteen years (Poling and others 2003). The number of acreage of commercial vineyards has increased greatly and the sales cost per ton has increased as well (Poling and others 2003). Muscadines are an excellent source

of dietary fiber, some essential minerals, vitamins B and C, iron, and are low in fat (Stanley 1997). They also contain the cancer-fighting compounds resveratrol and ellagic acid. Additionally, the grapes contain numerous other phenolic compounds that may contribute to the anticarcinogenic properties. These grapes that contain abundant phytochemicals are in increasing demand. More research is necessary to fully characterize both the phytochemical composition and the biological properties of extracts obtained from these grapes. Finding a new application for muscadine grapes and their byproducts can further expand the market for the grapes. With further research, the 900 – 1000 pounds of waste that are produced from each ton of muscadines processed can be reworked and used for other applications (Stanley 1997).

### **Phenolic compounds**

Phenolics are compounds with the basic structure consisting of an aromatic ring and a hydroxyl group. Phenolic compounds have been identified as antioxidants and so they may play a part in the disease fighting properties of muscadine grapes. These compounds have also been demonstrated to be anti-inflammatory, antimicrobial, and anticarcinogenic (Jang and others 1997, Pace-Asciak and others 1995, Rodríguez Vaquero and others 2005). Polyphenols can be further subclassified into groups including flavonoids, tannins, anthocyanins, catechins, and stilbenes. The category of polyphenols include compounds such as catechin, ellagic acid, epicatechin, gallic acid, quercetin and resveratrol (**Figure 1.1**).

The Western diet provides, on average, 50 mg of flavonoids per day (Linseisen 1997). Another study estimates that Americans consume about 1 g of polyphenols per day (Scalbert and Williamson 2000). Intake is subject to great variability depending on the food source as well as the bioavailability of the consumed food or extracts. No definitive relationship has been determined between the structures of the compounds and the antioxidant activity or anticarcinogenic strength. Some evidence supporting structure activity relationships have been established *in vitro* ((Heim and others 2002, Rice-Evans and others 1996). For example, the hydroxyl groups are responsible for the substantial radical scavenging activity by participating in the following reaction:



More research must be done to learn more about the mechanisms of effect.

The total amount of phenolic compounds in a sample can be measured with the Folin-Ciocalteu method developed by Singleton and Rossi (1965). This method has been extensively used to analyze muscadine and Cabernet franc grapes (Fuhrman and others 2005, Pastrana-Bonilla and others 2003, Vasantha Rupasinghe and Clegg 2007, Yi and others 1997, Yi and others 2005). A similar method developed by Swain and Hills (1959) has also been used frequently (Del Pozo-Insfran and others 2007, Lee and others 2005, Mertens-Talcott and others 2006). The Singleton and Rossi method is an official AOAC procedure. The Folin-Ciocalteu method measures the reducing capacity of a sample. Because of its convenience, simplicity, and reproducibility, this assay has

become routine for total phenolics analysis (Huang and others 2005). The results also correlate strongly with antioxidant activity.

The levels and types of phenolic compounds in a grape or in wine can vary depending on several environmental as well as processing factors. Not only do the concentrations of phenolic compounds vary from cultivar to cultivar, but also, the time of harvest, soil and climate conditions, cultivation methods, and the use of pesticides can all influence the composition of grapes (Brossaud and others 1999, Magee and Smith 2002, Prajitna and others 2007, Roldán and others 2003). Processing factors include grape crushing techniques, fermentation methods, and storage conditions (Gambutì and others 2004, Netzel and others 2003, Talcott and Lee 2002).

In the wine industry, it is important to identify the phenolic composition of grapes and wine because phenolic compounds play a large role in the quality of wines. Attributes such as color, flavor, stability, and aging behavior are all potentially affected (Mazza and others 1999, Netzel and others 2003). In fact, until recently, it was recommended that German vineyard owners, among others, minimize the phenolic content in their wines as lower concentrations of phenolics reduce cloudiness and negative taste such as astringency (Netzel and others 2003).

### **Phenolic compounds in Cabernet franc products**

Very little research has been done on the biological and compositional properties of Cabernet franc grapes. The flavonoid composition of Cabernet franc skins has been analyzed using high performance liquid chromatography (HPLC), but resveratrol and

ellagic acid contents were not investigated (Brossaud and others 1999). Cabernet franc skins, must, and wine were all analyzed for total phenolics, total flavonols, and total anthocyanins. Only anthocyanins were identified and quantified individually (Mazza and others 1999). In a study of commercial Cabernet franc wine, the concentrations of fifteen polyphenols were measured including resveratrol, but not ellagic acid (Soleas and others 1997). The effect of different vinification techniques on total antioxidant capacity and phenolic composition of Cabernet franc wine was determined. A combination of fermentation on skin and mash heating yielded the highest antioxidant capacity and highest levels of flavonoids and stilbenes (Netzel and others 2003). Again, ellagic acid content was not investigated. No studies were found that compared the phenolic compositions of muscadine skins and Cabernet franc skins. Furthermore, there are no known studies that contrasted the wines of each type.

### **Phenolic compounds in muscadine products**

Relative to the amount of information available on Cabernet franc grapes, there have been many studies on muscadine grapes, although the information on the phenolic composition of muscadines is relatively small compared to the more popular *V. vinifera* type grapes. The skins of Carlos and Noble cultivars were analyzed for free ellagic acid and ellagic acid glycosides (Lee and Talcott 2004). A complete analysis of phenolic compounds of Carlos and Noble whole grapes, skins, seeds, and leaves identified ellagic acid, myricetin, quercetin, kaempferol, and *trans*-resveratrol as the major phenolics (Pastrana-Bonilla and others 2003). Muscadine skins have also been evaluated for

anthocyanins (Yi and others 2005). Piceid has also been quantified in Carlos and Noble skins. Analysis of several other cultivars determined that the bronze colored (Carlos, e.g.) muscadines had greater total stilbene concentrations than that of the dark-skinned (Noble, e.g.) muscadines (LeBlanc and others 2007).

### ***Antioxidants***

Free radicals are highly reactive molecules that are harmful and damaging to our cells, leading to degenerative aging and contributing to diseases such as cancer and heart disease. The cells are damaged by chain reactions caused by the free radicals produced by oxidation reactions. Antioxidants are compounds that are capable of preventing or reducing the rate of oxidation of other compounds. All foods have various levels of antioxidant capacity. Those with a higher capacity may be more beneficial to our health than those with a lower capacity. The total antioxidant activity of foods can be determined by a multitude of methods such as the oxygen radical absorbance capacity assay or the Trolox equivalent antioxidant capacity assay.

For the analysis of muscadine and Cabernet franc grapes, including their skins, seeds, pulp, wine, and marc leftover from winemaking, the following methods have been used for the determination of total antioxidant capacity: 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Jacob and others 2008), ferric reducing ability of plasma (FRAP) assay (Vasanth Rupasinghe and Clegg 2007), oxygen radical absorbance capacity (ORAC) assay (Del Pozo-Insfran and others 2007, Lee and Talcott 2004, Lee and others 2005, Mertens-Talcott and others 2006, Talcott and Lee 2002, Yilmaz and Toledo 2004) and

the Trolox equivalent antioxidant capacity (TEAC) or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay (God and others 2007, Netzel and others 2003, Pastrana-Bonilla and others 2003). Other methods that are used on other types of foods include the copper reducing antioxidant activity (CUPRAC) assay, total oxidant scavenging capacity (TOSC) assay, and the total radical-trapping antioxidant parameter (TRAP) assay (Prior and others 2005). No group has compared two or more of the antioxidant activity methods, or evaluated all of the assays at once on muscadine and Cabernet franc grapes and grape products.

A few reviews on total antioxidant capacity methods for foods in general have been published (Frankel and Meyer 2000, Huang and others 2005, Prior and others 2005, Sánchez-Moreno 2002). None could recommend any one assay definitively and confidently. Despite the variability of the assays, the options can be narrowed down due to the advantages and disadvantages of each method, as well as availability of equipment and supplies.

The ORAC assay is based on the hydrogen transfer reaction mechanism. Since hydrogen atom transfer is important in the radical chain reaction, this method may be more biologically relevant as it measures chain breaking capacity of the antioxidants (Ou and others 2002, Prior and others 2005). Electron transfer based assays have the disadvantage of only measuring an antioxidant's ability to reduce. During an ORAC assay, a peroxy radical reacts with a fluorescent probe to form a nonfluorescent product, which is then quantitated by fluorescence. The ORAC method is applicable to a wide range of foods. An increasing number of food and supplement manufacturers have been

using this method to provide values on their nutrition labels (Bank and Schauss 2004). The United States Department of Agriculture (USDA) has already begun to develop a database of ORAC values of fruits, vegetables, nuts, rice bran, as well as dried fruit and cooked fruit and vegetables (Wu and others 2004). The most up-to-date compilation consists of values for 277 foods (USDA 2007). Although no standardized method exists for antioxidant capacity evaluation, the ORAC assay appears to be the most readily accepted and the most widely used for consumer awareness.

The ORAC assay measures the antioxidant capacity of an unknown sample through fluorescence intensity. With these continuous measurements, the rate at which 2,2'-azobis (2-amidino-propane dihydrochloride) (AAPH), a peroxy radical generator, breaks down the fluorescent indicator fluorescein can be determined. Decay curves are plotted and the area between the curve of the sample and the curve of the blank is calculated. In the presence of antioxidants, the fluorescein is protected by the antioxidants and the degradation is slowed. Since different antioxidants, combinations of antioxidants, and food matrices can affect the time of reaction, the ORAC assay is advantageous in that it takes into consideration various lag phases. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble vitamin E analog used as the standard. This is a common standard used in other antioxidant capacity assays as well. **Table 1.1** contains ORAC values, as well as total phenolics, of several everyday foods.

Many studies have been done analyzing the antioxidant capacity of wines and various parts of grapes, but not many have been done on grape skins specifically, as

opposed to the seeds or the whole grape. Grape skins contain many different phenolic compounds, which have been shown to be effective antioxidants, mainly due to their redox properties. Using *in vitro* antioxidant assays, the phenols show greater activity than vitamins E and C (Rice-Evans and others 1997).

### ***Anticarcinogenic properties***

Cancer encompasses a group of diseases distinguished by abnormal cells growing and spreading uncontrollably. If the cancer is not treated, the disease can be fatal. In 2008, it is estimated that 186,320 new cases of prostate cancer will affect men. Approximately 15% of those diagnosed will not survive the disease. The prostate is the leading site of new cancer cases in men with 25% of all cancers and is the second leading site of death, behind lung and bronchial cancer. Diagnoses are also significantly higher in African American men compared to white men with the death rate more than twice as high (ACS 2008).

Diet has become increasingly important as a means of preventing cancer (Cumming and Bingham 1998, Willett 1995). Overconsumption of calories and/or a high fat diet may contribute to the development of cancer. But, fruits and vegetables may actually reduce cancer risk. Because of their protective effects, fruits and vegetables may also be useful for cancer therapy. Diets with high consumptions of red meat and fat are associated with increases in prostate cancer risk whereas vegetables, especially salads and tomatoes, are associated with lower risk (Armstrong and Doll 1975, Cummings and Bingham 1998, Willett 1995). Epidemiological studies are not definitive, as no

significant associations with diets high in fruits and vegetables have been reported (Steinmetz and Potter 1991). Generally, a diet filled with fruits and vegetables is thought to be effective against all major types of cancers (Cummings and Bingham 1998).

The reason for the positive relationship between fruits and vegetables and cancer may be due to specific groups of compounds including carotenoids, flavonoids, phenols, and plant sterols. Muscadine and Cabernet franc grapes are rich in flavonoids and phenols (Yilmaz and Toledo 2004). Wine, in particular red wine, has recently received much publicity for its health benefits. The skins and seeds of these grapes have potential anticarcinogenic properties as well. Leftover grape skins and seeds from winemaking, known as grape marc, could be reworked to be added to food products or reworked to make supplements in order to provide anticancer benefits.

These antioxidants can affect cancer at several stages. First, antioxidants can scavenge free radicals and reduce the incidence of damage to nucleic acids, blocking the initiation of cancer cell formation. Cellular biochemical processes can also be modulated, such as the maintenance of calcium homeostasis and mitochondrial function. In a tertiary step, they can also modulate gene expression (Jacob 2008). Polyphenols also have the ability to sensitize tumor cells to chemotherapy and radiotherapy by way of inhibiting the processes at all stages of cancer (Garg and others 2005).

### **Cabernet franc grapes – in vitro anticancer activity**

There are no known studies of the anticancer activity of any type of Cabernet franc grapes. There have been studies on other varieties of red grapes and wines on

cancer, but not Cabernet franc specifically. A limited number of studies have analyzed the effects of grapes and wines on prostate cancer in particular. Grape seed extract, of *Vitis vinifera*, was tested on DU145 and LNCaP prostate cancer cell lines.

Concentrations of 10-100 µg/mL grape seed extract were applied and significantly inhibited cell growth and caused apoptosis in both cell lines (Agarwal and others 2000). In the DU145 cells, several molecular mechanisms were affected, including modulation of mitogenic signaling and modulation of cell-cycle regulators.

### **Muscadine grapes – in vitro anticancer activity**

Muscadine grapes have exhibited anticancer properties on several types of cancerous cells. Phenolic acid and anthocyanin fractions of red and white muscadine grape skins were found to inhibit HepG2 liver cancer cell growth as well as induce apoptosis (Yi and others 2006). In the analysis of MOLT-4 leukemia cells, Mertens-Talcott and others (2008) determined that red muscadine wines reduced cell viability and induced apoptosis. The group also observed cell cycle arrest in the G<sub>2</sub>/M phase.

Polyphenolic extracts of muscadine skins and pulp have also been shown to induce apoptosis, decrease cell number, and cause alterations in cell cycle kinetics in Caco-2 colon carcinoma cells (Mertens-Talcott and others 2006). Results were in agreement in another study, in regards to apoptosis and cell proliferation, which applied muscadine skin polyphenolic extract on Caco-2 and HT-29 colon carcinoma cells. Significant increases in DNA fragmentation of the cancer cells were observed with treatments as low as 10 µg/mL of anthocyanin fraction (Yi and others 2005). Muscadine grape extract has

also been demonstrated to suppress the growth of breast cancer and cervical cancer cell lines (Wedge 2002).

Muscadine grape skin extract showed several characteristic anticarcinogenic properties on *in vitro* prostate cancer growth. Treatments of Ison cultivar extracts exhibited antiproliferative effects on nontumorigenic prostate cancer cells without inhibiting growth in normal epithelial cells (Hudson and others 2007). Extracts targeted distinct pathways such as the mitogen-activated protein kinase survival pathway, which leads to apoptosis. The phosphatidylinositol 3-kinase-Akt pathway was also affected. These two signaling pathways have proven to play important roles in cell growth, survival, and inhibition of apoptosis in prostate cancer (Alvarez and others 1991, Cobb and Goldsmith 1995, Gonzalez and others 1991). Muscadine skin extract inhibited the Akt pathway, degraded the Akt protein, and decreased Akt expression levels. Interestingly, the same experimental methods were applied using resveratrol only, leading to different effects and different mechanisms of action on the prostate cells (Hudson and others 2007).

### **Muscadine grapes – other anticancer activities**

God and others (2007) showed that extracts of both red and white muscadine grapes possessed significant antioxidant activity, suppressed mutagenesis by a metabolically activated mutagen, and inhibited matrix metalloproteinase (MMP) activity. MMPs are enzymes that are important factors in tumor metastasis. These enzymes work to degrade the extracellular matrix as well as the basement membrane. Tate and others

(2004) also determined that muscadine pomace extracts inhibit MMP activity, specifically MMPs 2 and 9. Ellagic acid, the key compound in muscadines, effectively decreased MMPs 2 and 9 when added to DU-145 prostate cancer cells at a concentration of 50  $\mu\text{mol/L}$  (Losso and others 2004). Concentrations lower than 10  $\mu\text{mol/L}$  were not very successful at MMP inhibition.

### **Specific compounds with anticarcinogenic properties**

Resveratrol inhibits tumor initiation, promotion, and progression as well as angiogenesis and metastasis (Jang and others 1997). The investigation of the compound found that resveratrol induced phase II drug-metabolizing enzymes, inhibited cyclooxygenase and hydroperoxidase functions, and induced human promyelocytic leukemia cell differentiation which applies to anti-initiation, anti-promotion, and anti-progression activities, respectively (Jang and others 1997). Resveratrol can affect the cell cycle at several phases and transitions in many different kinds of cancers including human prostate, breast, colon, and lung carcinoma cells (Delmas and others 2006). Specifically, resveratrol enhanced radiosensitivity of human lung cancer cells due to the inhibition of nuclear factor-Kappa B (NF- $\kappa$ B) activation (Liao and others 2005). NF- $\kappa$ B is a transcription factor that affects apoptosis, proliferation, and inflammatory response by targeting the genes involved in those functions.

Another mechanism in which resveratrol may have chemopreventive effects is in its modulation of phosphoglycerate mutase B. The involvement with the metabolic enzyme indicates that resveratrol has a role in glycolysis and there may be a possible

involvement of thiol groups and consequently mediation of protein-protein interactions, in prostate cancer cells (Narayanan and others 2004). Surprisingly, in LNCaP prostate cancer cells, resveratrol had no effect on cell proliferation, while high concentrations ( $>10^{-7}$  M) were necessary for partial growth inhibition of PC3 cells (Kampa and others 2000). However, in DU145 cells, resveratrol was the most potent inhibitor compared to other flavonoids – (+)-catechin, (-)-epicatechin, and quercetin. Resveratrol has also proven to downregulate telomerase activity in colon tumor cells (Fuggetta and others 2006). The molecule also has been shown to interfere with several other particular pathways and inhibit activities important to the prevention of cancer (Han and others 2007).

Ellagic acid inhibits tumor initiation through several mechanisms. First, ellagic acid can inhibit the metabolic activation of polycyclic hydrocarbons, nitroso compounds, and aflatoxin B<sub>1</sub> into forms that induce DNA damage (Stoner and Mukhtar 1995). Ellagic acid also promotes carcinogen detoxification, scavenges reactive metabolites of carcinogens, and may also occupy sites in DNA that might otherwise react with carcinogens or their metabolites. Losso and others (2004) applied ellagic acid to Caco-2, MCF-7, Hs 578T breast, and DU145 cancer cells and observed anti-proliferative activity and apoptosis at dose concentrations of 1-100  $\mu\text{mol/L}$ . The induction of apoptosis was also associated with decreased adenosine triphosphate (ATP) production. ATP production is essential for cancer cell growth. Not only was ellagic acid effective on the various cancer cell lines, but ellagic acid also had no cytotoxic effect on normal human lung fibroblast cells.

Quercetin, (-)-epicatechin, and (+)-catechin showed significant antiproliferative effects on the prostate carcinoma cell lines LNCaP, PC-3, and DU145 (Kampa and others 2000). Interestingly, the LNCaP cells were less sensitive to the polyphenols than PC-3 while the compounds had varied results on DU145 cells. The growth inhibition was proposed to be mediated through the modulation of nitric oxide (NO) production. In Mia PACA-2 and BSp73AS pancreatic carcinoma cells, quercetin and *trans*-resveratrol, but not rutin, caused an increase in oligonucleosomal DNA fragmentation as well as increase in Annexin staining, with quercetin being the more potent compound (Mouria and others 2002). These are both defining characteristics of apoptosis.

In a study of more than thirty flavonoids, all of the compounds possessed anti-proliferative activity in the human colon cancer cell lines HT-29 and Caco-2, including rutin, kaempferol, quercetin, and myricetin (Kuntz and others 1999). Furthermore, almost all of the compounds inhibited growth without cytotoxicity. Knowles and others (2000) also showed results that kaempferol, quercetin, myricetin, as well as luteolin, apigenin, and genistein, substantially suppress proliferation of PC-3 prostate cancer cells. Not only did treatment of the cells with 100  $\mu$ M quercetin result in complete growth inhibition, but the compound's antiproliferative effects continued despite being removed from the culture medium. Agreeably, quercetin, kaempferol, and naringenin have also been demonstrated to possess antiproliferative properties, without cytotoxicity, against Hepa-1c1c7 liver cancer cells and LNCaP cells at concentrations ranging from 12.5 – 50  $\mu$ M (Campbell and others 2006). However, the corresponding glycones rutin, quercetrin, and naringin showed no cell growth inhibition. Quercetin, along with apigenin and

resveratrol, have exhibited potential for proteasome inhibitory activity (Yang and others 2008). Targeting proteasomes are a way to treat cancer since the ubiquitin-proteasome degradation pathway is involved in cell proliferation, apoptosis, angiogenesis, and other cellular processes.

