INHIBITION OF HUMAN ALDOSE REDUCTASE BY HIGH PHENOLIC AND HIGH ANTI-OXIDATIVE EXTRACTS OF MUSCADINE GRAPE FRACTIONS

by

LAUREN SNIPES

(Under the Direction of Diane K. Hartle)

ABSTRACT

Muscadines are berries of *Vitis rotundifolia*, a grape native to SE USA. Muscadine skins and seeds are sold as nutraceutical products because of their high antioxidant activity, anti-inflammatory and anti-cancer phytochemical profiles. Hyperglycemia leads to both induction and activation of aldose reductase (AR), the initial rate-limiting enzyme of the polyol pathway (conversion of glucose to sorbitol to fructose). Activation of the polyol pathway contributes to Advanced Glycation Endproduct (AGE) formation in organs with AR activity including lens of the eye, kidney, heart, nerves and liver. This pathway is implicated in diabetic cataract formation and neuropathies. Because the pharmacognosy of muscadine skins revealed many compounds with anti-AR activity, we tested the ability of muscadine skin and seed extracts to inhibit human recombinant AR (HRAR) *in vitro*. The results indicated that extracts of muscadine skins and seeds inhibited AR activity at low concentrations. Nutraceutical products made from muscadine skins and seeds may prove useful in inhibiting AGE formation via inhibition of the polyol pathway.
INDEX WORDS: Diabetes, diabetic complications, polyol pathway, aldose reductase, aldose reductase (AR) inhibitors, muscadine grape, antioxidant, total phenolic content, ferric reducing antioxidant power (FRAP)
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CHAPTER 1

INTRODUCTION

ALDOSE REDUCTASE AND THE POLYOL PATHWAY

Aldose reductase (AR) is a small monomeric protein that is a member of the aldo-keto reductase superfamily. It is composed of 315 amino acid residues and has many similarities to another NADPH-dependent oxidoreductase, aldehyde reductase. Aldose reductase is a widely expressed aldehyde-metabolizing enzyme present in most mammalian cells. The enzyme is located in the cytoplasm of most cells, but is often located in different cell types of a specific organ. For example, AR is highly expressed in the lens as compared to other tissues in the body [1, 2]. In the lens, aldose reductase is located only in the epithelium, while in the kidney it is contained in Henle's loop, collecting tubules, outer and inner medulla, but not in the cortex [3]. The distribution of aldose reductase from tissue to tissue also varies considerably. Aldose reductase exhibits broad substrate specificity, in that it reduces glucose as well as aromatic and aliphatic aldehydes. The sugar galactose actually serves as a better substrate for aldose reductase than glucose. Due to aldose reductase's greater affinity for galactose than glucose, increased levels of galactose are more rapidly reduced to galactitol (dulcitol) than glucose to sorbitol. Galactitol is not oxidized by sorbitol dehydrogenase; therefore intracellular levels of galactitol remain high and cause a greater hyperosmotic effect than glucose. The tissues in which
galactitol accumulates have a similar cellular pathology to those in diabetic tissues and galactosemic animal studies are important in studying the relationship between aldose reductase and diabetic complications [4, 5].

The polyol pathway was first discovered in the seminal vesicle by Hers in 1956. Hers was able to demonstrate the conversion of blood glucose to fructose as an energy source of sperm cells [2]. In 1959, Van Heyningen reported that sorbitol could be found in the diabetic rat lens [6]. Van Heyningen's research lead to recognition of the importance of the polyol pathway in the development of diabetic complications [2]. The polyol pathway is an alternate pathway for glucose metabolism and converts glucose to fructose using two enzymes, aldose reductase and sorbitol dehydrogenase, Figure 1.1. Aldose reductase is the rate-limiting enzyme in this pathway and it catalyzes the conversion of glucose to sorbitol using NADPH as its cofactor [2, 3]. Sorbitol is then oxidized to fructose by sorbitol dehydrogenase which utilizes NAD$^+$ as its cofactor [5].

**Polyol Pathway**

![Polyol Pathway Diagram](image)

**Figure 1.1** Polyol Pathway

Diabetes involves a high oxidative stress state with increased production of toxic aldehyde compounds and elevated blood glucose levels. Aldose reductase
is an important factor of hyperglycemic induced metabolic changes that lead to the development of secondary diabetic complications [7]. Although much research has been done in the past few years, the mechanism by which aldose reductase contributes to the development of diabetic complications remains uncertain.

There is an increased expression of aldose reductase under hyperosmotic stress. The increased flux of glucose through the polyol pathway directly increases the activity of aldose reductase, but reactive oxygen species indirectly activate aldose reductase as well [8]. Aldose reductase competes with hexokinase for the utilization of glucose in many tissues. Under normal physiological conditions hexokinase has a greater affinity for glucose than aldose reductase. In this circumstance glucose is phosphorylated into glucose-6-phosphate by hexokinase, which then is subject to glycolysis [2, 4]. Only 3% of glucose enters the polyol pathway under normal physiological conditions. However, under hyperglycemic conditions, hexokinase becomes saturated by elevated levels of glucose and it is forced into the polyol pathway. Hyperglycemia causes more than 30% of glucose to go into the polyol pathway. When glucose is forced into the polyol pathway there is an increase in the production of sorbitol. However, under hyperglycemic conditions sorbitol is produced more rapidly than it can be converted to fructose resulting in an accumulation of sorbitol [2, 3]. The intracellular accumulation of sorbitol is enhanced due to its polarity and cannot penetrate through the cell membranes. The accumulation of sorbitol in the cell produces a hyperosmotic effect which
results in an influx of fluids [4, 5]. This accumulation of sorbitol has been thought to cause osmotic vascular damage and lead to diabetic complications. Another obstacle resulting from the utilization of glucose through the polyol pathway is the rapid depletion of NADPH and NAD$^{+}$, which causes a change in redox state and metabolic imbalances [2, 7].

In the lens, the upsurge of sorbitol causes osmotic swelling which results in redox imbalance and protein insolubilization leading to cataract formation. The accumulation of sorbitol in the lens initiates hyperosmotic swelling which triggers the loss of membrane permeability and leakage of amino acids, glutathione, and myoinositol [3]. Water is drawn into the lens fibers due to the accumulation of sorbitol which then causes the lens to swell. The swelling increases the permeability to substances that are at a higher concentration in the lens than in the surrounding intraocular fluids. Therefore, concentrations of potassium, amino acids, glutathione, inositol, and ATP decrease, and sodium and chloride ion concentrations start to increase. This results in the lens membranes becoming permeable to all substances except the larger proteins. Under normal physiological conditions there is a continual growth of the lens within the eye. This normal lens growth is stunted as observed in cases of sugar cataracts due to a decrease in protein synthesis. Generally a high potassium/sodium environment is suitable for protein synthesis in the lens, however, in sugar cataracts the intracellular concentration of potassium is lowered and sodium is increased. This slows the process of protein synthesis in the lens accounting for the diminished new growth. Aldose reductase inhibitors can prevent or moderate
the complications of diabetic cataracts. Aldose reductase inhibitors can be administered orally, by injection, or as eye drops to diabetic patients. Efficacy of the aldose reductase inhibitors depends on the administration at the early stages of cataract formation and the strength of the inhibitor [3, 5].

In other target organs of diabetic complications, the depletion of cofactors NADPH and NAD$^+$ is thought to cause metabolic imbalances. The increased flux of glucose through this pathway under hyperglycemic conditions imposes a significant strain on the NADPH supply [2, 7]. NADPH is utilized for many reductive metabolic steps such as the detoxification of reactive oxygen species (ROS) and hyperoxides as seen in Figure 1.2 [9]. A large depletion of NADPH could affect the ability of the cell to protect itself from oxidative stress. Glutathione reductase also needs NADPH for glutathione to remain in the reduced form and with the increased use of the polyol pathway during hyperglycemia it is unable to do so. Glutathione reductase is an antioxidative enzyme; therefore the lack of NADPH may produce oxidative stress by inhibiting the reduction of oxidized glutathione [7, 9]. The depletion of NADPH and glutathione results in indirect formation of advanced glycation endproducts (AGEs) through the increased formation of oxidative stress [10]. The depletion of NADPH and NAD$^+$ would also decrease the generation of nitric oxide in endothelial cells leading to redox imbalances. The depletion of NADPH slows the production of nitric oxide from L-arginine by nitric oxide synthase. This causes the release of nitric oxide to be reduced and leads to the slowing of nerve conduction [2, 8].
Diabetes mellitus has become a growing epidemic that affects nearly 21 million Americans today. There are two major types of diabetes that affect most people. Insulin-dependent diabetes mellitus (IDDM) is known as type 1 diabetes and is an autoimmune disease that results in the destruction of pancreatic beta-cells and insulin deficiency in the patient. Insulin is the storage and anabolic hormone of the body that is responsible for allowing target tissues to take up glucose [11]. Type 1 diabetes usually develops during childhood and only accounts for 5-10% of all diagnosed cases of diabetes [12]. The cause of type 1 diabetes is not completely understood however, environmental factors which could trigger the initiation of pancreatic beta-cell destruction are thought to play a role as well as some genetic factors. Type 2 diabetes, also known as noninsulin-
dependent diabetes, is the most common form of the two types of diabetes. It accounts for 90-95% of all cases of diagnosed diabetes [12]. Type 2 diabetes is a progressive disease in which a person gradually develops a resistance to insulin [13]. In type 2 diabetes, insulin is produced by the beta-cells, but there are not enough functional insulin receptors to take up glucose for use by the cells. The actual cause of type 2 diabetes is unknown, but diet, lifestyle, and genetic factors are thought to play a role. Type 2 diabetes can develop at any age and obesity is common in patients with this type of diabetes. While the direct cause of diabetes is still unknown, there are many symptoms that can indicate one is developing diabetes. These symptoms include frequent urination, excessive thirst, extreme hunger, unusual weight loss, increased fatigue, irritability, and vision problems [11, 14].

Diabetes mellitus is a widespread disease and is one of the leading causes of blindness, kidney failure, heart attack, and amputation [15]. If left untreated, diabetes can cause many complications such as cardiovascular disease, hypertension, dental disease, retinopathy, cataracts, renal disease, neuropathies, diabetic ketoacidosis and amputations [16]. Because of their insulin-insensitivity, organs such as the lens, retina, nerves and kidney are target organs for these diabetic complications [17]. These complications arise from chronic hyperglycemia which causes oxidative stress in tissues and results in damage to blood vessels and peripheral nerves [7]. There are several well-researched theories addressing how hyperglycemia actually initiates the complications of diabetes. However, for this study we have chosen to focus on the aldose
reductase (polyol pathway) theory. This theory proposes that under hyperglycemic conditions, there is an elevated level of intracellular glucose which causes an increased flux of glucose through the polyol pathway. This results in an intracellular accumulation of sorbitol and fructose that causes hyperosmotic stress [1, 7, 18]. The excess glucose and fructose binds to –NH$_2$ groups on proteins to produce fructosamines and advanced glycation end products (AGEs). AGEs accumulate during the normal aging process, however, they build up to a greater extent during diabetes [19]. The formation of AGEs is in both intracellular and extracellular compartments in multiple organs. AGEs accelerate the aging of blood vessels and cells within organs which leads to major diabetic problems such as retinopathy, nephropathy, and neuropathy [20].

**STRUCTURE OF ALDOSE REDUCTASE**

The intracellular accumulation of sorbitol is thought to cause the hyperosmotic stress and cellular damage that leads to diabetic complications [5]. Therefore, aldose reductase inhibitors have been developed to help prevent and treat these diabetic complications. However, to date a successful aldose reductase inhibitor has not been developed. Aldose reductase inhibitors are developed so that they are able to prevent the reduction of glucose to sorbitol therefore reducing diabetic complications [15]. Over the years much research has been done to create an ideal aldose reductase inhibitor; however this has been extremely difficult due to the lack of knowledge about the enzyme itself. Most of the compounds developed as aldose reductase inhibitors are non-specific and tend to inhibit aldehyde reductase as well as aldose reductase. Developers want to avoid
inhibiting aldehyde reductase because it is an important enzyme that reduces aldehydes which my react with proteins and damage cells. Aldehyde reductase also plays a role in the excretion of drugs and the detoxification of 3-deoxyglucose [1]. Designing an aldose reductase inhibitor specific to aldose reductase remains difficult due to the structural and functional similarity of aldose reductase and aldehyde reductase [3, 15].

