PHENOLICS IN RED WINE POMACE AND THEIR POTENTIAL APPLICATION IN ANIMAL AND HUMAN HEALTH

A Thesis presented to
the Faculty of the Graduate School
at the University of Missouri

In Partial Fulfillment of the Requirements for the Degree

Master of Science

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AUGUST 2008

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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DEDICATION

To my parents, Kevin and Rebecca Spradling. Thank you for always believing in me, encouraging me to follow my dreams, and giving me the confidence to accomplish this. Also, to my five amazing sisters who have always given me wonderful support and inspiration.

ACKNOWLEDGEMENTS

I would like to extend my thanks to Dr. Joseph Awika for all of his dedication and support throughout this research. Through his encouragement and teaching he has made me a more confident scientist. Also, I would like to express my gratitude to Dr. Ingolf Gruen and Dr. Mark Ellersieck for giving me help, critiques, and guidance while completing this research.

I will always be grateful to Dr. Ellen Dierenfeld who inspired me to conduct research that can benefit exotic zoo animals. Through her, I discovered another area of research I am truly passionate about.

I appreciate all the hard work that JoAnn Lewis did to carefully edit and perfect my work; and to Elizabeth Melanson, Leonnard Ojwang, Liyi Yang, and Tom Jondiko of the Department of Food Science, for all of their encouragement and eagerness to lend a hand during the completion of my research.

I would like to also acknowledge Stone Hill Winery in Hermann, Missouri for donating the grape pomace directly after the wine production. Their generosity made my research ideas become reality.

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ABSTRACT

Grape pomace by-products, from wine-making, have some beneficial antioxidant compounds left in them after vinification and could be a cheap source of these compounds for value added products. One of the most common antioxidant groups found in grapes are the phenols. Grape pomace possesses a high amount of polyphenols, particularly flavonols. These antioxidants have the potential to reduce cardiovascular disease, prevent some cancers, have chemoprotective roles, reduce signs of skin aging, aid in glucose transport, and prevent other diseases such as iron storage disease (ISD).

In this research, five Missouri red wine pomace samples were evaluated; Chambourcin, Grenache, Michigan, Norton and Vincent varieties. They were analyzed for total phenolic content using the Folin-Ciocalteu assay, total condensed tannin content using the Vanillin-HCl assay, procyanidin degree of polymerization using normal-phase HPLC, iron-binding potential using the iron-binding phenolic capacity assay, and antioxidant activity using the ABTS and ORAC assays.

The results showed that the vinification method applied to the grape variety greatly affected the pomace properties and composition. The Vincent variety pomace,

which was lightly pressed during vinification, had the highest levels of total polyphenols (62.18 ± 4.53 mg gallic acid equivalents per gram), condensed tannins (120.24 ± 2.64 mg of catechin equivalents per gram), iron-binding phenolic capacity (120.23 ± 0.25 mg catechin equivalents per gram), and the highest antioxidant activity (914.42 ± 75.86 µM trolox equivalents per gram for the ORAC assay and 1605.52 ± 102.97 µM trolox equivalents per gram for the ABTS assay).

The Norton variety pomace, because it was heavily pressed during vinification, was found to have the lowest values in all assays. It had the lowest total polyphenols (25.12 ± 1.34 mg gallic acid equivalents per gram), condensed tannins (10.18 ± 0.41 mg of catechin equivalents per gram), iron-binding phenolic capacity (19.62 ± 0.22 mg catechin equivalents per gram), and the lowest antioxidant activity (365.31 ± 72.57 µM trolox equivalents per gram for the ORAC assay and 569.76 ± 18.87 µM trolox equivalents per gram for the ABTS assay). The Chambourcin, Grenache, and Michigan varieties had values between the two extremes due to their moderate pressing during vinification.

Red wine pomace, produced from lightly pressed grapes, has higher phenolic and antioxidant activities. This type of pomace has the greatest potential for applications that will benefit animal and human health.

CHAPTER 1

INTRODUCTION

1.1 Need for Research

Wine is one of the world's most popular alcoholic beverages. It has been produced and consumed throughout history for cultural, economical, social, religious, and, more recently, health reasons. Wine produced from grapes is constantly being investigated for its health benefits. The by-product of wine production is grape pomace, which includes grape skins, seeds, and sometimes stems and leaves. The pomace has the potential to be a rich source of the antioxidants that are becoming more and more popular in the food and health industries.

