

## ABSTRACT

VAUGHAN, TRIVETTE LASHUM. Antioxidant Properties of Muscadine Grape Products. (Under the direction of Dr. Leon Boyd.)

Phenolic compounds present in grapes and wines may play a positive role in human nutrition, related to their positive protective action towards reducing the risks associated with heart disease and the scavenging properties toward free radicals associated with degenerative diseases. Noble and carlos grapes were either cold pressed at room temperature or hot pressed at 43 C followed by collection of their free run juices and fermentation to wines. Resulting pomaces were dried at 37 C, 49 C, and 60 C by convection and vacuum ovens. Juices, wines and pomaces were tested for total phenol content and antioxidant capacity via the oxygen radical absorbance capacity assay (ORAC). Carlos grape skin, seed and delipidized seed extracts were analyzed to determine their antioxidant activity in a safflower oil model system. Carlos grape skin and seed were dried at 60 C and extracted using ethyl acetate and water. The antioxidant activity of carlos grape extracts were compared to tert-butyl hydroquinone (TBHQ) and propyl gallate (PG). Three levels (60, 180 and 300 milliequivalents of gallic acid) of extract were tested in an oil model system using the oxidative stability instrument (OSI) and conjugated diene, to measure levels of antioxidant activity. The objective of this research was to study the effects of processing on muscadine juices, wines and pomaces and to study the antioxidant properties of muscadine extracts in a lipid model system. Results of processing effects indicated that noble hot pressed juice and noble wine showed a significantly higher level of total phenols and total antioxidant capacity than for carlos samples. Total phenols indicated that all noble pomaces were significantly higher than carlos pomaces. Antioxidant capacity indicated no significant difference between

noble and carlos dried samples. Analysis by OSI showed that ethyl acetate seed extracts at a level of 300 milliequivalents of gallic acid was significantly higher than TBHQ and PG. Oxidative index values for water extracts were not significantly different from TBHQ and PG. Addition of both extracts into the oil model system using OSI index indicated increasing antioxidant activity with increasing concentration of extracts added.

# ANTIOXIDANT PROPERTIES OF MUSCADINE GRAPE PRODUCTS

by  
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APPROVED BY:

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Chair of Advisory Committee

## **DEDICATION**

I would like to thank my Mother and Father for their support throughout my lifetime. The both of you have been very supportive of my academic career. Thanks for all the love and guidance you have given to me over the years. I am truly blessed to have you as parents.

## **BIOGRAPHY**

Trivette L. Vaughan was born on November 17, 1973 in Ahoskie, NC. She is the daughter of Earl and Betty Vaughan and the granddaughter of Otis and Clarine Lassiter. She has one older brother and sister, Darrell Vaughan and Michelle Williams. She also has three younger sisters, Latoya, Chenay, and Jennifer Vaughan. After graduation from Hertford County High in 1992, she attended North Carolina Agricultural and Technical State University and obtained a B.S. degree in Biology in 1996 and a M.S. in Food and Nutrition in 2000. She then began working towards a M.S. degree in Food Science at NCSU under the direction of Dr. Leon Boyd.

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## LITERATURE REVIEW

### Vitis Species

#### Vitis vinifera

Vitis vinifera is the grape of antiquity often mentioned in the Bible. Most table wine and raisin grapes are produced from this grape species. The vinifera originated in the regions between and south of the Caspian and Black Seas in Asia Minor and has been carried from region to region by man in all temperature climates and has been grown more recently in subtropical climates (Winkler, 1974). There are hundreds of Vinifera cultivars. Vitis vinifera has also been crossed with American species to give rise to many of the hybrid grapes in eastern America. Vitis vinifera cannot tolerate extreme winter conditions and requires warm hot summers for the proper development of the fruit. In the southern vinifera grape growing is difficult due to temperature fluctuations during winter and humid summers of the eastern United States. Grape culture is best where there is moderate rain between blooming and harvesting. Different species of Vitis and Muscadinia are found in different regions of the world. Muscadina can be identified by its tight bark that does not shed, simple tendrils, nodes without a diaphragm and small clusterlets berries that detach as they mature. By contrast, Vinifera has forked tendrils, a bark that sheds, a diaphragm at the nodes, and elongated clusters with berries that adhere to the pedicels at maturity (Weaver, 1976). Some of the famous wines produced from Vitis vinifera are Merlot, Pinot Noir and Cabernet-Sauvignon. Except for Vitis vinifera, most Vitis species originated in the northern hemisphere and are especially common in North America.

### Vitis rotundifolia

The three American species of the subgenera Muscadinia are *V. rotundifolia* (in the southern US) and *V. munsoniana* (in central and southern Florida) and *V. popenoei* (New Mexico). These grape varieties are produced in the American south and most prevalent throughout the southeast. Muscadine grapes are best suited for the southern states from eastern Texas to the Atlantic seaboard (Weaver, 1976). *Vitis rotundifolia* is more commonly known as “muscadine”. Generally, these are small, loose clusters of large berries with a thick skin and dense pulp (Vine et al., 1997). The bronze skinned varieties are collectively referred to as “scuppernongs”. Cultivars in the *rotundifolia* species with the most commercial importance are the Carlos, Noble, and Magnolia. The Muscadine grape is more insect and disease resistant compared to the *vinifera* and have a natural immunity to *Botrytis cinerea Pers.* disease (Pezet et al., 1994). The advantage of the *V. labrusca* and *V. rotundifolia* are their resistance to fungal diseases and to winter killing.

### Hybrids

Hybrids can be produced by cross breeding different grape species to produce a more high quality grape that yields a higher quality wine. Some hybrids include the Concord, Catawba and the Delaware. French hybrids have been derived from crosses between *V. vinifera* and a number of wild American species. The French hybrids were developed to produce grapes that were resistant to phylloxera and to produce wine less fruity and more neutral in flavor. Phylloxera is an insect that attacks the foliage and the roots of the *vinifera*, which have no immunity to the disease. This was first identified and seen in Europe in the 1860's and spread from grapevine to grapevine in European

regions. Grapes can be divided into five main classes depending on their purpose. These include table varieties, wine grapes, raisin grapes, juice grapes and canning grapes. More than 90% of the world's grapes are used for wine, raisins and table use.

Wine is the result of fermenting grapes or some other fruit. For white wines, the juice is pressed out before fermentation. Most red wines are made by fermenting crushed grapes before they are pressed. Pink wines also known as blush wines are generally made by very limited skin contact during fermentation and then quickly pressed. These results in only a small amount of color extracted from the grape skins.

### **Mechanical Harvest and Quality of the Grape**

Wine grapes are picked by hand or mechanical equipment. The proper time to harvest wine grapes depends mainly on the kind of wine to be made. Grapes for dry wines should have high acidity and moderate sugar content. These grapes have a degree Brix ranging from 20-22 Brix. Grapes for sweet wines should be high in sugar content as possible which yields a degree Brix of 24 or higher (Jacob, 1950). Grapes are noted to be easier to harvest from about 10pm to 10am. During this time the berries are more turgid than at night. This makes removing the berry from the vine easier compared to the berry being soft and easy to bruise when harvested. The analysis of pH in making wine is significant during the ripening season because it can be used to indicate fruit ripeness. Normally a pH of 3.10 would indicate the beginning of ripening for most grape cultivars. A pH of 3.50 may signal the end of maturity and on the onset of over ripening. A pH of 3.3 can be considered ideal for most vineyards to harvest (Vine, 1992). Brix is a simple and inexpensive measurement of dissolved solids in grapes, musts and juice. Red grapes will have a slightly higher value of nonsugar Brix solids due to higher concentrations of

color pigments and glycerols. Normal Brix from grapes are usually in the range of 12° to 25° Brix, depending on the species and cultivar.

### Red Wine Production

Red wine vinification consists of three main classes of operation: alcoholic fermentation, extraction and malolactic fermentation. These operations are generally carried out in four stages:

1. Mechanical operations with grapes such as destemming and crushing.
2. Fermentation (alcoholic fermentation)
3. Separating the wine (draining and pressing)
4. The finishing transformations (malolactic fermentation)

### Crushing

Once grapes reach the winery they are put through a mechanical device that separates the berries from the stems, lightly ruptures the skins, and pumps the juice, skins, pulp and seeds into a fermenting tank (Martini, 1981). At this stage the grapes are now considered “must”. Shortly after crushing sulfur dioxide is added, the normal amount is about 75 to 100 mg/L (Martini, 1981). The sulfur dioxide added at this point serves to slow the growth of organisms, mainly yeast. This allows for the cultured yeast to start the fermentation.

### Alcoholic Fermentations

Different strains of *Saccharomyces cerevisiae* yeast are used for the fermentation of the red musts. The three most common strains of wine yeast used in fermentation are Montrachet, Pasteur champagne and Pasteur white champagne. Montrachet yeast is the most popular yeast used. It is available for both red and white wine fermentations and

may be called Montrachet Red and Montrachet White. This yeast works especially well in producing Chardonnay in barrel and stainless steel. It tolerates sulfur dioxide well but does not work well with high sugar levels (more than 23.5 %). Pasteur champagne yeast is the second most common yeast strain used. It was isolated in Champagne, France and is technically a mixed population culture. It is most commonly used in sparkling wine production because of its effectiveness in low temperatures and its tolerance of high alcoholic conditions. Pasteur white is used primarily for white wine fermentation because of its ability to ferment slowly and tolerate cold temperatures. Pasteur white yeast works better in stainless steel than wooden barrels. Red table wine grapes contain their pigments and tannins in the skins, therefore, red wines must be fermented on contact with skins in order to extract tannins and color (Martini, 1981).

During fermentation of the skins, the skins float to the top and form a “cap” on the juice. This can become a major problem in the fermentation of red wines. In order to extract the proper color and tannins and to keep the cap from overheating or becoming a source of overheating or becoming a source of spoilage or off flavors, the cap must be submerged frequently. Fermentation generates heat and red wines must have temperature control during the fermentation process and should not be allowed to become too hot or too cold. Ough and Amerine, 1966 have recommended temperature ranges of 18 to 29C and that cap temperatures not exceed liquid temperatures by over 6C. Extreme highs or lows in temperature can cause the fermentation to “stick” and it becomes difficult to start again. Stick or stuck fermentations refer to the fermentation process stopping before all the sugar has gone. Temperature has an effect on the degree of extraction from the skins and the seeds. Singleton and Esau, 1969 reported that increasing the temperature and the

ethanol content increased the extraction of seed tannins. Total phenols extracted into red wines by the skins and seeds usually show an increase with an increase in contact time, temperature, ethanol, sulfur dioxide content and maceration. The juice is usually drawn from the skins for one to five days after fermentation starts or between 9 and 0 Brix. For the production of slow aging wines, the skin contact time ranges from two to three weeks. After fermentation is complete the wine is separated from the lees to prevent the formation of hydrogen sulfide. The lees consist mainly of yeast cells and pulp and may be removed by racking or centrifuging. Racking is the process of siphoning the wine off the lees to allow for stabilization and clarification.

#### Malo-Lactic Fermentation

Malo-lactic fermentation is a type of secondary fermentation steps in which malic acid is converted to lactic acid with a reduction in total acidity and an increase in pH (Martini, 1981). Malo-lactic fermentation is desired for the production of premium red wine with more desirable flavor qualities. This secondary fermentation also ensures that fermentation will not take place in the wine bottle, which makes the wine cloudy and gassy with the development of off flavors and odors. This step ensures that fermentation has gone to completion before bottling.

#### Aging

After the fermentation process, the wine is ready for aging. The length of time red wines are aged is dependent on the type of wine desired. Aging is one of the most complex processes in winemaking. Newly fermented wine is cloudy, has a harsh taste and is without a pleasing bouquet that develops later during aging (Amerine et al., 1972). Bouquet is a sensory term relating to the entire fragrance of a wine. This fragrance is



comprised mainly of fruit aroma, fermentation flavors from yeast and or bacteria and wood essences (Vine et al., 1997). Red table wines improve in bouquet at about 1 to 4 years. White wines require little if any aging and are ready for bottling in only a year or two (Amerine et al., 1992). The idea of barrel aging is that bitter phenolic compounds that exist in the oak react with the fermenting juice proteins which result in more oak flavor extraction and less astringent tannin extraction. This results in a wine with a less fruity intensity and increased alcohol levels (Vine et al., 1997). During barrel aging the wine comes into contact with oxygen through the pores of the wood. The phenols in the wine can react with oxygen and other compounds from the barrel resulting in the development of the complex flavor and color of the wine. The three major groups of volatile oak extractives are oak lignin, oak polysaccharides and lactones gives rise to different flavors associated with barrel aging such as bitter almond, caramel, cedar, smoke, roast and tar (Vine et al., 1997).