Grape polyphenolic compounds have also been studied for their additive and synergistic interactions. When applying polyphenols to MOLT-4 leukemia cells, ellagic acid at 10  $\mu\text{mol/L}$  had no significant effect on cell proliferation and viability, but the combination of ellagic acid and resveratrol exhibited a more than additive interaction (Mertens-Talcott and Percival 2005). The combination of resveratrol and quercetin also had an additive effect. Effects on the cell cycle were also analyzed. The  $G_1$ , S,  $G_2$ , and M are the four phases of the cell cycle, in that order (**Figure 1.2**). The S phase is where DNA synthesis occurs and the M phase is where mitosis occurs. Kinases and cyclins are the signalers that tell the cell to move from the G phases (preparation) to the S or M phase. In  $G_0$ , the cells are temporarily or permanently out of the cell cycle. Cancer cells are unable to transition to  $G_0$  and will therefore repeat the cell cycle indefinitely. No synergistic effects were observed in the cell cycle analysis, but the combination of three compounds – quercetin, resveratrol, and ellagic acid – caused arrest in the  $G_0/G_1$  phase. Other treatments involving those compounds also caused arrest or delay in the  $G_0/G_1$  phase as well as in the S phase. It was not determined, though, whether or not the cells would normalize cell cycle progression after 48 hours. *Trans*-resveratrol and quercetin were also observed to have a more than additive effect on mitochondrial cytochrome c

release and caspase-3 activity in a human and a rat pancreatic carcinoma cell line (Mouria and others 2002).

Although quercetin and kaempferol were determined to modify different phases of the cell cycle (S phase and the G<sub>2</sub>/M phases, respectively), one study found no synergistic antitumorigenic effects on PC-3 prostate cancer cells (Knowles and others 2000). Yet, Campbell and others (2006) observed significant synergistic antiproliferative effects when quercetin, kaempferol, and naringenin were matched together against Hepa-1c1c7 liver cancer cells and LNCaP prostate cancer cells. The pairing of quercetin with ellagic acid displayed a synergistic interaction on the reduction of proliferation, the reduction of viability, and the induction of apoptosis in MOLT-4 leukemia cells (Mertens-Talcott and others 2003). No relationship could be determined between the structure (subclasses or type and location of substituents within each class) and the antiproliferative effects on Caco-2 and HT-29 colon cancer cells in an analysis of more than thirty common flavonoids (Kuntz and others 1999).

### **Specific compounds with anticancer activity in animals**

Quercetin has been shown to prevent metastatic cancer lesions, decrease growth of the primary tumor, and increase apoptosis in nude mice injected with the Mia PACA-2 pancreatic cell line (Mouria and others 2002). Compared to doses of (+)-catechin, *trans*-resveratrol, and gallic acid, quercetin was the most potent compound in the reduction of tumor in mice with skin cancer (Soleas and others 2002). Even though numerous studies on the chemopreventive activity of resveratrol have turned up positive results, there have

also been studies with no effects (Baur and Sinclair 2006). A study of resveratrol doses on breast cancer in mice showed no effects on growth or metastasis (Bove and others 2002). Yet, resveratrol had an effect on prostate cancer in transgenic mice. With a diet of 625 mg per kg for 23 weeks led to a decrease in cell proliferation, a decrease in the growth factor IGF-1, a down-regulation of the protein kinases ERK1 and 2, and an increase in the tumor suppressor estrogen receptor- $\beta$  (Harper and others 2007). These are all factors that slow the progression of prostate cancer. Chemically induced lung, liver, skin, and esophageal cancer in rodents have all been inhibited by ellagic acid (Stoner and Mukhtar 1995).

### **Epidemiological studies**

Several epidemiological studies have been done based on diet. Diet is undoubtedly extremely important for a generally healthy lifestyle. Many studies have revealed that diet may also be a significant factor in the prevention of cancer. Willett (1995) estimates that cancer deaths avoidable by dietary change range from 10% to 70%. Diet has been estimated to account for approximately 75% of cancers of the prostate with a range of 20%-80% (Willett 1995). A number of epidemiological studies have shown that the consumption of a flavonoid-rich diet may reduce the risk of breast, prostate, stomach, and lung cancers (Le Marchand 2002, Ren and others 2003). The high intakes of flavonoids were mainly from soy products, green and black tea, and apples. In a study of almost 10,000 Finnish men and women, Knekt and others (1997) observed an inverse relationship between the intake of flavonoids and the incidence of all sites of cancer

combined. The most significant association was between flavonoid intake and lung cancer. Another study revealed a significantly lower cancer incidence rate with higher quercetin intakes (Knekt and others 2002). The risk of prostate cancer specifically, was reduced at higher myricetin intakes. Sources of myricetin were attributed mainly to berries, while quercetin was provided primarily by apples and onions. Unfortunately, no data on wine consumption was collected as beer and liquors are preferred in Finland.

### **Other potential health benefits of polyphenol consumption**

Phenolic compounds have also proven to not only have anticarcinogenic properties, but also other disease fighting properties. These health benefits add to the growing list of advantageous aspects of phenolics. An animal study on hamsters demonstrated that a combination of catechin, quercetin, and resveratrol prevented the development of atherosclerosis (Auger and others 2005). The moderate doses fed to the hamsters were equal to that found in two glasses of red wine. Plasma cholesterol concentration and aortic fatty streak area were both reduced. Most likely due to their reducing capabilities, phenolics not only help protect against cardiovascular diseases, they also have anti-inflammatory properties, antiallergic activities, antidiabetic effects, and they may improve endothelium functions (Han and others 2007). Grape polyphenols may inhibit the enzymes that catalyze the release of histamine (Shi and others 2003). Histamine is the compound that causes inflammation and allergies. A Finnish epidemiological study revealed an inverse relationship between cerebrovascular disease and kaempferol, naringenin, and hesperetin intakes (Knekt and others 2002). Other

relationships included reduced incidence of lung cancer, asthma and type 2 diabetes associated with higher intakes of several different phenolic compounds. The mechanisms for these disease fighting properties may also be related to the anticancer properties.

### ***Bioavailability***

Another factor, which must be considered when testing grape extracts or specific compounds for biological properties, is their bioavailability. If the extracts contain phenolics that have poor bioavailability, then the anticancerous effect on the human body is low, even with promising results *in vitro*. In a study of ten human volunteers, *trans*-resveratrol was absorbed approximately twenty times more effectively than (+)-catechin when oral doses were ingested (Soleas and others 2001a). Along with (+)-catechin, quercetin was also poorly absorbed after oral doses in humans and animals (Soleas and others 2001b). The three compounds, resveratrol, (+)-catechin, and quercetin, have the same rate of absorption no matter the matrices (alcoholic, grape juice, vegetable juice), but the peak absorptions range from 10 – 40 nmol/L (Goldberg and others 2003). This range of concentrations is not sufficient compared to the circulating EC<sub>50</sub> (half maximal effective concentration) of 5 – 100 µmol/L necessary for beneficial health effects, such as antioxidant, anticancer, and anti-inflammatory effects, based on *in vitro* activity. These concentrations were determined from a small sample of subjects (twelve males).

Another study reports a 17% and 24% absorption rate of quercetin-3-rutinoside and quercetin aglycone, respectively, in humans (Hollman and others 1995). The volunteers had a 52% absorption rate of quercetin when consumed in the form of onion

rings which is a significant amount. Not only does phenol concentration of foods depend on season, preparation, and variety, among others, but the bioavailability is also highly dependent on the type of food (Hollman and others 1997). Despite promising results from foods like onions, bioavailability of phenols from grapes and wine may differ greatly.

The information on bioavailability of polyphenols found in grapes following consumption in animal models vary depending on the method of administration, method of detection, and the type of animal model (Schlachterman and others 2008). For example, daily doses of 50  $\mu\text{g}/\text{kg}$  body weight of resveratrol administered to rats led to serum levels that reached 8.0  $\mu\text{M}$  (Azios and Dharmawardhane 2005). Meanwhile, Asensi and others (2002) detected serum levels of less than 1  $\mu\text{M}$  in rats, mice, and rabbits given 20  $\text{mg}/\text{kg}$  resveratrol. More research is necessary in order to determine the most bioavailable or least bioavailable compounds for various types of cancer.

There are conflicting reports on whether or not the human consumption of red wine has the ability to reduce the oxidizability of low-density lipoproteins (LDL). Two studies involved the consumption of red wine over a period of two weeks. An 11% increase in the resistance of LDL to oxidation by an artificial azo radical initiator was reported in one study, and a significant increase in the resistance of LDL and plasma to lipid oxidation was reported in the other (Fuhrman and others 1995, Kondo and others 1994). Yet, de Rijke and others (1996) found no changes with *in vitro* LDL oxidizability in humans over a four week period of either red wine or white wine consumption.

Jacob and others (2008) suggest that specially bred high-polyphenol grape lines may be able to increase the polyphenol bioavailability due to the high concentrations.

Phenolic acids are among the most well absorbed polyphenols, followed by catechins, flavanones, and quercetin glucosides (Han and others 2007, Manach and others 2005, Williamson and Manach 2005), but they are absorbed by different kinetics. The least well-absorbed phenolic compounds are the proanthocyanidins and the anthocyanins, which are large molecular weight polyphenols. The bioavailability of proanthocyanidins is still uncertain due to the limited number of studies. To date, studies suggest that the absorption is limited to compounds with a low degree of polymerization and/or metabolites created in the colon (Cos and others 2003). The absorption of anthocyanins may be as low as one tenth that of quercetin (Prior 2003). More *in vivo* studies must be carried out in order to confidently determine the bioavailability of phenolic compounds.

## References

Agarwal C, Sharma Y, Agarwal R. 2000. Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: Modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis. *Mol Carcinog* 28:129-138.

Alvarez E, Northwood IC, Gonzalez FA, Latour DA, Seth A, Abate C, Curran T, Davis RJ. 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c- myc and c-jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. *J Biol Chem* 266(23):15277-15285.

[ACS] American Cancer Society. Cancer Facts and Figures 2008. Report. Atlanta (GA): American Cancer Society; 2008.

Armstrong B, Doll R. 1975. Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices. *Int J Cancer* 15:617-631.

Asensi M, Medina I, Ortega A, Carretero J, Bano MC, Obrador E, Estrela JM. 2002. Inhibition of cancer growth by resveratrol is related to its low bioavailability. *Free Radic Biol Med* 33:387-398.

Auger C, Teissedre PL, Gérain P, Lequeux N, Bornet A, Serisier S, Besançon P, Caporiccio B, Cristol JP, Rouanet JM. 2005. Dietary wine phenolics catechin, quercetin, and resveratrol efficiently protect hypercholesterolemic hamsters against aortic fatty streak accumulation. *J Agric Food Chem* 53:2015-2021.

Azios NG, Dharmawardhane SF. 2005. Resveratrol and estradiol exert disparate effects on cell migration, cell surface actin structures, and focal adhesion assembly in MDA-MB-231 human breast cancer cells. *Neoplasia* 7:128-140.

Bank G, Schauss A. 2004. Antioxidant testing: an ORAC update. *Nutr World* <http://www.nutraceuticalsworld.com/articles/2004/03/antioxidant-testing-an-orac-update>. Accessed 17 September 2008.

Baur JA, Sinclair DA. 2006. Therapeutic potential of resveratrol: the *in vivo* evidence. *Nat Rev Drug Discov* 5(6):493-506.

Bonilla F, Mayen M, Merida J, Medina M. 1999. Extraction of phenolic compounds from red grape marc for use as food lipid antioxidants. *Food Chem* 66:209-215.

Bove K, Lincoln DW, Tsan MF. 2002. Effect of resveratrol on growth of 4T1 breast cancer cells *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 291:1001-1005.

Brossaud F, Cheynier V, Asselin C, Moutounet M. 1999. Flavonoid compositional differences of grapes among site test plantings of Cabernet franc. *Am J Enol Vitic* 50(3):277-284.

Campbell JK, King JL, Harmston M, Lila MA, Erdman JW. 2006. Synergistic effects of flavonoids on cell proliferation in Hepa-1c1c7 and LNCaP cancer cell lines. *J Food Sci* 71(4):S358-S363.

Cobb MH, Goldsmith EJ. 1995. How MAP kinases are regulated. *J Biol Chem* 270:14843-14846.

Cos P, De Bruyne T, Hermans N, Apers S, Vanden Berghe D, Vlietinck AJ. 2003. Proanthocyanidins in health care: Current and new trends. *Curr Med Chem* 10:1345-1359.

Crowe A. The pomace predicament. *WineMaker* [Internet]. 2005 Aug [cited 2009 March 24]; [about 7 pages]. Available from: <http://www.winemakermag.com/stories/article/indices/21-fresh-grape-winemaking/678-the-pomace-predicament>

Cummings JH, Bingham SA. 1998. Diet and the prevention of cancer. *Brit Med J* 317:1636-1640.

De Rijke YB, Demacker PNM, Assen NA, Sloots LM, Katan MB, Stalenhoef AFH. 1996. Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. *Am J Clin Nutr* 63:329-334.

Delmas D, Lançon A, Colin D, Jannin B, Latruffe N. 2006. Resveratrol as a chemopreventive agent: A promising molecule for fighting cancer. *Curr Drug Targets* 7:423-442.

Del Pozo-Insfran D, Del Follo-Martinez A, Talcott ST, Brenes CH. 2007. Stability of copigmented anthocyanins and ascorbic acid in muscadine grape juice processed by high hydrostatic pressure. *J Food Sci* 72(4):247-253.

Frankel EN, Meyer AS. 2000. Review: The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J Sci Food Agr* 80:1925-1941.

Fuggetta MP, Lanzilli G, Tricarico M, Cottarelli A, Falchetti R, Ravagnan G, Bonmassar E. 2006. Effect of resveratrol on proliferation and telomerase activity of human colon cancer cells in vitro. *J Exp Clin Cancer Res* 25:189-193.

Fuhrman B, Lavy A, Aviram M. 1995. Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation. *Am J Clin Nutr* 61:549-554.

Gambutì A, Strollo D, Ugliano M, Lecce L, Moio L. 2004. *Trans*-resveratrol, quercetin, (+)-catechin, and (-)-epicatechin content in south Italian monovarietal wines: Relationship with maceration time and marc pressing during winemaking. *J Agric Food Chem* 52:5747-5751.

Garg AK, Buchholz TA, Aggarwal BB. 2005. Chemosensitization and radiosensitization of tumors by plant polyphenols. *Antioxid Redox Sign* 7:1630-1647.

God JM, Tate P, Larcom LL. 2007. Anticancer effects of four varieties of muscadine grape. *J Med Food* 10(1):54-59.

Goldberg DM, Yan J, Soleas GJ. 2003. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. *Clin Biochem* 36:79-87.

Gonzalez FA, Raden DL, Davis RJ. 1991. Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J Biol Chem* 266:22159-22163.

Han X, Shen T, Lou H. 2007. Dietary polyphenols and their biological significance. *Int J Mol Sci* 8:950-988.

Harper CE, Patel BB, Wang J, Arabshahi A, Eltoum IA, Lamartiniere A. 2007. Resveratrol suppresses prostate cancer progression in transgenic mice. *Carcinogenesis* 28(9):1946-1953.

Heim KE, Tagliaferro AR, Bobilya DJ. 2002. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J Nutr Biochem* 13:572-584.

Hollman PCH, de Vries JHM, van Leeuwen SD, Mengelers MJB, Katan MB. 1995. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am J Clin Nutr* 62:1276-1282.

Hollman PCH, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH, Katan MB. 1997. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett* 418:152-156.

- Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53:1841-1856.
- Hudson TS, Hartle DK, Hursting SD, Nunez NP, Wang TT, Young HA, Arany P, Green JE. 2007. Inhibition of prostate cancer growth by muscadine grape skin extract and resveratrol through distinct mechanisms. *Cancer Res* 67(17):8396-8405.
- Jacob JK, Hakimuddin F, Paliyath G, Fisher H. 2008. Antioxidant and antiproliferative activity of polyphenols in novel high-polyphenol grape lines. *Food Res Int* 41:419-428.
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275:218-220.
- Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM, Castanas E. 2000. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutr Cancer* 37(2):223-233.
- Knekt P, Järvinen R, Seppänen R, Heliövaara M, Teppo L, Pukkala E, Aromaa A. 1997. Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *Am J Epidemiol* 146(3):223-230.
- Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T, Aromaa A. 2002. Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 76:560-568.
- Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. 2000. Flavonoids suppress androgen-independent human prostate tumor proliferation. *Nutr Cancer* 38(1):116-122.
- Kondo K, Matsumoto A, Kurata H, Tanahashi H, Koda H, Amachi T, Itakura H. 1994. Inhibition of oxidation of low-density lipoprotein with red wine. *Lancet* 344:1152.
- Kuntz S, Wenzel U, Daniel H. 1999. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur J Nutr* 38:133-142.
- Leach JE, Barbosa P, Davis MJ, Hoel DG, Moffitt LJ, Power AG, Root TL, Schultz J, Splinter WE, Staskawicz BJ, et al. 2004. California agricultural research priorities: Pierce's disease. Washington, DC: National Academies Press.

- LeBlanc MR, Johnson CE, Wilson PW. 2007. Stilbene levels in the tissue and juice of muscadine grapes (*Vitis rotundifolia* Michx.). *Int J Fruit Sci* 6(2):87-100.
- Le Marchand L. 2002. Cancer preventive effects of flavonoids – a review. *Biomed Pharmacother* 56:296-301.
- Lee JH, Johnson JV, Talcott ST. 2005. Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. *J Agric Food Chem* 53:6003-6010.
- Lee JH, Talcott ST. 2004. Fruit maturity and juice extraction influences ellagic acid derivatives and other antioxidant polyphenolics in muscadine grapes. *J Agric Food Chem* 52:361-366.
- Liao HF, Kuo CD, Yang YC, Lin CP, Tai HC, Chen YY, Chen YJ. 2005. Resveratrol enhances radiosensitivity of human non-small cell lung cancer NCI-H838 cells accompanied by inhibition of nuclear factor-kappa B activation. *J Radiat Res* 46:387-393.
- Linseisen J, Radtke J, Wolfram G. 1997. Flavonoid intake of adults in a Bavarian subgroup of the national food consumption survey. *Z Ernährungswiss* 36:403-412.
- Liu SM. 1999. Anti-cancer agents: A treatment of cisplatin and their analogues [Internet]. London: Imperial College; c1999 [modified 2003 May 2; cited 2009 March 4]. Available from: <http://www2.mrc-lmb.cam.ac.uk/personal/sl/Html/Preface.html>
- Losso JN, Bansode RR, Trappey A, Bawadi HA, Truax R. 2004. In vitro anti-proliferative activities of ellagic acid. *J Nutr Biochem* 15:672-678.
- Magee JB, Smith BJ. 2002. Resveratrol content of muscadine berries is affected by disease control spray program. *Hort Sci* 37(2):358-361.
- Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* 81:230S-242S.
- Mazza G, Fukumotogi L, Delaquis P, Girard B, Ewert B. 1999. Anthocyanins, phenolics, and color of Cabernet franc, Merlot, and Pinot noir wines from British Columbia. *J Agric Food Chem* 47:4009-4017.
- Mertens-Talcott SU, Talcott ST, Percival SS. 2003. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *Nutr Cancer* 133:2669-2674.

Mertens-Talcott SU, Percival SS. 2005. Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. *Cancer Lett* 218:141-151.

Mertens-Talcott SU, Lee JH, Percival SS, Talcott ST. 2006. Induction of cell death in Caco-2 human colon carcinoma cells by ellagic acid rich fractions from muscadine grapes (*Vitis rotundifolia*). *J Agric Food Chem* 54:5336-5343.

Mertens-Talcott SU, Percival SS, Talcott ST. 2008. Extracts from red muscadine and Cabernet sauvignon wines induce cell death in MOLT-4 human leukemia cells. *Food Chem* 108:824-832.

Mouria M, Gukovskaya AS, Jung Y, Buechler P, Hines OJ, Reber HA, Pandol SJ. 2002. Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome c release and apoptosis. *Int J Cancer* 98:761-769.

Narayanan NK, Narayanan BA, Nixon DW. 2004. Resveratrol-induced cell growth inhibition and apoptosis is associated with modulation of phosphoglycerate mutase B in human prostate cancer cells: two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis and mass spectrometry evaluation. *Cancer Detect Prev* 28(6):443-452.

Netzel M, Strass G, Bitsch I, Könitz R, Christmann M, Bitsch R. 2003. Effect of grape processing on selected antioxidant phenolics in red wine. *J Food Eng* 56:223-228.

Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J Agric Food Chem* 50:3122-3128.

Pace-Asciak CR, Hahn SE, Diamandis EP, Soleas G, Goldberg DM. 1995. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin Chim Acta* 235:207-219.

Pastrana-Bonilla E, Akoh CC, Sellappan S, Krewer G. 2003. Phenolic content and antioxidant capacity of muscadine grapes. *J Agric Food Chem* 51:5497-5503.

Poling EB, Mainland CM, Bland WT, Cline B, Sorensen KA. 2003. Muscadine Grape Production Guide for North Carolina. Report. Raleigh (NC): North Carolina Cooperative Extension Service.

- Prajitna A, Dami IE, Steiner TE, Ferree DC, Scheerens JC, Schwartz SJ. 2007. Influence of cluster thinning on phenolic composition, resveratrol, and antioxidant capacity in Chambourcin wine. *Am J Enol Vitic* 58(3):346-350.
- Prior RL. 2003. Fruits and vegetables in the prevention of cellular oxidative damage. *Am J Clin Nutr* 78(3):570S-578S.
- Prior RL, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:4290-4302.
- Ren W, Qiao Z, Wang H, Zhu L, Zhang L. 2003. Flavonoids: Promising anticancer agents. *Med Res Rev* 23(4):519-534.
- Rice-Evans CA, Miller NJ, Paganga G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Bio Med* 20(7):933-956.
- Rice-Evans CA, Miller NJ, Paganga G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci* 2(4):152-159.
- Rodríguez Vaquero MJ, Alberto MR, Manca de Nadra MC. 2005. Antibacterial effect of phenolic compounds from different wines. *Food Control* 18(2):93-101.
- Roldán A, Palacios V, Caro I, Pérez L. 2003. Resveratrol content of *Palomino fino* grapes: Influence of vintage and fungal infection. *J Agric Food Chem* 51:1464-1468.
- Sánchez-Moreno C. 2002. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Tech Int* 8(3):121-137.
- Scalbert A, Williamson G. 2000. Dietary intake and bioavailability of polyphenols. *J Nutr* 130:2073S-2085S.
- Schlachterman A, Valle F, Wall KM, Azios NG, Castillo L, Morell L, Washington AV, Cubano LA, Dharmawardhane SF. 2008. Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model. *Trans Onc* 1(1):19-27.
- Shi J, Yu J, Pohorly JE, Kakuda Y. 2003. Polyphenolics in grape seeds – Biochemistry and functionality. *J Med Food* 6(4):291-299.
- Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16(3):144-158.