The worldwide amount of grape pomace, from the wine and juice industries, averages about seven to nine million tons per year (Baumgartel and others 2005). These waste materials contain biodegradable organic matter; however, their disposal generates huge amounts of industrial waste and creates serious environmental problems. The waste loads at the processing plants could be significantly reduced through by-product usage. A variety of processes are being developed aimed at converting waste materials into bio-fuels, food ingredients, or other value added products. However, due to lack of appropriate feasibility studies on the exploitation of such wastes, their utilization is still in its infancy (Makris and others 2007).

Often grape pomace is used as animal feed during the dry season but it is considered anti-nutritious due to its high polyphenolic content, therefore, its use in this area has much decreased. On the other hand, there are some captive animal species where

high polyphenolic content diets may be therapeutic in some instances. In humans, polyphenols are known to contribute various health benefits.

Exotic, captive animals in zoos across the United States are one of the largest tourist attractions and learning experiences available. It is now clearly recognized that certain captive animals are prone to hemosiderosis (excessive iron accumulation in the body), which can lead to chronic iron storage disease (ISD), medically referred to as hemochromatosis (Wood and others 2003). ISD can be caused by excessive dietary iron intake, excessive iron uptake from the gastrointestinal tract, hemolysis, or inadequate excretion of iron (Lowenstine and Munson 1998). As serum iron levels increase, lysosomes release ionic iron, damaging membranes. Cells are damaged and replaced; tissues and organs can become fibrotic, leading to ISD (Sheppard and Dierenfeld 2002).

It is believed many wild species are genetically susceptible to excessive iron accumulation. Their natural diets may contain low levels of iron and high levels of polyphenols, such as tannins, which chelate iron in the gastrointestinal tract and decrease intestinal iron absorption (Andrews and others 2005). In zoos, diets of captive animals are typically higher in iron, as well as in ascorbic acid which enhances iron absorption from the intestine.

Treatment of hemosiderosis, and prevention of ISD, in captive zoo species, as well as humans, has included reduction of dietary iron concentrations, phlebotomy, and therapeutic use of deferoxamine mesylate or deferiprone (Farina and others 2005). However, the use of natural dietary chelators, such as polyphenols, may be more practical in zoo animals.

Polyphenols are common plant constituents that share the same core element, the phenol group. They contribute to color, taste, nutritional value, and even structure of plants. Tannins are high molecular weight polyphenolics founds in higher plants including many plants used as foods (Hartzfeld and others 2002).

Historically, tannins have not been incorporated into the diets of captive animals because they bind to essential minerals and also lower digestive enzyme activity (Silanikove and others 1994). However increased antioxidant status caused by the use of tannins, and their ability to chelate iron, could have a beneficial effect on animal and human health; but if tannins should be incorporated in designed diets, low-cost and functional tannins sources, such as grape pomace should be tested (Clauss and others 2006).

Wine industry by-products, including grape seeds, skins, and stems, are very rich sources of antioxidant polyphenols compared with other agri-food solid wastes. Their exploitation as a source of value added products may therefore be cost-effective and merits an investigation (Makris and others 2007). Tannins found in the grape pomace may also provide a cost effective means to aid in the prevention of ISD in captive animals, as well as in humans.

1.2 Objectives of Research

Three objectives for this research were established:

1. To determine total polyphenols and proanthocyanidin content of wine pomace from various grape varieties.
2. To determine *in vitro* iron chelation capacity of the wine pomace extracts from various grape varieties.
3. To determine antioxidant activity of the wine pomace from various grape varieties.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Red-Grape Wine Pomace

2.1.1 Pomace Production & Polyphenols

The grape (*Vitis vinifera*) is one of the world's largest fruit crops with an annual production of 58-61 million metric tons (Murthy and others 2002). Grape growing plays a major role in the worldwide fruit production, with an international acreage of approximately 7.8 million hectares (OIV 2002), and is constantly growing.

Wine industry wastes account for almost 30% (w:w) of the grapes used for wine production (Makris and others 2007). Grape pomace, a remnant of the wine making process, is one of the most important residues of the wine industry. It consists of different amounts of grape, skin, pulp, seeds, and, if not removed, stems (Baumgartel and others 2005).