### White Wine Production

White wine is made by fermenting just the grape juice on its own without any extraction of the solid parts from the bunch. This is the major difference between the production of white wine and red wine. In red wine vinification racking and pressing are done after fermentation whereas white wine vinification requires separation of the skin, seeds and stems from the juice by draining and pressing before fermentation (Peynaud, 1984).

### Effects of Enzymes on Fermentation

The most common application of pectic enzymes in grape processing is the processing of grape juice and concentrates. These enzymes are used to break down the

natural pectin in grapes, which can reduce juice yields, inhibit clarification, and foul concentration equipment (Vine, 1981). Many larger wineries use pectic enzymes to increase juice yield and clarity. Commercial enzymes are typically crude fungal preparations, containing impurities such as extraneous enzymes, proteins, mucilage, and melanoidins (Martino et al., 1994). The application of pectic enzymes may however affect total anthocyanin levels. For example, in Pinot noir wines total monomeric anthocyanin differed between treatments over time (Wightman, 1997). Pinot noir wines treated with Rapidase EX Color (0.005% w/v) and Rohapect VRSL (0.010% w/v) did not differ significantly from the control in total monomeric anthocyanin content. Treatment with AR 2000 (0.005% w/v) and two concentrations of Cytolase PCL5 (0.005% w/v and 0.02% w/v) significantly reduced total anthocyanin. The main difference among the treatments was observed within the first two days of fermentation. Twenty-four hours after inoculation with yeast there was almost a two fold difference among treatments in the amount of anthocyanin extracted from the skins. Cytolase PCL5 (0.005%) treated musts contained the most anthocyanin and control the least. This change was due to the pectolytic enzymes breaking down the cell walls and releasing pigments from vacuoles within the skin. Other than anthocyanins, differences in phenolic compounds were also noted among treatments containing AR 2000 and Cytolase PCL5 (0.02% w/v).

The relative levels of quercetin glucoside, glucuronide and aglycon were determined in Pinot noir and Cabernet Sauvignon wines after fermentation. AR200 and Cytolase PCL5 showed an increase in the quercetin aglycon in both wines compared to the control samples. In Pinot noir wine, levels of caffeic acid increased significantly when treated with AR2000 compared to the control samples. Caftaric acid was

approximately the same as the control when treated with Rapidase EX Color. In Cabernet Sauvignon, caffeic acid showed a significant increase when treated with AR 2000. Other enzyme treatments were not as effective when compared to the control samples. In Pinot noir, treatments with AR2000 and Cytolase PCL5 (0.020%) in Pinot noir showed a significant increase in levels of trans-Resveratrol. In Cabernet Sauvignon, only AR 2000 showed a significant increase in levels of trans-Resveratrol.

### **Phenolic Components of Grapes and Wines**

Table wines are comprised of about 85% water and 12% ethyl alcohol (Vine, 1997). A major group of compounds found in a small portion of wine are polyphenols. The entire family of phenols, phenolics, and tannins are referred to as polyphenols. On the average, approximately 65% of grape polyphenols are found in the seeds; 22% in the stems; 12% in the skins and only 1% in the pulp (Vine, 1997). Polyphenols or phenolic compounds are categorized into two major categories, flavonoids and nonflavonoids. Flavonoids are large polymer molecules involved with wine color and tannins. Nonflavonoids are generally smaller molecules, which are primarily associated with oak flavor extracts such as cinnamic and vanillic acid (Vine, 1997). Flavonoids include anthocyanins, flavonols and flavanols and nonflavonoids consist of benzoic acids, hydroxycinnamic acids and Stilbenes. Polyphenolic compounds are very unstable compounds and their degradative reactions start as soon as the grape is crushed or pressed and continue throughout the wine making process and aging. These reactions lead to the formation new diverse products (Waterhouse and Ebler, 1998).

## **Effects of Wine Processing on Phenolic Compounds**

The content of total and individual phenolics in wines can be influenced by enological practices associated with the wine making process (Castellari et al., 2000). Wines made by skin fermentation with stem contact contained much higher polymeric phenols than those wines made by skin fermentation without stem contact (Kantz and Singleton, 1991). Auw et al determined the effect of several processing treatments including immediate press, hot press, and skin fermentation on the phenol composition and color of red wines and juices. Hot pressed wines involve heating the grapes to a certain temperature (43 C) and holding (10 minutes) before pressing to release the juice. Normal pressing does not involve any type of heat treatment prior to pressing. Immediate pressed wines and juices had the lowest phenol levels, whereas skin-fermented wines had higher levels of nearly all compounds than hotpress wines and juices (Auw et al., 1996). Resveratrol levels in wines would be expected to decrease with aging and with oxygen exposure along with other phenolics. Levels of resveratrol averaged 2.1 mg/L in Pinot noir wine from 1982 (Jeandet et al., 1995). Resveratrol is a phytoalexin produced by grape berries in response to fungal infection. Higher levels would be expected in years when there was a high fungal disease pressure in the vineyard (Siemann and Creasy, 1992). Wines are often aged in stainless steel tanks or oak barrels. During this stage many reactions can lead to the formation of polymeric pigments by direct condensation of anthocyanins with other flavonoids or a combination of pigments with acetaldehyde. These reactions cause a modification of phenolics with the production of highly colored compounds and reduce astringency. Studies by Castellari et al., 2000 on the change in phenolic compounds as a function of the oxygen supplied during storage showed that

adding oxygen to red wine during the maturation phase reduced total phenolic compounds and increased the red polymeric pigments. Results showed that oxygen plays a detrimental role in wine quality by significantly decreasing the amount of phenolic antioxidant compounds that are present in wine. Wightman et al., 1997 reported that processing enzymes showed an effect on anthocyanins and phenolics present in Pinot noir and Cabernet sauvignon wines. The enzymes AR 2000 at 0.005% and Cytolase PCL at 0.02% treated Pinot noir wines showed significantly less anthocyanins compared to the control. Two enzyme treated Cabernet sauvignon wines contained less anthocyanin than did the control. Trans resveratrol content increased in all enzyme treated wines and those that had the greatest resveratrol content also contained the greatest amount of quercetin acylcon.

In white wines, skin contact has also been shown to have a positive effect on phenolic content (Darias-Martin et al., 2000). In a variety of *V. vinifera*, it was reported that compared to direct vintage pressing, skin contact increased the levels of phenolics. Catechin increased by four times and total flavonoids increased from 3.11 to 16.5 mg/l. Non flavonoids were also extracted in greater quantity in the skin-fermented wine. Trans-caftaric acid increased from 65mg/l (control) to 115 mg/l. In general, increases in contact temperature and time seem to give wines with a higher pH, potassium and total phenolic levels (Singleton et al., 1980).

### **Effects of Processing on Sensory Quality**

Oak barrels have been commonly used in the aging of wine and spirits because of the positive effects they have on wine flavor (Perez-Prieto et al, 2002). These qualities include color stability, spontaneous clarification, and the development of a more complex

aroma. The complexity of aroma is increased because of the extraction of certain compounds present in the wood, which are transferred to wine during the aging period (Puech and Wood, 1998). Among the compounds which are released from the wood, and from a sensory point of view the most important compounds, are the oak lactones (*cis*- and *trans*- $\beta$ -methyl- $\gamma$ -octalactones) that are present in green wood (Puech and Moutounet, 1999). These compounds arise from lipid oxidation and are known to increase in concentration after the wood is toasted. Other important compounds are guaiacol and 4-methylguaiacol, which has a smoky aroma; which are lignin degradation products formed during toasting; and vanillin, a compound normally present in green wood but whose concentration is increased by seasoning and toasting (Weeks and Shefton, 1999). Furfuryl compounds (furfural, 5-methylfurfural and furfuryl alcohol) are other important compounds that can be of sensory importance. These compounds have a pleasant aroma and are formed from the degradation of hemicellulose during toasting (Chatonnet et al., 1989).

### **Biological Importance of Wine and Grape Components**

French Paradox: In 1991, 60 Minutes CBS News presented a scientific report entitled “The French Paradox.” It showed epidemiological data that compared dietary intakes and disease incidences in various countries including Brittan, France, United States and others. The report revealed that red wine consumption offered a degree of protection from atherosclerosis and cardiovascular diseases in the French population. The report revealed that according to the MONICA (Monitoring of Trends and Determinants in Cardiovascular Disease) data, the French population had lower death rates related to atherosclerosis ( Renaud and De Lorgeril, 1992). The death rates from the

coronary vascular disease (CVD) were much lower in the French population in spite of having dietary food consumption patterns similar to that of many developed nations that had high incidences of CVD. Scientist postulated that the polyphenolic compounds found in red wine acted as a source of dietary antioxidants that reduced the risk associated with the development of coronary vascular diseases (Kinchella et al., 1993).

Studies (German and Walzem, 2000; De Lorimier, 2000) have shown that high dietary intakes of cholesterol and saturated fats are directly associated with risk factors related to the development of coronary heart disease. This disease can be found world wide with higher mortality rates existing in more affluent populations. However, mortality rates for coronary heart disease are less in France where high fatty diets are similar to other countries. This has become known as the “French paradox”. Coronary heart disease can be defined as atherosclerosis of the coronary arteries. Atherosclerosis results in abnormally thickened regions called plaques on the vascular wall. As plaques develop, they narrow the arteries and decrease blood supply, which causes damage to the heart and brain. A clot may form on the abnormal surface of the plaque region and block the artery. This may eventually lead to a heart attack or stroke (Stanley and Mazier, 1999).

### **Biological Function of Selected Polyphenols**

Currently the “French Paradox” has stimulated interest to investigate whether polyphenols from grapes may offer antioxidant benefits to other body organs such as the liver, stomach and the brain. Additional studies under investigation are analyzing whether polyphenolic compounds from other botanical sources offer similar health benefits. Polyphenolic compounds are predominately found in vegetables, fruits, wine,

and tea. These compounds possess a common structural component of two benzene rings on either side of a 3-carbon ring. Multiple hydroxyl groups, methyl groups, and sugars attached to the common structure produce the various classes of Flavonoids (Figure 1). Flavonoids such as quercetin, epicatechin gallate, and catechin (flavan-3-ols) are found in grape skin and seeds (Sun et al., 2002). Several classes of Flavonoids have been shown to be potent antioxidants that have the capability to scavenge hydroxyl radicals (Auroma, 1998). Free radicals contribute to the onset of degenerative diseases such as coronary vascular disease, arthritis, and cirrhosis (Ishige et al., 2000). Thus, having flavonoid compounds to scavenge these free radicals can possibly prevent these diseases from rapidly occurring in the human body. Resveratrol, Catechin, Ellagic acid and Quercetin are compounds that are postulated to be responsible for the health benefits of grapes and wines.