- Soleas GJ, Dam J, Carey M, Goldberg DM. 1997. Toward the fingerprinting of wines: Cultivar-related patterns of polyphenolic constituents in Ontario wines. *J Agric Food Chem* 45:3871-3880.
- Soleas GJ, Yan J, Goldberg DM. 2001a. Ultrasensitive assay for three polyphenols (catechin, quercetin and resveratrol) and their conjugates in biological fluids utilizing gas chromatography with mass selective detection. *J Chromatogr B* 757:161-172.
- Soleas GJ, Yan J, Goldberg DM. 2001b. Measurement of *trans*-resveratrol, (+)-catechin, and quercetin in rat and human blood and urine by gas chromatography with mass selective detection. *Method Enzymol* 335:130-145.
- Soleas GJ, Grass L, Josephy PD, Goldberg DM, Diamandis EP. 2002. A comparison of the anticarcinogenic properties of four red wine polyphenols. *Clin Biochem* 35:119-124.
- Stanley D. 1997. America's first grape: The muscadine. *Agr Res Nov*:14-16.
- Steinmetz KA, Potter JD. 1991. Vegetables, fruit, and cancer. I. Epidemiology. *Cancer Cause Control* 2:325-357.
- Stoner GD, Mukhtar H. 1995. Polyphenols as cancer chemopreventive agents. *J Cell Biochem* S22:169-180.
- Swain T, Hillis WE. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J Sci Food Agric* 10:63-68.
- Talcott ST, Lee JH. 2002. Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. *J Agric Food Chem* 50:3186-3192.
- Tate P, God J, Bibb R, Lu Q, Larcom LL. 2004. Inhibition of metalloproteinase activity by fruit extracts. *Cancer Lett* 212:153-158.
- [USDA] United States Department of Agriculture. 2007 Nov. Oxygen radical absorbance capacity (ORAC) of selected foods – 2007. Beltsville (MD): United States Department of Agriculture. Available from: United States Department of Agriculture, Beltsville, MD 20705 or <http://www.ars.usda.gov/Services/docs.htm?docid=15866>
- University of Rochester Medical Center. Winemaking waste proves effective against disease-causing bacteria in early studies. *ScienceDaily* [Internet]. 2008 January 8 [cited 2009 January 30]. Available from: <http://www.sciencedaily.com/releases/2008/01/080102122256.htm>

Vasanth Rupasinghe HP, Clegg S. 2007. Total antioxidant capacity, total phenolic content, mineral elements, and histamine concentrations in wines of different fruit sources. *J Compos Anal* 20:133-137.

Vinismo [Internet]. c2007-2009 [modified 2009 14 January; cited 2009 January 30]. Available from: [http://vinismo.com/en/Cabernet\\_Franc](http://vinismo.com/en/Cabernet_Franc)

Wedge DE. 2002. Berries curb cancer cells. *Agr Res* June:23.

Willett WC. 1995. Diet, nutrition, and avoidable cancer. *Environ Health Persp* 103(suppl 8):165-170.

Williamson G, Manach C. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am J Clin Nutr* 81:243S-255S.

Wood M, McGinnis L. Core J. 2006. Grapes! Our never-ending crush. *Agr Res* Apr:4-8.

Wu X, Gu L, Holden J, Haytowitz DB, Gebhardt SE, Beecher G, Prior RL. 2004. Development of a database for total antioxidant capacity in foods: a preliminary study. *J Food Compos Anal* 17:407-422.

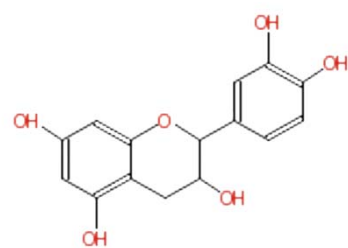
Yang H, Landis-Piowar KR, Chen D, Milacic V, Dou QP. 2008. Natural compounds with proteasome inhibitory activity for cancer prevention and treatment. *Curr Protein Pept Sc* 9:227-239.

Yi OS, Meyer AS, Frankel EN. 1997. Antioxidant activity of grape extracts in a lecithin liposome system. *J Am Oil Chem Soc* 74(10):1301-1307.

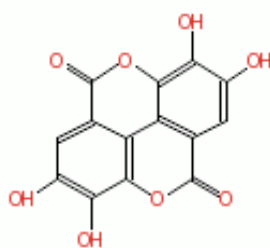
Yi W, Fischer J, Akoh CC. 2005. Study of anticancer activities of muscadine grape phenolics in vitro. *J Agric Food Chem* 53:8804-8812.

Yi W, Akoh CC, Fischer J, Krewer G. 2006. Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Res Int* 39:628-638.

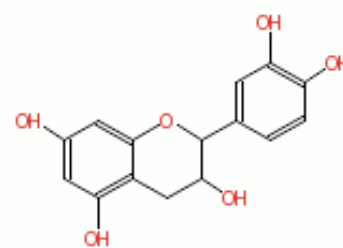
Yilmaz Y, Toledo RT. 2004. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J Agric Food Chem* 52:255-260.



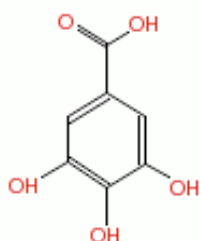
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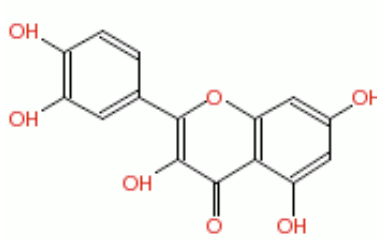
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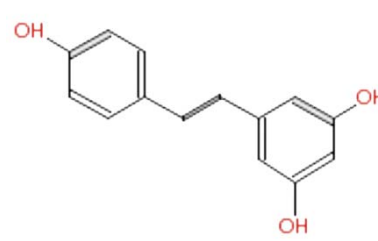
Epicatechin



Gallic acid



Quercetin



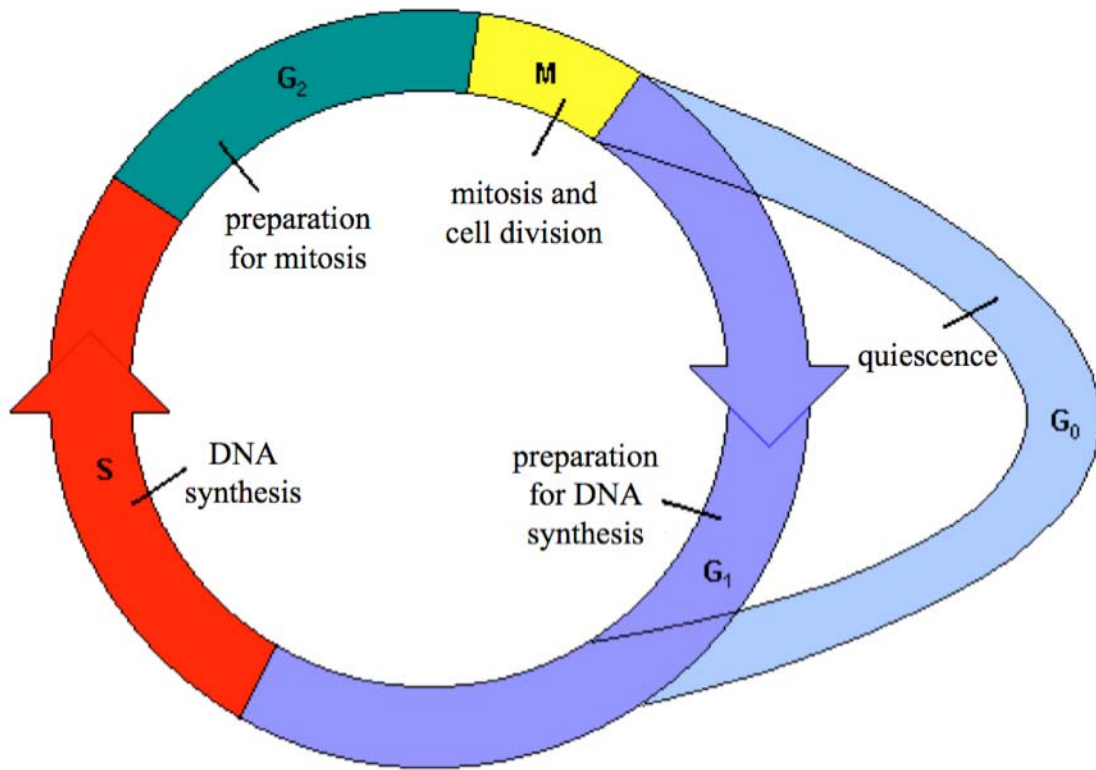
Resveratrol

**Figure 1.1:** Chemical structures of select phenolic compounds.

**Table 1.1:** Oxygen radical absorbance capacity and total phenolics of selected foods<sup>a</sup>

<b>Sample</b>	<b>ORAC</b> ( $\mu\text{mol Trolox equivalents}$ per 100 g)	<b>Total Phenolics</b> (mg gallic acid equivalents per 100 g)
Blueberries	6552	531
Broccoli	1362	337
Chocolate, dark	20823	1297
Table grapes, red	1260	177
Table grapes, green	1118	145
Table wine, red	3873	18
Table wine, white	392	20

<sup>a</sup> Table adapted from USDA 2007.



**Figure 1.2:** The cell cycle (Adapted from Liu 1999).

## CHAPTER 2. ANTIOXIDANT CHARACTERIZATION OF GRAPE SKIN EXTRACTS

### *Abstract*

The “French paradox” is a hypothesis that piqued interest in the advantages of drinking red wine. Despite high saturated fat intake, instances of heart disease in France is low. Red wine contains phenolic compounds that are powerful antioxidants and regular wine consumption may provide protective effects. Muscadine (cv. Carlos and Noble) and Cabernet franc grape skins contain a wide variety of phenolic compounds. Phenolics were extracted from freeze dried skins, picked from two harvests, with 80:20 methanol:water. Extracts were characterized by the Folin-Ciocalteu method, oxygen radical absorbance capacity (ORAC) assay, and by high pressure liquid chromatography (HPLC). Carlos skins had the lowest total phenols content and ORAC values while Noble skins had the highest. Carlos and Noble skin extracts contained high amounts of ellagic acid and low amounts of resveratrol. No ellagic acid was detected in Cabernet franc extracts, while resveratrol was detected in only the sample from the 2006 harvest. The phenolics found in grape skins may have nutraceutical applications.

## ***Introduction***

The “French paradox” is a hypothesis that piqued interest in the advantages of drinking red wine. Even though the French consume a diet containing large quantities of saturated fat, the mortality rate due to coronary heart disease is lower than that of most other developed countries (WHO 1989). Other risk factors of coronary heart disease include high blood pressure, high body-mass index, and cigarette smoking (in males). However, these factors are no lower in France than in other industrialized countries (WHO 1989). The hypothesis states that the inverse relationship is due to the protective effects of regular wine consumption (Renaud and de Lorgeril 1992). While many other factors could be contributing to this correlation, red wine is indeed full of phenolic compounds that are powerful antioxidants. These phenolics are extracted from the skins and seeds of grapes during winemaking. Muscadine and Cabernet franc grapes contain a wide variety of phenolic compounds.

Plants synthesize phytochemicals in response to stressful conditions in order to survive. Plants must cope with factors such as drought, salinity, nutritional deficiency, intense sunlight, pollutants, pathogens, insects, adverse climactic conditions, and phytophagy (Iriti and Faoro 2006). Phenolic compounds are secondary metabolites and therefore not active in the development, growth, and reproduction cycles. Phenolics can serve as antioxidants. Antioxidants are compounds that are capable of preventing or reducing the rate of oxidation of other compounds. This is extremely important as oxidation reactions create free radicals. These free radicals are damaging to cells and can

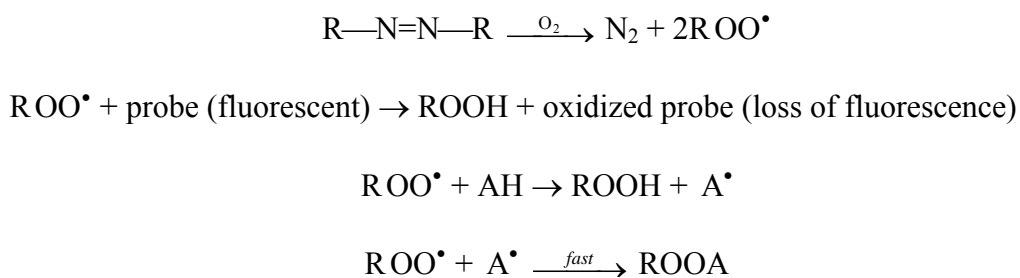
lead to diseases such as cancer. The structure of phenolics makes them the ideal compounds for free radical-scavenging activities (Rice-Evans and others 1997).

Phenolic compounds have been extracted from plant foods using ethanol, methanol, ethyl acetate, water, acetic acid, and acetone in a variety of combinations and proportions (Sun and others 2006). There is no one method that is optimized and standardized for the extraction of all classes of phenolic compounds in different plant foods. This is due to the wide array of structural differences and the food matrix (Mané and others 2007). For instance, 80% ethanol in water (v/v) was the most efficient extraction solvent for all phenolics in peanut skin, while 60% or 70% methanol in water was optimal for grape seed extraction (Yilmaz and Toledo 2006, Yu and others 2005). Yet, 70% ethanol extracted the least amount of catechins in tea compared to boiling water and 80% methanol, while pure methanol was optimal for extraction of catechins in grape seeds (Kallithraka 1995, Khokhar and Magnusdottir 2002). Pekić and others (1998) determined that the solvent system 90% ethyl acetate in water, was the most efficient for extraction of proanthocyanidins in grape seeds. A mixture of 90% methanol in acetic acid was the most successful solution to extract free phenolic acids in several types of plant food (Mattila and Kumpulainen 2002).

The total amount of phenolic compounds in a sample can be measured with the Folin-Ciocalteu method, developed by Singleton and Rossi (1965). The Folin-Ciocalteu method is an electron transfer reaction and measures the reducing capacity of a sample. More specifically, the method is based on the reaction of the hydroxyl groups of the phenolic compounds with the phosphomolybdate (Folin-Ciocalteu) reagent. Because of

its convenience, simplicity, and reproducibility, this assay has become routine for total phenolics analysis (Huang and others 2005). The results also correlate well with antioxidant activity.

While not a standardized assay, the most widely accepted method for measuring antioxidant capacity is the ORAC assay. A database of the antioxidant capacity of numerous foods, determined by ORAC, has been compiled by the USDA, which allows for comparison (USDA 2007). The ORAC assay is a hydrogen atom transfer reaction and measures the rate at which 2,2'-azobis (2-amidino-propane dihydrochloride) (AAPH), a peroxy radical generator, breaks down the fluorescent indicator fluorescein and a nonfluorescent product is formed (**Figure 2.1**) (Ou and others 2001). In the presence of antioxidants, the fluorescein is protected by the antioxidants and the degradation is slowed.



**Figure 2.1:** Reaction occurring during the ORAC assay where A is the antioxidant and R OO<sup>•</sup> is the peroxy radical (Prior and others 2005).

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble vitamin E analog used as the standard. This is a common standard used in other antioxidant capacity assays as well.

Since different antioxidants, combinations of antioxidants, and food matrices can affect the time of reaction, the ORAC assay is advantageous in that it takes into consideration various lag phases. The ORAC assay is also advantageous in that it is readily automated and can be applied to lipophilic antioxidants. A drawback of this method is that the assay is only measuring the inhibition of one of the most common types of reactive oxygen species – peroxy radicals. Antioxidants in a food may react differently to different radical or oxidant sources. For instance, carotenoids work well at quenching singlet oxygen, while most other phenolics are ineffective (Prior and others 2005). However, the ORAC assay has been modified to accommodate for the activities against two of the other most common free radicals such as the peroxynitrite (NORAC) and hydroxyl radicals (HORAC) (Bank and Schauss 2009, Ou and others 2002). ORAC methods are also being developed for several other oxidants including singlet oxygen, hydroperoxide, and the superoxide anion (Bank and Schauss 2009).

Phenolic compounds have been shown to not only be cardioprotective (Hung and others 2000) and powerful antioxidants (Frankel and others 1993, Mayer and others 1997), but also anti-inflammatory (Pace-Asciak and others 1995), anti-carcinogenic (Jang and others 1997), anti-atherosclerosis (Han and others 2007), and even anti-bacterial and anti-allergic (Ren and others 2003). The phenolics found in grape skins can function as multipurpose antioxidants having usage in nutraceuticals to possibly prevent and treat diseases.

The objectives of this study were to determine the total phenolic contents and antioxidant capacities of Cabernet franc grape skin extract and two cultivars of

muscadine grape skin extracts. The purpose of this study was also to identify the phenolic compounds present in the skin extracts through high pressure liquid chromatography (HPLC) and HPLC-time of flight mass spectrometry.

### ***Materials and methods***

#### **Samples**

Carlos and Noble muscadine grapes and Cabernet franc grapes were provided by the North Carolina State University research farms in Castle Hayne, North Carolina and Reidsville, North Carolina, respectively. Methanol was purchased from Fisher Scientific (Pittsburgh, PA).

The skins were manually separated from the seeds and pulp, then freeze dried using a VirTis Genesis 25XL freeze dryer (Gardiner, NY) at -70°C. The dried skins were then ground into a fine powder using a coffee grinder (Krupps, Medford, MA) and stored in a -20°C freezer.

Noble and Cabernet franc grapes from the 2007 harvest were also pressed and made into wine as described by Amerine and others (1980). Wine extracts were also prepared by reducing a 500 mL aliquot of wine on a rotary evaporator down to 5 mL (Büchi, Switzerland). The water bath temperature did not exceed 40°C. Wine and wine

extracts were stored in a refrigerator and analyzed at full strength or diluted appropriately.

### **Extraction of grape skins**

The extraction procedure was similar to that of Tsanova-Savova and others (2005). A 12 mL aliquot of 80% aqueous methanol was added to 2 g of dried grape skin powder. The mixture was vortexed for one minute and then sonicated in a Branson 1200 ultrasonic cleaner (Danbury, CT) for twenty minutes. Halfway into the sonication, the mixture was vortexed for an additional twenty seconds. Following sonication, the mixture was centrifuged for twenty minutes at setting 90 on a IEC Centra-4B centrifuge (International Equipment Company, Needham Heights, MA). The supernatant was then pipetted into an amber vial and stored in a -20°C freezer until analysis.

### **Folin-Ciocalteu total phenolics assay**

Anhydrous sodium carbonate was obtained from EMD Chemicals (Gibbstown, NJ) and gallic acid and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO).

The improved procedure using Folin-Ciocalteu reagent described by Singleton and Rossi (1965) was followed. Briefly, samples were prepared by appropriate dilutions with deionized water. Gallic acid standard solutions were made with deionized water in concentrations ranging from 0 – 500 mg gallic acid per L of solution or milliequivalents (meq) of gallic acid. Following mixing, the tubes were left to incubate at room

temperature, in the dark, for two hours. The absorbances of the solutions were read at 765 nm using a Spectronic Genesys 2 spectrophotometer by Spectronic Analytical Instruments (Leeds, UK). The entire procedure was carried out under yellow lighting.

All samples were analyzed in duplicate. Statistical analyses were done using one way ANOVA and the Fisher's LSD post hoc test with the program XLSTAT (New York, NY).

### **Oxygen radical absorbance capacity (ORAC) assay**

Dibasic and monobasic phosphate buffers were purchased from MP Biomedicals (Solon, Ohio). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Fisher (Fair Lawn, NJ) and fluorescein sodium from Riedel-de Haën (Seelze, Germany). AAPH (2,2'-azobis (2-amidinopropane) dihydrochloride) was purchased from Wako Chemicals (Richmond, VA).