Research continues on the enzyme aldose reductase and crystallographic structures for human aldose reductases have even been determined. These images show that aldose reductase has a tertiary structure very similar to that of aldehyde reductase. These structures also show that aldose reductase has a \((\beta/\alpha)_8\) barrel structural motif with a large hydrophobic active site. The active site of the enzyme is located at the center of the barrel and is where the cofactor NADPH binds. The inhibitor binds to the active site on top of the nicotinamide ring of NADPH. Previous research with inhibitors show that compounds with different chemical structures can interact with aldose reductase in different conformations. This flexibility of the enzyme structure makes it hard for researchers to use a theoretical approach in predicting how inhibitors will bind to aldose reductase. Researchers have also found a ‘specificity pocket’ on the enzyme in which inhibitors that are more effective against aldose reductase than aldehyde reductase bind [2]. However, a specific aldose reductase inhibitor that does not interfere with other compounds in the target organs has not been discovered.
ALDOSE REDUCTASE INHIBITORS

Aldose reductase inhibitors may be useful agents to prevent and reduce the effects of diabetic complications. Due to their potential benefits many aldose reductase inhibitors have been developed by pharmaceutical companies. Compounds such as flavonoids, benzopyrans, spirohydantoins, alkanoic acids, alkaloids, nonsteroidal anti-inflammatory agents, quinines, and oxamines of aromatic aldehydes have all been proven to show some aldose reductase inhibition [3]. Therefore aldose reductase inhibitors can be categorized into six main classes:

1. Compounds containing five-membered cyclic imides
2. Carboxylic acid derivatives
3. Phenolic compounds
4. Aryl sulfonyl nitromethanes
5. Amino acid derivatives
6. Other compounds

Sorbinil was one of the first promising ARIs to be developed by the pharmaceutical company Pfizer. Sorbinil is a cyclic imide (spirohydantoin) compound that was thought to improve nerve conduction velocity in diabetic patients. Research done on diabetic rats proved that sorbinil could lower glomerular polyol accumulation and normalize the concentration of sorbitol in the lens through oral administration [21, 22]. However, when tested in humans sorbinil only produced modest results with no significant improvements. Sorbinil also had an adverse effect of a hypersensitivity reaction in the early weeks of
therapy [2, 15]. Fidarestat and minalrestat were two ARIs that were derived from sorbinil. While these compounds were more potent than sorbinil they were ineffective and their adverse effects outweighed their benefits. Imirestat was another cyclic imide used for aldose reductase inhibition and was able to normalize sorbitol levels in the nerve, retina and lens tissues. Despite its effectiveness, imirestat was discontinued due to its toxicity [15].

The carboxylic acid derivatives are another class of ARIs that have been studied extensively. Compounds in this group have a flexible carboxylic acid moiety which is an important factor in the interaction with aldose reductase in physiological conditions. Alrestatin is one of the more well known inhibitors of this group and proved to be stable when given orally. In initial studies alrestatin proved to be somewhat effective in diabetic patients suffering from neuropathy. However in later trials alrestatin was not as effective as previously determined [3, 15].

Epalrestat was another carboxylic acid derivative that showed initial positive results. Epalrestat was tested in patients with diabetic neuropathy and was able to normalize the symptoms of peripheral neuropathy while showing few adverse effects. Epalrestat also showed positive effects on vascular smooth muscle cells under hyperglycemic conditions. Epalrestat is the only ARI on the market today, although it is only marketed in Japan by Ono Pharmaceutical [15]. Ponalrestat was developed as an ARI and while it showed promising effects in vitro, it failed to show enough effectiveness in clinical trials. The clinical trials showed that ponalrestat could not penetrate the nerve at the appropriate doses.
Figure 1.3: Structures of selected synthetic aldose reductase inhibitors, Tolrestat and Epalrestat
**Figure 1.4:** Structures of selected synthetic aldose reductase inhibitors, Zenarestat and Sorbinil
and was found to have no significant effect in double-blind placebo-controlled studies [8]. Tolrestat was an ARI developed by Wyeth Ayerst to help treat and prevent diabetic neuropathy. Tolrestat was an orally administered drug that was 6 times more potent than alrestatin. However it showed only modest improvements in human diabetic neuropathy and had numerous side effects such as hepatic necrosis, dizziness and rash. Therefore Tolrestat was removed from the market in 1996 [2, 15].

Zenarestat is an ARI in the carboxylic acid derivative group that again showed promise but its clinical trials were halted due to the toxic effects it produced in patients. Zenarestat was an ARI that was developed by Pfizer to treat diabetic neuropathy. Zenarestat showed therapeutic effects at high doses of 1200 mg/day, however treatment at these doses also caused renal toxicity in patients [15]. Zopolrestat was another ARI that was developed by Pfizer to treat nephropathy, neuropathy, and cardiomyopathy. Zopolrestat's clinical trials were terminated after producing little change in nerve fiber density of diabetic patients. While this seems to be the most extensively studied group of ARIs, there have not been any marketable ARIs in the United States to come out of this group. There is great theoretical potential for ARIs having a flexible carboxylic acid moiety; however in clinical trials they proved to be less active than the cyclic imides [15].

Flavonoids are the most widely studied natural products group of ARIs. Flavonoids are derived from natural substances such as plants and show great promise as ARIs [23]. They have proven to be effective ARIs and are thought to
be less toxic than the synthetic compounds developed as ARIs. There are several sub-groups of flavonoids that are studied for their aldose reductase inhibitory potential such as flavonols, flavanones, ellagic acids, flavanonols, isoflavones, and chlacones. Flavonoids are polyphenolic compounds which consist of fifteen carbon atoms and a chromane ring that is connected to a second benzene ring [15].

Flavonoids are known for their potent antioxidant activity. They were initially discovered as ARIs in 1975, when they were found to show AR activity against rat lens aldose reductase [24]. Quercetin is one of the most studied flavonoid inhibitors of aldose reductase. Quercetin has been shown to decrease the accumulation of sorbitol in the lens. Previous studies with rat lens aldose reductase and human lens aldose reductase proved that quercetin had IC\textsubscript{50} values of 5 x 10^{-6} M [24, 25]. However quercetin is poorly absorbed when administered orally [15]. The glycosides of quercetin also showed good aldose reductase inhibitory activity in rat lens. Isoquercitrin (quercetin 3-O-glucoside) had 45 percent inhibition at 1 x 10^{-6} M and hyperoside (quercetin 3-O-galactoside) had an IC\textsubscript{50} of 1 x 10^{-6} M [24, 25]. Quercitrin is a flavonol which appears to be more potent than quercetin. While quercitrin is not considered a selective ARI, it did show much higher inhibition against aldose reductase than aldehyde reductase [1]. \textit{In vitro} studies using rat lens aldose reductase showed quercitrin as a potent inhibitor having an IC\textsubscript{50} of 1 x 10^{-7} M [24]. Flavonols guaijaverin and desmanthin-1 also have aldose reductase inhibition similar to that of quercitrin. Desmanthin-1 studies showed that it had aldose reductase
inhibition equivalent to that of the synthetic ARI, epalrestat [26]. Myricetin also showed good aldose reductase inhibition, having 55 percent inhibition at $1 \times 10^{-5}$ M [24]. Isoflavones, flavan-3-ols and stilbenes were tested for inhibition against aldose reductase, however they were not as potent as the flavones, flavonols and flavanone compounds tested [15, 26]. Chalcones are another type of flavonoids that have been examined as potential ARIs. Different chalcone compounds have been proven to decrease the accumulation of sorbitol in red blood cell, sciatic nerves and the lens. 3, 4, 2’, 4’-tetrahydroxychalcone was found to be the most effective ARI of the chalcones [1]. Ellagic acid is another well known ARI that has shown high inhibitory activity against aldose reductase [23]. The aldose reductase inhibitory effects of ellagic acid were tested in vitro using isolated erythrocyes, lens and sciatic nerve. This study showed that ellagic acid had an IC$_{50}$ of $2.4 \times 10^{-6}$ M, while epalrestat, a known synthetic aldose reductase inhibitor, had an IC$_{50}$ of $4.2 \times 10^{-6}$ M. There was also an in vivo study done on ellagic acids ability to inhibit aldose reductase in the lens and sciatic nerve. This study proved that ellagic acid had significant inhibitory activity similar to that of epalrestat [17]. Due to the extensive research done on flavonoids as ARIs, it has been shown that factors such as structural features, electronic environment and the position of the hydroxyl and other substituents can affect the potency of the flavonoids as ARIs. Therefore others have been able to determine structural requirements for ARIs. In previous studies it has been determined that flavones and flavonols having hydroxyl groups at the meta or para positions of the phenyl ring show greater aldose reductase inhibitory
Figure 1.5: Structure of selected subgroups of flavonoids, the flavonols and anthocyanins
activity. Flavonoids have great potential to be effective ARIs, however, they do have one drawback in that they are poorly absorbed through oral administration. Since many flavonoids are highly water soluble they cannot diffuse through cellular membranes under physiological conditions. However, some compounds such as phenolic aglycones can passively diffuse through cellular membranes with ease due to their hydrophobic properties [15, 26].

The aryl sulfonyl nitromethanes and the amino acids are two groups of ARIs that have not been researched as extensively as the first three groups of ARIs. An aryl sulfonyl nitromethane that has been investigated for aldose reductase inhibition is IC 222155. This compound has been shown to prevent hyperalgesia in diabetic rats. N-substituted amino acids are another group of ARIs that have been recently studied as a type of aldose reductase inhibition. N-[4-(Benzoylamino)phenylsulfonyl]glycine is a selective ARI that has been investigated as a potential ARI. This compound is believed to be able to prevent diabetic complications of the eye such as cataracts and retinopathy. Studies of this compound have shown that it has unique aldose reductase binding sites and it selectively inhibits aldose reductase. However, research has shown that this compound does not exhibit the free carboxylate anion and therefore reduces its permeability and uptake in physiological conditions [15]. These two groups of ARIs show potential in being useful ARIs, but they lack the research regarding their potency and toxicity in humans.
CHEMISTRY OF THE MUSCADINE

The muscadine grape (*Vitis rotundifolia*) is a grape that is native to the southeastern United States and is known for thriving in the warm, humid climate which kills other species of grapes. The muscadines have a unique, thick skin that most other species of grape do not have; this thick skin allows the muscadine to grow under the harsh conditions [20, 27]. The muscadine is also different from other species of grapes in that it has 20 pairs of chromosomes, while the American and European species have only 19 pairs of chromosomes. This extra genetic information is responsible for the muscadine’s unique spectrum of phytochemicals [20]. The muscadine contains many powerful phytochemicals that are capable of fighting diseases such as diabetes, cancer, heart disease, and arthritis. Many recent studies have shown that free radicals are the main cause of degenerative and neurological diseases [28]. Research has shown that flavonoids are capable of donating hydrogen-atoms to peroxyl radicals [29]. The plant antioxidants act as oxygen quenchers, free radical scavengers, peroxide decomposers, and enzyme inhibitors [28]. Diabetes is a disease which causes the increased production of free radicals. The muscadines antioxidant effects help protect the tissues against free-radical damage [20]. Due to the muscadines wide spectrum of phytochemicals it is thought to be a potential ARI.

The muscadine contains high levels of anthocyanins, flavanols, flavonols, and ellagic acid [20]. The most abundant of the flavonoids in the muscadine are the anthocyanins. The polyphenolics, anthocyanins, flavonols, and phenolic acids
are the greatest contributors to the total antioxidant capacity [30]. The muscadine skin and seeds contain most of the phytochemicals that are important to the muscadine, however the pulp has a high concentration of vitamin C [20, 31]. The chemical constituents observed in the skin and seed are very different. Ellagic acid was the most abundant phenolic found in the skins followed by myricetin, quercetin, kaempferol, and trans-resveratrol [28]. Ellagic acid is a phenolic compound that is unique to the muscadine and is a potent ARI [20, 27]. The phenolics found in the seed were epicatechin, catechin, and gallic acid, with epicatechin being the most plentiful phenolic. Research shows that muscadine seeds have a higher antioxidant capacity than other parts of the fruit. This is thought to be due to the high levels of epicatechin and catechins found in the seeds [28]. Ultimately the muscadine was the chosen fruit for this project due to its numerous phytochemicals which are good ARIs. The muscadine contains ellagic acid, kaempferol, myricetin, myricetin glycosides, quercetin, quercetin glycosides, chlorogenic acid, and epicatechin, all of which inhibit aldose reductase [20].