Over the past years there has been increasing interest in natural antioxidants and their role in human health and nutrition. The fact that the oxidative process plays host to many degenerative diseases and contribute significantly to the risk of human aging and cancer has focused the interest on this subject (Larrauri et al., 1997). The protection that fruits and vegetables provide against diseases such as cancer and cardiovascular diseases has been attributed to the various antioxidants contained in them (Wang, 1996). The majority of the antioxidant capacity of a fruit or vegetable may be from compounds other than Vitamin C, Vitamin E or beta-carotene. For example, some flavonoids, which include flavones, isoflavones, flavonones, anthocyanins, catechin and isocatechin, are components of the human diet that show strong antioxidant activities (Wang, 1996). Plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating



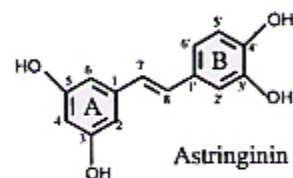
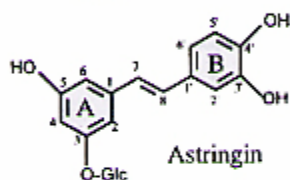
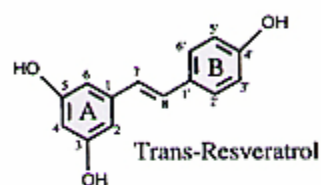
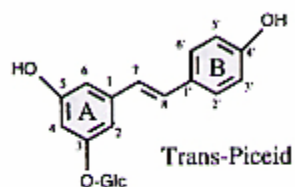
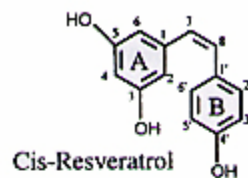
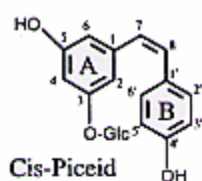
antioxidants and singlet oxygen quenchers. The flavanols particularly the Catechin and Catechin-gallate ester family, and the flavonols quercetin, kaempferol, and their glycosides are constituents of the beverages prepared from green and black teas and red wines. Part of the antioxidant capacity of fruits may be from flavonoids. Flavonoids are low molecular weight polyphenolic compounds that are widely distributed in fruits and vegetables (Hertzog et al., 1992). Many flavonoids such as kaempferol, quercetin, luteolin myricetin, and catechin have been shown to have antioxidant, anti-inflammatory, anti-allergic, anticancer and anti-hemorrhagic properties. Hertzog (1992) measured the flavonoids content of fruit, vegetables and beverages in the Dutch diet and related the baseline intake of dietary flavonoids to coronary heart disease mortality and the incidence of myocardial infarction in the Zutphen Elderly Study during a five-year follow up period. Their research showed that the intake of flavonoids was significantly inversely related to mortality from coronary heart disease and of borderline significance with the incidence of a first fatal or nonfatal myocardial infarction (Hertzog et al., 1993).

The total antioxidant capacity of some common fruits and fruit juices is measured by using the oxygen radical absorbance assay (ORAC) values of common commercial fruit juices by Wang, et al, 1996). Grapes contain a large amount of polyphenols, which include the phenolic acids, flavonoids, anthocyanins and proanthocyanidins (Lu et al., 1999) (Figure 1). These phenolic compounds are associated with such activity as antioxidant activity via, free radical scavengers, inhibition of lipoprotein oxidation, reduced oxidation of low-density lipoproteins, and the potential to reduce risk factors associated with the development of heart disease. Red wine consumption has been shown to cause a decrease in platelet aggregation as well as an increase in high-density

lipoprotein cholesterol that is independent of the alcohol content of wine (Sieigneur et al., 1990).

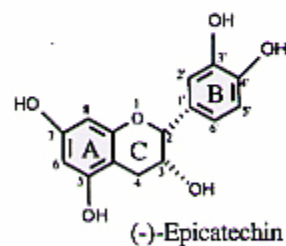
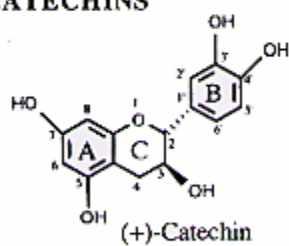
## NON-FLAVONOIDS

### STILBENES



## FLAVONOIDS

### CATECHINS



### ANTHOCYANINS

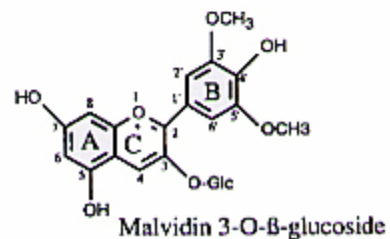
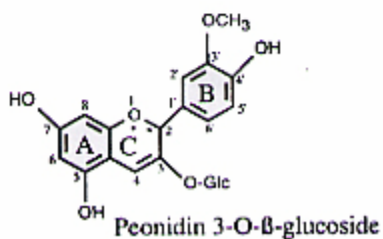


Figure 1. Basic structure of polyphenolic compounds: non-flavonoids, Flavonoids and anthocyanins.

Frankel et al (1993) provided additional evidence that the phenolic substances found in red wine inhibited oxidation of low-density lipoprotein.

### Stilbenes

Resveratrol (*trans*- 3, 5, 4'-trihydroxystilbene) has been identified as a major constituent of various fruits and vegetables products especially in grapes and wines. This compound occurs naturally in grapes and a variety of medicinal plants where it functions as a phytoalexin that protects against bacterial and fungal infections and other stress factors (Bertilli et al., 1996). There has been much interest in *trans*-resveratrol in wine for its protective effect against heart disease since it inhibits platelet aggregation (Kimura et al., 1995). It also functions to inhibit the oxidation of low-density lipoproteins, reduces the levels of triacylglycerol and protects the liver from lipid peroxidation (Shan et al., 1990). Higher concentrations of resveratrol are found in red wine compared to white wines, primarily because resveratrol is found in the skin of grapes and red wines are fermented on the skins (Goldberg et al., 1995). Goldberg et al, 1995 reported that for California and Canadian red wines made from Pinot noir grapes seem to have higher *trans*-resveratrol concentrations than Cabernet Sauvignon. It was concluded that wines made from the Pinot noir grapes have higher levels of *trans*-resveratrol concentrations relative to those from other cultivars regardless of the country or region of origin. High levels of *trans* resveratrol were uniformly found in Pinot noir wines from other regions such as Australia, Alsace and the Loire Valley. This shows that climate and territory do not influence the *trans* resveratrol concentrations that this particular grape yields on vinification (Goldberg et al., 1995). Wines made from Pinot noir grapes have higher *trans*-resveratrol concentrations than those from Cabernet Sauvignon and Chardonnay is

in the order Pinot noir < Chardonnay < Cabernet Sauvignon (Jeandet et al, 1992). The higher levels of trans-resveratrol found in Pinot noir wines compared to Cabernet Sauvignon and Chardonnay may be due to the higher content of leaves included in the fermentation process (Jeandet et al., 1992) This shows that the trans resveratrol content of leaves does not reflect the content of the berry skins or of the wine that is made from the grape. A second possible conclusion is that the trans resveratrol content of wines made from Cabernet Sauvignon grape showed fluctuations that appeared to be temperature dependent. Cabernet Sauvignon wines made from California, Australia, and South America have lower concentrations than those wines made in Bordeaux and Ontario. This difference can be explained by the cooler and more humid conditions in Bordeaux and Ontario.

#### Procyanthocydins

Proanthocyanidins are the polymerization products of flavan-3-ols to oligomers. The flavan-3-ol monomers, (+) –catechin and (-) – epi-catechin, as well as their dimers are found in grapes and wines (Pekic et al, 1997). Catechin monomers are constituents of grapes with the grape seed being the natural reservoir ( Katalinic, 1999). Among the polyphenols, epigallocatechin gallate (EGCG) has been studied as a preventive substance for carcinogenesis (Yanaga et al., 2002). A chemopreventive effect was seen in a group of mice treated with EGCG *in vitro* and *in vivo* using mouse mammary epithelial cells RIII/MG. In the *in vitro* experiment, Catechin containing 50% or more EGCG significantly inhibited the growth of pre cancerous RIII/MG cells. Control treatments of RII/ MG cells containing no catechins formed tumor development in 13 weeks whereas by the 20<sup>th</sup> week, 40% of the cells were tumorous. In the group treated with 1%

Catechin, no tumor was detected even in the 20<sup>th</sup> week. This study showed that EGCG inhibited growth in the mouse epithelial carcinogenesis model RII/MG and induced apoptosis. These results suggest that Catechin can be used as a chemopreventive substance.

### Ellagic Acid

Ellagic acid is a polyphenols found in a wide variety of fruits and nuts such as strawberries, grapes and walnuts. It is the major phenolic constituent in distilled beverages. It exhibits antimutagenic, antioxidant, and anti inflammatory activity in bacterial and mammalian systems. The plant derived polyphenols inhibit hydroxy radical induced lipid peroxidation in rat liver microsomes in a dose and concentration dependent manner (Priyadarsini et al., 2002). The concentrations of ellagic acid varied from 2 to 30 uM. In an *in vitro* lipid oxidation model system the extent of lipid peroxidation was found to decrease with an increase in the concentration of ellagic acid. At 2 uM ellagic acid concentration, 42% inhibition was obtained, whereas 24.8 uM ellagic acid exhibited 76% protection. The amount of ellagic acid required to inhibit lipid peroxidation by 50% was determined to be 3.14 uM. This study showed that ellagic acid acted as a good antioxidant even at very low concentrations (Zafrilla et al., 2001). Other studies show that ellagic acid arrested gastrointestinal (GI) tumor cells, inhibited overall cell growth, and caused apoptosis in tumor cells (Baratabab et al., 1999).

Hassonna et al, 1997 showed that ellagic acid offered better protection against oxidative stress compared to vitamin E. Cozzi et al, 1995 observed cytogenic protection against hydrogen peroxide damage by ellagic acid could be attributed to it's scavenging reactive oxygen species (ROS) or free radicals.

### Quercetin

Quercetin is a natural and abundant flavonoid compound found in apples, onions, tea and red wine. Currently the compound is used for treatment of allergic conditions such as asthma, hay fever, and hives (Frankel et al, 1993). Clinical test have shown quercetin to be effective against inflammatory conditions such as gout, pancreatitis and prostatitis. The Mayo Clinic study indicated quercetin as having a significant activity against the androgen receptor in the human prostate cancer cell lines. The biological effects of androgens in the prostate are mediated by the androgen receptor. An activated androgen receptor can turn on or off critical genes, which affect the biology and pathology of the prostate (Frankel et al., 2001). Androgens are involved in the development, progression and growth of prostate cancer. ROS may be involved in a number of diseases such as cancer and arteriosclerosis. Antioxidants therefore may be of central importance in the prevention of these diseases.

### **Extraction and Identification of Selected Antioxidants**

Extraction of plant phenols have be carried out using various solvents such as methanol, ethanol, ethyl acetate, acetone and water at different concentrations (Pekic et al., 1997). Proanthocydins such as catechin and epicatechin are well soluble in ethyl acetate. The seeds of grapes have a higher content of proanthocyanidins than the rest of the grape according to Bourzeiz et al., 1986. The yield of proanthocyanidins was 2.3 times higher using ethyl acetate (9:1) than the yield using acetone and water (2:3). It was concluded that ethyl acetate and water selectively extracts proanthocyanidins from grape seeds. Extractions of the grape pomace have been carried out using ethanol and water

(80:20). The pomace extracts yielded compounds quercetin, catechin, gallic acid and kaempferol (Foo, 1999). From this study trans caftaric acid was the most abundant compound present. Although there are numerous methods for extraction of phenolic compounds from plant material, an optimized and validated method for extraction of a variety of phenolic compounds in different plant foods is lacking (Matilla et al., 2002). A common feature of extraction methods is their suitability to only one plant material for certain phenolic acids or certain forms of phenolic acids. Soluble phenolic acids have been extracted using mixtures of methanol, acetone, and or water, cold or hot methanol, or mixtures of methanol, water, and acetic acid. Bound phenolic acids have been released using acid hydrolysis or alkaline hydrolysis or both methods. The aglycon form makes the compound more active as an antioxidant compared to the glucoside form. Extraction of resveratrol from wine has been done using ethyl acetate followed by solvent evaporation and filtration using C-18 SPE cartridge (Goldberg, 1995) and cation exchange resins (Lima et al., 1999). Results showed the successful identification of resveratrol and its glucosides when analyzed using reverse phase high performance liquid chromatography. Typically a reverse phase C 18 column is used for identification along with a dual solvent system.

Other researchers (Lamuela-Raventos et al., 1995) have identified resveratrol and other compounds from direct injecting aliquots of wine onto the HPLC system after filtration through Whatman inorganic Anopore membrane filters (Anodisc 2um). In this study several filters were compared including ones made from nylon, PVDF and polysulfone. These retained more than 60% of trans resveratrol. The Whatman anodisc did not retain any of the compounds.



## Assays to Measure Antioxidant Capacity

Methods to measure the antioxidant properties of compounds can be grouped into two general categories; those direct methods that determine the ability of the compound to sequester free radicals and secondly, indirect methods that measure the ability of an unknown compound or antioxidant to delay the oxidation of an oil in a model system or food. Direct methods include the TRAP-FRAI, ORAC and Total Phenols. Indirect methods include Oxidative Stability Index (OSI), Conjugated Dienes (CD), Peroxide Value (PV) and Thioburbituric acid reactive substance (TBARS).

Methods have been developed to measure the total antioxidant capacities of various fruits, vegetables and wines Cao and Prior (1999). The total peroxy radical trapping parameter or TRAP assay of Wanyer et al., 1985, was the most widely used assay of antioxidant capacity during the past two decades (Cao and Prior, 1999). The major problem with the TRAP assay is with the oxygen electrode end point in that the oxygen electrode will not maintain its stability over the period of time required.