The improved ORAC procedure using fluorescein described by Ou and others (2001) was followed. Briefly, phosphate buffer was prepared with dibasic and monobasic phosphate buffers and deionized water to create a solution with a final pH of 7.4. The buffer was prepared fresh every two weeks and stored in the refrigerator. All further dilutions were made using this buffer. Trolox working standards were made in various concentrations ranging from 0  $\mu\text{M}$  to 12.5  $\mu\text{M}$  to create a standard curve. The working standards were prepared fresh daily. A 10 nM fluorescein solution was prepared daily from a stock solution stored in the refrigerator. Sample extracts were diluted

accordingly. Using a black 96 well plate (Greiner Bio-One Monroe, NC), each solution was added to each well as follows:

*blank* = 130  $\mu$ L buffer + 60  $\mu$ L fluorescein solution

*standard* = 70  $\mu$ L buffer + 60  $\mu$ L Trolox solution + 60  $\mu$ L fluorescein solution

*sample* = 70  $\mu$ L buffer + 60  $\mu$ L sample + 60  $\mu$ L fluorescein solution

The plate was then incubated for 15 minutes at 37°C in darkness. A 153 mM solution of AAPH was prepared fresh daily, immediately prior to use. This solution was added in 60  $\mu$ L aliquots to each well after incubation. The plate was then analyzed using a Tecan Safire<sup>2</sup> microplate reader (Männedorf, Switzerland) coupled with Magellan (Version 6.1) software. Readings were taken every minute for eighty cycles with five seconds of shaking in between. The instrument was held at a temperature of 37°C. The entire procedure was carried out under yellow lighting.

The final ORAC values were calculated using a regression equation between Trolox concentration and the net area under the fluorescein decay curve.

$$\text{AUC} = 0.5 + \left( \frac{R_2}{R_1} \right) + \left( \frac{R_3}{R_1} \right) + \dots + 0.5 \left( \frac{R_n}{1} \right)$$

Where AUC = area under the curve

$R_1$  = fluorescence reading at the initiation of the reaction

$R_n$  = last reading

$$\text{AUC}_{\text{net}} = \text{AUC}_{\text{sample/standard}} - \text{AUC}_{\text{blank}}$$

A standard curve was plotted and the concentration of the sample was then calculated from the equation of the line.

All samples were analyzed in duplicate. Statistical analyses were done using one way ANOVA and the Fisher's LSD post hoc test with the program XLSTAT (New York, NY).

### **High pressure liquid chromatography (HPLC)**

A 6 mL aliquot of the sample extract, prepared as described in the Extraction section, was added to a Strata-X-SPE column (Phenomenex, Torrance CA). The column was pre-loaded with 1 mL methanol and 1 mL of HPLC grade water. The 6 mL sample was allowed to flow through the packed bed by gravity. Then, 4 mL of 5% methanol in HPLC grade water was added to wash the bed. After the solution finished flowing, a vacuum was applied for 1 minute. A 4 mL aliquot of methanol was then added and the eluent was collected. The collection tube was dried under nitrogen in a water bath set at 40°C (about 1 hour). The sample was then reconstituted in 300 µL of methanol and transferred to an Amicon Ultrafree-MC tube with 0.22 µm filter (Millipore, Bedford, MA). The sample was centrifuged for 30 minutes using a Costar Model 10 mini centrifuge (Corning, Lowell, MA). The filtrate was stored in a -20°C freezer until analysis.

Extracts were transferred to HPLC vials and diluted appropriately for injection into the instrument. The analysis was done using a Waters 996 photodiode array detector, Waters 717 plus autosampler, Waters 501 and 510 pumps, and a Waters

automated gradient controller (Milford, MA). Two solvents were used: 0.1% trifluoroacetic acid in HPLC grade water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). For each 50  $\mu$ L sample injection, the following gradient was run:

<u>Time (min)</u>	<u>%A</u>	<u>%B</u>
0	95	5
10	75	25
17	50	50
27	0	100
35	100	0
40	95	5

The extract was separated using a Waters Spherisorb 5 $\mu$ m ODS2 C18 column (250 mm x 4.6 mm).

All samples were analyzed in triplicate. Statistical analyses were done using one way ANOVA and the Fisher's LSD post hoc test with the program XLSTAT (New York, NY).

### **High pressure liquid chromatography – Time of flight mass spectrometry (HPLC-TOF-MS)**

A 5  $\mu$ L injection of the solution prepared as described in the HPLC section was injected on to an Agilent Series 1100 liquid chromatogram (HPLC) (Agilent Technologies, Inc., Santa Clara, CA) and separated using a C18 column (Restex Ultra Aqueous, 100 mm x 2.1 mm, (Restex Corp., Bellefonte, PA)). The HPLC was interfaced directly to a Leco Unique Time of flight mass spectrophotometer (TOF-MS) (Leco Corp, St. Joseph, MO). The flow rate was 0.4 mL/min using a gradient of 0.1 % formic acid in water (A) and 50/50 v/v acetonitrile/methanol (B) as the mobile phase. The gradient

program was as follows:

<u>Time (min)</u>	<u>%A</u>	<u>%B</u>
0	90	10
30	5	95
35	5	95
40	95	5

The column was heated to 30°C and the autosampler was held at 10°C. The MS used a high flow electrospray ionization (ESI) source in the negative mode. The ESI voltage was -3500 V with a desolvation temperature of 300°C. The nebulizer pressure was 375 kPa using nitrogen as a desolvation gas at 7 L min<sup>-1</sup>. The interface temperature was 100°C. The nozzle was set to -160 V and the skimmer was set to -60 V. The data was acquired at 1.56 spectra sec<sup>-1</sup> using ChromaTOF software (Version 4.0, Leco Corp.).

## ***Results and Discussion***

### **Folin-Ciocalteu total phenolics assay**

The total phenolic contents of the grape skin extracts are shown in **Figure 2.2**. The total phenol values ranged from 12-27 mg gallic acid equivalents (GAE) per gram of freeze dried skin sample. Generally, the skins of the muscadine cultivar Noble, had a greater concentration of phenolic compounds than the Cabernet franc skins. Carlos extracts, on the other hand, varied significantly between the two harvest years and cannot be compared definitively to the other two samples. This difference between years is not surprising as many environmental factors can influence the phenolic content including time of harvest and climate conditions. The Cabernet franc samples were least affected by the year to year seasonal effects. Total phenolics concentration in Cabernet franc

grape skins reach their maximum when fully ripened (Mazza and others 1999).

Compared to other total phenol values found in literature (**Table 2.1**), the results all fall within the range with the 2007 Carlos skin extract on the very low end.

The total phenol values for the Noble and Cabernet franc wines (**Figure 2.4**) were well within the range relative to the literature values (**Table 2.1**). The Noble wine extract produced a total phenolics value of approximately ten times that of the wine, while the Cabernet franc wine extract led to a value of about sixteen times that of its respective wine. When comparing the relationship of phenolic values between Noble and Cabernet franc, the ratio of Noble skins (2007) versus Cabernet franc skins (2007) is 1.57 while the ratio of the wines is 1.80. These ratios are very similar. The higher amount of phenolic compounds in the Noble wine may be due to a higher concentration in the seeds, juice, and pulp compared to Cabernet franc grapes, as both grapes were fermented for the same period of time.

### **Oxygen radical absorbance capacity assay**

The antioxidant capacity of a food is a strong indicator of the potential health benefits it could possibly provide. In **Figure 2.3**, the antioxidant capacities for the grape skin samples are shown. The ORAC values ranged from 110 - 354  $\mu\text{mol}$  Trolox equivalents (TE) per gram of freeze dried grape skin. The Noble skins had the highest ORAC values in both harvests. The Cabernet franc and Carlos samples had similar antioxidant capacities with Cabernet franc being slightly higher. When compared to

literature values (**Table 2.2**), the ORAC for Carlos is much lower, on a dry weight basis, than the reference. This is likely due to yearly differences such as temperature and rainfall. The others cannot be directly compared as the skins of Noble were only analyzed by TEAC and there is no data on the skins of Cabernet franc.

The Noble wine did not have a significantly higher ORAC value than the Cabernet franc wine (**Figure 2.5**). This follows the same relationship as the total phenolics assay produced. The ORAC value of the Cabernet franc wine extract was higher than the Noble wine extract despite having similar total phenol concentrations and having been fermented for the same period of time. This may be indicative of the varied phenolic profiles of the two types of grape skins.

There is a strong correlation (0.9906) between the total phenol values and the ORAC values. The correlation is weaker with Carlos skins alone. Carlos is the only golden grape of all the samples analyzed and it is likely to contain different phenolic compounds than the red skinned varieties. The phenolics that Carlos skins contain may not have as strong of an antioxidant capacity as those found in Noble and Cabernet franc skins. Both Noble and Cabernet franc grapes are red-skinned and thus contain colored pigments such as anthocyanins, which also act as antioxidants, in addition to the normal array of phenolic compounds.

### **High pressure liquid chromatography (HPLC)**

Muscadines and Cabernet franc grapes both contain a variety of phenolic compounds, yet the profiles are very different. Ellagic acid and resveratrol are the

primary compounds in muscadine grapes and Cabernet franc grapes, respectively. Ellagic acid and resveratrol peaks were identified by HPLC by comparing both retention times and characteristic spectra to that of standards. Select chromatograms are presented in **Figures 2.6 – 2.9** and spectra of ellagic acid and resveratrol are presented in **Figures 2.10 and 2.11**, respectively. Concentrations determined by HPLC in both types of skins for each harvest are displayed in **Figures 2.12 and 2.13**. Literature values for both compounds as found in all parts of the grapes are shown in **Table 2.3**. No ellagic acid was detected in Cabernet franc skin extracts. This was an expected result as ellagic acid is a polyphenol often found in *V. rotundifolia*. Carlos and Noble skin extracts contained high amounts of ellagic acid with the Noble extract from 2007 reaching 268.7 µg/g dry weight. There was a large variation between harvests for the Noble extracts. Again, this is likely due to seasonal differences.

Resveratrol contents were low in the muscadine skin extracts with all values under 15 µg/g dry weight. In 2006, an average of 70.26 µg/g dry weight was detected in the Cabernet franc sample. On the contrary, no resveratrol was detected in the 2007 skin extract. This is surprising as the 2006 sample contained a significant amount of resveratrol. Although the 2006 Cabernet franc skin extract had high amounts of resveratrol, the ORAC value was lower than the muscadine extracts. Yet, resveratrol has a peroxy radical scavenging capacity more than seven times that of ellagic acid (Yilmaz and Toledo 2004). This implies that the other compounds in muscadines create a distinct profile with a better antioxidant capacity than that of Cabernet franc skins. The profile

could include flavonols such as catechin or epicatechin, which also have very high peroxy radical scavenging capacities compared to ellagic acid.

Literature values for resveratrol and ellagic acid in grape skins can be found in **Table 2.3**. It is to be noted again that all literature values found were on a fresh weight basis. Using an approximate conversion to dry weight (Pastrana-Bonilla and others 2003), it is estimated that the results for the concentrations of both ellagic acid and resveratrol fall on the low end of the range found in literature. For Noble skins, the pattern is the same. The lower values may be due to seasonal variations or harvesting times. No literature values for Cabernet franc skins could be found, but the high concentration relative to the muscadines is expected as resveratrol is one of the primary compounds in *V. vinifera*.

The ORAC values do not follow the same trend as ellagic acid concentration nor resveratrol concentration. Although ellagic acid is the main phenolic compound of muscadine skins and resveratrol is that of Cabernet franc skins, there are clearly other compounds with significant antioxidant capacities. Several other phenolic compounds have been identified in these skins in other studies, including kaempferol, myricetin, quercetin, and cyanidin (**Table 2.7**).

### **High pressure liquid chromatography – Time of flight mass spectrometry (HPLC-TOF-MS)**

HPLC-TOF-MS was used to analyze samples from the 2007 harvest. Carlos and Noble skin samples had 25 and 19 peaks, respectively, while the Cabernet franc skin

extract produced only 11 peaks (**Figures 2.14 – 2.16**). Not only did the number of peaks in the chromatograms differ greatly, but also the pattern of peaks. Notably, the Cabernet franc extract showed no significant peaks with retention times below 15 minutes, while the muscadine extracts both had several. Surprisingly, the Carlos skin extract contained more peaks, despite being a golden skin. White grapes lack anthocyanins, which are responsible for the red and blue hues of grape skins. The Carlos and Noble muscadine extracts contained several peaks with the same unique mass and retention time.

Despite the numerous peaks, few could be identified. Most of the phenolic compounds typically found in muscadine and *V. vinifera* grape skins and other grape parts were not detected by HPLC-TOF-MS. Possible identifications are shown in **Tables 2.4 – 2.6**. The lack of peaks may be due to the degradation of compounds over time. Several other phenolic compounds have been identified in grape skins of all types in other studies, including protocatechuic acid, malvidin, and peonidin (**Table 2.8**).

The composition of grape seeds may also be of use for the application of reworking winemaking waste into supplements or treatments (Shi and others 2003). Although grape seeds have a higher antioxidant capacity than grape skins, the skins have a very different compositional profile than grape seeds. Grape seeds have greater amounts of flavan-3-ols compared to grape skins (Escribano-Bailo'n and others 1995). The major dimer of Merlot and Cabernet Sauvignon skins and seeds is B<sub>2</sub>, but the skins contained none of the B<sub>4</sub> dimer while the seeds had relatively large concentrations (de Freitas and others 2000). This pattern has also been reported in other studies of *V. vinifera* grapes (de Freitas and Glories 1999). In general, hydroxycinnamates in wine

come from the juice of the grape, flavonoids from the skins or seeds, and hydroxybenzoates from degradation of fermented material during aging (Singleton 1992). Winemaking byproducts contain a wide variety of phenolic compounds from skins and seeds that can be extracted and used for supplements.

### ***Further work***

In order to further characterize muscadine and Cabernet franc grapes, more research must be done to identify the complete phenolic compound profiles of the skins. Analyses should be done on samples encompassing a few different harvests for a more in depth understanding of the profiles. With data from over several years, the range of variability due to seasonal differences can be determined. Grape skin samples could be subject to acid hydrolysis to free compounds that may exist primarily in the glucosidic or glycosidic form. The study could also be expanded to the analysis of a golden colored cultivar of *V. vinifera*, so that a dark and light cultivar of both *V. vinifera* and *V. rotundifolia* can be compared with one another. There is no doubt that the antioxidant capacity of muscadine and Cabernet franc grapes is powerful, but the basis for the activity can be defined even further.

### ***Conclusion***

The skins of muscadines and Cabernet franc grapes both have high antioxidant capacities, but very different phenolic compounds. While a primary compound of muscadine skin is ellagic acid, Cabernet franc skins contain no ellagic acid, but high

amounts of resveratrol. Grapes of *V. vinifera* have been receiving much attention for their resveratrol contents, but Noble grapes of *V. rotundifolia* were determined to have a significantly higher ORAC value. With Carlos grapes being a golden grape, its ORAC value still rivaled that of Cabernet franc skins. Both HPLC and HPLC-TOF-MS revealed that the phenolic compound profiles of the muscadines were more complex than that of Cabernet franc. Muscadine grape skins could be a valuable tool in the prevention of diseases as they contain many health-promoting compounds.

## **References**

- Amerine MA, Berg HW, Kunkee RE, Ough CS, Singleton VL, Webb AD. 1980. Technology of wine making. 4<sup>th</sup> ed. Westport (CT): AVI Publishing, Inc.
- Bank G, Schauss A. An ORAC update. In: Oracwatch.org [Internet]. Haines Falls (NY): Oracwtach.org; c2009 [cited 2009 February 20]; [about 3 p]. Available from: [http://www.oracwatch.org/what\\_orac.php](http://www.oracwatch.org/what_orac.php)
- Cliff MA, King MC, Schlosser J. 2007. Anthocyanin, phenolic composition, colour measurement and sensory analysis of BC commercial red wines. *Food Res Int* 40:92-100.
- De Freitas VAP, Glories Y. 1999. Concentration and concentrational changes of procyanidins in grape seeds and skin of white *Vitis vinifera* varieties. *J Sci Food Agric* 79:1601-1606.
- De Freitas VAP, Glories Y, Monique A. 2000. Developmental changes of procyanidins in grapes of red *Vitis vinifera* varieties and their composition in respective wines. *Am J Enol Vitic* 51(4):397-403.
- Escribano-Bailo'n MT, Guerra MT, Rivas-Gonzalo JC, Santos-Buelga C. 1995. Proanthocyanidins in skins from different grape varieties. *Lebensm Unters Forsch* 200:221-224.
- Frankel EN, Waterhouse AL, Kinsella JE. 1993. Inhibition of human LDL oxidation by resveratrol. *Lancet* 341:1103-1104.
- Han X, Shen T, Lou H. 2007. Dietary polyphenols and their biological significance. *Int J Mol Sci* 8:950-988.
- Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53:1841-1856.
- Hung LM, Chen JK, Huang SS, Lee RS, Su, MJ. 2000. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. *Cardiovasc Res* 47:549-555.
- Iriti M, Faoro F. 2006. Grape phytochemicals: A bouquet of old and new nutraceuticals for human health. *Med Hypotheses* 67:833-838.
- Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275:218-220.

- Kallithraka S, Garcaviaguera C, Bridle P, Bakker JI. 1995. Survey of solvents for the extraction of grapeseed phenolics. *Phytochem Anal* 6:265-267.
- Khokhar S, Magnusdottir SG. 2002. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *J Agric Food Chem* 50:565-570.
- LeBlanc MR, Johnson CE, Wilson PW. 2007. Stilbene levels in the tissue and juice of muscadine grapes (*Vitis rotundifolia* Michx.). *Int J Fruit Sci* 6(2):87-100.
- Lee JH, Johnson JV, Talcott ST. 2005. Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. *J Agric Food Chem* 53:6003-6010.
- Lee JH, Talcott ST. 2002. Ellagic acid and ellagitannins affect on sedimentation in muscadine juice and wine. *J Agric Food Chem* 50:3971-3976.
- Lee JH, Talcott ST. 2004. Fruit maturity and juice extraction influences ellagic acid derivatives and other antioxidant polyphenolics in muscadine grapes. *J Agric Food Chem* 52:361-366.
- Mané C, Souquet JM, Ollé D, Verriés C, Vèran F, Mazerolles G, Cheynier V, Fulcrand H. 2007. Optimization of simultaneous flavanol, phenolic acid, and Anthocyanin extraction from grapes using an experimental design: Application to the characterization of Champagne grape varieties. *J Agric Food Chem* 55:7224-7233.
- Mattila P, Kumpulainen J. 2002. Determination of free and total phenolic acids in plant-derived foods by HPLC with diode-array detection. *J Agric Food Chem* 50:3660-3667.
- Mayer AS, Yi OS, Person DA, Waterhouse AL, Frankel EN. 1997. Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes. *J Agr Food Chem* 45:1638-1643.
- Mazza G, Fukumoto L, Delaquis P, Girard B, Ewert B. 1999. Anthocyanins, phenolics, and color of Cabernet franc, Merlot, and Pinot Noir wines from British Columbia. *J Agric Food Chem* 47:4009-4017.
- Mertens-Talcott SU, Percival SS, Talcott ST. 2008. Extracts from red muscadine and Cabernet sauvignon wines induce cell death in MOLT-4 human leukemia cells. *Food Chem* 108:824-832.
- Netzel M, Strass G, Bitsch I, Könitz R, Christmann M, Bitsch R. 2003. Effect of grape processing on selected antioxidant phenolics in red wine. *J Food Eng* 56:223-228.

Ou B, Hampsch-Woodill M, Prior RL. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* 49:4619-4626.

Ou B, Hampsch-Woodill M, Flanagan J, Deemer EK, Prior RL, Huang D. 2002. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J Agric Food Chem* 50:2772-2777.

Pace-Asciak CR, Hahn SE, Diamandis EP, Soleas G, Goldberg DM. 1995. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin Chim Acta* 235:207-219.

Pastrana-Bonilla E, Akoh CC, Sellappan S, Krewer G. 2003. Phenolic content and antioxidant capacity of muscadine grapes. *J Agric Food Chem* 51:5497-5503.

Pekić B, Kovač V, Alonso E, Revilla E. 1998. Study of the extraction of proanthocyanidins from grape seeds. *Food Chem* 61:201-206.

Prior RL, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* 53:4290-4302.

Ren W, Qiao Z, Wang H, Zhu L, Zhang L. 2003. Flavonoids: Promising anticancer agents. *Med Res Rev* 23(4):519-534.

Renaud S, de Lorgeril M. 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 339(8808):1523-1526.

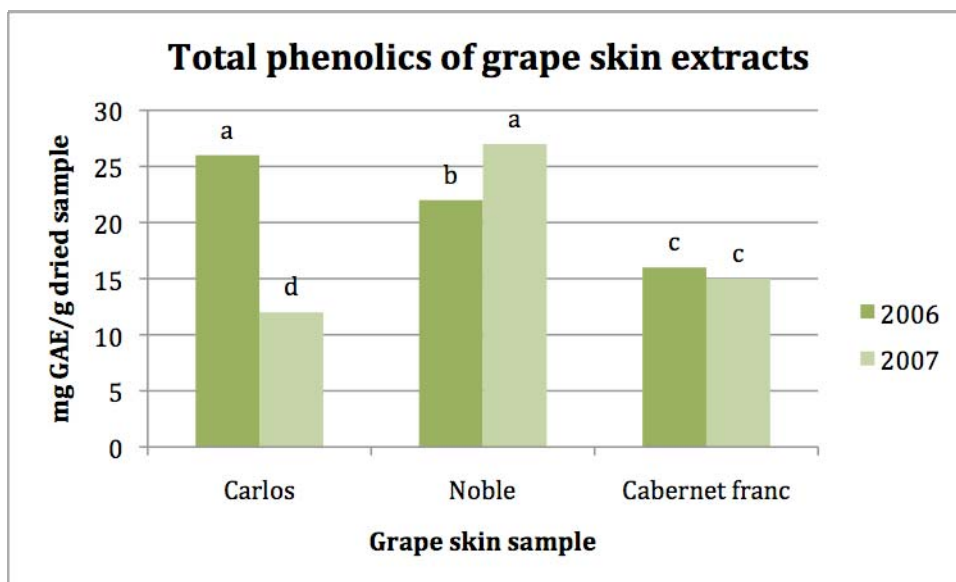
Rice-Evans CA, Miller NJ, Paganga G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci* 2(4):152-159.