During the vinification process of red wine, phenolic compounds are transferred from solid parts of the grape cluster into wine. The rate of transfer depends on various factors including phenolic concentration of grapes, level of pressing, maceration time, fermentation contact time, temperature, and alcohol levels. The seeds contain the highest concentrations of phenolic compounds and most of these compounds are monomeric flavan-3-ols (catechins) and procyanidins (Sun and others 1999). Both grape skins and seeds contain monomeric, oligomeric, and polymeric proanthocyanidins; the mean degree of polymerization being higher for skin flavanols (Torres and Bobet 2001).

The phenolic compounds of wine, and particularly the flavanols (i.e. catechins), have been the center of attention in recent studies since their relation to the beneficial effects of a moderate consumption of wine was observed; often known as the "French Paradox" (Gonzalez-Paramas and others 2004). Phenolic compounds present in red wine cause an increase in serum total antioxidant capacity when ingested which thereby inhibit low-density lipoprotein (Yildirim and others 2005), and reduce the risk of cardiovascular disease. The antioxidative properties may also exert a chemopreventive role toward degenerative diseases (Ruberto and others 2005), as well as act as preventative agents against skin cancer and other diseases (Torres and others 2002).

2.1.2 Current Uses

Some industrial uses currently under investigation for wine pomace waste include use as animal feed, as possible nutritive ingredients for value added products, in the production of citric acid, and the use of anthocyanins from grape skins as colorants (Lu and Yeap Foo 1998). Pomace can, and has, been used as animal feed, especially in the dry season. Its use is limited due to its very low nutritional value and its antinutritional factors (Sanchez and others 2002). Pomace has also been used as a fertilizer, though not very successfully (Su and Silva 2006).

Spirits from fermented grape pomace are very popular in Mediterranean countries. Several countries produce traditional distilled alcoholic beverages from different raw materials. Very popular are those produced from grape marc, such as the Greek *tsipouro* and *tsikoudia*, the Italian *grappa*, the Portuguese *bagaceiras* and

aguardiente, and lastly the French *au-de-vie de marc* (Gerogiannaki-Christopoulou and others 2006). Pomace has also been used in the production of traditional foods such as molasses and vinegar (Ozkan and others 2004).

2.1.3 Ongoing Research Opportunities & Future Uses

Disposal of industrial solid wastes has become an increasingly serious problem. Disposal sites have become more difficult to locate and more expensive to operate. Emphasis is being placed more and more on recovery and use (Rice 1976). At present, only minimum amounts of these wastes are up-graded or recycled (Ruberto and others 2005). Because world population is in continuous growth and natural resources are consequently limited, studies dealing with the utilization of renewable sources have attracted great interest in recent years (Pinelo and others 2005).

The recovery of wine by-products from agricultural industries to be converted into value added products is a good example. The using of this waste is of great importance, not only because of its significant health benefits, but also because it could exploit a large amount of the wine industry wastes (Louli and others 2003). Between 5 and 9 million tons per year are shipped to disposal sites; consequently, their environmental impact can be detrimental (Schieber and others 2001).

A variety of health-promoting products obtained from by-products of the grape and wine industry are being introduced into the market (Gomez-Plaza and others 2006). By-products obtained from the juice and wine industry can be a source of new value added products such as phenolic antioxidant supplements or ingredients for food

processing. It has been reported that polyphenols in grape pomace have a higher antioxidant capacity than in wine (Su and Silva 2006). Red grape pomace, consisting of pressed skins, disrupted cells from the grape pulp, seeds, and stems, could be a rich source of antioxidants for foods, cosmetics, and pharmaceuticals (Louli and others 2003).

Some wine by-products are now being sold to the rapidly growing dietary supplement industry (Shrikhande 2000). These products are being marketed for prevention of cardiovascular disease, cancers, and other diseases. However, there is currently no use of grape pomace in the therapeutic prevention of ISD. Addition of natural iron-chelators in the diet of captive animals, as well as humans, which are susceptible to ISD has been suggested (Sheppard and Dierenfeld 2002). Therefore, using grape pomace as an inexpensive source of tannins would not only benefit the health of animals and humans but also the environment by decreasing the amount of waste in landfills.