The ORAC assay depends on the detection of chemical damage to  $\beta$ -phycoerythrin ( $\beta$ -PE) through the decrease in its fluorescence. The fluorescence of PE is highly sensitive to the conformation and chemical stability of the protein (Cao and Prior, 1999). Under normal conditions the loss of fluorescence in the presence of radical species (RS) is an index of oxidative damage of the protein. The inhibition of the RS action by an antioxidant is reflected in the protection against the loss of PE fluorescence in the ORAC assay and is a measure of its antioxidant capacity against the RS. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve of the sample as compared to that of the blank in which no

antioxidant is present to a standard (Trolox), whose concentration is known (Ou et al., 2001). However, there are three major limitations of the ORAC when  $\beta$ -PE is used as the probe. The first is that  $\beta$ -PE produces inconsistency from lot to lot, which results in variable reactivity to peroxy radical (Cao and Prior, 1999). Secondly,  $\beta$ -PE is not photo stable and after exposure to excitation light for certain time, it can be photo bleached. This activity was observed in a 96 well plate reader where the fluorescence signal was found to decline dramatically without the addition of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). Lastly, it has been observed that  $\beta$ -PE interacts with polyphenols due to the nonspecific protein binding. Due to these disadvantages the use of fluorescein (FL) (3', 6' – dihydroxyspiro [isobenzofuran-1 [3H], 9'[9H]-xanthen]-3-one as the fluorescent probe is being used to replace  $\beta$ -PE.

Free radicals are generated by (AAPH). The damage caused by formation of free radicals is measured by a decrease in the fluorescence of fluorescein. In this assay Trolox the control standard, which is a water-soluble analog of Vitamin E is used to protect the fluorescein from oxidative damage caused by AAPH. Results are measured by calculating the differences in area under the curve of fluorescein between a blank and an unknown with a comparison to a standard such as Trolox. Results are expressed as micromoles of Trolox equivalents per gram or milliliter of sample (Wang, 1996). Unlike the other antioxidant activity methods, the improved ORAC assay using FL provides a direct measure of the hydrophilic chain-breaking antioxidant capacity against peroxy radicals.

## Total Phenols

Several factors affect the amount of total phenol found in a wine. Skin and seed contact time, ethanol concentration, fermentation temperature, agitation of juice and skins, intensity of pressing, grape variety, and their total inherent phenol content all affect the final total phenol content of the wine. There are several methods for estimating the total phenol content of wines that include precipitation with heavy metals, precipitation by the addition of organic compounds, oxidation under controlled conditions, and formation of colored products with various chemical elements. Of these methods only two have survived: the Neubauer-Lawenthal method and the Folin-Denis method. The first was for some time the official method of the AOAC but was replaced by the Folin-Denis method. More recently the Folin-Ciocalteu reagent replaced the Folin-Denis reagent method. The main differences in the two reagents are the use of lithium sulfate, longer heating time in the reagent preparation, and the presence of hydrochloric acid in the Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent contains a higher percentage of molybdate in the complex and is more easily reduced. The lithium prevents precipitation, which caused problems using the Folin-Denis reagent. Lithium substitutes for sodium and its salts are more soluble. The sulfate ion may also improve the solubility of the salt complexes. The response of the test depends on the phenol present in the sample. The number of  $-OH$  groups or potentially oxidizable group controls the amount of color formed. Neither the ORAC nor Total Phenol assay identifies a particular phenolic compound that is responsible for the activity antioxidant in the sample (Amerine and Ough, 1980). However, of the two assays the ORAC does measure the ability of a given sample to sequester free radicals in an *in vitro* model system.

### Oxidative Stability Index

The active oxygen method (AOM) also known as the Swift test of the American Oil Chemists' Society is an accelerated method for assessing the oxidative stability of fats and oils. The principle of this method is that aging and rancidification of a fat are greatly accelerated by aeration in a tube held at a constant elevated temperature. Air is bubbled through heated oil between 98 and 100 °C for different times. The peroxide values are determined and plotted against time, and the induction period is determined from the resulting graph. Over the years this method has been used extensively but certain difficulties and deficiencies have been identified. For example, the end point is determined by the amount of peroxides in the oxidized oil and the reaction is extremely susceptible, during the rapid oxidation phase, to variations in the oxygen supply. An automated version of the AOM method is the Oil Stability Instrument (OSI) and Rancimat are now being used. The Rancimat method uses a commercial apparatus. The OSI, which is a computer assisted instrument. The OSI methods may be considered an automated version of the AOM, since both use the principle of accelerated oxidation. The OSI and Rancimat methods automatically and continuously measure the changes in conductivity caused by ionic volatile organic acids, mainly formic acid, whereas in the AOM, peroxide values are determined. Organic acids are stable oxidation products that are produced when oil is oxidized by a stream of air bubbled through it. In the OSI and Rancimat methods, oxidation proceeds slowly at first because during the induction period, formic acid is released slowly. The end point is selected to coincide with the point at which the rapid rise in conductance begins (Shahidi and Wanasundara, 1998).

### Conjugated Dienes

Polyunsaturated fatty acid oxidation is accompanied by an increase in the ultraviolet absorption of the product. Lipids that contain methylene-interrupted dienes or polyenes show a shift in their double-bond position during oxidation that is due to isomerization and conjugate formation (Logani, 1980). At 234 nm, conjugated dienes show an intense absorption, which is similar to conjugated trienes that absorb at 268 nm. According to Farmer and Sutton, (1946) the absorption increases due to formation of conjugated dienes and trienes is proportional to the uptake of oxygen and formation of peroxides during the early stages of oxidation. St Angelo et al., (1972) studied the autoxidation of peanut butter by measuring the peroxide value and absorption increase at 234nm due to the formation of conjugated dienes. Shaidi et al., (1994) and Wanasundara et al, 1995 found that conjugated dienes and peroxide value of edible oils correlate well during their oxidation. These authors concluded that the conjugated diene method may be used as an index of stability of lipids in place of or in addition to, peroxide value. However, the presence of compounds such as phospholipids, absorbing in the region of conjugated diene formation may interfere with such determination.

### Peroxide Value

A classical method used for the quantification of hydroperoxides is the determination of peroxide value (PV). The hydroperoxide content is generally referred to as PV is determined by an idometric method. This theory is based on the reduction of the hydroperoxide group (ROOH) with iodide (I<sup>-</sup>). The amount of iodine (I<sub>2</sub>) released is proportional to the concentration of peroxide present. Released I<sub>2</sub> is assessed by titration against a standardized solution of sodium (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) using a starch indicator. Some

potential drawbacks of this method are absorption of iodine at unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be titrated (Gray, 1978). Although determination of peroxide value is common, the usefulness of this measure is generally limited to the initial stages of lipid oxidation.

### TBARS

One of the oldest and most frequently used test for assessing lipid oxidation in foods and other biological systems is the 2-Thiobarbituric Acid Value (TBA). This value is expressed as milligrams of malonaldehyde (MA) equivalents per kilogram of sample or as micromoles of MA equivalents per gram of sample. Malonaldehyde is a minor product of oxidation of polyunsaturated fatty acids. This reacts with the TBA reagent to produce a pink complex with an absorption maximum at 530 to 532 nm. There are numerous methods for determining TBA values. The TBA test may be performed directly on the sample, its extracts or distillate. In the extraction method, TBA reactive substances (TBARS) are extracted from food material into an aqueous medium before color development with the TBA reagent. The main disadvantages of both of these methods are the long assay time and possibility of artifact formation from browning reaction products (Shahidi and Wanasundara, 1998).

### **Extraction and Identification of Individual Antioxidants**

High Performance Liquid Chromatography is an improved analytical separation technique. This method can be used for the analysis of any compound with solubility in a mobile phase (Nielsen, 1994). HPLC is normally used as an analytical technique to identify and determine the different phenolic compounds (flavonoids, nonflavonoids and Stilbenes) based on retention time. HPLC conditions are critical for the identification of

compounds. The pH of the mobile phases and the elution program is critical for isolation and identification of compounds. A variety of columns have been utilized for the chromatography of flavonoids and nonflavanoids, including silica, alumina, C-8 and C-18 (Strack, 1979; Hertzog, 1992). The use of a photodiode array UV-visible detector is also necessary in order to identify and determine different classes of phenolic compounds present in a sample. With this instrument identification can be based on a maximum absorbance at a certain wavelength as well as retention time.

### **Gas Liquid Chromatography**

The mechanism behind gas liquid chromatography is that solutes are fractionated as a result of differential migration through a closed tube of stationary phase or adsorbent. This method has been used to separate mixtures of naturally occurring phenolic compounds and is also useful for particular applications with flavonoids (Harborne, 1975). In order to increase volatility, phenols are generally chromatographed as their trimethylsilyl ethers. For the separation of substituted phenols, it is necessary to use several types of columns, because closely related structures may have identical retention times on one or both columns. Irvine and Saxby, 1969 found it necessary to use three stationary phases in order to resolve and identify the 38 phenols present in tobacco leaf samples. Gas liquid chromatography is ideally suited for the separation of a wide variety of high molecular weight and/or less stable compounds such as proteins, plant pigments and antioxidants (Snyder and Kirkland, 1974).

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## **ABSTRACT**

**VAUGHAN, TRIVETTE LASHUM. Effects of Processing on the Antioxidant Activity of Muscadine Juices, Wines, and Pomace (Under the direction of Dr. Leon Boyd.)**

The objectives of this study were to determine the effects of processing on the polyphenolic content and antioxidant capacity of muscadine products including juices, wines, and pomaces. Noble and Carlos muscadine grapes were either cold pressed at room temperature or hot pressed at 43 C followed by collection of their free run juices and fermentation to wines. The resulting pomaces were dried at 37 C, 49 C, and 60 C by convection and vacuum ovens. Juices, wines and pressed pomaces were tested for total phenol content and antioxidant capacity via the oxygen radical absorbance capacity assay (ORAC). Results indicated that Noble hot pressed juice and Noble wine showed a significantly higher level of total phenols and total antioxidant capacity than for Carlos samples and that all Noble samples showed a significantly higher level for antioxidant activity. For phenolic content, all Noble pomaces were significantly higher than Carlos pomaces. Antioxidant capacity values indicated no significant difference between noble and Carlos dried samples, however for all samples dried at 60 C showed the highest phenolic content.

## INTRODUCTION

### **Effects of Processing on the Antioxidant Activity of Muscadine Juices, Wines, and Pomace**

Phenolic compounds present in grapes and wines may play a positive role in human nutrition, by reducing the risks associated with coronary heart disease and by reducing free radicals associated with several degenerative diseases. Phenolics are accumulated in the most lignified parts of the grape, especially in the seeds (Kovac et al., 1995). Different processing techniques and enological practices can affect the level of phenolic compounds present in grapes and wines. Processing techniques such as enzyme treatment, application of temperature, and skin contact can alter the phenolic content present in wine (Wightman et al., 1997). Studies by Wightman et al., 1997 showed that the addition of enzymes and different concentration of enzymes can affect the levels of anthocyanins present in Pinot noir wine. Total monomeric anthocyanin differed between treatments over time (Wightman, 1997). Pinot Noir wines treated with Rapidase EX Color (0.005% w/v) and Rohapect VRSL (0.010% w/v) did not differ significantly from the control in total monomeric anthocyanin content. Treatment with AR 2000 (0.005% w/v) and two concentrations of Cytolase PCL5 (0.005% w/v and 0.02% w/v) significantly reduced total anthocyanin (Wightman, 1997). The content of total and individual phenolics in wines can be influenced by enological practices associated with the wine making process (Castellari et al., 2000). Wines made by skin fermentation with stem contact contained much higher polymeric phenols than those wines made without stem contact (Kantz and Singleton, 1991).

Enological practices such as increased light exposure has been correlated with increased phenol content (Kliewer, 1977). Several studies have correlated high

temperatures with decreased phenolics (Buttrose et al, 1971; Faragher, 1986). It has also been shown that sudden heat stress during grape ripening can cause large decreases in anthocyanin content of grape berries (Kliewer, 1977). The phenolic content of grapes is highly variable and individual compounds may change in response to different environmental conditions such as sun exposure, soil conditions, and rainfall (Crippen et al., 1986).