As the antioxidant properties of the muscadine are being discovered many commercial vineyards have taken interest in developing muscadine food supplementation and nutraceutical products. The skins and seeds are not used in the juicing process; therefore many vineyards have leftover skins and seeds that could be used for nutraceutical products. Dried, powdered muscadine skin, seed and pomace can be encapsulated and taken as a food supplement. Since most of the antioxidants are found in the skins and seeds these capsules would
have a high nutraceutical value. These products would be beneficial in that they have a long shelf life and they are highly concentrated in phytochemicals. The muscadine products are being processed today to extract the highly concentrated antioxidants of the seed. This product can then be encapsulated and sold as a nutraceutical product. However these pills need to be standardized so that the consumer is assured the quality of the product. A good way to standardize these products would be to test them for their total phenolic and antioxidant contents. This would require two simple, inexpensive assays, the Folin-Ciocalteu total phenolic assay and the ferric reducing antioxidant power (FRAP) assay. However, many commercialized vineyards which are producing these products do not have a standardization method [20].

DETERMINATION OF TOTAL PHENOLICS

Phenolics are a group of secondary plant metabolites that are ubiquitous among the plant kingdom [32]. Phenolics are compounds that contain a phenol ring and there are more than 8,000 structures known [33]. Phenolics can be separated into two categories, flavonoids and non-flavonoids. Flavonoids are the most widely distributed group of phenolics and consist of flavanols (flavan-3-ols), flavonols, and anthocyanins. Flavanols include compounds such as simple monomeric catechins and proanthocyanidins. Flavanols are usually found in the skin and include compounds such as quercetin, myricetin and kaempferol. Anthocyanins are the most abundant flavonoids found in the muscadine and consist of cyanidins with a sugar molecule attached [20, 27]. Anthocyanins consist of compounds such as cyanidin 3-O-glucoside [20, 34]. The
nonflavonoids include hydroxycinnamic acids (e.g. caffeic acid), benzoic acids (e.g. gallic acid), hydrolysable tannins (e.g. ellagitannins), and stilbenes (e.g. reseveratrol) [34]. Recent studies have shown that polyphenols have antioxidant and free-radical scavenging properties [35]. Many of the antioxidants found in food are phenols [36]. Due to their antioxidant activity phenolics are capable of ultraviolet protection, pigmentation, disease resistance and nodule production [32]. The daily intake for phenolics ranges from 20 mg to 1 g [33]. Since phenolics have many antioxidant functions it is important to find a reliable method that can measure them accurately.

The Folin-Ciocalteu Micro Method for Total Phenolics was used to determine the total phenolic content of the samples tested in this project. This method is based on the Slinkard and Singleton total phenol method [37], however the volumes have been reduced. The Folin-Ciocalteu method is the most commonly used method for measuring total phenolics [38]. Other methods for total phenolic determination include high-performance liquid chromatography (HPLC), permanganate titration, colorimetry with iron salts and ultraviolet (UV) absorbance. Due to the numerous phenolic compounds the isolative method of HPLC is difficult to apply and results are difficult to interpret [36]. The permanganate titration method is not ideal because it is difficult to standardize and subject to interferences from sugars. The method of colorimetry with iron salts is not as accurate because monophenols usually do not react, and vicinal diphenols and vicinal triphenols turn different colors. The UV method also has problems such as interference from other compounds that absorb at similar
wavelengths. The Folin-Ciocalteu method is a much more simpler method that produces comparable and reproducible results [36]. The Folin-Ciocalteu assay is a colorimetric method that uses conventional spectrophotometric detection at a wavelength of 765 nm. The method is based on the oxidation of polyphenolic compounds with phosphomolybdic and phosphotungstic acids in a basic medium. This results in a color change and the resulting color intensity is proportional to the concentration of polyphenols. The total phenolic values are derived from a calibration curve that is made using a series of standard solutions [38]. Many compounds can be used to create the standard solutions including tannic acid, gallic acid, catechins, tyrosine and others. Gallic acid is the most commonly used standard because it is inexpensive, soluble in water and stable in dry form [36]. The Folin-Ciocalteu assay is reproducible and reliable, which makes it an ideal method to standardize nutraceutical products in the muscadine industry.

**DETERMINATION OF TOTAL ANTIOXIDANT POWER**

There is an increasing interest in antioxidant activity in foods. Antioxidants play an important biological role in the body in that they defend against reactive oxygen species (ROS). ROS are harmful byproducts made during cell aerobic respiration that cause oxidative damage to lipids, proteins and nucleic acids [39, 40]. ROS have been known to play a role in diseases such as coronary artery disease, diabetes and cancer. Antioxidants are capable of preventing the formation of radicals, scavenging them, or promoting their decomposition [41]. Therefore antioxidants in the diet may help maintain an adequate antioxidant
status to prevent or stop the destructive nature of ROS [39]. Due to the number of different antioxidants and their chemical diversity, it is difficult to measure individual antioxidants [39, 41].

There have been several methods developed for the measurement of total antioxidant capacity such as the oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and trolox equivalent antioxidant capacity (TEAC) [42]. These major antioxidant capacity assays can be divided into two categories, hydrogen atom transfer (HAT) based assays and single electron transfer (SET) based assays. [43]. The HAT-based assays measure an antioxidant’s ability to quench free radicals by hydrogen atoms [42]. This occurs when a peroxyl radical is generated and then a hydrogen atom is taken from an antioxidant [39]. HAT reactions are usually solvent and pH dependent, however some are temperature dependent. The SET-based assays involve a potential antioxidant that transfers one electron to reduce a compound [42]. While many assays have been developed to test antioxidant capacity, the ORAC and FRAP methods are two of the most commonly used methods.

The ORAC is a HAT-based assay that measures the antioxidant inhibition of peroxyl radical induced oxidations. The assay consists of a peroxyl radical that reacts with a fluorescent probe to form a nonfluorescent product. The antioxidant capacity is then calculated using the decreased rate and the amount of product formed over time [42]. A major drawback of this assay is that it is temperature sensitive and temperature control must be monitored throughout the assay plate.
This is important because even small temperature differences can decrease the reproducibility of the assay. Another drawback is that this assay requires specialized equipment for fluorescence that may not be available in many analytical labs [42].

The FRAP is a SET-based assay that uses antioxidants as reductants in a redox-linked assay. It involves the reduction of a ferric tripyridyltriazine (Fe$^{3+}$TPTZ) complex to ferrous tripyridyltriazine (Fe$^{2+}$) by a reductant at a low pH [40]. The ferrous form has an intense blue color which can be monitored at 593 nm [40]. The FRAP assay is a nonspecific reaction in that any half reaction with a lower redox potential than the ferric/ferrous half-reaction will force Fe$^{3+}$ to Fe$^{2+}$. The absorbance then is directly related to the reducing power of the antioxidants. The FRAP can be used to measure the total antioxidant capacity on a variety of samples such as biological fluids, aqueous as well as ethanolic extracts of drugs, food and plants [44].

The FRAP assay was the chosen method for the determination of total antioxidant capacity for this project. The FRAP assay was chosen because it is an inexpensive, simple, speedy and reproducible assay that does not require specialized equipment. Many other tests used today measure the ability of antioxidants in a sample to inhibit the oxidative effects generated in the reaction mixture. However many of these methods have a lag phase, which requires specialized equipment, and are extremely time-consuming. These methods also may not be as sensitive as the FRAP assay. The FRAP assay does not require pretreatment of samples or use a lag phase [44]. The FRAP assay can be
performed with automated, semi automated, or manual methods and does not require any specialized equipment [41, 42]. All of these aspects make the FRAP method ideal for commercialized vineyards to use as a standardization test for their nutraceutical products. Results have proven that the FRAP values correlate well with the total phenolic values measured by the Folin-Ciocalteu assay [45]. The FRAP assay is a sensitive, reproducible method for measuring the total antioxidant capacity.

There were three main objectives of this project that were studied and analyzed. The first objective was to test the hypothesis that based upon the known chemistry of the muscadine, muscadine extracts will have significant aldose reductase inhibition. The second objective was to create a training manual that could be used to train a non-scientist technician in the muscadine industry to do muscadine total phenolic and FRAP assays for quality control. The third objective was to determine if a direct correlation exists between the phenolic content and antioxidant values in processed muscadine fractions and products.
References


CHAPTER 2

INHIBITION OF HUMAN RECOMBINANT ALDOSE REDUCTASE BY MUSCADINE GRAPE EXTRACTS

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1 Snipes, Lauren, Phillip Greenspan, James L. Hargrove, Diane K. Hartle, to be submitted to the Journal of Agricultural and Food Chemistry
Abstract

Muscadines are berries of *Vitis rotundifolia*, a grape native to SE USA. Muscadine skins and seeds are sold as nutraceutical products because of their high antioxidant activity, anti-inflammatory and anti-cancer phytochemical profiles. Hyperglycemia leads to both induction and activation of aldose reductase (AR), the initial rate-limiting enzyme of the polyol pathway (conversion of glucose to sorbitol to fructose). Activation of the polyol pathway contributes to Advanced Glycation Endproduct (AGE) formation in organs with AR activity including lens of the eye, kidney, heart, nerves and liver. This pathway is implicated in diabetic cataract formation and neuropathies. Because the pharmacognosy of muscadine skins revealed many compounds with anti-AR activity, we tested the ability of muscadine skin and seed extracts to inhibit human recombinant AR *in vitro*. Ethanol was removed from a 1:2 (wt/v) 50% ethanolic extract of muscadine skins. Water was then removed by freeze-drying. A dose/response relationship for inhibition of AR was established using various dilutions of the dried muscadine skin and seed extracts. Complete AR inhibition for the muscadine skin extracts occurred at 625 and 1250 µg/mL with the IC$_{50}$ at approximately 35.3 and 66.9 µg/mL. Complete AR inhibition for the muscadine seed extract occurred at 625 µg/mL and the IC$_{50}$ at 105 µg/mL. Conclusion: Extracts of muscadine skins and seeds showed AR inhibition activity at low concentrations. Nutraceutical products made from muscadine skins and seeds may prove useful in inhibiting AGE formation via inhibition of the polyol pathway as well as by direct inhibition of glycation.
2.1 Introduction

Diabetes is a disease that affects over 250 million people worldwide and occurs when the body does not produce enough insulin or the cells are not able to take up glucose because of insulin resistance. Chronic hyperglycemia accelerates aging in many organs. Insulin-insensitive organs such as the lens, retina, nerves and kidney are particularly vulnerable target organs during chronic hyperglycemia. Complications include cataracts, retinopathy, neuropathy and nephropathy. Even with pharmacotherapeutic regimens, chronic hyperglycemia is difficult to control [1, 2]. In addition a far greater number of people are classified as pre-diabetic or metabolic syndrome patients. While not overtly diabetic these conditions also involve hyperglycemia. Therefore, dietary and drug strategies that can decrease the aging effects of hyperglycemia may be clinically useful.

One major target for controlling complications of hyperglycemic states is to decrease the ability of excess glucose to enter the polyol pathway. Aldose reductase (AR) is the key enzyme in the polyol pathway that catalyzes conversion of intracellular glucose to sorbitol, which is then oxidized to fructose by sorbitol dehydrogenase [3]. This net conversion of glucose into fructose constitutes the polyol pathway of glucose metabolism [4]. Under hyperglycemic conditions the polyol pathway accelerates the formation of sorbitol in insulin-insensitive tissues. In many tissues aldose reductase directly competes with hexokinase for the utilization of glucose [3]. Under normal physiological conditions most of the cellular glucose is phosphorylated into glucose-6-
phosphate by hexokinase and only 3% of glucose enters the polyol pathway. However, under hyperglycemic conditions, hexokinase is saturated by high levels of glucose which causes 30% of total glucose to enter the polyol pathway [5, 6]. This causes sorbitol to be formed more rapidly than it is converted to fructose, resulting in an overflow of polyol pathway products and a rapid depletion of NADPH [5, 7]. The intracellular accumulation of sorbitol occurs due to its poor penetration through cellular membranes and slow metabolism by sorbitol dehydrogenase. The accumulation of sorbitol and its metabolites result in osmotic swelling, loss of membrane integrity and eventual cataract formation [8, 9]. Furthermore, when the cell converts sorbitol to fructose for disposition, this leads to formation of Advanced Glycation Endproducts (AGEs). Aldose reductase inhibition may therefore decrease the rate of diabetic complications in organs that express aldose reductase.

Studies over the past several years have proved that AR plays a significant role in the development of the diabetic cataract, retinopathy, neuropathy and nephropathy. It is now well known that AR is the key enzyme in the polyol pathway and is responsible for catalyzing the conversion of glucose to sorbitol [10]. Hyperglycemic conditions lead to hyperosmotic stress in the cell. The accumulation of sorbitol leads to hydration of the cell, loss of membrane permeability and leakage of amino acids, glutathione and myoinositol [6]. While AR has been identified as a principal cause of these complications, an effective, non-toxic aldose reductase inhibitor (ARI) has not been developed. Many synthetic ARIs have been developed, however, they have been ineffective or
possess toxic effects. Therefore many researchers have now turned toward generally recognized as safe natural products such as flavonoids for ARIs [11].