Rodríguez Montealegre R, Romero Peces R, Chacón Vozmediano C, Martínez Gascueña J, García Romero E. 2006. Phenolic compounds in skins and seeds of ten grape *Vitis vinifera* varieties grown in a warm climate. *J Food Comp Anal* 19:687-693.

Shi J, Yu J, Pohorly JE, Kakuda Y. 2003. Polyphenolics in grape seeds – biochemistry and functionality. *J Med Food* 6(4):291-299.

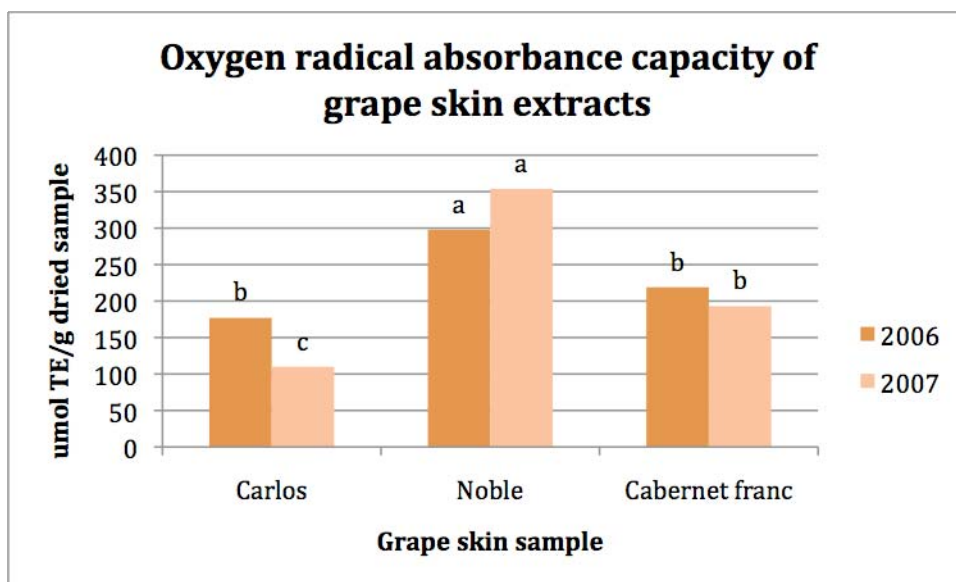
Singleton VL. 1992. Wine composition, in *Proceedings: Potential health effects of components of plant foods and beverages in the diet*, University of California, Davis, August 14-15.

- Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 16(3):144-158.
- Soleas GJ, Dam J, Carey M, Goldberg DM. 1997. Toward the fingerprinting of wines: Cultivar-related patterns of polyphenolic constituents in Ontario wines. *J Agric Food Chem* 45:3871-3880.
- Sun B, Ribes AM, Leandro MC, Belchior AP, Spranger MI. 2006. Stilbenes: Quantitative extraction from grape skins, contribution of grape solids to wine and variation during wine maturation. *Anal Chim Acta* 563:382-390.
- Talcott ST, Lee JH. 2002. Ellagic acid and flavonoid antioxidant content of muscadine wine and juice. *J Agric Food Chem* 50:3186-3192.
- Tsanova-Savova S, Ribarova F, Gerova M. 2005. (+)-Catechin and (-)-epicatechin in Bulgarian fruits. *J Food Compos Anal* 18:691-698.
- [USDA] United States Department of Agriculture. 2007 Nov. Oxygen radical absorbance capacity (ORAC) of selected foods – 2007. Beltsville (MD): United States Department of Agriculture. Available from: United States Department of Agriculture, Beltsville, MD 20705.
- [WHO] World Health Organization. 1989. World health statistics annual. Geneva: World Health Organisation.
- Yi W, Akoh CC, Fischer J, Krewer G. 2006. Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Res Int* 39:628-638.
- Yi OS, Meyer AS, Frankel EN. 1997. Antioxidant activity of grape extracts in a lecithin liposome system. *IAOCS* 74(10):1301-1307.
- Yilmaz Y, Toledo RT. 2004. Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *J Agric Food Chem* 52:255-260.
- Yilmaz Y, Toledo RT. 2006. Oxygen radical absorbance capacities of grape/wine industry byproducts and effect of solvent type on extraction of grape seed polyphenols. *J Food Compos Anal* 19:41-48.
- Yu J, Ahmedna M, Goktepe I. 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem* 90:199-206.



<sup>a</sup> GAE = gallic acid equivalents. Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 2.2:** Total phenolics for each grape skin extract from 2006 and 2007 harvests as determined by the Folin-Ciocalteu assay.<sup>a</sup>



<sup>a</sup> TE = Trolox equivalents. Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 2.3:** Antioxidant capacity for each grape skin extract from 2006 and 2007 harvests as determined by the ORAC assay.<sup>a</sup>

**Table 2.1:** Reference values for total phenolics in muscadine (cv. Carlos and Noble) and Cabernet franc grape parts and wine.<sup>a</sup>

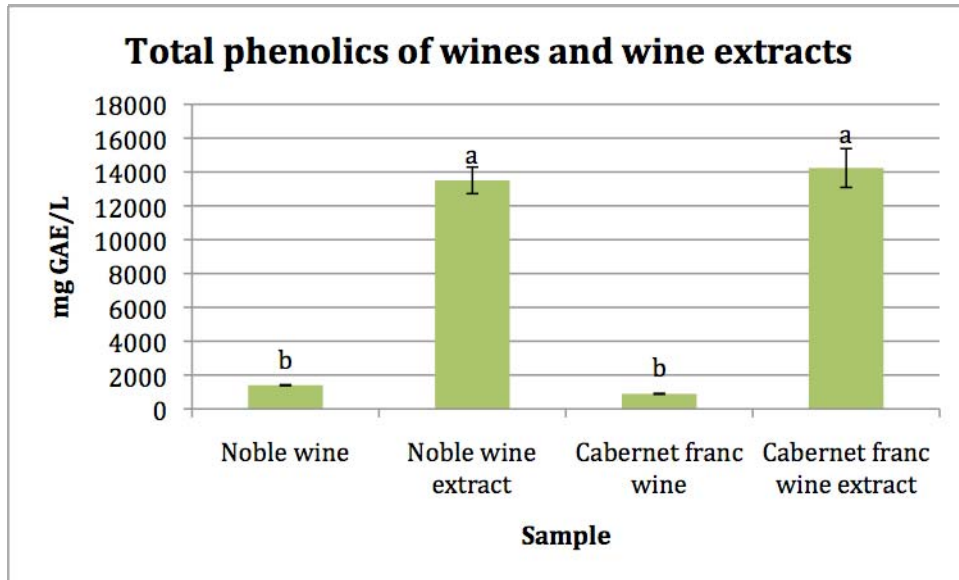
<b>Sample</b>	<b>Type</b>	<b>Total Phenolics (mg GAE/100 g FW)<sup>b</sup></b>	<b>Est. Total Phenolics (mg GAE/g DW)<sup>d</sup></b>
Muscadine (cv. Carlos)	skin	253.0 – 545.6	14.1 – 30.5
	seed	1920.3	36.1
	pulp	25.1 – 73.8	1.8
	whole	307.9	17.7
	wine	189 – 248 <sup>c</sup>	n/a
Muscadine (cv. Noble)	skin	309.0 – 355.1	22.9 – 26.3
	seed	2685.3	45.1
	pulp	33.4 – 84.8	2.7 – 7.0
	whole	425.7	28.2
	wine	298 – 1860 <sup>c</sup>	n/a
Cabernet franc	skin	46.2 – 100.9	-
	whole	136.5	-
	wine	86.2 – 119.3; 932 <sup>c</sup>	n/a
	must	16.3 – 96.5	-

<sup>a</sup> Sources: Cliff and others 2007, Lee and Talcott 2004, Mazza and others 1999, Mertens-Talcott and others 2008, Pastrana-Bonilla and others 2003, Talcott and Lee 2002, Yi and others 1997.

<sup>b</sup> All values reported on a fresh weight basis.

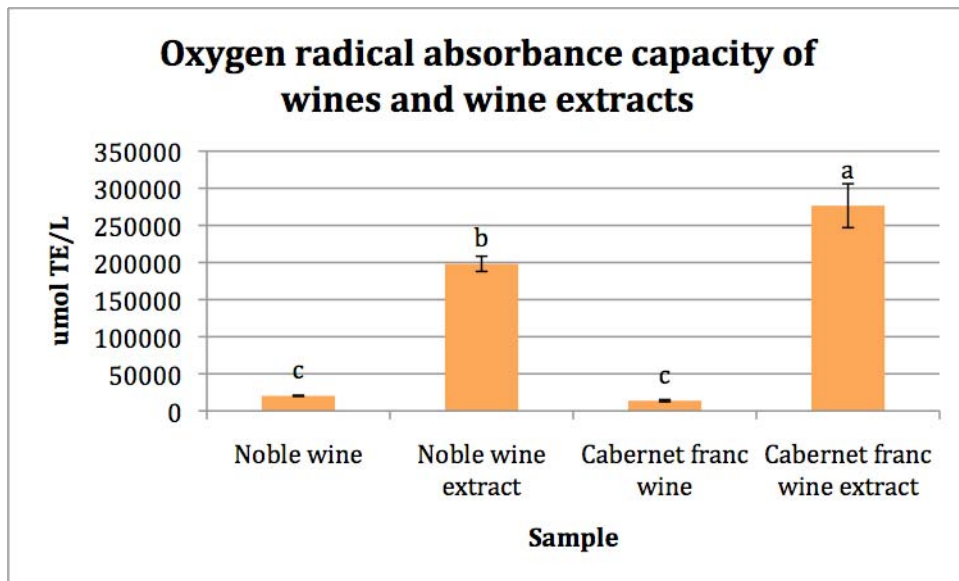
<sup>c</sup> Per L.

<sup>d</sup> Estimated values on a dry weight basis calculated from previous data (Pastrana-Bonilla and others 2003).



<sup>a</sup> GAE = gallic acid equivalents. Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 2.4:** Total phenolics for each wine and wine extract from the 2007 harvest as determined by the Folin-Ciocalteu assay.<sup>a</sup>



<sup>a</sup> TE = Trolox equivalents. Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 2.5:** Antioxidant capacity for wines and wine extracts from the 2007 harvest as determined by the ORAC assay.<sup>a</sup>

**Table 2.2:** Reference values for antioxidant capacity in muscadine (cv. Carlos and Noble) and Cabernet franc grape parts and wine as determined by the oxygen radical absorbance capacity assay and/or Trolox equivalent antioxidant capacity assay.<sup>a</sup>

Sample	Type	Method	Total Antioxidant Capacity <sup>b</sup>
Muscadine (cv. Carlos)	skin	ORAC; TEAC	86.2 µmol/g; 14.9 µM/g
	seed	TEAC	204.6 µM/g
	pulp	TEAC	3.4 µM/g
	whole	TEAC	18.2 µM/g
Muscadine (cv. Noble)	skin	TEAC	12.4 µM/g
	seed	TEAC	234.7 µM/g
	pulp	ORAC; TEAC	14.3 µmol/g; 2.1 µM/g
	whole	TEAC	27.8 µM/g
	wine	ORAC	24.3 µmol/mL
Cabernet franc	wine	TEAC	9.3-11.9 mmol/L

<sup>a</sup> Sources: Lee and Talcott 2004, Mertens-Talcott and others 2008, Netzel and others 2003, Pastrana-Bonilla and others 2003.

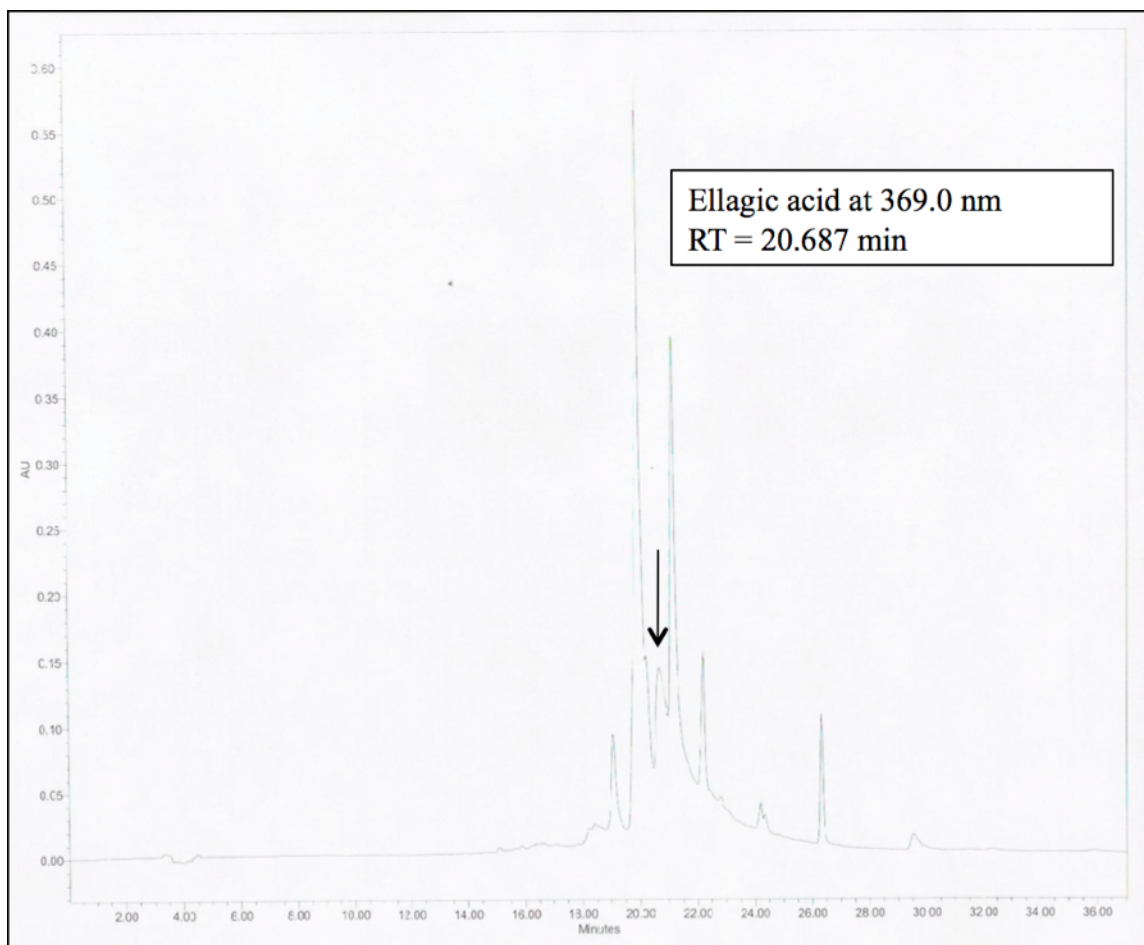
<sup>b</sup> All values reported on fresh weight basis in Trolox equivalents.

**Table 2.3** Reference values for ellagic acid and resveratrol in muscadine (cv. Carlos and Noble) and Cabernet franc grape parts and wine.<sup>a</sup>

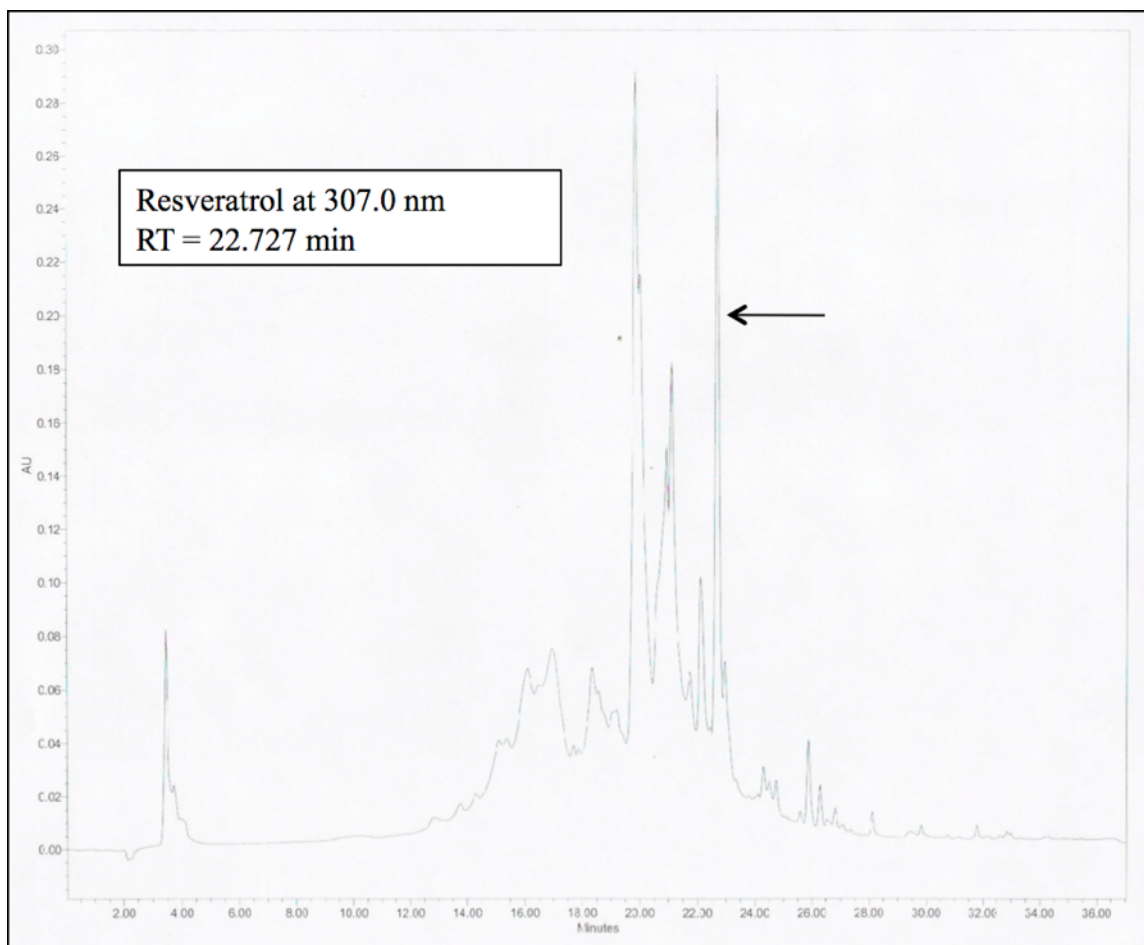
Sample	Type	Ellagic Acid (mg/100g FW)	Resveratrol (mg/100g FW)
Muscadine (cv. Carlos)	skin	0.8 – 19.7	0.2 – 0.4
	pulp	0.3	-
	whole	6.4	0.1
	wine	1.6 – 2.3 <sup>b</sup>	-
Muscadine (cv. Noble)	skin	7.6 – 14.6	0.1-11.3
	pulp	0.9	-
	whole	6.8	0.1
	wine	nd – 56.0 <sup>b</sup>	0.2 <sup>b</sup>
Cabernet franc	wine	-	0.71 <sup>b</sup>

<sup>a</sup> Sources: LeBlanc and others 2007, Lee and Talcott 2002, Lee and Talcott 2004, Mertens-Talcott and others 2008, Pastrana-Bonilla and others 2003, Soleas and others 1997, Talcott and Lee 2002.

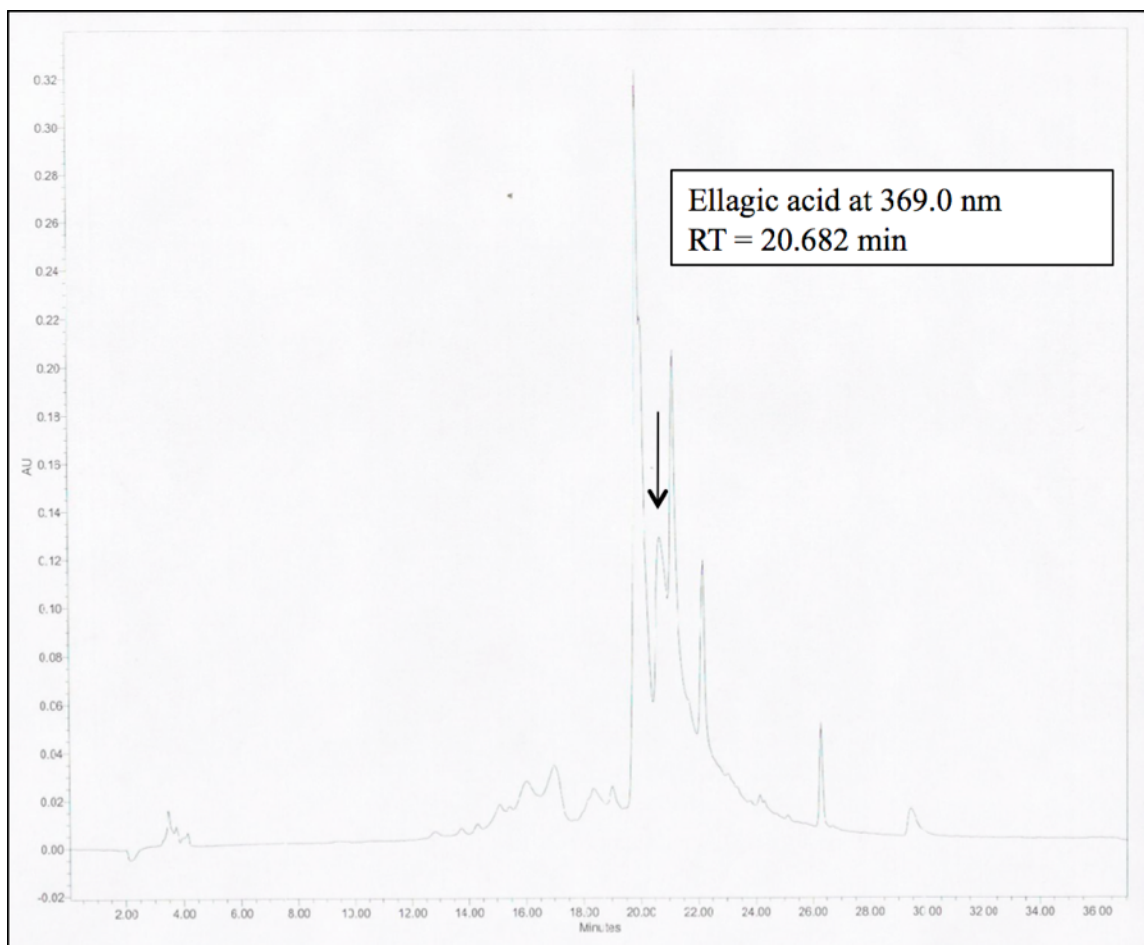
<sup>b</sup> Per L.