Other studies show that there are several process techniques that can affect the color and composition of red wines. Such processes as the application of immediate press, hot press and skin fermentation can affect the phenol composition and color of red wines and juices. Wines fermented from immediate press juices contained less phenolic compounds, followed by hot pressed juices, with skin fermented wines containing the highest level of phenols (Auw et al, 1996).

In white wines, skin contact has also been shown to have a positive effect on phenolic content (Darias-Martin et al., 2000). In a variety of *V. vinifera*, it was reported that compared to direct pressing, skin contact increased the levels of phenolics. Catechin increased by four times and total flavonoids increased from 3.11 to 16.5 mg/l. Non flavonoids were also extracted in greater quantity in the skin-fermented wine. Trans-caftaric acid increased from 65mg/l (control) to 115 mg/l. In general, increases in contact temperature and time seem to result in wines having a higher pH, potassium, and total phenolic levels (Singleton et al., 1980).

The objective of this research was to determine the effects of selected processing techniques on antioxidant retention of muscadine juices, wines and the resulting pomace. As the spent pomace of muscadine represents a significant portion of the total grape



mass, a secondary objective was to determine how these antioxidant nutrients could be recovered.

Some muscadine are resistant to fungal attack, which is theoretically tied to increased levels of antioxidant compounds including Stilbenes. The application of processing techniques to further extract more phenolic compounds into the processed products would increase consumer acceptance of muscadine products.

## MATERIALS AND METHODS

### Processing of grapes:

Muscadine cultivars Carlos and Noble, were obtained from the North Carolina State University research farm at Castle Hayne, NC. The grapes were chilled overnight at 4 C prior to pressing. A crusher (Presque Wine Cellars, Northeast, PA) was used followed by the addition of 75 ppm SO<sub>2</sub> added as potassium bisulfite. Crushed grapes were then pressed using a stainless steel bladder press lined with pressing cloth (Presque Wine Cellars, Northeast PA). Juices obtained at room temperature are termed “cold press” whereas juices in which mild temperature of 43 C was applied prior to pressing are designated as “hot press” juices. Aliquots from both the cold press and hot press juices which were obtained prior to pressing are referred to as “free run” juices.

Juices obtained by cold press and hot press were fermented to wines by the addition of Montrachet Yeast (Red Star- Universal Foods, Milwaukee, WI) at a level of 1 gram per gallon. Approximately 2 kg each of muscadine grape was pressed at room temperature (cold press) to obtain a free press juice followed by the addition of Montrachet Yeast (Red Star- Universal Foods, Milwaukee, WI) to obtain the fermented wine.

### Juice and Wine Production from Enzyme Treated Carlos and Noble Cultivars

Carlos grapes were crushed mechanically and the pectic enzyme Rapidase was added to the must at a ratio of 0.003 ml/kg of crushed weight. All juice samples were pressed using a stainless steel bladder press with a pressing cloth (Presque Isle Wine Cellars, Northeast, PA). Juice samples were collected immediately after pressing and after settling for 36 hours overnight at refrigeration temperature (4 C). The samples were

flushed with nitrogen for approximately one minute and stored at freezer temperature (-20 C) and stored up to 30 days for future analysis. The remaining juice was taken to 21 Brix, inoculated with Montrachet active dry wine yeast (Red Star- Universal Foods, Milwaukee, Wi) and allowed to ferment at room temperature. From the Carlos grape juice three samples were analyzed that included Carlos free run juice, Carlos cold pressed juice and Carlos wine.

From the Noble grape, the following samples were collected: immediate pressed juice, hot pressed juice and Noble wine. Noble grapes were treated the same as the carlos, with the exception that aliquots of pressed juice was heated to 43 C, cooled to room temperature and brought to a brix of 22<sup>0</sup> prior to fermentation. From the Noble grape, the following samples were collected: free run juice, hot pressed juice and wine. Samples were analyzed for total phenols and antioxidant activity using the ORAC assay.

#### Grape Pomace:

Carlos and Noble grape pomaces were dried using convection (Blue Electric Company, Blue Island, Ill) and vacuum (Fisher Isotemp Vacuum Oven, Pittsburgh, PA) ovens at 37 C, 49 C and 60 C. Approximately 4 grams of dried pomace and approximately 1 gram of dried seeds were ground in a coffee grinder (Mr. Coffee, Cleveland, Ohio) and extracted using methanol and water (80:20). The mixture was homogenized using a Tissumizer (Tekmar Co., Cincinnati, OH) for 1 minute. The mixture was allowed to sit for 20 minutes. Afterwards the mixture was filtered using Whatman #2 filter paper. The remaining solvent was evaporated under nitrogen using a rotary evaporator (Buchi, Rotavapor (R), Switzerland) at less than 40 C. The concentrated material was redissolved in 2.5 ml of methanol. All extraction solvents and

buffer solutions were of analytical grades obtained from Fisher Scientific (Pittsburgh, PA). The extracted material was flushed with nitrogen and stored at 2 C until analyzed. Samples were analyzed for total phenols and antioxidant capacity using the oxygen radical absorbance capacity (ORAC) assay.

### **Measurement of Antioxidant Activity**

#### Total Phenol Assay

The potential total phenol content of samples was determined using the Folin-Ciocalteu Method (Amerine and Ough, 1980). A calibration curve was prepared ranging from 0, 1, 2, 3, 5 and 10 milliequivalents of gallic acid. Samples were diluted to fall within the calibration curve. The absorbance was measured at 734 nm using a 260 UV-Visible Recording Spectrophotometer (Schimadzu Scientific Instruments, Norcross, GA).

#### ORAC Assay

The ORAC assay (Cao and Prior, 2001) was used to measure the protective effect of grape pomace extracts by assessing the area under the fluorescence decay curve. Samples were compared to that of a blank in which no sample was present and to a standard, Trolox 0.05 mM. (Sigma Chemical Company, St. Louis, MO). Samples used were diluted at 5ul to 5000ul to fall within 5% of the initial reading. A 0.02 mM concentration of Fluorescein (Sigma Chemical Company, St. Louis, MO) was used as the fluorescent material and all samples were prepared in  $\text{KH}_2\text{PO}_4$  buffer with a pH of 7. The cytoflour ( Perspective Biosystems, Framingham, MA) parameters were set at an emission of 565 nm and an excitation at 540 nm. Samples were read every 5 minutes for 45 minutes.

### Statistical Analysis

Each experiment was replicated twice with duplicate samples run for each analysis. Analysis of Variance (ANOVA) was conducted to determine significant difference between treatments for each of the assays performed. An alpha level  $p < 0.05$  was used in the analysis. Treatment differences were determined using Tukey's Test (SAS, Cary, NC)

## **RESULTS AND DISCUSSION**

### Total Phenols for Juices and Wines

The content of total and individual phenolics in wines can be influenced by practices such as hot and cold pressing and fermentation into wine. Differences in processing techniques can affect antioxidant levels in juices and wines. Figure 1 shows the total phenols for Carlos and Noble juices and wines. Carlos samples showed a significantly lower phenolic content compared to Noble samples with the exception of noble free run juice. The free run juice was collected before pressing; therefore phenolics contained in the pomace were not sufficiently released. Even though Noble hot pressed juices was not significantly higher than Noble free run juice, it has been shown that the application of heat can increase the phenolic content (Auw et al., 1996). Our study showed an increase of approximately 43% in total phenols due to the application of heat. Noble wine samples showed the highest level of phenols due to the fermentation process. This is expected since Carlos wine or white wine are obtained from pressed juice and the juice is not allowed to ferment on the skins.

### ORAC Values for Juices and Wines

The oxygen radical absorbance capacity (ORAC) indicates the same trend for Carlos and Noble samples (Figure 2). In this study the process of making Noble wine from juice involved the application of pressing and heating. These two processing methods used for Noble wine increased the phenolic content and ORAC value compared to Carlos samples. Talcott and Lee (2002) observed the same trend in phenolic content and ORAC values for muscadine juices and wines fermented in a similar manner. However it is important to note that in our study, the Noble hot pressed juice obtained an

antioxidant capacity that was not significantly different from that of the fermented Noble wine.

#### Total Phenols for Dried Pomace

Drying of wine by-products may be an essential step in the processing of grape pomace into a nutraceutical product. For the drying conditions studied (convection and vacuum) there was no significant difference in the two drying conditions for Carlos and Noble grapes. Figure 3 shows the total phenol values for convection and vacuum dried pomaces dried at 37 C, 49 C, and 60 C. For the temperatures studied, pomaces processed at 60 C showed the highest level of phenols although not significantly different from the other temperatures. It has been reported that phenolic antioxidants exhibit significant decomposition at temperatures above 60 C (Larrauri et al., 1997). Makkar and Singh, 1991 reported a decreased content of total proanthocyanidins in cassava and Leucaena leaves when heated to 90 C for 24 hours. In this study a temperature of 60 C may have caused degradation of phenolic compounds that were present. Polyphenolic compounds are very unstable compounds and their degradative reactions start as soon as the grape is crushed or pressed and continues throughout the wine making process and aging (Waterhouse and Ebler, 1998). Noble grapes (convection and vacuum) showed a significantly higher phenolic content compared to Carlos grapes (Figure 4). Noble grapes have been reported to have higher total phenol than Carlos due to the presence of anthocyanin pigments.

#### ORAC Values for Dried Pomace

The antioxidant capacity of dried pomaces is shown in figure 4. Results of the ORAC assay indicated no significant difference between Carlos and Noble dried

pomaces (Figure 5). In this study pomaces were extracted using methanol and water (80:20). Extractions using methanol and water may not have been as effective at extracting bound phenolic compounds from the pomace as solvents containing acid. Bound polyphenolic compounds, present as glucosides have been released using acid hydrolysis or alkaline hydrolysis or both methods (Goldberg, 1995). In this study pomace did not undergo any type of hydrolysis. This may explain the insignificant difference for ORAC values among Carlos and Noble pomace extracts. Even though there are numerous extraction methods for phenolic compounds from plant material, an optimized method for extraction of a variety of phenolic compounds is lacking (Matilla et al., 2002).

It can be concluded that at higher temperatures (60 C), there is a greater retention of total phenols resulting in a greater expression of antioxidant activity as indicated by ORAC values. However the presence of polyphenol oxidases may decrease the amount of antioxidants present. Therefore at the temperature used in this experiment (37 C, 49 C, and 60 C), the polyphenol oxidase may not have been deactivated quick enough to prevent degradation of polyphenols.

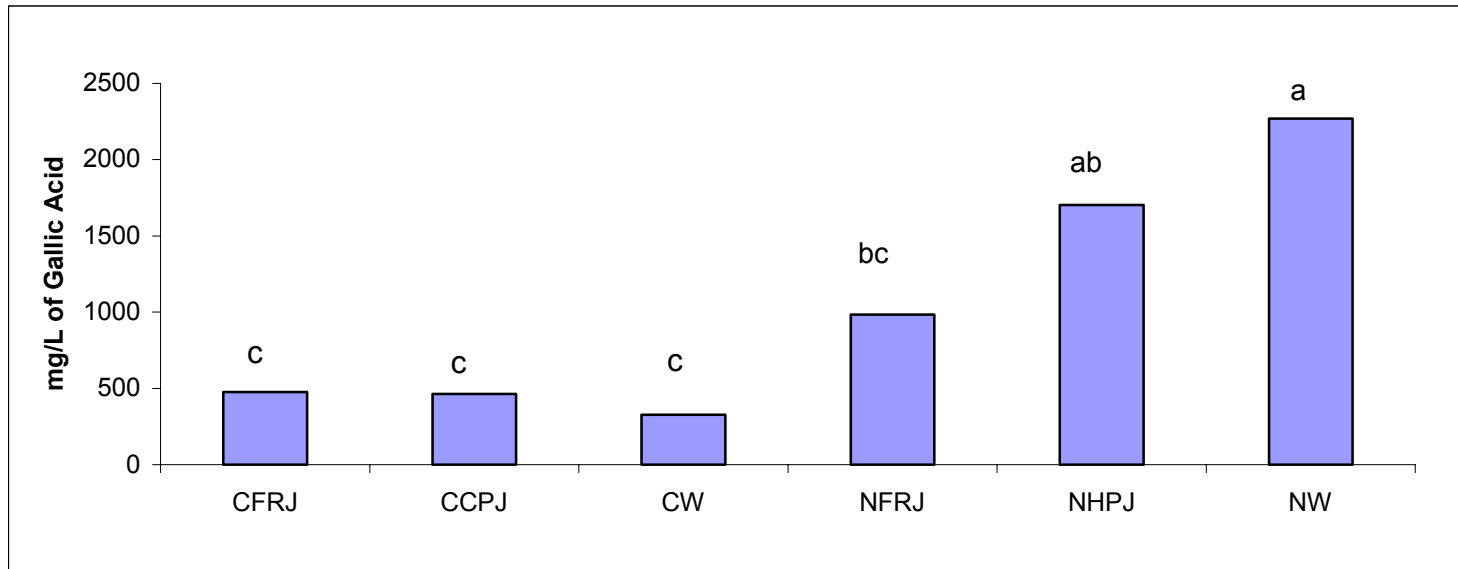
Further studies comparing the variety and ratio of solvents used to extract phenolic compounds from grape and plant material needs to be examined. Different classes of phenolic compounds may be readily extracted using one solvent compared to another. Seeds of grapes were shown to yield higher phenolics when extracted using ethyl acetate, which is a food grade solvent compared to methanol or ethanol (Foo, 1999). Extraction of grape pomace using ethyl acetate and water selectively extracts proanthocyanidins at least 2 times higher than using acetone, methanol and water (Foo,



1999).