Flavonoids are phytochemicals that have been widely studied and proven to have aldose reductase inhibition [1]. In recent studies, we test the ability of muscadine grape skin and seed extracts to inhibit aldose reductase. Muscadine grapes (Vitis rotundifolia) are grown commercially in the SE USA. Muscadines are a separate species of grapes and are distinguished by 20 pairs of chromosomes, instead of 19 pairs by Vitis vinifera (wine and table) grapes. The muscadine contains high levels of flavonoids, flavan-3-ols, flavonols, anthocyanins, anthocyanidins, oligomeric proanthocyanidins, proanthocyanidins, ellagic acid and ellagitannins which contribute to its high phenolic and antioxidant values. This extra genetic information is probably responsible for the unique spectrum of phytochemicals in muscadines. Many muscadine phytochemicals are individually known aldose reductase inhibitors, e.g., ellagic acid, kaempferol, myricetin, myricetin glycosides, quercetin, quercetin glycosides, chlorogenic acid and epicatechin [12]. These compounds exist in relatively high concentrations. Muscadine seeds and skins are currently being used for nutraceutical product manufacturing. The purpose of this study was to test the collective aldose reductase inhibition activity of extracts of muscadine skins and seeds.

2.2 Materials and Methods

**Chemicals and Reagents.** DL-glyceraldehyde, lithium sulfate, β-NADPH, quercetin, Folin-Ciocalteu reagent, gallic acid, sodium carbonate, sodium acetate, glacial acetic acid, TPTZ (2,4,6-tri[2-pyridyl]-s-triazine), ferric chloride,
and ferrous sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Human Recombinant aldose reductase (HRAR) was purchased from VWR International (West Chester, PA).

**Preparation of Muscadine Extracts.** The muscadine skin extract was a generous gift of R. Dixon Phillips (University of Georgia) and the muscadine seed powder was obtained from Muscadine Product Corporation, LLC (Wray, GA).

**Extract 1 (Muscadine skin extract with 50% EtOH).** Ethanol was removed from a 1:2 (wt/vol) 50% ethanolic extract of powdered muscadine skins. Water was then removed by freeze-drying. The dried muscadine skin was then prepared in a 10% (wt/vol) 50% ethanol extract which was then stirred for 2 h. The extract was then centrifuged to remove the precipitate.

**Extract 2 (Muscadine skin extract with H₂O).** Ethanol was removed from a 1:2 (wt/vol) 50% ethanolic extract of muscadine skins. Water was then removed by freeze-drying. A 10% (wt/vol) muscadine skin extract was prepared with the addition of water. The extract was then stirred for 2 h and then centrifuged to remove the precipitate.

**Extract 3 (Muscadine seed extract with 50% EtOH).** A sample of 1g of dried muscadine seeds was made into a powder using a commercial coffee grinder. Then a 1:5 dry wt/vol 50% EtOH extract was made and stirred for 2 h. The extract was then centrifuged to remove the precipitate.

**Determination of Total Phenolics.** The total phenolic content of the muscadine extracts were determined using the Folin-Ciocalteu assay described by Slinkard and Singleton [13], using gallic acid as a reference phenolic standard.
Absorbance was measured at 765 nm in a Beckman DU 600 series spectrophotometer. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight.

**Ferric Reducing Antioxidant Power (FRAP) Assay.** FRAP values were determined using a modified version of the Benzie and Strain method[14], using purchased ferrous sulfate as the reference standards. Absorption was measured at 593 nm on a Beckman DU 600 series spectrophotometer. The FRAP assay is used for the direct testing of antioxidant capacity and is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex to the ferrous form. The “antioxidant power” was determined at the absorbance of 593 nm after six minutes of incubation. The results were then expressed as mmol of ferrous sulfate formed/gram of dry wt. of grape skin or seed using a standard curve constructed with known ferrous sulfate. Results were expressed as millimoles of Fe$^{2+}$ equivalents per gram of dry weight.

**Enzyme Inhibitory Assay.** Aldose reductase activity was assayed using a Beckman DU 600 Series spectrophotometer by determining the decrease in the concentration of NADPH at 340 nm. The reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.2), 0.4 M lithium sulfate, 0.15 mM β-NADPH, 10 mM DL-glyceraldehyde, and 5 µL of human recombinant aldose reductase (HRAR) (VWR) in a total volume of 1.0 mL. The reaction was started by addition of the enzyme, and the initial reaction rate was measured for 5 minutes [15]. The reference blank contained all of the above except the substrate DL-glyceraldehyde. The effects of inhibitors were determined by adding them to the
reaction mixture. A dose-response curve was then constructed and the concentration of muscadine fraction necessary for 50% inhibition of HRAR activity (IC<sub>50</sub>) was estimated.

2.3 Results

**Total Phenolic Content and FRAP Values of Muscadine Skin and Seed Extracts.** The phenolic content of the muscadine extracts is shown in Table 1. Extracts 1-3 each contained high total phenolic concentrations (58.3, 24.8 and 37.9 mg/g, respectively). This study reveals a strong correlation between FRAP activity and total phenolics, found in Table 1. Extract 1 had the highest FRAP value of 26.3 mmol/g as well as the highest phenolic value. Extract 2 had the lowest FRAP value of 12.7 mmol/g as well as the lowest phenolic value. Extract 3 had a FRAP value of 18.4 mmol/g.

**Table 2.1:** Phenolic content and FRAP values of the muscadine extracts

<table>
<thead>
<tr>
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<th>Total Phenolic Content (mg/g)</th>
<th>FRAP Value (mmol/g)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µg of dry wt/ml)</th>
</tr>
</thead>
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<tr>
<td>Extract 1</td>
<td>58.3</td>
<td>26.3</td>
<td>35.3</td>
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<tr>
<td>(Muscadine skin extract w/ 50% EtOH)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extract 2</td>
<td>24.8</td>
<td>12.7</td>
<td>66.9</td>
</tr>
<tr>
<td>(Muscadine skin extract w/ H&lt;sub&gt;2&lt;/sub&gt;O)</td>
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<td></td>
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<tr>
<td>Extract 3</td>
<td>37.9</td>
<td>18.4</td>
<td>105</td>
</tr>
<tr>
<td>(Muscadine seed extract w/ 50% EtOH)</td>
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**Inhibitory Activity of Muscadine Skin and Seed Extracts.** The inhibitory activities of muscadine skins and seed extracts were evaluated and compared to an inhibition produced by purified quercetin, a standard polyphenolic that is a
known aldose reductase inhibitor. A dose/response relationship for inhibition of aldose reductase was established for extracts of both dried muscadine skins and seeds. Extract 1 proved to be the most potent inhibitor with an IC\textsubscript{50} of 35.3 µg/ml followed by Extract 2 and Extract 3 with IC\textsubscript{50} values of 66.9 µg/ml and 105 µg/ml, respectively, Figures 2.1, 2.2, 2.3. Extract 1 was prepared in 50% EtOH which for our purposes draws out the most phenolics. This preparation was then compared to the water extract (Extract 2), while this also was a skin preparation the water did not yield as many phenolics as the 50% EtOH extract. Lastly we tested a seed extract (Extract 3) to compare to the skin extracts. While this showed some inhibition it was not as effective as the skin extracts. Our results indicate that muscadine skin and seed extracts are effective AR inhibitors at low food supplement concentrations and can therefore be useful in hyperglycemic states to inhibit the polyol pathway.

2.4 Discussion

In this study, muscadine grape skin and seed extracts were studied as sources of aldose reductase inhibition activity. The muscadine has many AR inhibiting phytochemicals in its seeds and skins, therefore these parts of the muscadine were chosen to extract and test. While there is some overlap in the chemical composition of skin and seed fractions, there are also differences in the species of phenolics within each fraction. The skins have high levels of ellagic acid, reseveratrol complex and the aglycons of myricetin, quercetin, and kaempferol. The muscadine seed has high levels of catechins, epicatechin,
Inhibition of Aldose Reductase by Quercetin

IC50 = 3.68 µg/mL

Figure 2.1: Inhibition of HRAR by purified quercetin

Inhibition of Aldose Reductase by Muscadine Skin Extracts

IC50 = 35.3 µg/mL
IC50 = 66.9 µg/mL

Figure 2.2: Inhibition of HRAR by muscadine skin extracts
Inhibition of Aldose Reductase by Muscadine Seed Extract

![Graph showing inhibition of aldose reductase by muscadine seed extract.](image)

IC50 = 105 µg/mL

Figure 2.3: Inhibition of HRAR by muscadine seed extract

IC50 Concentrations of Muscadine Products on Aldose Reductase

<table>
<thead>
<tr>
<th>Product</th>
<th>IC50 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin Skin H2O Extract</td>
<td>3.68</td>
</tr>
<tr>
<td>Muscadine Skin 50% EtOH Extract</td>
<td>1.66</td>
</tr>
<tr>
<td>Muscadine Seed 50% EtOH Extract</td>
<td>2.08</td>
</tr>
<tr>
<td>Muscadine Seed 50% EtOH Extract</td>
<td>3.89</td>
</tr>
</tbody>
</table>

Figure 2.4: IC50 concentrations of extracts according to the total phenolics
ellagic acid, gallic acid and oligomeric proanthocyanidins (OPCs) [16].

All of the muscadine extracts showed potent AR inhibition with the muscadine skin with 50% EtOH (Extract 1) being the most potent inhibitor. The muscadine skin with H₂O extract (Extract 2) was the next most potent inhibitor followed by the muscadine seed with 50% EtOH extract (Extract 3). The IC₅₀ values of extracts 1, 2, and 3 were 35.3, 66.9, and 105 µg/mL respectively. After calculating their inhibition according to their total phenolic content the muscadine extracts proved to be slightly more powerful than quercetin, a known ARI, Figure 2.4.

Our results show that both muscadine grape skins and seeds have potent AR inhibitory activity. Since both are fractions of a GRAS (Generally Recognized as Safe) fruit that is without toxicity, the fractions may prove useful for nutraceutical and functional food/beverage products to help hyperglycemic patients. These nutraceutical products can then be used to prevent or delay the onset of diabetic complications. Unlike the history of synthetic ARIs, muscadine nutraceutical products are effective while being non-toxic to humans and can be easily obtained all year round.
2.5 References


CHAPTER 3

TECHNICAL MANUAL FOR ANTIOXIDANT AND TOTAL PHENOL CONTENT IN QUALITY CONTROL OF NATURAL PRODUCT PROCESSING

Introduction

The purpose of this project was to identify easy and economical assays that could be used by muscadine processors for onsite quality control of products. Many commercialized muscadine vineyards have started developing many new muscadine nutraceutical products. Many commercialized muscadine vineyards are processing grapes for juice and wine production and the seeds and skins for nutraceutical products. In developing and running these processing lines, there is a need for quality control assays to follow total phenolic and antioxidant capacity to insure a high quality product.

The seeds and skins of the muscadine possess most of the medicinal value and can be processed into food supplements. The skins and seeds can be dried, powdered and encapsulated. These products provide the consumer with a much more phytochemically concentrated product than a whole fruit. As vineyards produce these nutraceutical supplements from waste pomace, it is important that these products be monitored for quality control to optimize processing, storage conditions and shelf life.
The criteria for these assays were (1) economical, (2) simple, (3) fast. Two assays that meet these criteria are the Folin-Ciocalteu Micro Method for Total Phenolics and the Ferric Reducing Antioxidant Power (FRAP) assay.

The Folin-Ciocalteau phenolic assay is used to determine the total phenolics of samples such as muscadine skin or seed extract. The FRAP assay is an effective method to assess the total antioxidant power of a sample. The nutraceutical grade endproducts need to be standardized to total phenolic content and need to be high in antioxidant power. Dried muscadine skins, seeds and pomace powders and extracts of these fractions need to be tested for total phenolics and antioxidant power during processing and storage. These assays take relatively little time and are ideal for the multiple sampling necessary from batch to batch, fraction to fraction and for various dilutions of extracts.