2.2 Tannins

2.2.1 Overview

Phenolic compounds are among the most widely distributed plant secondary products and are found in many plants used as food and feed (Hagerman and Robins 1993). Flavonoids are a large class of polyphenolic compounds that contain several phenolic hydroxyl groups attached to ring structures designated A,B and C. Phenolic compounds are multifunctional antioxidants with chain-breaking and metal-chelating activities in the same molecule (Khokhar and Owusu Apenten 2003). Structural

variations within the rings subdivide the flavonoids into several families; flavonols, flavones, flavanols, and isoflavones (**Figure 1**). Of importance for this research are the polyflavan-3-ols, often known as condensed tannins. These tannins lack the 2,3-double bond and the 4-one structure (Rice-Evans and others 1997).

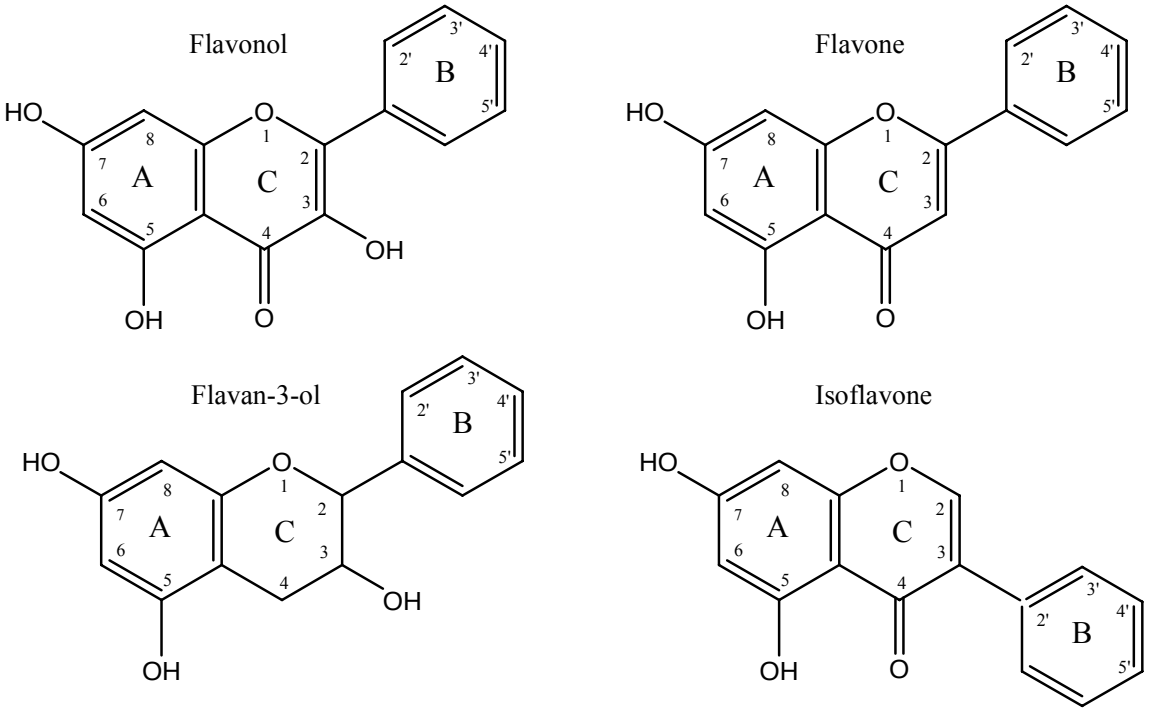


Figure 1: Structures of the flavonoids. The basic structure consists of the fused A and C rings, with the phenyl B ring attached though its 1' position to the 2-position of the C ring.

Tannins are water-soluble phenolic metabolites of plants with a molecular weight of 500 or greater and with the ability to precipitate proteins from aqueous solutions (Gupta and Haslam 1980). They are present in many plant foods (Al-Mamary and others 2001). Condensed tannins (also known as proanthocyanidins), and hydrolyzable tannins are the most widely occurring (Deaville and others 2007). It is generally assumed that

tannins are not absorbed, and their effects are confined to the digestive tract. Since tannins strongly inhibit digestive enzymes, the antinutritional effects of dietary tannin are often related to inhibition of digestion (Mehansho and others 1987).

2.2.2 Condensed & Hydrolyzable Tannins

The three principle groups of tannins are the condensed tannins (proanthocyanidins), the hydrolyzable tannins, and the phlorotannins. Phlorotannins are only founds in marine algae and brown algae and not widely consumed by humans (Hagerman and Robins 1993). Tannins are considered secondary metabolites and irregularly distributed substances that have no specific metabolic function. Physiochemically, tannins are complex polymers and for this research are conveniently divided into two major types; condensed and hydrolyzable tannins (Mehansho and others 1987).