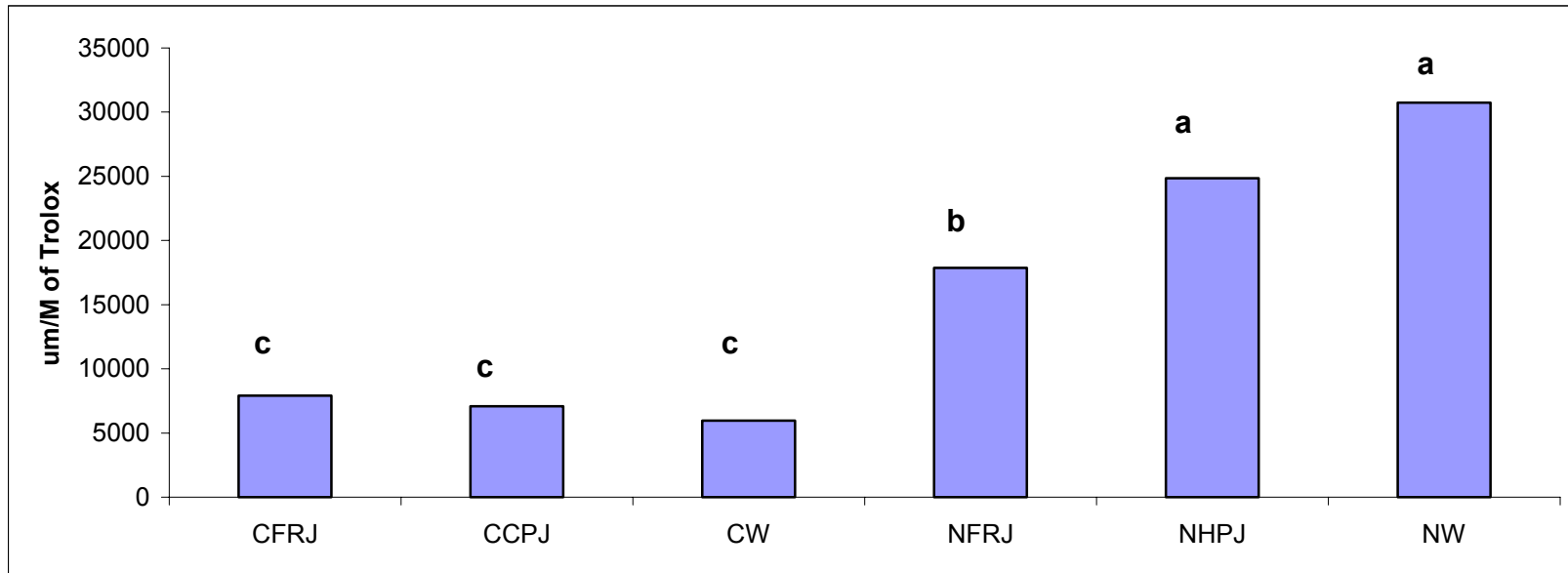
Noble hot pressed juice and noble wines showed no significant differences for their ability to scavenge free radicals. Therefore the non alcoholic drinking population, including children, young adults and others can consume the juice without the potential ill effects of alcohol. The other major advantage of the hot processing is the saving on processing cost associated with the fermentation and conversion of the juice to wine.

Figure 1. Total Phenols for Muscadine Juices and Wines



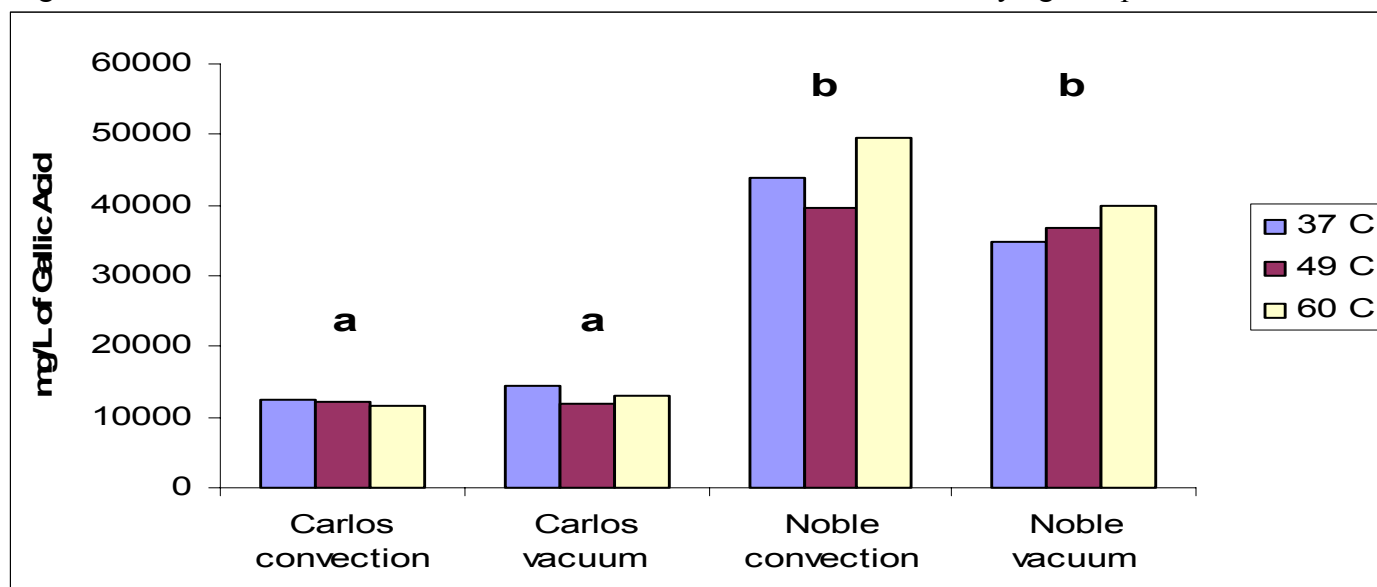
CFRJ= Carlos Free Run Juice; CCPJ= Carlos Cold Press Juice; CW= Carlos Wine  
NFRJ= Noble Free Run Juice; NHPJ= Noble Hot Press Juice; NW= Noble Wine  
Means with the same letter were not significantly different at  $p < 0.05$

Figure 2. ORAC of Muscadine Juices and Wines



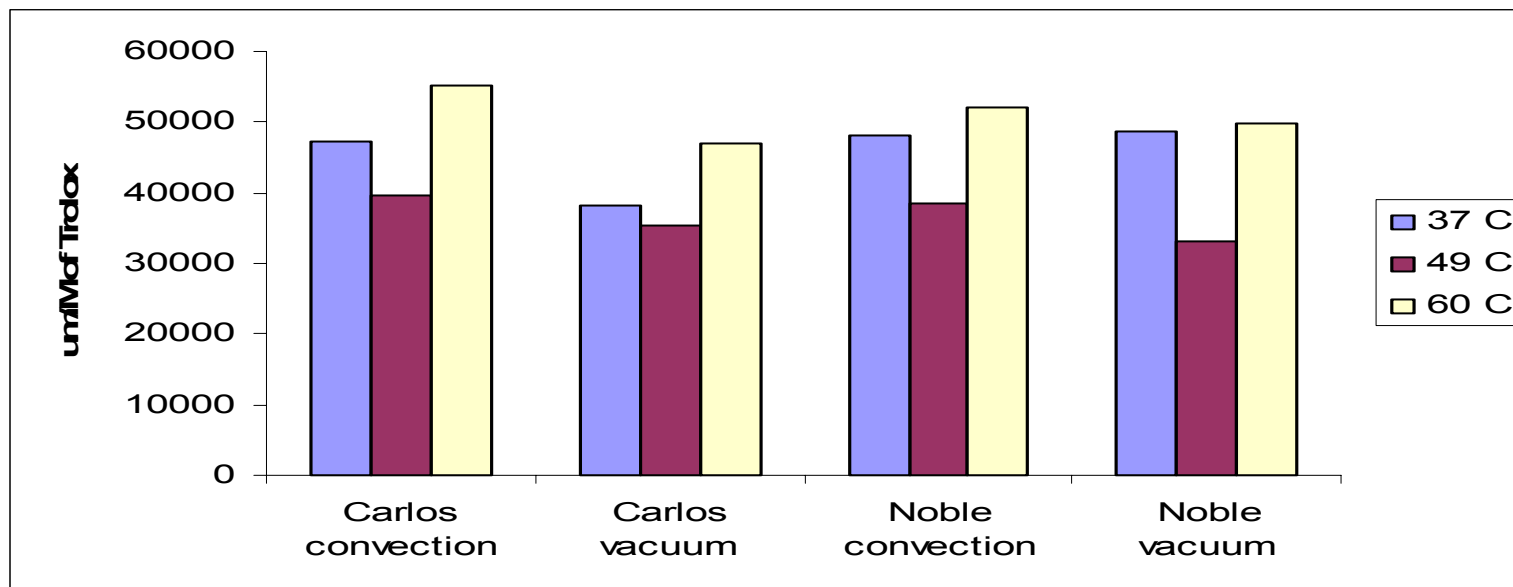
CFRJ= Carlos Free Run Juice; CCPJ= Carlos Cold Press Juice; CW= Carlos Wine  
NFRJ= Noble Free Run Juice; NHPJ= Noble Hot Press Juice; NW= Noble Wine  
Means with the same letter were not significantly different at  $p < 0.05$

Figure 3. Total Phenols for Carlos and Muscadine Dried Pomace at Three Drying Temperatures



Means with the same letter were not significantly different at  $p < 0.05$

Figure 4. ORAC of Carlos and Noble Dried Pomace produced at Three Drying Temperatures



Means were not significantly different at  $p < 0.05$

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## ABSTRACT

### **VAUGHAN, TRIVETTE LASHUM. Antioxidant Properties of Muscadine Extracts in a Lipid Model System (Under the direction of Dr. Leon Boyd)**

Carlos grape skin, seed and delipidized seed extracts were analyzed to determine their antioxidant activity in a safflower oil model system. Carlos grape skin and seeds were dried at 60 C and extracted using ethyl acetate and water. Grape seeds were delipidized using the soxlet extraction method. Three levels (60, 180 and 300 milliequivalents of gallic acid) of extract were tested in an oil model system using the oxidative stability instrument (OSI) and conjugated diene, to measure levels of antioxidant activity. The antioxidant activity of Carlos grape extracts were compared to that of tert-butyl hydroquinone (TBHQ) and propyl gallate (PG). Analysis using OSI showed that seed extracts in ethyl acetate at a level of 300 milliequivalents of gallic acid was significantly higher than TBHQ and PG. OSI values for water extracts were not significantly different from TBHQ and PG. Conjugated diene values for the ethyl acetate extract showed that the extract was not effective in preventing oxidation. By contrast, the water extracts were somewhat effective in reducing the formation of conjugated dienes. Addition of both extracts into the oil model system using OSI index indicated increasing antioxidant activity with increasing concentration of extracts added. The ethyl acetate extracts contained antioxidant activity equivalent to or greater than commercial antioxidants TBHB and PG when added at higher levels, whereas the addition of water extracts showed limited increases in antioxidant activity with increases in concentration.