The cost to start up a lab and run these assays is affordable by most commercial vineyards or product manufacturers. It is especially economical considering the high cost of commercial analytical laboratory analysis. Also the cost for necessary chemicals and equipment is relatively low making the maintenance cost of the lab fairly low. The cost for the equipment and chemicals needed to start up a lab can range between $2,000-10,000, depending on the equipment purchased. The spectrophotometer is the main piece of equipment that is needed and these can be ordered from VWR International from $4,000-8,000, however even more inexpensive spectrophotometers can be purchased either new or used in the $1,000-1,500 range. These simple machines may be fully adequate for small sample volume needs. The equipment needed to start
up a lab can be ordered from VWR International (www.vwr.com). Most of the equipment can be ordered online, however, the customer must fill out some company identification forms prior to ordering and this may take a couple of days before being approved. The chemicals for these assays can be ordered from Sigma-Aldrich (www.sigmaaldrich.com). Again, some forms must be completed by the company before ordering and this may take a couple of days for them to be approved.

**Total Phenolic Content Assay**

The Folin-Ciocalteu Micro Method for Total Phenolics is based on the Slinkard and Singleton method [1], but the volumes have been scaled down for this application. The Folin-Ciocalteu method is the most common method used for measuring total phenolics. The Folin-Ciocalteu method is a colorimetric method that uses conventional spectrophotometric detection at a wavelength of 765 nm. The method is based on the oxidation of polyphenolic compounds with phosphomolybdic and phosphotungstic acids in a basic medium. The oxidation results in a color change and the resulting color intensity is proportional to the concentration of polyphenols [2]. The Folin-Ciocalteu method is a reliable and reproducible method to use for quality control of muscadine nutraceutical products.

**Antioxidant Power Assay**

The FRAP method chosen for this manual is a modified version of the methods by Benzie and Strain [3]. The FRAP assay is used for the direct testing of antioxidant capacity and is based on the reduction of a ferric 2,4,6-tripyridyl-s-
triazine complex to the ferrous form. The ferrous form creates an intense blue color which can be monitored at 593nm. The absorbance then is directly related to the reducing power of antioxidants [3]. The FRAP assay was the chosen method for the determination of total antioxidant capacity for this project because it is an inexpensive, simple, speedy and reproducible assay that does not require specialized equipment. The FRAP assay can be performed with automated, semi-automated, or manual methods.

**Methods for Total Phenolic Content Assay**

The equipment and materials for this assay can be ordered online or by telephone from VWR International and Sigma-Aldrich. However, ordering online is very simple and it allows the customer to choose from a variety of products and gives a detailed description of each item.

**Supply Companies**

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<tr>
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</tr>
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<td><a href="http://www.vwr.com">www.vwr.com</a></td>
<td><a href="http://www.sigmaaldrich.com">www.sigmaaldrich.com</a></td>
</tr>
<tr>
<td>1-800-932-5000</td>
<td>1-800-521-8956</td>
</tr>
</tbody>
</table>

**Equipment and Supplies** (suggested supplies, VWR catalog number)

- UV/VIS spectrophotometer
- Set of plastic cuvettes (appropriate for the spectrophotometer chose)
- 6 – 100 mL volumetric flasks
1 – 10 mL graduated cylinder
1 – 100 mL graduated cylinder
1 – 1000 mL graduated cylinder
1 – 1000 mL beaker or volumetric flask
1 – 2-20 µL pipet (VWR digital pipettors, variable volume, 40000-250)
1– 20-200 µL pipet (VWR digital pipettors, variable volume, 40000-254)
1 – 1000 µL pipet (VWR digital pipettors, variable volume, 40000-256)
Filtering funnel (VWR analytical funnels, 30252-913)
Filter paper (VWR filter paper,18.5cm, 28310-140)
Parafilm (Parafilm M, 52858-000)

**Chemicals** (Sigma-Aldrich catalog number)

Gallic Acid (G-7384)

Ethanol, molecular biology 190 proof (E7148)

Sodium Carbonate (S1641)

Folin-Ciocalteu reagent, 2N (F9252)

Distilled water

**Methods**

**Gallic Acid Stock Solution**

Weigh 0.500 g of gallic acid and measure 10 mL of ethanol, and then dissolve the 0.500 g of gallic acid in the 10 mL of ethanol. Dilute this solution to 100 mL with distilled water, using a graduated cylinder. This solution will keep up to two weeks if refrigerated.
**Sodium Carbonate (Na₂CO₃) Solution**

Weigh 200 g of Na₂CO₃ and completely dissolve in 800 mL of distilled water.

Bring this solution to a boil, then let cool and add a few crystals of Na₂CO₃. Let this solution sit for 24 hours then filter and dilute to 1L with distilled water. This solution can also be kept at room temperature to keep it from precipitating out of solution.

**Preparation of Standards** (in concentrations of 0, 50, 100, 150, 250, and 500 mg/L)

- **0 mg/L standard stock solution** – distilled water, this will be used as the blank
- **50 mg/L standard stock solution** – measure 1 mL of the gallic acid solution and put into a 100 mL volumetric flask. Then dilute to volume (100 mL) with distilled water.
- **100 mg/L standard stock solution** – measure 2 mL of the gallic acid stock solution and put into a 100 mL volumetric flask. Then dilute to volume with distilled water.
- **150 mg/L standard stock solution** – measure 3 mL of the gallic acid stock solution and put into a 100 mL volumetric flask. Then dilute to volume with distilled water.
- **250 mg/L standard stock solution** – measure 5 mL of the gallic acid stock solution and put into a 100 mL volumetric flask. Then dilute to volume with distilled water.
500 mg/L standard stock solution—measure 10 mL of the gallic acid stock solution and put into a 100 mL volumetric flask. Then dilute to volume with distilled water.

1. After making these solutions, pipet 20 µL of each standard stock solution into separate cuvettes. Be sure to change pipet tips after transfer of each solution to avoid contaminating one solution with a more concentrated solution.

2. Next pipet 1.58 mL of water into each cuvette. This can be done using the 1000 µL pipet by transferring 1000 µL and 580 µL to each cuvette.

3. Next add 100 µL of the Folin-Ciocalteu reagent (Sigma), and mix well.

4. Then wait between 30 sec and 8 min, and then add 300 µL of the sodium carbonate solution, and cover with a parafilm square and invert to mix.

5. Leave the solutions at 20ºC (68ºF) for 2 hours or 40ºC (104º F) for 30 min and then determine the absorbance. It is also acceptable to leave the solutions at room temperature for 2 hours.

6. The absorbance is to be read at 765 nm subtracting the absorbance of the blank (the 0 mg/L solution). Before reading the absorbances of the solutions the instrument should be zeroed out by reading the blank first.

Extracts and Dilutions

Extraction methods for muscadine products may vary, however, two simple extraction procedures for extracting muscadine products involve using 50% ethanol (EtOH) and distilled water. Previous work has proved that a 1:10 dilution
using 50% EtOH is an effective method for extracting dried muscadine products. Another efficient extraction method is using a 1:10 distilled water dilution to extract muscadine products. After making these extracts, they should then be diluted to put into the reaction mixture. Usually a series of dilutions is made to determine what dilution will fit best on the standard curve. Here are some experimental ranges for the dilution factors of muscadine products.

**Table 3.1:** Experimental ranges for dilutions of muscadine products for total phenolic content assay

<table>
<thead>
<tr>
<th>Muscadine Products</th>
<th>Dilution range for purple muscadine products</th>
<th>Dilution range for white muscadine products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skins</td>
<td>1/8-1/16</td>
<td>1/4-1/8</td>
</tr>
<tr>
<td>Seeds</td>
<td>1/16-1/32</td>
<td>1/32-1/64</td>
</tr>
<tr>
<td>Wines</td>
<td>1/8-1/16</td>
<td>0-1/2</td>
</tr>
<tr>
<td>Juices</td>
<td>1/2-1/4</td>
<td>0-1/2</td>
</tr>
</tbody>
</table>

Due to the fact that the total phenolic assay is a colorimetric method, the standard curve cuvettes should appear clear to dark blue. Therefore, one can visually tell if the sample will land within the range of the standard curve before reading them in the spectrophotometer. Triplicates should be done for each sample.

To create the standard curve, plot the absorbance at 765 nm vs. concentration. This curve will be used to determine the concentrations of the samples. Microsoft Excel® is an excellent program to use when plotting the data. After plotting the data, a trend line (linear) should be added. One should also select “display equation on chart” and “display R-squared value on chart.”
The R-squared value is not required, but the closer this value is to 1.0, the more accurate is your standard curve.

**Table 3.2:** Example of concentrations and absorbance’s for a standard curve for the total phenolics assay

<table>
<thead>
<tr>
<th>X (concentration)</th>
<th>Y (absorbance at 765 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.001</td>
<td>0.0247</td>
</tr>
<tr>
<td>0.002</td>
<td>0.1131</td>
</tr>
<tr>
<td>0.003</td>
<td>0.1506</td>
</tr>
<tr>
<td>0.005</td>
<td>0.2510</td>
</tr>
<tr>
<td>0.010</td>
<td>0.5484</td>
</tr>
<tr>
<td>0.015</td>
<td>0.8013</td>
</tr>
</tbody>
</table>

**Figure 3.1:** Example of a standard curve for total phenolics assay

When testing samples follow steps 1-7, substituting the 20 µL of the gallic acid solution with your sample. Most samples must to be diluted in order for them to
fall in the range of the curve. After obtaining this value, it needs to be multiplied by the dilution factors in order to determine the amount of total phenols per unit sample.

**Calculations**

To determine the total phenolic content in your sample follow steps 1-7 and get the absorbance of your sample. Then make sure the absorbance of the sample falls within the range of your standard curve (if not, then the sample needs to be diluted until the absorbance falls within the range of the curve). Usually you can see if your sample is within the range of the standard curve. After doing this take the absorbance of your sample and enter it into the equation found on your standard curve.

Ex. Equation of standard curve \( y = 54.489x - 0.0104 \)

Absorbance of sample = 0.1791

Plug in the absorbance for \( y \)

\[
0.1791 = 54.489x - 0.0104
\]

Then solve for \( x \)

\[
x = 0.0035
\]

If you have to dilute the sample, then multiply \( x \) by the dilution factor to get the total phenolic content of your sample.

Ex. 1/16 dilution factor \( x = 0.0035 \) mg of phenol in assay

\[
\frac{0.0035}{20 \, \mu l} \times \frac{1000 \, \mu l}{1 \, mL} \times \frac{10 \, mL}{1 \, g} \times 16 = 28 \, mg/g
\]

\[
mg \, of \, phenol \, in \, assay \times 1000 \, \mu l \times extract \, dilution \times total \, dilution = mg/g
\]

\[
vol \, of \, sample \, in \, assay \times 1 \, mL \times \frac{mg}{g} \, of \, sample
\]
The 10 mL/1 g in the above equation represents the dilution of the extract. In this example a 1:10 dilution was used, however if a different dilution is used the numbers need to be adjusted accordingly.

If you are testing liquids, then the calculation would be done slightly different, e.g.

Ex. 1/8 dilution factor absorbance of sample = 0.2517

\[
0.2517 = 54.489x - 0.0104 \\
x = 0.0048 \text{ mg of phenol in assay}
\]

\[
\frac{0.0048}{20 \mu l} \times 1000 \mu l \times 8 = 1.92 \text{ mg/mL} = 1920 \text{ mg/L}
\]

\[
\frac{\text{mg of phenol in assay} \times 1000 \mu L \times \text{total dilution of sample}}{\text{vol of sample in assay} \times 1 \text{ mL}} = \text{mg of phenol in assay/g}
\]

**Methods for FRAP Assay**

**Equipment and Supplies** (suggested supplies, VWR catalog number)

- UV/VIS spectrophotometer
- Disposable plastic cuvettes (appropriate for the chosen spectrophotometer)
- Water Bath (37°C)
- pH meter
- pH standards
- 50mL beakers (3)
- Glass stirring rod
- Distilled water
- Timer
- Eppendorf tubes (1.5mL)
- 10mL Test tubes (47729-576)
25 or 50mL Graduated cylinder
10mL Graduated cylinder
1000mL Volumetric flask
2-20µL pipet (VWR digital pipettors, variable volume, 40000-250)
20-200µL pipet (VWR digital pipettors, variable volume, 40000-254)
100-1000µL pipet (VWR digital pipettors, variable volume, 40000-256)

**Chemicals** (Sigma-Aldrich catalog number)
- Sodium acetate 3H₂O (S-2889)
- Glacial acetic acid (A6283)
- HCl (hydrochloric acid) (920-1)
- TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) (T1253)
- Ferric chloride (F7134)
- Ferrous sulfate (215422)

**Methods for Solutions**

**Acetate Buffer, 300 mM, pH 3.6**

The acetate buffer is one of the main ingredients in the FRAP reagent. A 300 mM, pH 3.6 solution of acetate buffer is required for this assay and can be made up very simply. First 3.1 g of sodium acetate is weighed out and then dissolved in an adequate amount of distilled water. Next this solution is mixed with 16 mL of glacial acetic acid and diluted with distilled water to 1 L. After diluting the solution to 1 L the solution’s pH must be checked. If the solution is not already at pH 3.6 then a few drops of HCl can be added to make the solution more acidic or
a few drops of NaOH (sodium hydroxide) can be added to make it more basic.