Condensed tannins are oligomeric and polymeric flavan-3-ols linked through acid-labile carbon-carbon bonds (**Figure 2**). They are ubiquitous and present as the second most abundant natural phenolic. The flavan-3-ol units are linked mainly through the C4—C8 bond, but the C4—C6 bond also exists. The proanthocyanidin monomers consisting exclusively of (epi)catechin are known as procyanidins and are the most common found in nature (Gu and others 2003) (**Figure 2**). The dimers, trimers, and oligomers, occur in red wine, grape seeds, apples, and cocoa (**Figure 3**) etc. Tannins can play a considerable role in the browning phenomena of apple and apple juices. Moreover,

in fruit juices and fermented beverages (wine, cider, beer), procyanidins are responsible for some organoleptic criteria such as bitterness and astringency (Guyot and others 1996).

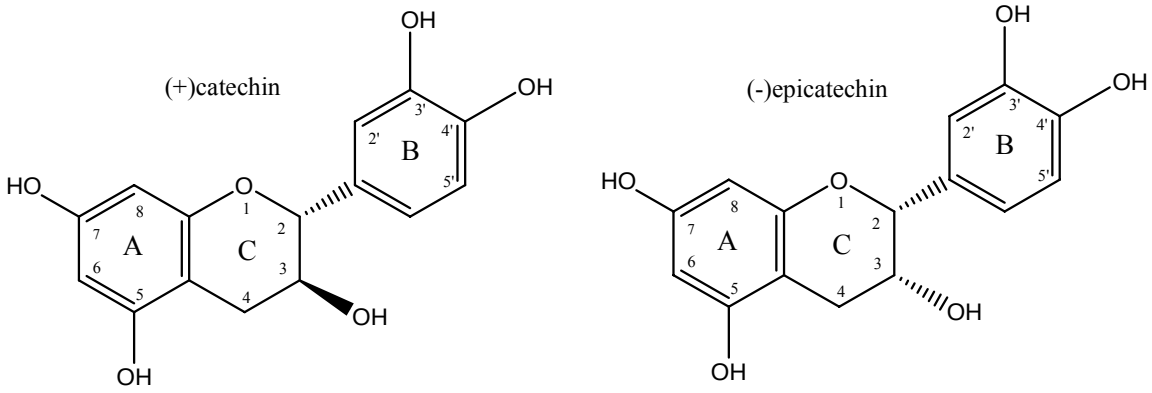


Figure 2: Structures of common condensed tannin monomers found in food.