## INTRODUCTION

### **Antioxidant Properties of Muscadine Extracts in a Lipid Model System**

Oxidation of the lipid components in foods by the free radical chain reaction of lipids is a major problem in the food industry. The extent of lipid oxidation in foods depends on the chemical structure of the fatty acids, food processing methods, and the temperature at which the foods are cooked and or stored. The addition of antioxidants to foods that contain fats and oils is one of the most efficient ways to prevent oxidation. (Tian and White, 1994). The stability or resistance of foods to oxidation will also depend on the antioxidants present in the food (Aruoma, 1996). Many food antioxidants such as tert-butyl hydroquinone (TBHQ), propyl gallate (PG), butylated hydroxytoluene (BHT) and hydroxyanisole (BHA) are chain-breaking inhibitors of lipid oxidation. Though commercial antioxidants are highly effective in preventing oxidation in lipid containing foods, questions have been raised as to their safety. Some antioxidants such as TBHQ have been banned from use in selected European markets whereas others such as BHT and BHA have been questioned due to their possible connections with cancer (Nobuji, 2000). The use of these compounds is increasingly limited out of safety considerations, and their replacement by natural antioxidants is being advocated (Aeschbach et al., 1994).

Food manufacturers are interested in natural antioxidants to act as replacements for the synthetic antioxidants currently used by the industry to control the formation of off flavors and rancidity, especially in fat based products (Aeschbach et al., 1994; Decker and Xu, 1998). The utilization of natural antioxidants from various plant materials has been studied by numerous investigators (Murcia et al., 2001; Du and Yen, 1997; Tian and

White, 1994; Chen et al., 1998; and Kaitaranta, 1992). The ground or aqueous extracts of cereals and oilseeds has been shown to prevent lipid oxidation at room temperature as well as at elevated temperatures. (Tian and White, 1994). This research concluded that oat flour increased the stability of oils, fats, margarine and mayonnaise.

Oat extracts that were placed in a soybean model system showed that the oak antioxidant provided better protection in the dark at accelerated room temperature when compared to TBHQ. (Tian and White, 1996). Peroxide values of cottonseed oil treated with 0.005%, 0.02%, and 0.03 of oat extract and 0.02% of TBHQ stored at 30 C in the dark over 35 days were also studied. The induction period of the oils that contained oak extracts at all levels was much longer than was the induction period of the control (Tian and White, 1994).

Of the many natural sources of antioxidants, seeds contain a variety of polyphenolic compounds that traditionally has been shown to be effective in protecting the lipids within the seed from oxidation (Murcia and Martinez- Tome, 2001). These polyphenolic compounds include flavonoids, phospholipids, phenolic acids, stilbenes, catechins and others (Chen et al., 1998).

Resveratrol is a phenolic compound of the stilbene family that is present in wines and various parts of the grape, including the skin and seed. Murcia and Martinez-Tome, 2001 compared the antioxidant activities and prooxidant activities of resveratrol with other antioxidants, BHT, BHA, PG, sodium tripolyphosphate (TPP), alpha tocopherol and vanillin. The ability of these compounds to inhibit lipid peroxidation was as follows: BHA>resveratrol>PG>TPP>vanillin>phenol>BHT> alpha tocopherol.

Several Mediterranean and tropical fruits have been analyzed in order to assess their antioxidant capacity compared with that of commercial food additives (BHA, BHT, and PG).

Some of the fruits studied were plum, apricot, white grape, red grape, apple, watermelon and orange. Mediterranean and tropical fruits showed very good scavenger activity against hydroxy radicals. Refined olive oil was used to examine the protective effect of tropical fruits using the Rancimat method. Watermelon showed a significantly ( $p < 0.05$ ) greater protection compared to the other fruit (Murcia et al., 2001). However, PG was the most effective ( $p < 0.05$ ).

The objective of this research was to study the antioxidant activity of Muscadine grape extracts (seed, skin and delipidized seed) when placed in an oil model system compared to commercial antioxidants TBHQ and PG. As the Carlos cultivar represents the most popular type of muscadine grape used to make wines, it was chosen for study because of its abundance as a raw material having the potential to become an asset or a liability to the wine industry. A second objective was the application of a food grade solvent extraction system to test the potential usefulness of our extracts in a lipid-containing food.

## MATERIALS AND METHODS

Carlos grape skin and seed were dried at 60 C for 24 hr in a drying oven (Fisher Scientific Isotemp Oven Model 630 G, Pittsburgh, PA). Approximately 10 grams of Carlos skin and seed was extracted using ethyl acetate and water (150:150). The mixture was filtered using cheesecloth and separated out into two phases: ethyl acetate and water. Each portion was filtered again by vacuum filtration. The ethyl acetate phase was evaporated using a rotary evaporator (Buchi Rotavapor (R), Switzerland) at less than 40 C. The sample was then redissolved in 25 ml of ethyl acetate. The water phase was freeze-dried and redissolved in 25 ml of water. The same procedure was carried out using 10 grams of dried Carlos grape seeds that were delipidized using the Soxlet extraction method (Wilkes et al., 2000).

### Total Phenols

Each extract was analyzed for total phenols using the Folin-Ciocalteu method as described by Amerine and Ough, 1980. Using gallic acid as a reference standard, aliquots representing 60, 180 and 300 milliequivalents of gallic acid were then added to each treatment. The commercial standards TBHQ and PG were kept constant at 200 ppm which was equivalent to 60 milliequivalents of gallic acid.

### Conjugated Dienes

Grape extracts at levels of 60, 180 and 300 milliequivalents of gallic acid were added to 5g of tocopherol stripped safflower oil (Sigma Chemical Company, St. Louis, MO). Tert-butyl hydroquinone (TBHQ) and propyl gallate (PG) were added at a level of 60 milliequivalents per liter of gallic acid. Samples were vortexed (Fisher Brand Vortex

Genie 2, Pittsburgh, PA) and placed in a drying oven (Fisher Scientific Isotemp Oven Model 630 G, Pittsburgh, PA) at approximately 60 C. Samples were removed at day 0, 2, and 4 for measurement of conjugated dienes. An aliquot of 200  $\mu$ l was removed and placed in 1 ml of isooctane and vortexed. Absorbance was measured at 234 nm using a 260 UV-Visible Recording Spectrophotometer (Schimadzu Scientific Instruments, Norcross, GA). The concentration of samples was based on the standard (+) -9-hydroxyoctadecadienoic acid (HODE) (Sigma Chemical Co., St. Louis, MO) as described by Frankel and Huang, 1996.

#### Oxidative Stability Index

The skin, seed and delipidized seed were added at 60, 180 and 300 milliequivalents of gallic acid to 5 grams of safflower oil. Commercial antioxidants PG and TBHQ were added at 60 milliequivalents per liter of gallic acid. The temperature of the OSI was set at 100 C and oxygen was bubbled through the mixture at 8 psi. The Folin-Ciocalteu method (Amerine and Ough, 1980) was conducted on all extracts to determine total phenols which were then used to determine aliquots added to each model system. Sixty milliequivalents of extract was found to be equal to the addition of 200 ppm of PG and TBHQ. The onset of oxidation was recorded and the antioxidant index was calculated as the ratio of induction time of the samples/control.

#### Statistical Analysis

The entire experiment was replicated three times. Analysis of Variance (ANOVA) was conducted to determine significant difference between treatments for each of the assays performed. An alpha level of  $p < 0.05$  was used in the analysis. Treatment differences were determined using Tukey's Test (SAS, Cary, NC).

## RESULTS AND DISCUSSION

### Conjugated Dienes

Tables 1, 2 and 3 show the (HODE) concentrations of conjugated dienes for ethyl acetate extracts added at 60, 180 and 300 milliequivalents of gallic acid. Tert-butylhydroquinone (TBHQ) and propyl gallate (PG) were used at a concentration of 60 milliequivalents of gallic acid or 200 ppm. Tocopherol stripped safflower oil was used as the control. Results at level one (60 milliequivalents of gallic acid) (Table 1) showed that at day 0 all samples were statistically the same except for the seed extract which had a higher HODE concentration. At day 2 there was an increase in HODE concentration for all samples. The seed extracts and commercial antioxidants were not significantly different from the control at day 2. The same pattern was observed at day 4 with the exception of the seed samples, which were significantly higher than the other samples. At level one there was no antioxidant effect seen in the grape pomace extracts. Similar patterns were observed through tables 1, 2, and 3 in that none of the ethyl acetate treatments were as effective as the commercial antioxidants TBHQ and PG in preventing the oxidation of the safflower oil model system

Tables 4, 5 and 6 show the (HODE) concentrations of conjugated dienes for water extracts added at 60, 180 and 300 milliequivalents of gallic acid. The commercial antioxidants (TBHQ) and (PG) were used at a concentration of 60 milliequivalents of gallic acid.

Extracts at Day 0, level 1 (Table 4) showed no significant difference between treatments. At day 2 grape extracts showed some effect when compared to the control and were statistically the same as TBHQ and PG. At day 4, only the skin extract was as

effective as PG. Level 2 (Table 5) showed the same pattern as for day 0 and day 4, there was no significant difference among the samples and the control. At day 2 samples were significantly lower than the control. Extracts at 300 milliequivalents of gallic acid showed no significant difference at day 0. At day 2 all samples were significantly lower than the control. In some instances Carlos grape extracts were just as effective as the two commercial antioxidants TBHQ and PG.

### Antioxidant Index

The antioxidant value is a ratio of induction time of the treatment/control. Tables 7 and 8 show the OSI value and antioxidant index values for the 3 levels of grape extract added. The addition of ethyl acetate extracts (Table 7) showed the highest OSI time and antioxidant index value for seed extracts at 300 milliequivalents of gallic acid which was significantly higher than TBHQ and PG. As the concentration of grape extracts increased, the OSI induction time increased as well.

Ethyl acetate extractions (table 7) had higher OSI values and antioxidant index values than to water-extracted samples (Table 8). The same trend was observed for the grape seed and delipidized seed. The seed extracts for ethyl acetate and water extracts showed higher induction times than delipidized seed and skin extracts. Ethyl acetate seed extracts showed a significant increase in OSI induction time (Table 7) as the concentrations were increased. Delipidized seed and skin extracts showed slight increases in induction time between levels but were not significantly different from each other at levels 1 and 2. Lipid material from seeds via soxhlet extraction, phenolic compounds such as ellagic acid and catechins may have been removed with the lipid material. This

trend was also seen with the water extracts (Table 8) although the increases were not significant.

### Discussion

For the assays studied, conjugated dienes and OSI induction time the trends were not the same. Conjugated diene data showed that water extracts were more effective antioxidants than ethyl acetate extracts. Data from OSI induction time indicated that ethyl acetate extracts were more effective than water extracts. In the oil model system the water extracts were not soluble in the oil. This created an oil system surrounded by water, which contained the phenolic antioxidants. These phenolic compounds were able to prevent oxidation because they were located on the interface of the lipid system. Because ethyl acetate is soluble in oil, ethyl acetate-soluble antioxidants became part of the oil; therefore the phenolic compounds were dispersed within the oil system. This prevented the phenolic compounds from interacting at the interface of the air-oil system and contributed to the formation of conjugated dienes. Extracts that were analyzed using OSI induction time were subjected to a more stressful environment compared to extracts used to measure conjugated dienes. Water extracts placed on OSI were subjected to additional heat and oxygen. The additional heat and oxygen may have evaporated and precipitated the water-soluble antioxidants from the oil. The phenolic compounds present in the water were no longer able to prevent oxidation at the interface of the air-oil system. This could explain why the water extracts in the conjugated diene assay (60 C) were more effective compared to the OSI assay (100 C). The addition of ethyl acetate extracts in the OSI assay was more effective than in the conjugated diene assay. The additional heat and oxygen could have allowed for the separation of the ethyl acetate-soluble antioxidants to



be present at the air-oil interface providing a layer of protection not seen with the water-soluble antioxidants.

Negi et al., 2003 studied the antioxidant activities of pomegranate peel extracted using methanol, ethyl acetate and water. Water extracts showed less antioxidant activity compared to the other extracts. Strong antioxidant activities were seen using ethyl acetate at levels of 50 and 75 µg/ml concentrations. The study also concluded that all extracts showed an increase in antioxidant activity with an increase in dose except water extracts. In the study, as levels of extracts were increased so did the antioxidant index values for samples extracted using ethyl acetate. A similar trend was also observed in the case of freeze-dried olive extracted in methanol and water (McDonald et al., 2001).