After a correct pH is obtained the solution can be stored at 4°C (39°F).

**Dilute HCl**

A 40 mM HCl solution needs to be made so that it can be used to dissolve the TPTZ. To make this solution take 1.46 mL of concentrated HCL (11 M) and dilute with distilled water to 1 L. This solution can be stored at room temperature.

The next two solutions need to be made fresh each time a FRAP assay is run.

**TPTZ, 10 mM**

To make a 10 mM TPTZ solution, dissolve 0.031 g of TPTZ into 10 mL of 40 mM HCL. This solution dissolves better in a water bath at 50°C (122°F), however if there are problems dissolving the TPTZ a few drops of concentrated HCl will help.

**Ferric Chloride, 20 mM**

To make a 20 mM ferric chloride solution, dissolve 0.054 g of FeCl$_3$ in 10 mL of distilled water. The FeCl$_3$ dissolves easily in the water and does not need to be mixed in the water bath.

**Preparation of Standards**

First a stock 1 mM ferrous sulfate (FeSO$_4$7H$_2$O) solution must be prepared. This is done by weighing out 0.278 g of FeSO$_4$7H$_2$O and dissolving it in an adequate amount of distilled water (less than 1 L). Then this mixture must be diluted up to equal exactly 1 L using distilled water. Next a series of standards are made using this final stock ferrous sulfate solution and distilled water.
Table 3.3: Preparation of standards in the FRAP assay from a 1 mM stock solution of FeSO₄

<table>
<thead>
<tr>
<th>Standard Concentration in Assay (mM)</th>
<th>FeSO₄₇H₂O Stock Solution (mL)</th>
<th>Distilled Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>0.2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>0.4</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>0.6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>0.8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

After making these solutions 0.2 mL aliquots of each standard can be taken and stored in eppendorf tubes in the freezer at -20ºC (-4ºF). These standards can be stored for approximately 2 weeks before they need to be made up again. This will save time if running tests daily.

**FRAP Methods**

1. Turn on the water bath and set to 37ºC (99ºF) and set the standards out to defrost at room temperature.

2. Prepare the FRAP reagent by mixing 25 mL of acetate buffer with 2.5 mL of the TPTZ solution and 2.5 mL of the FeCl₃ solution. This solution should be kept covered (use aluminum foil around the bottle to block out light) in an appropriate container and put in the water bath at 37ºC.

3. Set the spectrophotometer to read at 593 nm wavelength.

4. After making the FRAP reagent and letting the standards thaw, set up three rows, each containing 7 cuvettes. In the first cuvette put 10 µL of
distilled water, this will be the blank for each the standards. Then pipet 10 
µL of each standard into the other 6 cuvettes. Be sure to change pipet 
tips after transfer of each solution. This creates triplicate standard curves.

5. Next put 30 µL of distilled water into each cuvette.

6. Set the timer for 6 minutes and then pipet 300 µL of the FRAP reagent into 
each cuvette. After each cuvette has received 300 µL of the FRAP 
reagent start the timer.

7. While waiting, the blank should be read and then the 6 other cuvettes can 
be put into the spectrophotometer after allowing at least 6 minutes for the 
colorimetric reaction to come to completion.

8. At 6 minutes 340 µL of distilled water should be put into each cuvette then 
run immediately after. The absorbance of each standard should then be 
written down.

9. Repeat steps 4-8 with 3 sets of standards.

10. Then repeat steps 4-8 using the unknown product samples instead of the 
standards. Each sample should also be run in triplicate.

**Extractions and Dilutions**

Extraction methods for muscadine products may vary, however, two simple 
extraction procedures for extracting muscadine products involve using 50% 
ethanol (EtOH) and distilled water. Previous work has proved that a 1:10 dilution
using 50% EtOH is an effective method for extracting dried muscadine products. Another efficient extraction method is using a 1:10 distilled water dilution to extract muscadine products. After making these extracts, they should then be diluted to put into the reaction mixture. Usually a series of dilutions is made to determine what dilution will fit best on the standard curve. Here are some experimental ranges for the dilution factors of muscadine products.

**Table 3.4**: Experimental ranges for dilutions of muscadine products for FRAP assay

<table>
<thead>
<tr>
<th>Muscadine Products</th>
<th>Dilution for purple muscadine products</th>
<th>Dilution for white muscadine products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skins</td>
<td>1/32-1/64</td>
<td>1/16-1/32</td>
</tr>
<tr>
<td>Seeds</td>
<td>1/64-1/128</td>
<td>1/128-1/256</td>
</tr>
<tr>
<td>Wines</td>
<td>1/16-1/32</td>
<td>1/4-1/8</td>
</tr>
<tr>
<td>Juices</td>
<td>1/4-1/8</td>
<td>1/2-1/4</td>
</tr>
</tbody>
</table>

Due to the fact that the total phenolic assay is a colorimetric method, the standard curve cuvettes should appear clear to dark blue. Therefore, one can visually tell if the sample will land within the range of the standard curve before reading them in the spectrophotometer. Triplicates should be done for each sample.

**Analysis**

To determine the FRAP value of the unknown sample follow steps 1-10 and find the absorbance of each sample. Then make sure the absorbance of each sample falls within the absorbance range of the standard curve. If not, the
sample needs to be diluted and rerun through steps 1-10 until the final sample absorbance falls within the range of the curve. After obtaining absorbance readings take the absorbance of each sample and plug it into the equation found on the standard curve.

To draw the standard curve, plot the absorbance at 593 nm vs. concentration in mmol/L. Since all standards and unknown samples are run in triplicate, these triplicates are averaged. This curve will be used to determine the concentrations of the samples. Microsoft Excel® is an excellent program to use when plotting the data. One can also use linear graph paper to draw the standard curve. After plotting the data, a linear trend line should then be drawn through the best fit of the points. One should also select “display equation on chart” and “display R-squared value on chart.” The R-squared value is not required, but the closer this value is to 1.0, the more accurate the curve will be. The curve should be totally linear within the range of the standards suggested above.

**Table 3.5**: Example of concentrations and absorbances for the standard curve of a FRAP assay

<table>
<thead>
<tr>
<th>X (concentration mmol/L)</th>
<th>Y (absorbance at 593 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0247</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0542</td>
</tr>
<tr>
<td>0.4</td>
<td>0.1137</td>
</tr>
<tr>
<td>0.6</td>
<td>0.1742</td>
</tr>
<tr>
<td>0.8</td>
<td>0.2387</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3012</td>
</tr>
</tbody>
</table>
Figure 3.2: Example of a standard curve for a FRAP assay

Ex. Equation of standard curve \( y = 0.3039x - 0.0053 \)

Absorbance of unknown sample = 0.1971

Since the absorbance for \( y \) is 0.1971, the equation to be solved to get the unknown concentration of \( x \) is:

\[
0.1971 = 0.3039x - 0.0053
\]

Then solve for \( x \)

\[
x = 0.67
\]

If the concentration of the unknown sample is 0.67 mmol in assay one must multiply by dilution factors to get the concentration in initial product.

If the initial product was extracted from a dry product, the extraction
dilution factor must also be used to multiply back to original concentration in dry product, 

Ex. 1/32 dilution factor, and $x = 0.67$

$$0.67 \times 32 \times 0.34 = 7.29 \text{ mmol/g}$$

The 0.34 is calculated based on the dilution of the extract, again this is based on a 1:10 dilution and the fact that the total volume of the cuvette was diluted 1:2.

For a liquid sample (like a juice or wine requiring no extraction factor) the calculation would be done in the following way.

Ex. 1/32 dilution factor $x = 0.67$

$$\frac{0.67 \times 32}{2} = 10.72 \text{ mmol/L}$$
References


CHAPTER 4

CORRELATION OF TOTAL PHENOLIC CONTENT AND ANTIOXIDANT POTENTIAL IN MUSCADINE PRODUCTS AND SPECIALTY VINEGARS

Snipes, Lauren, Phillip Greenspan, James L. Hargrove, Diane K. Hartle, to be submitted to the Journal of Agricultural and Food Chemistry

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2 Snipes, Lauren, Phillip Greenspan, James L. Hargrove, Diane K. Hartle, to be submitted to the Journal of Agricultural and Food Chemistry
Abstract

The muscadine grape (*Vitis rotundifolia*) is a berry enriched with phytochemicals that are capable of fighting diseases such as diabetes, cancer, arthritis and heart disease. Due to this knowledge muscadine skins and seeds are sold as nutraceutical supplements. The muscadine skins and seeds have high levels of anthocyanins, flavanols, flavonols, and ellagic acid. Thus it is important to be able to measure these phytochemicals in the muscadine for quality control in the muscadine industry. Due to the high phenolic content and antioxidant capacity of the muscadine we used the Folin-Ciocalteu assay and the ferric reducing antioxidant power (FRAP) assay to determine the levels of phytochemicals in the muscadine. The muscadine seeds proved to have the highest total phenolic content and FRAP values ranging from 44.3-74.4 mg/g and 27.4-41.4 mmol/g respectively. The skins which are rich in anthocyanins and ellagic acid also proved to have relatively high total phenolic and antioxidant values. The skins total phenolic content ranged from 11.2-28.8 mg/g and the FRAP values ranged from 6.9-12.8 mmol/g. Various muscadine and commercial juices, wines and vinegars were also tested for their total phenolic content and antioxidant capacity. We also wanted to prove that there was a significant correlation between these two assays that could be used in quality control. The FRAP value had a positive correlation with the total phenolic contents for all the muscadine products. Therefore one could potentially determine the FRAP value of a product based on its total phenolic content.
4.1 Introduction

The muscadine contains many powerful phytochemicals that are capable of fighting diseases such as diabetes, cancer, heart disease and arthritis. The muscadine contains high levels of anthocyanins, flavanols, flavonols and ellagic acid [1]. The most abundant of the flavonoids in the muscadine are the anthocyanins. The polyphenolics, anthocyanins, flavonols and phenolic acids are the greatest contributors to the total antioxidant capacity [2]. The muscadine skin and seed contain most of the phytochemicals that are important to the muscadine [1, 3]. The chemical constituents observed in the skin and seed are very different. Ellagic acid is the most abundant phenolic found in the skins followed by myricetin, quercetin, kaempferol and trans-resveratrol [4, 5]. The phenolics found in the seed are epicatechin, catechin and gallic acid, with epicatechin being the most plentiful phenolic. Research shows that muscadine seeds have a higher antioxidant capacity than other parts of the fruit, which is due to the high levels of epicatechin and catechins found in the seeds [4]. As the antioxidant properties of the muscadine are being discovered many commercial vineyards have taken interest in developing muscadine food supplementation and nutraceutical products. Dried, powdered muscadine skin, seed and pomace can be encapsulated and taken as a food supplement. Since most of the antioxidants are found in the skins and seeds these capsules would have a high nutraceutical value. These products would be beneficial in that they have a long shelf life and they are highly concentrated in phytochemicals. The muscadine extracts that are being processed today are made to extract the highly
concentrated antioxidants of the seed. This product can then be encapsulated and sold as a nutraceutical product. However these capsules need to be standardized so that the consumer is assured the quality of the product. A good way to standardize these products would be to test them for their total phenolic and antioxidant contents. This would require two simple, inexpensive assays, the Folin-Ciocalteu total phenolic assay and the ferric reducing antioxidant power (FRAP) assay. However, many commercialized vineyards which are producing these products do not have a standardization method [1].

The ferric reducing antioxidant power (FRAP) assay and the Folin-Ciocalteu total phenolics assay are two single electron transfer (SET) based assays that are used to measure the total phenolics and the total antioxidant capacity, respectively. The Folin-Ciocalteu assay is a commonly accepted and validated method for measuring the total phenolics of a sample. This assay has proven to be accurate and reproducible and has a basic standardized method [6]. However, there are several methods that are used to determine the total antioxidant capacity of a sample.