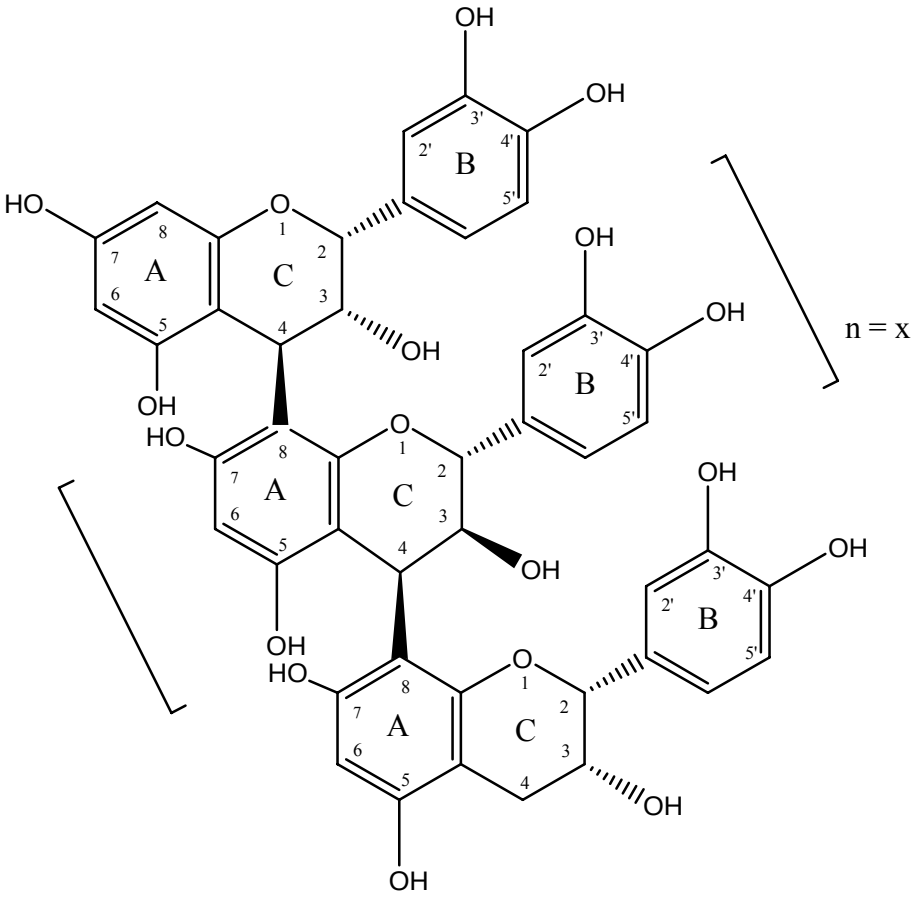


Figure 3: Structure of a simple condensed tannin.

Hydrolyzable tannins, or tannic acids, are composed of gallic acid or its condensation product ellagic acid, esterified to the hydroxyl groups of glucose (Al-Mamary and others 2001; Deaville and others 2007) (**Figure 4**). The galloyl moieties of the hydrolyzable tannins as well as the monomeric condensed catechins are greatly responsible for chelating and radical scavenging properties of these compounds (Heim and others 2002).

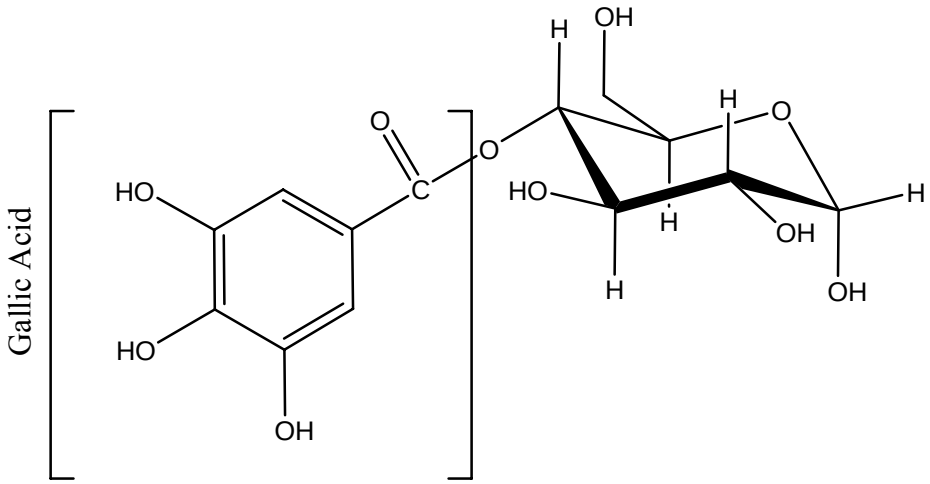


Figure 4: Structure of a hydrolyzable tannin; gallic acid and glucose.

2.2.3 Tannin Sources

Tannins are found in the leaves, fruits, bark, and wood of most trees as well as many other plants (Scalbert and others 1989). They are very abundant in vascular plants where they serve many useful functions, including the control of seed germination and morphogenesis, insect feeding deterrence, and protection against pathogenic attack (Moran and others 1997). In plants, these compounds also protect against ultraviolet radiation and herbivores (Harborne and Williams 2000). Both environmental and seasonal factors cause variation in plant tannin concentrations. Soil mineralogy, pH, water, and amount of sunlight affect the amount of tannin synthesized in plants in nature (Gaffney and others 2004). Therefore, obtaining a consistent source of dietary tannins from produce can be problematic.

In the human diet, tannins are mostly concentrated in fruits, vegetables, wines, teas and cocoa (Heim and others 2002). Consumption of tannin-rich foods and beverages

is associated with the sensation, known as astringency, of dryness or roughness in the mouth (Bacon and Rhodes 2000).