Proanthocyanidins such as catechin and epicatechin are quite soluble in ethyl acetate according to Bourzeiz et al., (1986). This could explain the higher antioxidant index values obtained with ethyl acetate compared to water extracts. The yield of proanthocyanidins was 2.3 times higher using ethyl acetate than using acetone, methanol and water. The study concluded that ethyl acetate and water selectively extracts proanthocyanidins from grape skins and seeds. The extraction of grape pomace yielded compounds such as quercetin, catechin, gallic acid and kaempferol (Foo, 1999). The antioxidant activity shown by pomegranate and olive peels was due to the presence of polyphenols that include ellagic acid, tannins and gallic acid (Gil et al., 2000). The overall results of the ethyl acetate extracts in this study showed that grape pomace may be used as bio preservatives in food applications and nutraceuticals. Future studies will identify the specific compounds responsible for the antioxidant activity and their relative

free radical scavenging activity using the oxygen radical absorbance capacity (ORAC) assay.

**Table 1: Concentration <sup>\*\*</sup> (µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Ethyl Acetate at Level 1\*\*\***

	<b>Day 0</b>	<b>Day 2</b>	<b>Day 4</b>
<b>Safflower Oil (Control)</b>	<b>1.87* <sup>a</sup></b>	<b>4.98 <sup>bcd</sup></b>	<b>3.27 <sup>c</sup></b>
<b>TBHQ #</b>	<b>1.89 <sup>a</sup></b>	<b>3.03 <sup>c</sup></b>	<b>3.45 <sup>c</sup></b>
<b>PG #</b>	<b>2.10 <sup>a</sup></b>	<b>2.90 <sup>c</sup></b>	<b>2.84 <sup>c</sup></b>
<b>Seed***</b>	<b>3.79 <sup>b</sup></b>	<b>5.16 <sup>d</sup></b>	<b>4.21 <sup>d</sup></b>
<b>Delipidized Seed***</b>	<b>2.47 <sup>a</sup></b>	<b>5.57 <sup>d</sup></b>	<b>4.47 <sup>d</sup></b>
<b>Skin***</b>	<b>2.30 <sup>a</sup></b>	<b>4.04 <sup>d</sup></b>	<b>3.39 <sup>c</sup></b>

**\*Means with the same letter are not significantly different at p<0.05**

**\*\*Hydroxyoctadecadienoic acid (HODE) equivalents**

**\*\*\*60 milliequivalents of Gallic Acid**

**# 60 milliequivalents of Gallic Acid**

**Table 2: Concentration\*\* (µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Ethyl Acetate at Level 2\*\*\***

	Day 0	Day 2	Day 4
Safflower Oil (Control)	1.87* <sup>a</sup>	4.98 <sup>d</sup>	3.27 <sup>c</sup>
TBHQ #	1.89 <sup>a</sup>	3.03 <sup>c</sup>	3.45 <sup>d</sup>
PG #	2.10 <sup>a</sup>	2.90 <sup>c</sup>	2.84 <sup>c</sup>
Seed***	2.95 <sup>b</sup>	4.33 <sup>d</sup>	5.34 <sup>e</sup>
Delipidized Seed***	2.90 <sup>b</sup>	4.63 <sup>d</sup>	4.40 <sup>d</sup>
Skin***	2.77 <sup>b</sup>	4.02 <sup>d</sup>	4.03 <sup>d</sup>

\*Means with the same letter are not significantly different at  $p < 0.05$

\*\*Hydroxyoctadecadienoic acid (HODE) equivalents

\*\*\*180 milliequivalents of Gallic Acid

# 60 milliequivalents of Gallic Acid

**Table 3: Concentration\*\* (µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Ethyl Acetate at Level 3\*\*\***

	Day 0	Day 2	Day 4
Safflower Oil (Control)	1.87* <sup>g</sup>	4.98 <sup>bc</sup>	3.27 <sup>de</sup>
TBHQ #	1.89 <sup>g</sup>	3.03 <sup>ef</sup>	3.45 <sup>de</sup>
PG #	2.10 <sup>fg</sup>	2.90 <sup>ef</sup>	2.84 <sup>efg</sup>
Seed ***	3.00 <sup>ef</sup>	6.06 <sup>a</sup>	4.15 <sup>cd</sup>
Delipidized Seed ***	2.97 <sup>ef</sup>	5.27 <sup>ab</sup>	4.11 <sup>cd</sup>
Skin ***	3.24 <sup>de</sup>	5.35 <sup>ab</sup>	4.93 <sup>bc</sup>

\*Means with the same letter are not significantly different at  $p < 0.05$

\*\*Hydroxyoctadecadienoic acid (HODE) equivalents

\*\*\*300 milliequivalents of Gallic Acid

# 60 milliequivalents of Gallic Acid

**Table 4: Concentration\*\* (µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Water Acetate at Level 1\*\*\***

	Day 0	Day 2	Day 4
<b>Safflower Oil (Control)</b>	<b>1.87*<sup>f</sup></b>	<b>4.98<sup>a</sup></b>	<b>3.27<sup>bc</sup></b>
<b>TBHQ #</b>	<b>1.89<sup>f</sup></b>	<b>3.03<sup>bcde</sup></b>	<b>3.45<sup>b</sup></b>
<b>PG #</b>	<b>2.10<sup>f</sup></b>	<b>2.90<sup>cde</sup></b>	<b>2.84<sup>cde</sup></b>
<b>Seed ***</b>	<b>1.86<sup>f</sup></b>	<b>3.11<sup>bcde</sup></b>	<b>3.33<sup>bc</sup></b>
<b>Delipidized Seed ***</b>	<b>1.83<sup>f</sup></b>	<b>2.93<sup>cde</sup></b>	<b>3.15<sup>bcd</sup></b>
<b>Skin ***</b>	<b>1.71<sup>f</sup></b>	<b>2.71<sup>de</sup></b>	<b>2.62<sup>e</sup></b>

**\*Means with the same letter are not significantly different at p<0.05**

**\*\*Hydroxyoctadecadienoic acid (HODE) equivalents**

**\*\*\*60 milliequivalents of Gallic Acid**

**# 60 milliequivalents of Gallic Acid**

**Table 5: Concentration\*\* (µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Water at Level 2\*\*\***

	<b>Day 0</b>	<b>Day 2</b>	<b>Day 4</b>
<b>Safflower Oil (Control)</b>	<b>1.87*<sup>a</sup></b>	<b>4.98<sup>c</sup></b>	<b>3.27<sup>b</sup></b>
<b>TBHQ #</b>	<b>1.89<sup>a</sup></b>	<b>3.03<sup>b</sup></b>	<b>3.45<sup>b</sup></b>
<b>PG #</b>	<b>2.10<sup>a</sup></b>	<b>2.90<sup>b</sup></b>	<b>2.84<sup>b</sup></b>
<b>Seed ***</b>	<b>2.32<sup>a</sup></b>	<b>2.84<sup>b</sup></b>	<b>3.94<sup>b</sup></b>
<b>Delipidized Seed ***</b>	<b>2.18<sup>a</sup></b>	<b>2.97<sup>b</sup></b>	<b>3.41<sup>b</sup></b>
<b>Skin ***</b>	<b>2.02<sup>a</sup></b>	<b>3.40<sup>b</sup></b>	<b>2.93<sup>b</sup></b>

**\*Means with the same letter are not significantly different at p<0.05**

**\*\*Hydroxyoctadecadienoic acid (HODE)**

**\*\*\*180 milliequivalents Gallic Acid**

**# 60 milliequivalents of Gallic Acid**

**Table 6: Concentration <sup>\*\*</sup>( µg/ml) of Conjugated Dienes in Safflower Oil Extracted with Water at Level 3\*\*\***

	<b>Day 0</b>	<b>Day 2</b>	<b>Day 4</b>
<b>Safflower Oil (Control)</b>	<b>1.87* <sup>a</sup></b>	<b>4.98 <sup>c</sup></b>	<b>3.27 <sup>ab</sup></b>
<b>TBHQ #</b>	<b>1.89 <sup>a</sup></b>	<b>3.03 <sup>ab</sup></b>	<b>3.45 <sup>ab</sup></b>
<b>PG #</b>	<b>2.10 <sup>a</sup></b>	<b>2.90 <sup>ab</sup></b>	<b>2.84 <sup>ab</sup></b>
<b>Seed ***</b>	<b>2.39 <sup>ab</sup></b>	<b>3.23 <sup>ab</sup></b>	<b>4.40 <sup>c</sup></b>
<b>Delipidized Seed ***</b>	<b>2.64 <sup>ab</sup></b>	<b>3.11 <sup>ab</sup></b>	<b>3.50 <sup>ab</sup></b>
<b>Skin ***</b>	<b>2.60 <sup>ab</sup></b>	<b>3.77 <sup>d</sup></b>	<b>4.04 <sup>d</sup></b>

**\*Means with the same letter are not significantly different at p<0.05**

**\*\*Hydroxyoctadecadienoic acid (HODE) equivalents**

**\*\*\*300 milliequivalents of Gallic Acid**

**# 60 milliequivalents of Gallic Acid**



**Table 7. Antioxidant Index for Ethyl Acetate Extractions in an Oil Model System**

	<b>OSI (hours)*</b>	<b>Antioxidant Index **</b>
<b>Safflower Oil (Control)</b>	<b>3.40</b>	<b>1.00<sup>f</sup></b>
<b>TBHQ</b>	<b>13.16</b>	<b>3.87<sup>cd</sup></b>
<b>PG</b>	<b>10.04</b>	<b>2.95<sup>d</sup></b>
<b>Seed—60 ppm</b>	<b>9.18</b>	<b>2.7<sup>d</sup></b>
<b>Seed—180 ppm</b>	<b>16.93</b>	<b>4.98<sup>b</sup></b>
<b>Seed—300 ppm</b>	<b>24.71</b>	<b>7.26<sup>a</sup></b>
<b>Delipidized Seed—60 ppm</b>	<b>4.03</b>	<b>1.19<sup>ef</sup></b>
<b>Delipidized Seed—180 ppm</b>	<b>5.93</b>	<b>1.74<sup>ef</sup></b>
<b>Delipidized Seed—300 ppm</b>	<b>9.5</b>	<b>2.79<sup>d</sup></b>
<b>Skin—60 ppm</b>	<b>4.25</b>	<b>1.25<sup>ef</sup></b>
<b>Skin—180 ppm</b>	<b>6.30</b>	<b>1.85<sup>e</sup></b>
<b>Skin—300 ppm</b>	<b>13.08</b>	<b>3.84<sup>c</sup></b>

<sup>a-f</sup> Means with the same letter are not significantly different at p<0.05.

\* Oxidative Stability Index

\*\*Milliequivalents of Gallic Acid

**Table 8. Antioxidant Index for Water Extractions in an Oil Model System**

	<b>OSI (hours)*</b>	<b>Antioxidant Index **</b>
<b>Safflower Oil (Control)</b>	<b>3.40</b>	<b>1.00<sup>f</sup></b>
<b>TBHQ</b>	<b>13.16</b>	<b>3.87<sup>cd</sup></b>
<b>PG</b>	<b>10.04</b>	<b>2.95<sup>d</sup></b>
<b>Seed—60 ppm</b>	<b>6.00</b>	<b>1.76<sup>de</sup></b>
<b>Seed—180 ppm</b>	<b>7.10</b>	<b>2.08<sup>cd</sup></b>
<b>Seed—300 ppm</b>	<b>8.30</b>	<b>2.44<sup>c</sup></b>
<b>Delipidized Seed—60 ppm</b>	<b>5.40</b>	<b>1.59<sup>de</sup></b>
<b>Delipidized Seed—180 ppm</b>	<b>5.50</b>	<b>1.62<sup>de</sup></b>
<b>Delipidized Seed—300 ppm</b>	<b>6.70</b>	<b>1.97<sup>cde</sup></b>
<b>Skin—60 ppm</b>	<b>5.63</b>	<b>1.66<sup>de</sup></b>
<b>Skin—180 ppm</b>	<b>5.28</b>	<b>1.55<sup>e</sup></b>
<b>Skin—300 ppm</b>	<b>5.10</b>	<b>1.50<sup>e</sup></b>

<sup>a-f</sup> Means with the same letter in common are not significantly different at p<0.05.

\*Milliequivalents of Gallic Acid

\*\*Oxidative Stability Index

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