The FRAP assay and the oxygen radical absorbance capacity (ORAC) assay are two of the more common assays used to determine the total antioxidant capacity. The FRAP assay is one of the more commonly used methods for measuring total antioxidant capacity and has proven in previous studies to have a strong correlation with the total phenolics in fruit and vegetable samples [7]. However, the ORAC became known as the industry standard, but it has its limitations. The ORAC assay is a hydrogen atom transfer (HAT) based reaction.
that is composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant [6]. The ORAC assay uses a fluorescent probe and the changes in its intensity to determine free radical damage [8]. One of the problems of the ORAC assay is the high equipment expense and the lengthy preparation and assay time. Fluorescent markers that are required for this assay are expensive and not available in many analytical laboratories [9]. A second problem of the ORAC is that it only tests one species of free radicals, the oxygen free radicals. There are many other free radicals that cause damage such as nitrogen free radicals and sulfur free radicals. Temperature also plays a critical role in the new ORAC assay. The polypropylene plate has poor thermal conductivity which can cause temperature differences in the external well of the microplate and result in decreased reproducibility of the assay [8, 9].

The FRAP is a single electron transfer (SET) based assay that uses antioxidants as reductants in a redox-linked assay. The FRAP assay measures the ability of a compound to reduce Fe$^{3+}$ to Fe$^{2+}$. Fe$^{2+}$ is a pro-oxidant that can react with H$_2$O$_2$ to produce OH•, a harmful free radical. The theory of the FRAP assay is that antioxidants such as ascorbic acid and uric acid reduce both reactive species and Fe$^{3+}$, so their ability to reduce Fe$^{3+}$ will reflect their ability to reduce reactive species [10, 11]. The FRAP assay involves the reduction of a ferric tripyridyltriazine (Fe$^{3+}$TPTZ) complex to ferrous tripyridyltriazine (Fe$^{2+}$) by a reductant at a low pH [10]. The ferrous form has an intense blue color which can be monitored at 593 nm [10, 12]. The FRAP assay is a nonspecific reaction in that any half reaction with a lower redox potential than the ferric/ferrous half-
reaction will force Fe\textsuperscript{3+} to Fe\textsuperscript{2+}. The absorbance is then directly related to the reducing power of the antioxidants. The FRAP assay can be used to measure the total antioxidant capacity on a variety of samples such as biological fluids, aqueous as well as ethanolic extracts of drugs, food, and plants [13]. The FRAP assay was chosen to determine the total antioxidant capacity because it is an inexpensive, simple, speedy and reproducible assay that does not require specialized equipment. Also research has been done that proves there is a correlation between the FRAP and ORAC assays that is extrapolatable to the phenolic fractions [14]. Therefore by using the Folin-Ciocalteu assay to determine the total phenolic content and the FRAP assay to determine the total antioxidant capacity it is possible to estimate the ORAC values from these results.

In the present study, the FRAP and total phenolic values were determined for various muscadine seeds, skins, wines and juices. Both assays were chosen because of utility in muscadine fractions, simplicity of the assays, and economy of the assays for small scale field processors. The hypothesis was to prove a positive correlation between the total phenolic content and FRAP assays in muscadine products and demonstrate a quality control mechanism transferable to processing plants at muscadine vineyards.
4.2 Materials and Methods

**Chemicals and Reagents**

Ferrous sulfate, 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), ferric chloride, sodium acetate, glacial acetic acid, Folin-Ciocalteu reagent, gallic acid, and sodium carbonate were purchased from Sigma Chemical Co. (St. Louis, Mo)

**Sample Preparation**

**Muscadine Seed Extracts**

The muscadine seeds were handpicked out of fresh Noble and Carlos muscadine grapes of two different varieties of commercial muscadine grapes. The muscadine seed products were from Duplin Winery (Rose Hill, NC) and Muscadine Products Corporation (Wray, GA). The seeds were then made into powder using a commercial coffee grinder. A 1:10 dry wt/vol 50% EtOH extract was made and stirred for 2 h. The extract was then centrifuged to remove the precipitate.

**Muscadine Seed Product Extracts**

The muscadine seed products were obtained from Muscadine Products Corporation (Wray, GA), Duplin Winery (Rose Hill, NC), and Post Familie Winery (Altus, AR). A 1:10 dry wt/vol 50% EtOH extract was made and stirred for 2 h. The extract was then centrifuged to remove the precipitate.

**Muscadine Skin Extracts**

The muscadine skins peeled from fresh Noble and Carlos muscadine grapes. The skins were crushed into a powder using a mortar and pestle. A 1:10 dry
wt/vol 50% EtOH extract was then made using the skin powder and stirred for 2h. The extract was then centrifuged to remove the precipitate.

**Determination of Total Phenolics.** The total phenolic content of the muscadine extracts was determined using the Folin-Ciocalteu assay described by Slinkard and Singleton [15], using gallic acid as a reference phenolic standard. Absorbance was measured at 765 nm in a Beckman DU 600 series spectrophotometer. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight.

**Ferric Reducing Antioxidant Power (FRAP) Assay.** FRAP values were determined using a modified version of the Benzie and Strain method [13], using purchased ferrous sulfate as the reference standard. Absorbance was measured at 593 nm on a Beckman DU 600 series spectrophotometer. The FRAP assay is used for the direct testing of antioxidant capacity and is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex to the ferrous form. The “antioxidant power” was determined at the absorbance of 593 nm after six minutes of incubation. The results were then expressed as mmol of ferrous sulfate formed/gram of dry wt. of grape skin or seed using a standard curve constructed with known ferrous sulfate. Results were expressed as millimoles of Fe$^{2+}$ equivalents per gram of dry weight.

**4.3 Results**

Various muscadine seeds were analyzed for total phenolics and antioxidant capacity [4]. **Figures 4.1** represents the total phenolic values and FRAP values of individual muscadine seed samples that were handpicked out of fresh Noble
Total Phenolics and FRAP Values of Noble and Carlos Muscadine Seeds

Figure 4.1: Total phenolic content and FRAP values of Noble and Carlos muscadine seed samples
(red) and Carlos (white) muscadine grapes of two different varieties of commercial importance to wine juice and nutraceutical product processors. The phenolic value of the seeds ranged from 44.3-74.4 mg/g with a mean of 60.5 ± 8.9 mg/g. The seeds of the Carlos muscadine grapes tended to have a higher phenolic value than those of the Noble muscadine grapes. The Carlos muscadine grape seeds had a mean phenolic value of 66.4 ± 5.2 mg/g, while the Noble muscadine grape seeds had a mean phenolic value of 52.3 ± 5.7 mg/g. The FRAP values also followed this trend in that the Carlos muscadine grape seeds had higher FRAP values than the Noble muscadine grape seeds. The Carlos muscadine grape seeds had a mean FRAP value of 37.1 ± 4.9 mmol/g and the Noble muscadine grape seeds had a mean FRAP value of 30.1 ± 1.8 mmol/g. The total FRAP value of the muscadine seeds had a mean of 34.2 ± 5.2 mmol/g.

Figure 4.2 represents various dried powdered muscadine seed and seed capsules from various vineyards. There were three different samples of freeze dried seeds which all had high phenolic and FRAP values. The freeze-dried seeds had a mean phenolic value of 54.2 ± 4.8 mg/g and a mean FRAP value of 25.5 ± 0.1 mmol/g. There were also five different kinds of muscadine grape seed capsules, however their values had a wide range. Grape seed capsule 2 had the highest phenolic and FRAP value of 78.4 mg/g and 41.1 mmol/g respectively. Grape seed capsule 1 had the lowest phenolic and FRAP values of 29.1 mg/g and 13.7 mmol/g. There were also several samples of dried powdered seed that were obtained from vineyards in the Southeast, however their phenolic and
Figure 4.2: Total phenolic content and FRAP values of muscadine seed products
Table 4.1: Sources for Figure 4.2, total phenolic content and FRAP values of muscadine seed products

<table>
<thead>
<tr>
<th>Muscadine Seed Products</th>
<th>FRAP Values (mmol/g)</th>
<th>Phenolic Content (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Freeze Dried Carlos Seeds, Duplin Winery</td>
<td>25.4</td>
<td>51.2</td>
</tr>
<tr>
<td>2- Freeze Dried Carlos Seeds, Duplin Winery</td>
<td>25.7</td>
<td>59.7</td>
</tr>
<tr>
<td>3- Air Dried Carlos Seeds, Duplin Winery</td>
<td>6.7</td>
<td>13.4</td>
</tr>
<tr>
<td>4- Freeze Dried Carlos Seeds, Duplin Winery</td>
<td>25.5</td>
<td>51.6</td>
</tr>
<tr>
<td>5- Seed Powder dried at 120ºF, Muscadine Products Corporation</td>
<td>14.7</td>
<td>35.6</td>
</tr>
<tr>
<td>6- Seed Powder dried at 100ºF, Muscadine Products Corporation</td>
<td>11.9</td>
<td>30.8</td>
</tr>
<tr>
<td>7- Wet Carlos Seed, Duplin Winery</td>
<td>15.2</td>
<td>33.1</td>
</tr>
<tr>
<td>8- Seed Powder, Muscadine Products Corporation</td>
<td>18.7</td>
<td>39.2</td>
</tr>
<tr>
<td>9- Grape seed capsule, Nature's Pearl, Inc</td>
<td>13.7</td>
<td>29.1</td>
</tr>
<tr>
<td>10-Grape seed capsule, Post Familie</td>
<td>41.1</td>
<td>78.4</td>
</tr>
<tr>
<td>11-Grape seed capsule, Nutra-Grape</td>
<td>27.9</td>
<td>60.8</td>
</tr>
<tr>
<td>12- Grape seed capsule, Duplin Winery</td>
<td>21.8</td>
<td>61.6</td>
</tr>
<tr>
<td>13- Grape seed capsule, Muscadine Products Corporation</td>
<td>13.7</td>
<td>40.4</td>
</tr>
</tbody>
</table>
Figure 4.3: Correlation between the total phenolic content and antioxidant (FRAP) values of the total muscadine seed data.
FRAP values were not as high as the freeze dried seeds. The phenolic value of the powdered seeds was 35.2 ± 4.2 mg/g, while the FRAP value was 15.1 ± 3.4 mmol/g. The muscadine seed products with the highest phenolic and FRAP values were grape seed capsules 2, 3, and 4, however the freeze dried seeds also had a high phenolic and FRAP content.

We hypothesized that the total phenolic content of muscadine fractions would correlate positively with the FRAP values. To test this hypothesis, all of the phenolic muscadine seed data obtained over a period of months was plotted against the muscadine seed FRAP data as shown in Figure 4.3. There was a significant correlation between the muscadine seed phenolic data and the muscadine seed FRAP data. The phenol versus FRAP graph had an $R^2$-value of 0.8103 which proved the correlation between the two assays.

The data in Figure 4.4 represents the data from muscadine grape seed skins which have been peeled off of individual muscadine grapes as well as a sample of dried powdered muscadine grape skin. The grape skin data was quite different from the seed data in that the Noble muscadine skins had the higher phenol and FRAP values than the Carlos muscadine skins. The mean phenolic value for the muscadine red skins was 25.7 ± 3.4 mg/g, and the mean phenolic value for the muscadine Carlos skins was 13.6 ± 1.4 mg/g. There was not as much of a difference between the FRAP values of the Noble and Carlos muscadine skins as there was in the seeds, yet there was a slightly higher value for the Noble skins than the Carlos skins. The Noble muscadine skins had a FRAP mean of 12.0 ± 1.1 mmol/g, and the Carlos muscadine grape skins had a
**Total Phenolic and FRAP Values of Muscadine Skins**

![Graph](image)

**Figure 4.4:** Total phenolic content and FRAP values for muscadine grape skin samples
Correlation of Total Phenolic Content with Antioxidant (FRAP) Values of Muscadine Skins

![Graph showing correlation between total phenolic content and FRAP values](image)

$R^2 = 0.719$

**Figure 4.5:** Correlation between the total phenolic content and antioxidant (FRAP) values of the muscadine skin samples
mean FRAP value of 8.0 ± 0.9 mmol/g. The dried powdered muscadine skin sample had a phenolic value of 21.9 mg/g and a FRAP value of 7.8 mmol/g. There was also a relationship between the total phenolic values of the skins and the FRAP values of the skins as seen in Figure 4.5. This correlation was not as strong as with the seeds, but it still had a good correlation with a $R^2$-value of 0.719.