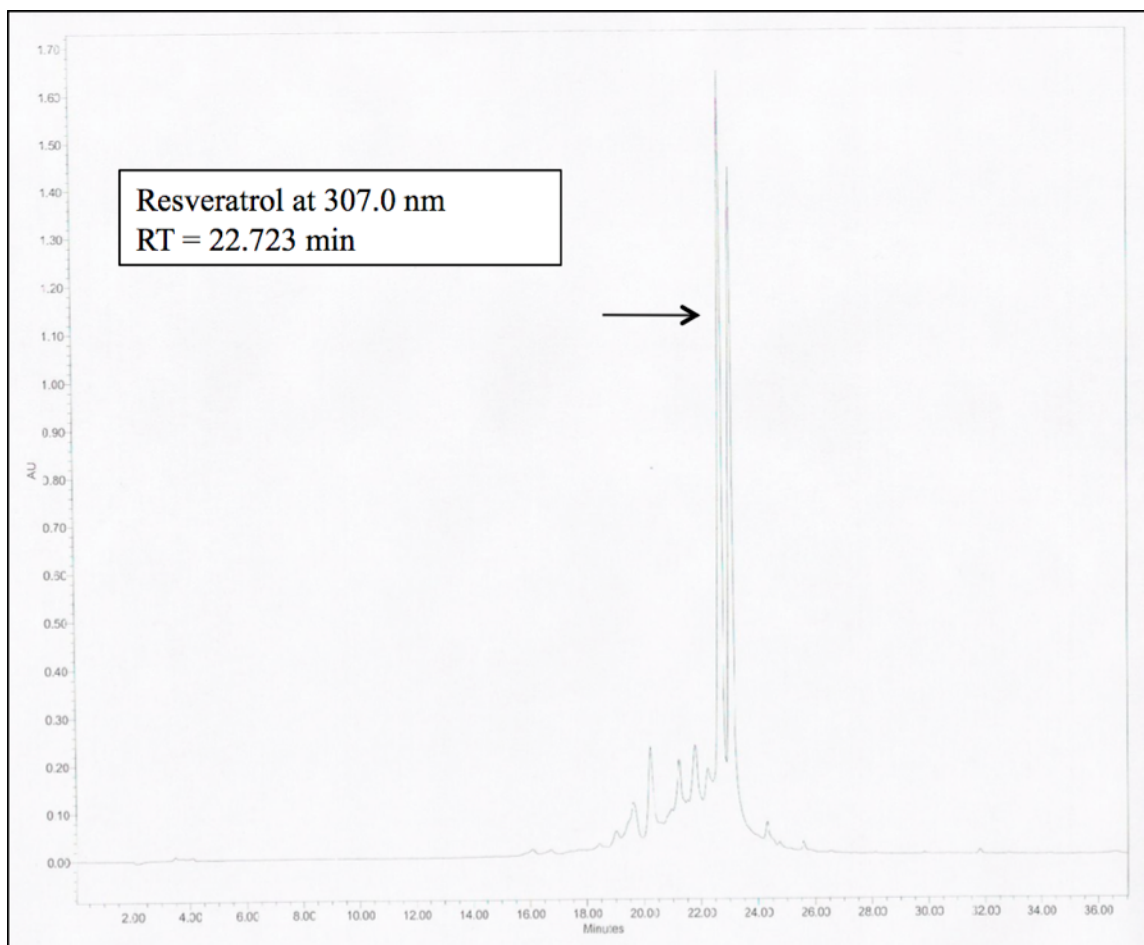
**Figure 2.6:** HPLC chromatogram of muscadine grape (cv. Carlos) skin extract from 2006 harvest.



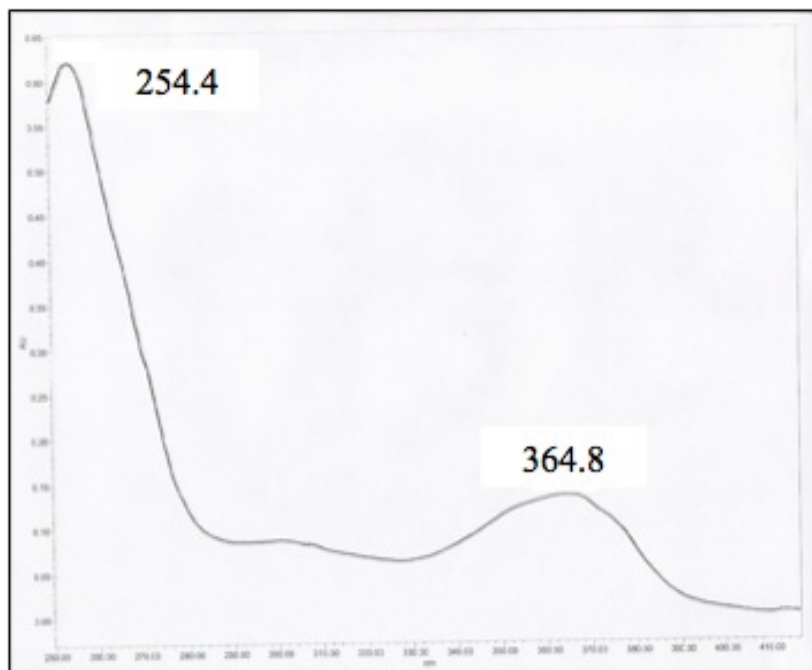
**Figure 2.7:** HPLC chromatogram of muscadine grape (cv. Noble) skin extract from 2006 harvest.



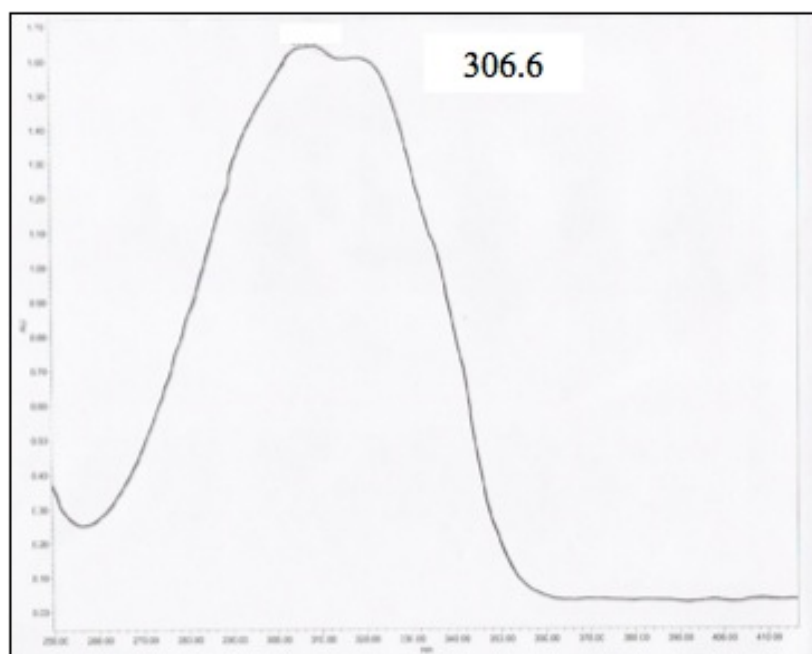
**Figure 2.8:** HPLC chromatogram of muscadine grape (cv. Noble) skin extract from 2006 harvest.



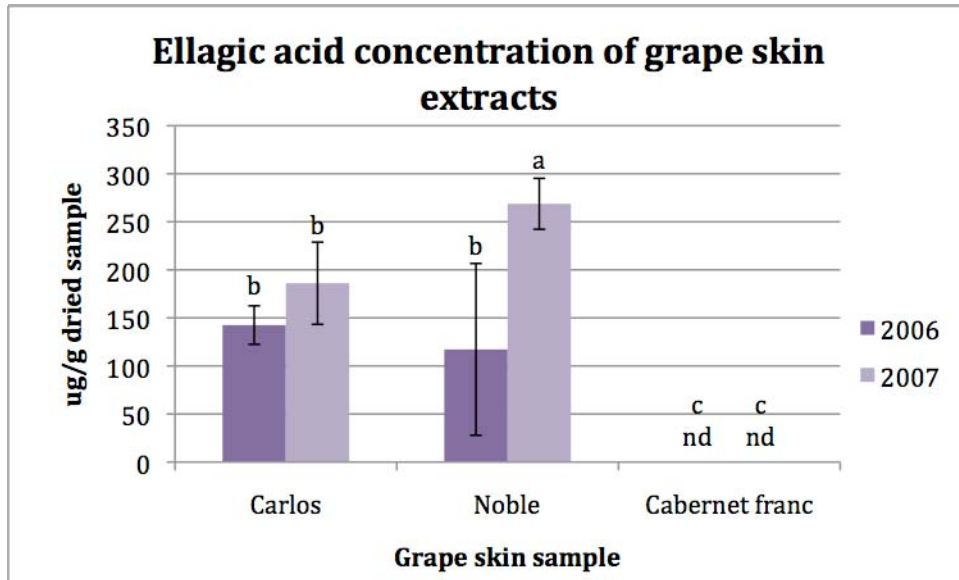
**Figure 2.9:** HPLC chromatogram of Cabernet franc skin extract from 2006 harvest.



**Figure 2.10:** Characteristic HPLC spectrum of ellagic acid.

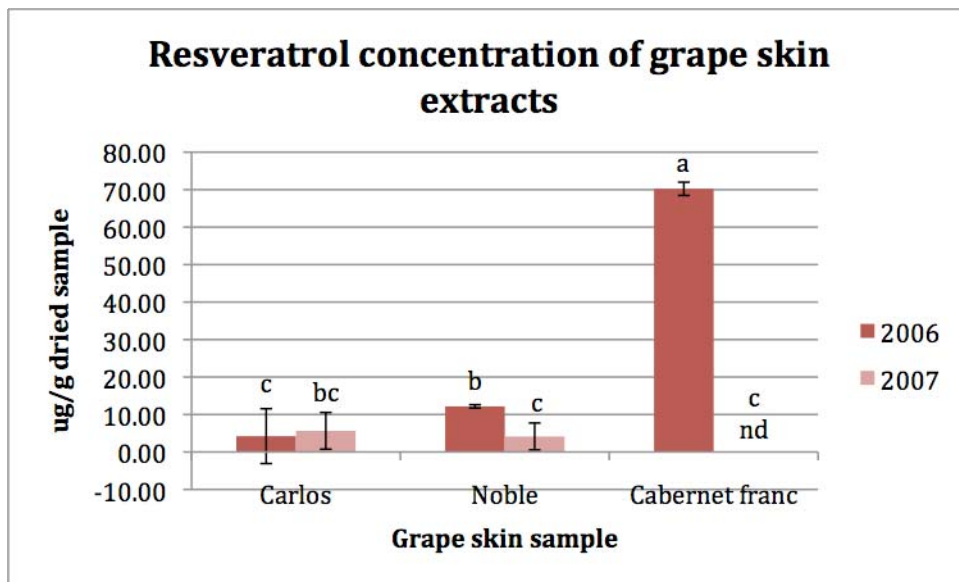


**Figure 2.11:** Characteristic HPLC spectrum of resveratrol.



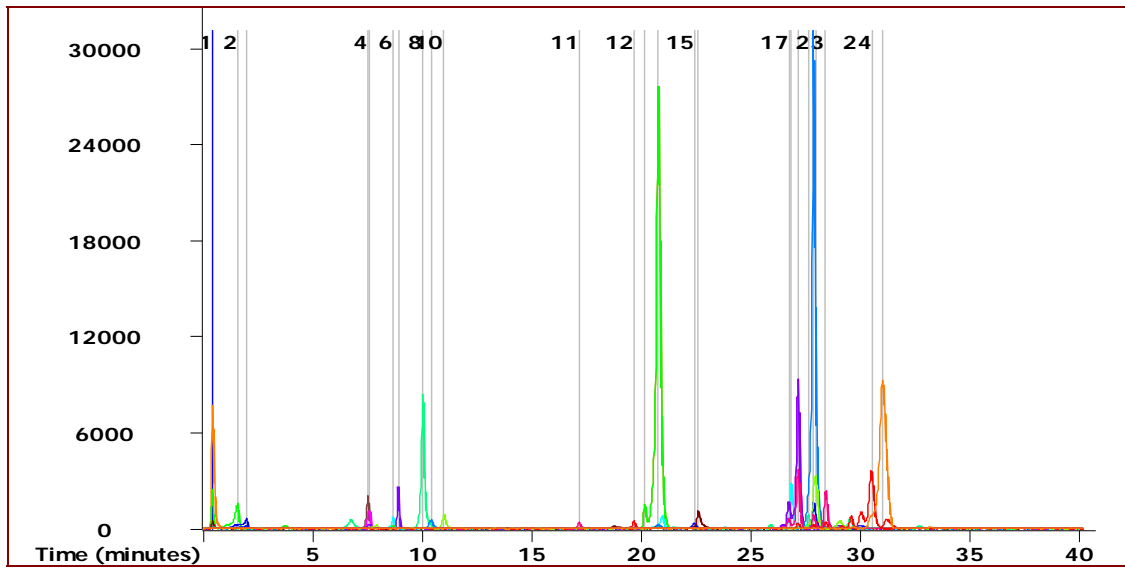
<sup>a</sup> Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 2.12:** Ellagic acid concentrations for each grape skin extract from 2006 and 2007 harvests as determined by high pressure liquid chromatography.<sup>a</sup>



<sup>a</sup> Means with the same letter are not significantly different ( $p < 0.05$ ).

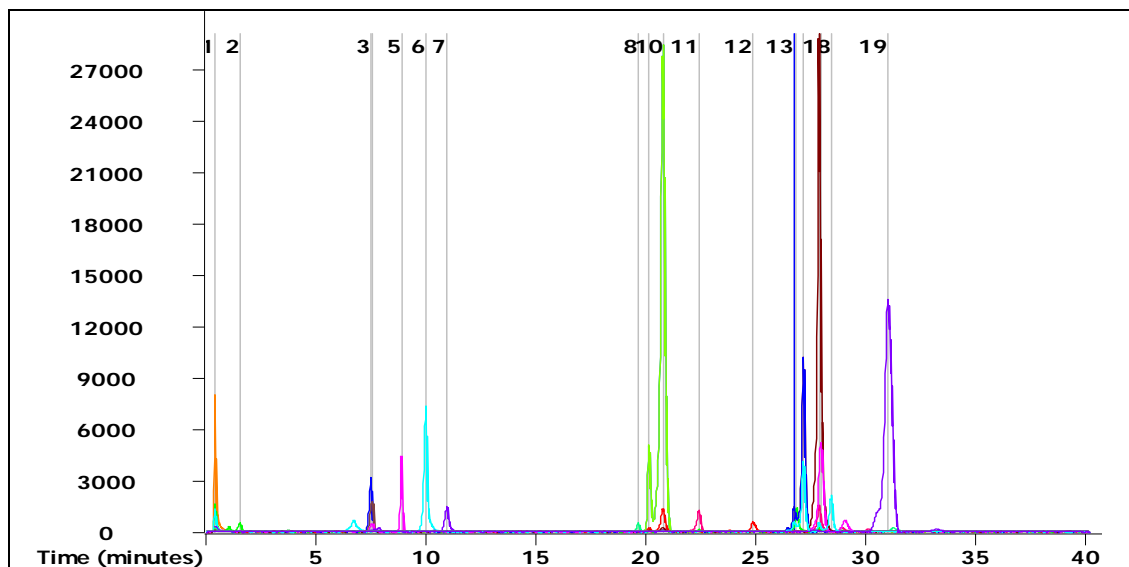
**Figure 2.13:** Resveratrol concentrations for each grape skin extract from 2006 and 2007 harvests as determined by high pressure liquid chromatography.<sup>a</sup>



**Figure 2.14:** HPLC-TOF-MS chromatogram of muscadine grape (cv. Carlos) skin extract from 2007 harvest.

**Table 2.4:** Possible identifications of peaks from HPLC-TOF-MS chromatogram of muscadine grape (cv. Carlos) skin extract from 2007 harvest.

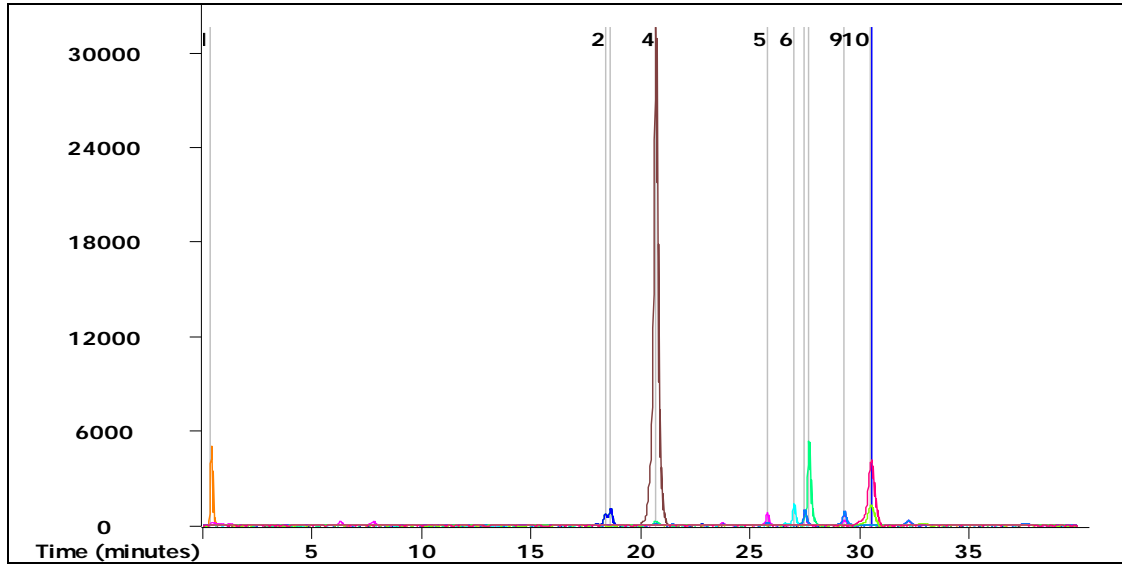
Peak no.	Unique mass	Retention time (min)	Tentative identification
1	113.0234	0.43	unknown
2	289.0757	1.57	catechin or epicatechin
3	475.1945	1.98	unknown
4	483.2562	7.52	digalloylglucose
5	507.2552	7.58	syringetin 3-galactoside/3-glucoside or laricitrin 3-glucuronide
6	453.1421	8.69	unknown
7	509.2672	8.91	unknown
8	343.0505	10.03	unknown
9	269.0828	10.40	apigenin or genistein
10	271.1008	10.99	naringenin
11	311.2049	17.15	caftaric acid
12	339.2396	19.65	unknown
13	455.3603	20.14	unknown
14	455.3614	20.77	unknown
15	429.3276	22.42	unknown
16	281.2499	22.58	unknown
17	819.5461	26.72	unknown
18	432.3661	26.83	unknown
19	819.5446	27.14	unknown
20	239.0621	27.62	unknown
21	446.3822	27.84	unknown
22	758.5573	27.95	unknown
23	821.5556	28.40	unknown
24	824.5797	30.50	unknown
25	621.4508	31.01	unknown



**Figure 2.15:** HPLC-TOF-MS chromatogram of muscadine grape (cv. Noble) skin extract from 2007 harvest.

**Table 2.5:** Possible identifications of peaks from HPLC-TOF-MS chromatogram of muscadine grape (cv. Noble) skin extract from 2007 harvest.

<b>Peak no.</b>	<b>Unique mass</b>	<b>Retention time (min)</b>	<b>Tentative identification</b>
1	133.0185	0.43	unknown
2	289.0763	1.57	catechin or epicatechin
3	483.2582	7.52	unknown
4	507.2601	7.60	syringetin 3-galactoside/3-glucoside or laricitrin 3-glucuronide
5	509.2699	8.91	unknown
6	343.0519	10.00	unknown
7	271.1039	10.98	unknown
8	339.2455	19.65	unknown
9	455.3629	20.14	unknown
10	455.3644	20.78	unknown
11	429.3309	22.40	unknown
12	459.3484	24.86	unknown
13	819.5539	26.72	unknown
14	432.3708	26.85	unknown
15	819.5528	27.17	unknown
16	446.3878	27.87	unknown
17	758.5635	27.95	unknown
18	821.5677	28.42	unknown
19	621.4554	30.99	unknown



**Figure 2.16:** HPLC-TOF-MS chromatogram of Cabernet franc grape skin extract from 2007 harvest.

**Table 2.6:** Possible identifications of peaks from HPLC-TOF-MS chromatogram of Cabernet franc grape skin extract from 2007 harvest.

<b>Peak no.</b>	<b>Unique mass</b>	<b>Retention time (min)</b>	<b>Tentative identification</b>
1	113.0231	0.40	unknown
2	255.2354	18.42	unknown
3	255.2370	18.64	pterostilbene
4	455.3637	20.72	unknown
5	165.0414	25.78	unknown
6	819.5458	27.01	unknown
7	239.0629	27.50	unknown
8	446.3878	27.70	unknown
9	239.0650	29.30	unknown
10	623.4453	30.50	unknown
11	621.4587	30.56	unknown

**Table 2.7:** Reference values of phenolics detected in muscadine (cv. Carlos and Noble) and Cabernet franc grape skins<sup>c</sup>

<b>Sample</b>	<b>Compounds (mg/100g FW)</b>					
<i>* phenolic acids</i>	<b>caffeic acid</b>	<b><i>p</i>-coumaric acid</b>	<b>ferulic acid</b>	<b>protocatechuic acid</b>	<b>syringic acid</b>	<b>vanillic acid</b>
Cabernet franc	n/a <sup>a</sup>	n/a	n/a	n/a	n/a	n/a
Muscadine (cv. Carlos)	n/a	n/a	n/a	n/a	n/a	n/a
Muscadine (cv. Noble)	n/a	n/a	n/a	n/a	n/a	n/a
<i>* flavonoids</i>	<b>catechin</b>	<b>epicatechin</b>	<b>kaempferol</b>	<b>myricetin</b>	<b>quercetin</b>	
Cabernet franc	n/a	n/a	n/a	n/a	n/a	
Muscadine (cv. Carlos)	n/a	n/a	0.4	19.6	1.3	
Muscadine (cv. Noble)	n/a	n/a	0.3	4.8	0.5	
<i>* flavonoids</i>	<b>rutin</b>	<b>naringenin</b>	<b>isorhamnetin</b>			
Cabernet franc	n/a	n/a	n/a			
Muscadine (cv. Carlos)	n/a	n/a	n/a			
Muscadine (cv. Noble)	n/a	n/a	n/a			
<i>* flavonoids</i>	<b>cyanidin</b>	<b>delphinidin</b>	<b>malvidin</b>	<b>pelargonidin</b>	<b>peonidin</b>	<b>petunidin</b>
Cabernet franc	n/a	n/a	n/a	n/a	n/a	n/a
Muscadine (cv. Carlos)	n/a	n/a	n/a	n/a	n/a	n/a
Muscadine (cv. Noble)	69.2	145.0	n/a	n/a	n/a	107.0
<i>* stilbenes and others</i>	<b>ellagic acid</b>	<b>gallic acid</b>	<b>piceid</b>	<b>resveratrol</b>		
Cabernet franc	n/a	n/a	n/a	n/a		
Muscadine (cv. Carlos)	0.8-19.7	n/a	14.7 <sup>b</sup>	0.2-0.4		
Muscadine (cv. Noble)	7.6-14.6	n/a	4.5-8.8 <sup>b</sup>	0.1-11.3		

<sup>a</sup> n/a: data not available

<sup>b</sup> Dry weight basis

<sup>c</sup> Sources: LeBlanc and others 2007, Lee and Talcott 2004, Pastrana-Bonilla and others 2003.

**Table 2.8:** Reference values of phenolic compounds detected in muscadine and *V. vinifera* varieties<sup>d</sup>

Sample	Compounds (mg/100g FW)					
<i>* phenolic acids</i>	<b>caftaric acid</b>	<b>coutaric acid</b>	<b>ferulic acid</b>	<b>protocatechuic acid</b>	<b>syringic acid</b>	<b>vanillic acid</b>
<i>V. vinifera</i> (red)	0.49-0.95	0.32-1.0	n/a	0.15-0.24	n/a	n/a
<i>V. vinifera</i> (white)	0.24-3.1	0.19-1.8	n/a	n/a	n/a	n/a
Muscadine (purple)	n/a <sup>a</sup>	n/a	n/a	n/a	n/a	n/a
Muscadine (golden)	n/a	n/a	n/a	n/a	n/a	n/a
<i>* flavonoids</i>	<b>catechin</b>	<b>epicatechin</b>	<b>kaempferol</b>	<b>myricetin</b>	<b>quercetin</b>	
<i>V. vinifera</i> (red)	0.85-2.5	0.62-1.3	n/a	n/a	n/a	
<i>V. vinifera</i> (white)	trace-2.3	trace-0.83	n/a	n/a	n/a	
Muscadine (purple)	n/a	n/a	0.2-0.6	1.8-7.1	0.9-3.0	
Muscadine (golden)	n/a	n/a	0.2-3.0	4.1-16.4	0.9-3.8	
<i>* flavonoids</i>	<b>rutin</b>	<b>naringenin</b>	<b>isorhamnetin</b>			
<i>V. vinifera</i> (red)	n/a	n/a	n/a			
<i>V. vinifera</i> (white)	n/a	n/a	n/a			
Muscadine (purple)	n/a	n/a	n/a			
Muscadine (golden)	n/a	n/a	n/a			
<i>* flavonoids</i>	<b>cyanidin</b>	<b>delphinidin</b>	<b>malvidin</b>	<b>pelargonidin</b>	<b>peonidin</b>	<b>petunidin</b>
<i>V. vinifera</i> (red)	n/a	n/a	n/a	n/a	n/a	n/a
<i>V. vinifera</i> (white)	n/a	n/a	n/a	n/a	n/a	n/a
Muscadine (purple)	detected <sup>b</sup>	detected	detected	n/a	detected	detected
Muscadine (golden)	detected	detected	detected	n/a	detected	detected

**Table 2.8 Continued**

<i>* stilbenes and others</i>	<b>ellagic acid</b>	<b>gallic acid</b>	<b>piceid</b>	<b>resveratrol</b>
<i>V. vinifera</i> (red)	n/a	n/a	7.2 <sup>c</sup>	65.7 <sup>c</sup>
<i>V. vinifera</i> (white)	n/a	n/a	n/a	n/a
Muscadine (purple)	6.2-22.2	detected	n/a	nd-0.2
Muscadine (golden)	19.7-21.1	n/a	n/a	nd-0.2

<sup>a</sup> Data not available.

<sup>b</sup> Detected = found, but in units unconvertible to mg/100g FW or DW

<sup>c</sup> Dry weight basis.

<sup>d</sup> Sources: Lee and others 2005, Pastrana-Bonilla and others 2003, Rodriguez Montealegre and others 2006, Sun and others 2006, Yi and others 2006.

### **CHAPTER 3. ANTICARCINOGENIC CHARACTERIZATION OF GRAPE SKIN EXTRACTS**

#### ***Abstract***

Close to 1.5 million Americans were diagnosed with cancer last year and approximately 186,320 of those new cases can be associated with prostate cancer. Grape skins are rich in phytochemicals, which have not only been shown to be anti-inflammatory and anti-atherosclerosis, but may also be effective anticancer agents. The anticarcinogenic activity of muscadine and Cabernet franc skin extracts was determined against LNCaP prostate carcinoma cells with the XTT proliferation assay and caspase-3 assay to determine apoptosis. Treatments of 5 mg/mL grape skin extract were effective at inhibiting proliferation and inducing apoptosis. Antiproliferative effects of less concentrated treatments of 1-2 mg/mL were varied depending on season and cultivar. None of the 1-2 mg/mL treatments significantly induced apoptosis. Further characterization must be done on other types of anticancer activities of these extracts, but these activities reveal that muscadine and Cabernet franc grape skins could possibly be used as effective supplements for cancer prevention.

## ***Introduction***

Close to 1.5 million Americans were expected to be diagnosed with cancer last year (2008) and approximately 186,320 of those new cases can be associated with prostate cancer (ACS 2008). Approximately 15% of those diagnosed with prostate cancer will not survive the disease. Diagnoses are also significantly higher in African American men compared to white men with the death rate more than twice as high. With so many Americans affected by the disease, new possibilities for treatments and preventions are necessary. Proper diet and nutrition are key factors in the prevention of cancer. Research suggests that one-third of the cancer deaths are likely due to obesity, lack of physical activity, and poor nutrition (ACS 2008). Antioxidants found in high concentrations in fruits and vegetables may reduce several risk factors associated with cancer. Many of the antioxidants have anti-inflammatory properties as well as free radical scavenging activities.

Grapes contain high amounts of phenolic compounds, which have high antioxidant capacities (Rice-Evans and others 1997). The skins in particular contain a large amount of phenolic compounds, more so than the pulp or juice. Both muscadine grapes and Cabernet franc grapes have high antioxidant capacities and contain various phenolic compounds as evident by ORAC, total phenol, and HPLC analysis. Antioxidants have the potential to be effective cancer fighting compounds (Kaur and Kapoor 2001, WCRF/AICR 2007).

Anticarcinogenic characteristics can be determined by antiproliferation and apoptosis assays. These two common assays are indicative of the anticarcinogenic

properties of a sample. The effects of a treatment can be quantified by a cell proliferation and viability assay, which measures the number of healthy cells and the number of cells that are dividing in culture (Wyllie and others 1998). The tetrazolium salt sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) is applied to cells as a labeling reagent. Metabolically active, viable cells cleave the yellow XTT salt and subsequently form an orange formazan dye. The orange dye is then spectrophotometrically quantified and cell proliferation and viability in response to grape skin extracts can be determined.

Apoptosis is when a cell undergoes programmed cell death. The ability to undergo apoptosis is important and part of a normal, healthy cell's life cycle (Bowen and others 1998). When cells become unwanted or unnecessary, they are eliminated through apoptosis (**Figure 3.1**). Cancer cells, on the other hand, proliferate rapidly and uncontrollably due to the lack of apoptosis. Caspase-3 is a protease that is vital to the apoptosis process. The activation of caspase-3 triggers a cascade of proteolytic activity leading to apoptosis of the cell. Thus, apoptosis can be detected by testing for increases in caspase-3 as well as other similar caspase proteases. Observing caspase-3 activity in cells differentiates apoptosis from necrosis. Necrosis is the premature destruction of cells caused by a catastrophic toxic or traumatic event (Bonfoco and others 1995). The substrate Z-DEVD-AMC is weakly fluorescent in the ultraviolet range, but when cleaved by caspase-3 and other similar proteases, the substrate produces a bright-blue fluorescent color. The Z in Z-DEVD-AMC refers to a benzyloxycarbonyl group, while DEVD refers to the amino acid sequence Asp-Glu-Val-Asp, and AMC refers to 7-amino-4-

methylcoumarin. In a cancer study, caspase-3- expression was reduced in prostatic tumors obtained directly from patients, compared to the normal prostate, implying that the pattern of caspase-3 expression may be indicative of prostate cancer progression and could be used as a target for treatment (Winter and others 2001).

The objective of this study was to evaluate the effects of muscadine and Cabernet franc grape skin extracts on LNCaP prostate cancer cells. Effects were determined based on two anticancer mechanisms – antiproliferation and induction of apoptosis.

### ***Materials and methods***

#### **Cell culture**

LNCaP human prostate carcinoma cell line, 0.25% trypsin-0.53 mM EDTA solution, and fetal bovine serum were purchased from ATCC (Manassas, VA). RPMI-1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate was also purchased from ATCC. Penicillin-streptomycin (10,000 units/mL penicillin and 10,000 µg/mL streptomycin) and phosphate buffered saline, pH 7.4, were from Invitrogen (Carlsbad, CA).

The LNCaP cells were maintained in the RPMI-1640 media, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. The media was changed every two to three days and the cells were passaged into 75 cm<sup>2</sup> vented tissue culture flasks every two to four days. The cells were kept in a 37°C humidified Fisher Scientific Isotemp incubator (Pittsburgh, PA) containing 5% carbon dioxide.

## **Extraction**

Phenolic compounds of the grape skin samples were extracted as done previously for antioxidant assays (page 43). After the methanol-water extraction, samples were placed under nitrogen for three hours in order to concentrate them as much as possible. All samples were then brought to a 4 mL volume with methanol.

## **Cell proliferation assay**

Cell proliferation kit II from Roche Diagnostics (Indianapolis, IN) was used to determine the effects of treatments on LNCaP cell proliferation and viability. The protocol provided was followed with no exceptions. Briefly, approximately  $1 \times 10^4$  cells were grown in each well of a 96 well clear microplate. Following a 4 hour incubation period, treatments were applied after aspiration of media. The final concentrations of the treatments in the wells were 1:100, 1:250, and 1:500 dilutions of grape skin extract. The dilutions were made using media. Blanks contained treatments and media with no cells and controls contained cells and media with no treatment. Controls with media containing 1% methanol were also monitored to ensure that the toxic effect was not due to the extraction solvent. The 1:100 dilution of grape skin extract contains the highest concentration of methanol (1%) of all the treatments. After an additional 24 and 48 hour incubation periods, the XTT labeling mixture was added. The absorbance was measured after a third and final incubation of 4 hours using a Multiskan MCC reader from Thermo Electron Corporation (Waltham, MA) at 450 nm with a reference wavelength at 690 nm.

All samples were analyzed in duplicate. Statistical analyses were done using one way ANOVA and the Fisher's LSD post hoc test with the program XLSTAT (New York, NY).

### **Apoptosis assay**

The EnzChek® Caspase-3 assay kit #1 from Invitrogen (Eugene, OR) was used to determine the effects of treatments on LNCaP apoptosis. The protocol provided was followed. Briefly,  $7 \times 10^5$  cells were plated in each well of a 12 well plate. Following a 4 hour incubation, the media was aspirated and treatments were applied. The final concentrations of the treatments in the wells were 1:100, 1:250, and 1:500 dilutions of grape skin extract. The dilutions were made using media. Controls contained cells and media with no treatment. Controls with media containing 1% methanol were also monitored to ensure that the toxic effect was not due to the extraction solvent. After an additional 24 hour incubation period, the cells were harvested, washed, and lysed. The supernatants were plated in a 96 well black plate and a solution containing Z-DEVD-7-amino-4-methylcoumarin (AMC) substrate was added. The Z-DEVD-AMC yields a bright blue-fluorescent product upon proteolytic cleavage. The fluorescence was measured with a Cytofluor™ II fluorescence multi-well plate reader from Applied Biosystems (Foster City, CA) using a  $360 \pm 40$  nm excitation and emission detection at  $460 \pm 40$  nm after a 30 minute incubation.

All samples were analyzed in duplicate. Statistical analyses were done using one way ANOVA and the Fisher's LSD post hoc test with the program XLSTAT (New York, NY).

### ***Results and Discussion***

Effects on cell proliferation is a common way to determine an anti-carcinogenic property of an extract. After 24 hours, only some of the 2006 extracts showed significant effects on LNCaP prostate cancer cells (**Figure 3.2a**). Treatment 1 is a 1:100 dilution of grape skin extract, which translates to 5 mg grape skin powder per mL solvent, whereas treatment 3 is 1 mg powder per mL. Treatments 1 and 2 of the Carlos and Cabernet franc skin extracts decreased proliferation significantly compared to the controls. The most concentrated Noble extract treatment also had an effect on proliferation compared to the control and the other Noble treatments, although the result was not significantly different than the 1% methanol control. Results from the XTT proliferation assay after 48 hours of incubation reveal that almost all of the 2006 extracts had a significant impact on the growth of LNCaP prostate cancer cells (**Figure 3.2b**). Only the 1 mg/mL Noble treatment had no significant effects on proliferation.

For the 2007 samples, the 5 mg/mL Carlos and Cabernet franc skin extracts were the only treatments to have a significant effect on LNCaP cell proliferation after 24 hours, similarly to the 2006 results (**Figure 3.3a**). After 48 hours, treatment 1 of Carlos extract and treatments 1 and 2 of the Noble and Cabernet franc extracts all had effects on LNCaP cells (**Figure 3.3b**). Although Noble skin extracts had the highest ORAC values,

the extracts did not necessarily have the greatest effects on cell proliferation, compared to the other grape skins. The 2006 Cabernet franc skin extract contained a large amount of resveratrol, but its effects on cell proliferation were not extremely different than Carlos or Noble skins, which have very low concentrations of resveratrol. The effects on cell proliferation also did not follow the pattern of ellagic acid concentrations. No ellagic acid was detected in Cabernet franc skin extracts, yet its effects are significant on cell proliferation.

Induction of apoptosis is a second mechanism that may be indicative of the action by which grape skin extracts are anticarcinogenic. The results of the caspase-3 assay (**Figure 3.4**) show that all muscadine and Cabernet franc samples had an effect, but only the most concentrated treatments induced significant apoptosis of the LNCaP cells compared to the control, except for the 2006 Cabernet franc sample. Neither muscadine nor Cabernet franc extracts induced significant apoptosis at treatments 2 and 3 compared to the control or the 1% methanol control. The pattern of apoptosis induction does not seem to correlate with resveratrol or ellagic acid concentrations, nor total phenol or ORAC values. This reveals the complexity of the phenolic compounds profiles of the grape skins.

In another study, skin extract from the muscadine grape cultivar Ison, induced apoptosis in over 75% of prostate cancer cells at the much lower dose of 20  $\mu\text{g}/\text{mL}$ , but this did not include LNCaP cells (Hudson and others 2007). Furthermore, the cells were growth factor starved for 24 hours prior to the addition of treatments. Low doses of 10-100  $\mu\text{g}/\text{ml}$  of *V. vinifera* grape seed extract induced apoptosis in LNCaP cells, but seeds

contain a much higher concentration of phenolics and have much higher ORAC values than skins in both muscadines and Cabernet franc grapes (**Tables 2.1** and **2.2**) (Agarwal and others 2000).

Since the extracts were in methanol and methanol is cytotoxic, no treatments contained more than 1% methanol. A 1% methanol control was tested to ensure that the results from both assays were due to the extracts themselves and not the solvent. Of the most concentrated treatments, the 2007 Noble extract was not different than the 1% methanol control.

A limited number of studies have analyzed the effect of grape extracts on prostate cancer cells. Muscadine grape skin extract of the cultivar Ison significantly inhibited tumor cell growth in several cell lines including RWPE-1, WPE2-NA22, WPE2-NB14, and WPE1-NB26, which represent different stages of prostate cancer progression (Hudson and others 2007). Antiproliferative effects were also apparent on LNCaP and DU-145 cells. The extract did not inhibit the growth of PrEC cells, which are normal epithelial cells of the prostate. The phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase survival pathways were targeted by the skin extract, which caused considerable apoptosis in the cancer cell lines. Doses of 10-100 µg/ml grape seed extract (*V. vinifera*) added to DU145 and LNCaP cells significantly inhibited cell growth and induced apoptosis in a dose-dependent and time-dependent manner (Agarwal and others 2000). The extract also had an effect on mitogenic signaling of DU145 cells including the inhibition of constitutive activation of phospho-extracellular signal-

regulated protein kinase (ERK) 1 and 2. ERK 1 and 2 are signals for cell growth and lead to the uncontrollable growth of prostate tumors.

Specific compounds commonly found in grapes have also been added to prostate cancer cells to analyze for anticarcinogenic properties. Resveratrol has been shown to induce differential expression of genes in LNCaP prostate cancer cells. At  $10^{-5}$  M, resveratrol downregulated prostate specific antigen (PSA) as well as other genes (Hsieh and Wu 2000; Narayanan and others 2003). Resveratrol also blocked the G<sub>1</sub>-to-S phase progression in several prostate cancer cell lines (RWPE-1, WPE2-NA22, WPE2-NB14, WPE1-NB26) with no effects on normal prostate cells (Hudson and others 2007). The cell cycle was affected through increased expression of p21 and decreased expression of cyclins and cyclin-dependent kinases. Strong antiproliferative characteristics were evident when  $2.5 \times 10^{-5}$  M of resveratrol was applied to LNCaP, PC-3, and DU-145 cells (Hsieh and Wu 1999). Again, resveratrol interrupted the G<sub>1</sub>-to-S transition, but in PC-3, DU-145, and JCA-1 cell lines and not LNCaP. Another study on DU-145 cells observed apoptosis induced by resveratrol. The mechanism was due to an increase in cellular p53 content, increased p53 binding to DNA, and was also dependent on the activation of mitogen activated protein kinase (MAPK) (Lin and others 2002).