Muscadine wines and juices were also tested in this project for their total phenolic content and FRAP values. Commercial Concord and Niagara grape juices were tested to compare to the muscadine juices, Figure 4.6. The commercial grape juices had higher phenolic and FRAP values, but the muscadine juices had acceptable values as well. The higher phenolic and FRAP values of the commercial grape juices is most likely due to the addition of vitamin C in these varieties. The commercial red grape juices had total phenolics of 2120 and 1380 mg/L with FRAP values of 11.2 and 7.6 mmol/L, respectively. The muscadine red grape juices had total phenolics of 1000 and 430 mg/L with FRAP values of 6.9 and 5.3 mmol/L. The commercial white grape juices also had higher phenolic and FRAP values than the muscadine white juices. Despite the lower values of the muscadine grape juice, the muscadine red wine had high levels of phenols and antioxidants. The muscadine red wine has levels of phenolics in the range of 1320-1140 mg/L with FRAP values in the range of 11.7-8.0 mmol/L. The white muscadine wines did not have high phenolic or FRAP values. There was also a relationship of phenolic content to antioxidant capacity
Total Phenolic and FRAP Values for Grape Juices

**Figure 4.6**: Total phenolic content and FRAP values of various grape juices

**Table 4.2**: Total phenolic and FRAP values of various grape juices

<table>
<thead>
<tr>
<th>Grape Juice</th>
<th>FRAP Value (mmol/L)</th>
<th>Phenolic Content (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welch’s Purple Grape Juice</td>
<td>11.2</td>
<td>2120</td>
</tr>
<tr>
<td>Sam’s Purple Grape Juice</td>
<td>7.6</td>
<td>1380</td>
</tr>
<tr>
<td>Muscadine Red Grape Juice, Muscadine Products Corporation</td>
<td>6.9</td>
<td>1000</td>
</tr>
<tr>
<td>Welch’s White Grape Juice</td>
<td>5.7</td>
<td>887</td>
</tr>
<tr>
<td>Muscadine Red Grape Juice, Nature’s Pearl, Inc</td>
<td>5.3</td>
<td>430</td>
</tr>
<tr>
<td>Sam’s White Grape Juice</td>
<td>3.9</td>
<td>540</td>
</tr>
<tr>
<td>Muscadine White Grape Juice, Nature’s Pearl Inc</td>
<td>2.4</td>
<td>333</td>
</tr>
<tr>
<td>Muscadine White Grape Juice, Muscadine Products Corporation</td>
<td>0.9</td>
<td>233</td>
</tr>
</tbody>
</table>
Figure 4.7: Correlation between the total phenolic content and antioxidant (FRAP) values of various grape juices
Total Phenolic and FRAP Values of Muscadine Wines

![Bar chart showing total phenolic content and FRAP values of muscadine wines](chart.png)

**Figure 4.8:** Total phenolic content and FRAP values of muscadine wines

**Table 4.3:** Total phenolic and FRAP values of the muscadine wines

<table>
<thead>
<tr>
<th>Muscadine Wines</th>
<th>FRAP Value (mmol/L)</th>
<th>Phenolic Content (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notchaway Red Muscadine Wine, Still Ponds Vineyard and Winery</td>
<td>11.7</td>
<td>1320</td>
</tr>
<tr>
<td>Notchaway Red Muscadine Wine, Still Ponds Vineyard and Winery</td>
<td>10.9</td>
<td>1180</td>
</tr>
<tr>
<td>Resveratrol Muscadine Grape Wine</td>
<td>8.0</td>
<td>1140</td>
</tr>
<tr>
<td>Carolina Carlos, Duplin Winery</td>
<td>2.3</td>
<td>270</td>
</tr>
<tr>
<td>Notchaway White Muscadine Wine, Still Ponds Vineyard and Winery</td>
<td>2.2</td>
<td>347</td>
</tr>
<tr>
<td>Plantation White Muscadine Wine, Still Ponds Vineyard and Winery</td>
<td>1.7</td>
<td>180</td>
</tr>
</tbody>
</table>
Figure 4.9: Correlation between the total phenolic content and antioxidant (FRAP) values of muscadine wines
among the juices and wines, **Figure 4.7**. The correlation between the total phenolic and FRAP values of the juices proved to be stronger than that of the skins and the seeds with and \( R^2 \)-value of 0.9013. The correlation between the phenolic content and FRAP values of the muscadine wines was more significant having an \( R^2 \)-value of 0.9601.

The final samples that were tested in this project were a variety of vinegars. There was a wide variety of vinegars tested ranging from various wine vinegars to the commonly used distilled white vinegars. The balsamic vinegars had the highest overall phenolic and antioxidant content as seen in **Figure 4.10**. The balsamic vinegars had total phenolic values ranging from 860-2590 mg/L and FRAP values ranging from 4.1-12.0 mmol/L, **Table 4.4**. The two muscadine red wine vinegars also had high phenolic values of 1250 and 993 mg/L and FRAP values of 10.1 and 5.1 mmol/L. As in other products, the muscadine white wine vinegars had much lower phenolic and antioxidant contents than the muscadine red wine vinegars. The persimmon, sherry, red wine and blueberry vinegars had respectable phenolic and FRAP values, but they were not as potent as the balsamic and muscadine red wine vinegars. The malt vinegar had a high phenolic content of 847 mg/L, but a lower FRAP value of 1.7 mmol/L. The distilled white vinegars were the only vinegars to show no phenolic or antioxidant content. There was a noticeable relationship between the phenolic and antioxidant values of the vinegars, **Figure 4.11**. This set of data had the a high correlation between the total phenolics and antioxidant values having a \( R^2 \)-value of 0.8994.
Figure 4.10: Total phenolic content and FRAP values of specialty vinegars
Table 4.4: Sources for Figure 4.10, total phenolic and FRAP values of the vinegars

<table>
<thead>
<tr>
<th>Vinegars</th>
<th>FRAP Value (mmol/L)</th>
<th>Phenolic Content (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- Balsamic Vinegar, Bella Famiglia</td>
<td>12.0</td>
<td>2590</td>
</tr>
<tr>
<td>2- Monan Federzon Balsamic Vinegar of Modena</td>
<td>11.9</td>
<td>2550</td>
</tr>
<tr>
<td>3- Noble Muscadine Wine Vinegar (stock), Casa Perdido</td>
<td>10.1</td>
<td>1250</td>
</tr>
<tr>
<td>4- Noble Muscadine Wine Vinegar, Casa Perdido</td>
<td>5.1</td>
<td>993</td>
</tr>
<tr>
<td>5- Red Wine Vinegar, Bellino</td>
<td>4.8</td>
<td>791</td>
</tr>
<tr>
<td>6- White Balsamic Vinegar, Bella Famiglia</td>
<td>4.5</td>
<td>860</td>
</tr>
<tr>
<td>7- Balsamic Vinegar of Modena, Newman’s Own</td>
<td>4.1</td>
<td>1120</td>
</tr>
<tr>
<td>8- Persimmon Vinegar</td>
<td>3.4</td>
<td>460</td>
</tr>
<tr>
<td>9- Sherry Vinegar, Columela</td>
<td>2.8</td>
<td>547</td>
</tr>
<tr>
<td>10- Blueberry Wine Vinegar, Casa Perdido</td>
<td>2.1</td>
<td>547</td>
</tr>
<tr>
<td>11- Apple Wine Vinegar, Casa Perdido</td>
<td>1.8</td>
<td>363</td>
</tr>
<tr>
<td>12- Malt Vinegar, Casa Perdido</td>
<td>1.7</td>
<td>847</td>
</tr>
<tr>
<td>13- White Muscadine Wine Vinegar, Casa Perdido</td>
<td>0.9</td>
<td>172</td>
</tr>
<tr>
<td>14- Magnolia Muscadine Wine Vinegar, Casa Perdido</td>
<td>0.8</td>
<td>160</td>
</tr>
<tr>
<td>15- Apple Cider Vinegar, Harris Teeter</td>
<td>0.7</td>
<td>222</td>
</tr>
<tr>
<td>16- Cucumber Wine Vinegar, Casa Perdido</td>
<td>0.5</td>
<td>350</td>
</tr>
<tr>
<td>17- Garlic Wine Vinegar, Progresso</td>
<td>0.4</td>
<td>142</td>
</tr>
<tr>
<td>18- Lemon Vinegar</td>
<td>0.3</td>
<td>75</td>
</tr>
<tr>
<td>19- Distilled White Vinegar, Avondale</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20- Organic Distilled White Vinegar, Spectrum Naturals</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Organic Rice Vinegar, Marukan</td>
<td>0</td>
<td>110</td>
</tr>
</tbody>
</table>
Correlation of Total Phenolic Content with Antioxidant (FRAP) Values of Vinegars

Figure 4.11: Correlation between the total phenolic content and antioxidant (FRAP) values of specialty vinegars
4.4 Discussion

After analyzing several sets of data for total phenolic and antioxidant content, the results showed a positive correlation between the two assays in products that are known to be high in natural phenolics without added ingredients. The correlation between the total phenolic content and the antioxidant capacity of fruits and vegetables has also been proven in previous studies. Studies have also shown a positive correlation between the total phenolic content and ORAC values [14]. Since many commercial vineyards do not have analytical labs set up in their corporation, this information could prove valuable to them. A muscadine vineyard could set up a lab to produce the total phenolic assay, since it is technically less demanding than the ORAC and FRAP assays. The muscadine vineyard could then use the values they get for their total phenolic values to estimate the FRAP and ORAC values. The phenolic values could be produced for each batch and therefore the FRAP and ORAC values could be predicted for each batch. The cheaper total phenolic content assay and FRAP assay will allow producers to obtain batch to batch and lot to lot data. Currently many producers only have one-time ORAC values from one batch. Setting up a quality control lab will allow producers to keep current total phenolic and antioxidant capacity data on hand instead of older data from a previous batch.

The total phenolic content and FRAP assay are two assays that would be excellent methods for muscadine grape vineyards to use for standardizing their nutraceutical and food supplementation products. The Folin-Ciocalteu assay and the FRAP assay would be excellent assays to use for quality control in the
muscadine industry. The products produced by the vineyards today need to be monitored for quality control and these are simple, reproducible methods that would provide valuable information for the muscadine vineyards. The assays use inexpensive equipment and chemicals, and can be easily set up and run. It is also apparent that these are large ranges of total phenolic and antioxidant potential values within a product sample range. This further supports the need for producers, manufacturers and consumers to have data concerning lot numbers of product. Reasons for ranges are a combination of natural influences on the grape as well as processing and storage conditions. Therefore it is important to the vineyards to keep current batch to batch records on hand.
4.5 References


CHAPTER 5

CONCLUSION

The first contribution to this thesis involved finding that muscadine skin and seed extracts possess considerable aldose reductase inhibition activity. The muscadine skin extracts proved to be more potent than quercetin, a bioflavonoid with known aldose reductase inhibitor activity. The seed extract was almost equally as effective as quercetin. This is an important new observation due to the fact that the pharmaceutical industry has not been able to find an effective, non-toxic aldose reductase inhibitor. The muscadine skins and seeds are non-toxic and could be used to produce food supplement products to help pre-diabetics and diabetics prevent the complications of diabetes, assuming bioavailability is appropriate.

The second contribution made to this thesis was the creation of a training manual for non-scientist technicians to use in quality control labs in the muscadine industry. A vineyard can set up a small lab at a low cost and analyze their products onsite, rather than sending them off to a professional laboratory to be analyzed at a high cost. Hundreds of samples obtained during handling and processing can be run for a very low cost. This would create useful marketing data, and can be obtained at considerable economy. The Folin-Ciocalteu assay is a simple, reproducible assay for the determination of total phenolics which can be done by a trained employee. The FRAP assay is a quick, reproducible
method for determination of total antioxidant capacity. While the FRAP is a bit more technically demanding than the total phenolics assay, it is a reliable assay that can be done by a trained employee.

The third contribution made to this thesis was determining a correlation between the total phenolic content and the antioxidant capacity of muscadine products. The phenolic fraction of muscadine skins and seed is of interest for nutraceutical development. The antioxidant value, measured by the FRAP assay, correlates positively with total phenolic content. The correlation could be useful to standardize products based on the phenol and FRAP results. The vineyards need immediate results during the processing of tons of pomace and working out extraction procedures. The FRAP and total phenolic assays are desirable assays for the vineyards to set up, however, the FRAP method is more labor-consuming than the total phenolic content assay. Due to the nature of the bulk material and the content of the extracts, the phenol assay results correlate linearly with the FRAP assay results. Therefore, knowing this relationship for muscadine products, the vineyard can extrapolate the predictable range of the FRAP value from the total phenolic content values. While this may not be appropriate for the analytical laboratory, this may prove to be a suitable “field” method in the muscadine industry. Samples can then be saved and both total phenolic content and FRAP assays can be rerun under laboratory conditions when time permits. The total phenolic assay proves to be the easiest possible method for the vineyard to use in scaling up processing of muscadine fractions for nutraceutical product manufacturing.