Treatment of PC-3 cells with 100  $\mu$ M quercetin, kaempferol, and luteolin led to complete growth inhibition (Knowles and others 2000). Genistein, apigenin, and myricetin had significant inhibition while naringenin and rutin had very little antiproliferative effects. A 25  $\mu$ M dose of kaempferol caused a block in the advance from the G<sub>2</sub> phase to the M phase and a 100  $\mu$ M dose of quercetin caused an increase in the

proportion of cells in the S phase. Yet, apoptosis did not occur even at 100  $\mu\text{M}$  doses of quercetin or kaempferol. Thus, the growth inhibition was due to the effects of the treatments on the cell cycle. Campbell and others (2006) observed similar results in LNCaP cells with doses as low as 12.5  $\mu\text{M}$  of quercetin, kaempferol, naringenin causing growth inhibition, while rutin did not. Synergistic effects were also noted. Ellagic acid at 1-100  $\mu\text{mol/L}$  had significant antiproliferative effects on DU-145 cells and induced apoptosis as a result of decreased ATP production (Losso and others 2004). Catechin and epicatechin have also proven to be antiproliferative against LNCaP and PC-3 cells (Kampa and others 2000). One of the mechanisms may be the modulation of the nitric oxide that is produced by prostate cancer cells in high amounts (Klotz and others 1998). Nitric oxide synthases can be detected using immunohistochemistry.

### ***Further work***

The anticarcinogenic effects of muscadine and Cabernet franc grape skin extracts are promising. More research needs to be done in the future to determine the mechanisms of the anticarcinogenic properties. Other prostate cancer cell lines can be used (ex. DU145 and PC-3) in order to determine whether or not the extracts can be used for prostate cancer in general. In order to determine just how extensive the power of the extracts is, other types of cancer cells need to be analyzed as well. The efficacy of the extracts as treatments or methods of prevention is also based on the bioavailability of the phenolic compounds. More studies must be done to analyze the absorption and excretion

of compounds. Effects on cancer cells and the bioavailability of grape skin treatments may be tested *in vivo* in animals for further analysis after *in vitro* research.

### ***Conclusion***

With over a million Americans being diagnosed with cancer every year and no cure, new possibilities for treatments are welcome. Grape skins contain high concentrations of antioxidants. Antioxidants have been shown to have anticarcinogenic properties. Treatments of 5 mg dried grape skin powder/mL of both muscadine and Cabernet franc grapes had significant effects on LNCaP prostate cancer cell proliferation and were able to induce apoptosis. Seasonal variability is great though and consistency in composition year to year would make using grape skin extract as a cancer treatment challenging. Further characterization must be done on other types of anticancer activities of these grape skin extracts to identify the compounds that have the greatest effect, but these two activities reveal that muscadine and Cabernet franc grape skins could possibly be used as effective supplements to aid in cancer prevention.

## **References**

Agarwal C, Sharma Y, Agarwal R. 2000. Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: Modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest and apoptosis. *Mol Carcinog* 28:129-138.

[ACS] American Cancer Society. *Cancer Facts and Figures 2008*. Report. Atlanta (GA): American Cancer Society; 2008.

Bonfoco E, Krainc D, Ankarcona M, Nicotera P, Lipton SA. 1995. Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci USA* 92:7162-7166.

Bowen ID, Bowen SM, Jones AH. 1998. *Mitosis and apoptosis: Matters of life and death*. London: Chapman & Hall.

Campbell JK, King JL, Harmston M, Lila MA, Erdman JW. 2006. Synergistic effects of flavonoids on cell proliferation in Hepa-1c1c7 and LNCaP cancer cell lines. *J Food Sci* 71(4):S358-S363.

Hsieh TC, Wu JM. 1999. Differential effects on growth, cell cycle arrest, and induction of apoptosis by resveratrol in human prostate cancer cell lines. *Exp Cell Res* 249:109-115.

Hsieh TC, Wu JM. 2000. Grape-derived chemopreventive agent resveratrol decreases prostate-specific antigen (PSA) expression in LNCaP cells by an androgen receptor (AR)-independent mechanism. *Anticancer Res* 20(1A):225-228.

Hudson TS, Hartle DK, Hursting SD, Nunez NP, Wang TT, Young HA, Arany P, Green JE. 2007. Inhibition of prostate cancer growth by muscadine grape skin extract and resveratrol through distinct mechanisms. *Cancer Res* 67(17):8396-8405.

Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM, Castanas E. 2000. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutr Cancer* 37(2):223-233.

Kaur C, Kapoor HC. 2001. Antioxidants in fruits and vegetables – the millenium's health. *Int J Food Sci Tech* 36:703-725.

Klotz T, Bloch W, Volberg C, Engelmann U, Addicks K. 1998. Selective expression of inducible nitric oxide synthase in human prostate carcinoma. *Cancer* 82:1897-1903.

Knekt P, Kumpulainen J, Järvinen R, Rissanen H, Heliövaara M, Reunanen A, Hakulinen T, Aromaa A. 2002. Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* 76:560-568.

Knowles LM, Zigrossi DA, Tauber RA, Hightower C, Milner JA. 2000. Flavonoids suppress androgen-independent human prostate tumor proliferation. *Nutr Cancer* 38(1):116-122.

Lin HY, Shih AI, Davis FB, Tang HY, Martino LJ, Bennett JA, Davis PJ. 2002. Resveratrol induced serine phosphorylation of p53 causes apoptosis in a mutant p53 prostate cancer cell line. *J Urol* 168(2):748-755.

Losso JN, Bansode RR, Trappey A, Bawadi HA, Truax R. 2004. In vitro anti-proliferative activities of ellagic acid. *J Nutr Biochem* 15:672-678.

Narayanan BA, Narayanan NK, Re GG, Nixon DW. 2003. Differential expression of genes induced by resveratrol in LNCaP cells: P53-Mediated molecular targets. *Int J Cancer* 104:204-212.

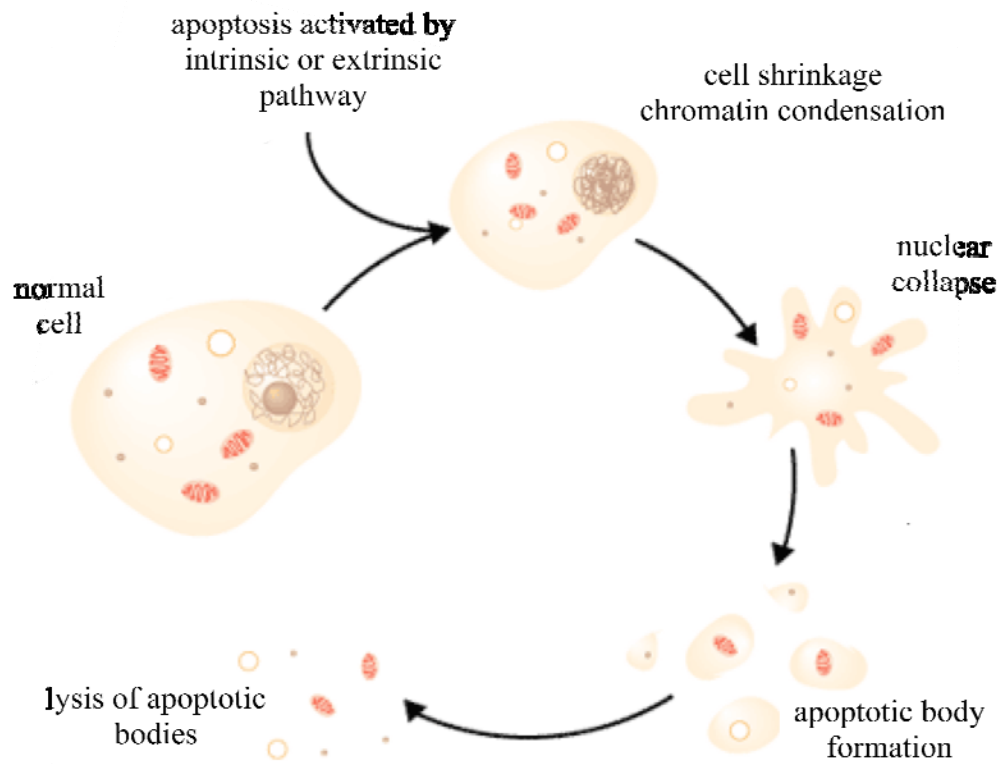
Rice-Evans CA, Miller NJ, Paganga G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci* 2(4):152-159.

[WCRF/AICR] World Cancer Research Fund / American Institute for Cancer Research. 2007. *Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective*. Washington DC: AICR.

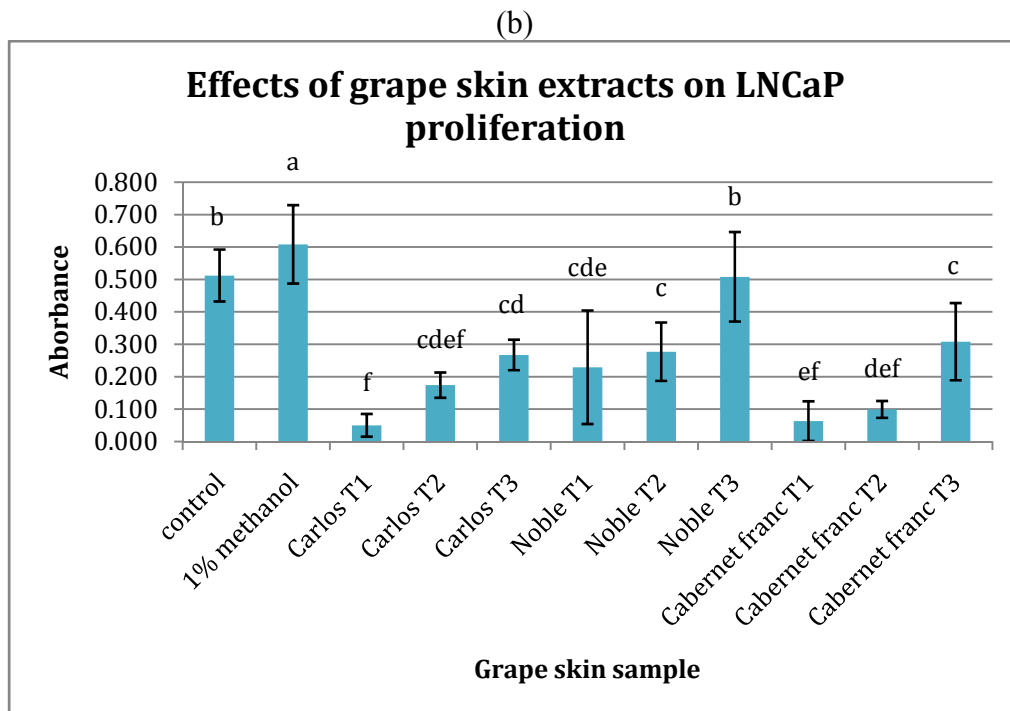
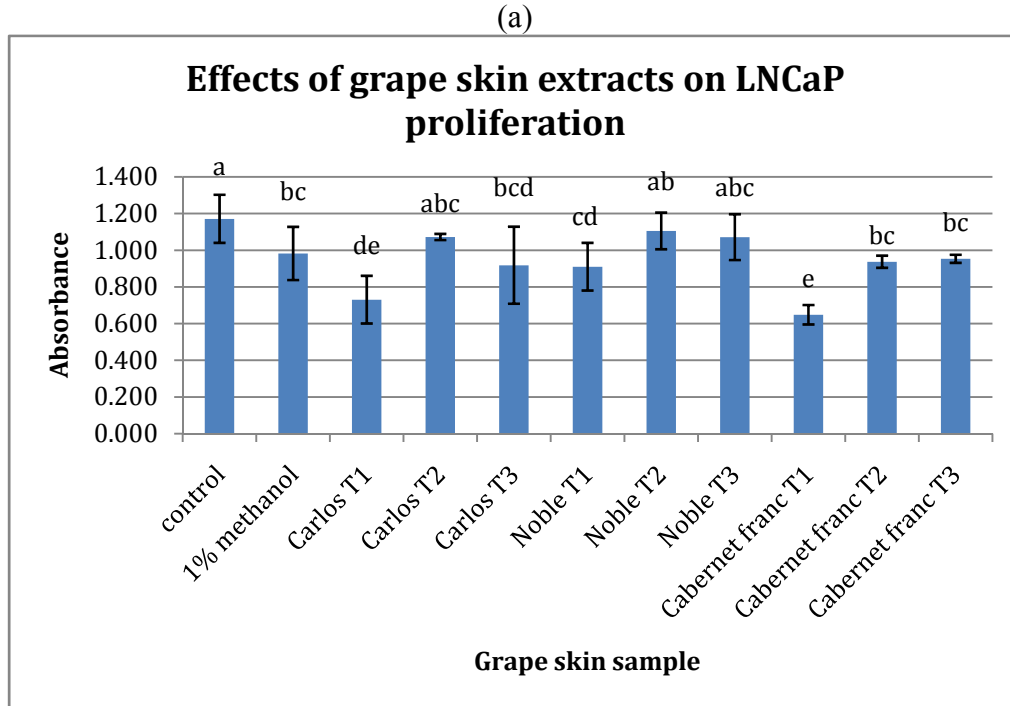
Winter R, Kramer A, Borkowski A, Kyprianou N. 2001. Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. *Cancer Res* 61:1227-1232.

Wyllie A, Donahue V, Fischer B, Hill D, Keeseey J, Manzow S. 1998. Cell proliferation and viability. In: *Apoptosis and cell proliferation*. 2<sup>nd</sup> ed. Boehringer Mannheim; p. 64-67.

Yau P. 2004. Apoptosis. *The Science Creative Quarterly* [Internet]. August [cited 2009 March 4]; 3 [about 7 pages]. Available from: <http://www.scq.ubc.ca/apoptosis/>.

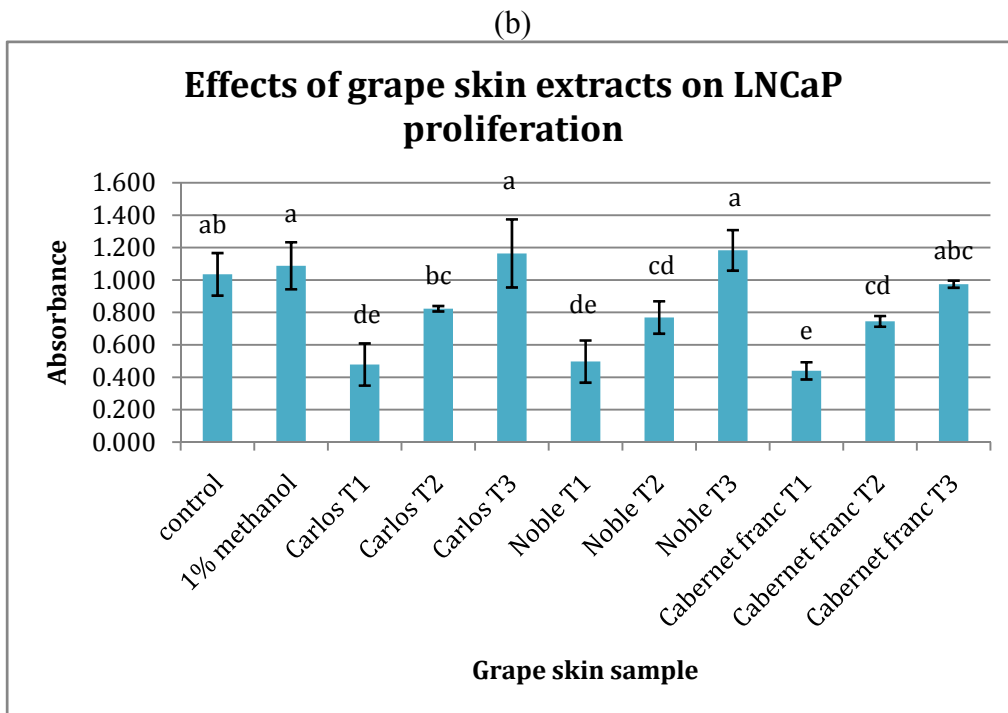
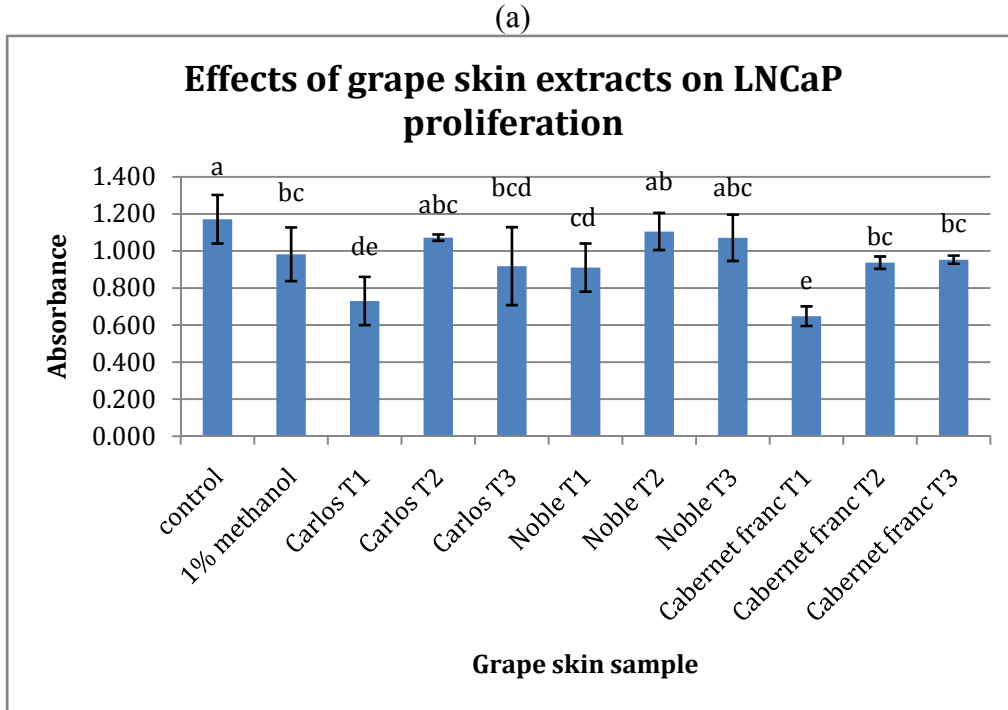


**Figure 3.1:** A cell going through the apoptosis process (Adapted from Yau 2004).



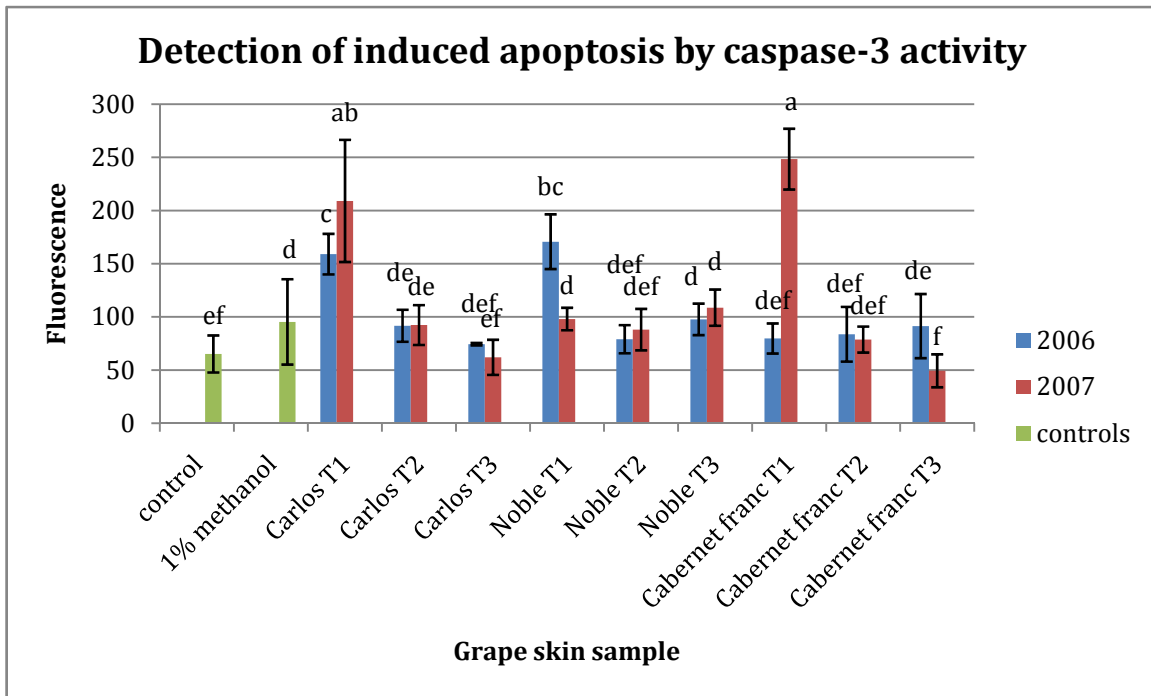
<sup>a</sup> Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figures 3.2a and 3.2b:** Effects of muscadine and Cabernet franc grape skin extracts from 2006 harvest on LNCaP proliferation after 24 (a) and 48 (b) hours of treatment.<sup>a</sup>



<sup>a</sup> Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figures 3.3a and 3.3b:** Effects of muscadine and Cabernet franc grape skin extracts from 2007 harvest on LNCaP proliferation after 24 (a) and 48 (b) hours of treatment.<sup>a</sup>



<sup>a</sup> Means with the same letter are not significantly different ( $p < 0.05$ ).

**Figure 3.4:** Detection of induced apoptosis by caspase-3 activity on LNCaP prostate cancer cells treated with muscadine and Cabernet franc grape skin extracts.<sup>a</sup>