2.2.4 Health Benefits of Tannins

Polyphenolic flavonoids exhibit a wide range of biological, pharmacological, and chemoprotective properties as free radical scavengers in preventing oxidation and cancer initiation (Ayed and others 1999; Yi and others 2006). They have been shown to have anticarcinogenic and antimutagenic potential, antimicrobial properties (Bacon and Rhodes 2000), as well as aid in glucose transport for Type II Diabetics (Liu and others 2005). Their cardio-protective effects stem from their ability to inhibit lipid peroxidation, chelate redox-active metals, and attenuate other processes involving reactive oxygen species. The average human consumption of tannins in the U.S. is estimated to be at least 1 g/day and could therefore be a significant source of dietary antioxidants (Pierpoint 1990).

One of the relationships between flavanols and their antioxidant activity is related to their transition metal-chelating potential. Phenolic compounds are potent inhibitors of iron absorption. Tannins containing catechol (proanthocyanidins) or galloyl (tannic acid) groups, with at least two adjacent hydroxyls, have these marked iron-binding properties (Brune and others 1991). They chelate iron by forming insoluble complexes with iron ions in the gastrointestinal lumen, thereby making the iron unavailable for absorption (Brune and others 1991). It is suggested that iron binds to polyphenols via the *ortho*

dihydroxy (catechol) (**Figure 5**) or trihydroxy-benzene (galloyl) group (Khokhar and Owusu Apenten 2003).

The propensity for metal chelation, particularly iron and copper supports the role of polyphenols as preventative antioxidants in terms of inhibiting transition metal-catalyzed free radical formation The catechol moiety in the B ring of flavanols is where the greatest antioxidant activity takes place (Rice-Evans and others 1997).

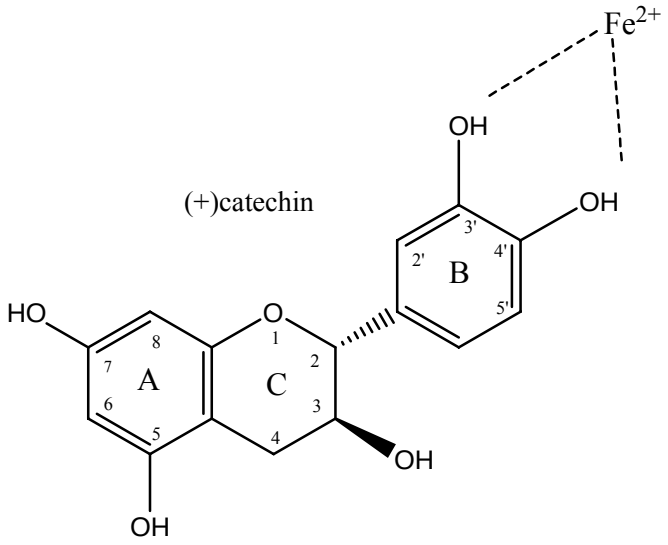


Figure 5: Structure of a condensed tannin monomer, catechin, binding to a ferric ion at the *O*-diphenolic group in the 3',4'-dihydroxy position of the B ring.

2.3 Tannins in the Animal Kingdom

2.3.1 Tannins in Nutrition

As expected from their effectiveness as plant defense chemicals, tannins exhibit a variety of antinutritional effects when present in the diet. In humans, tannins reportedly decrease protein utilization and perturb mineral absorption and diminish total body sodium (Mehansho and others 1987). The presence of tannins has been associated with lower nutritive value and lower biological availability of macromolecules like proteins and carbohydrates, amino acids, vitamins, and minerals in some animal species (Makkar 1989). The most important negative effect of tannins is associated with their formation of complexes with dietary proteins before their breakdown by pancreatic enzymes. The deleterious effects of the use of high tannins sorghums in pig and piglet feeding trials were evident by a decrease in growth performance and an increase in nitrogen in the excreta (Lizardo and others 1995).

Although behavioral responses are somewhat equivocal, tannins do not seem to be strongly selected against when found in native foods eaten by many species, and a number of animals have developed physiological mechanisms for dealing with high levels of dietary tannins (Robbins 1993). Some wild animal species, such as lemurs and rhinoceros, which ingest large amounts of dietary tannins, have developed a defense against tannin overload via the secretion of proline-rich salivary proteins (Mehansho and others 1987). There is evidence that salivary proline-rich proteins, from different herbivorous and omnivorous mammals, may have differing tannin binding specificities;

with proline-rich protein binding specifically to different types of tannin (Hagerman and Robins 1993).

In captive lemurs, hemosiderosis is linked to a diet containing little or no tannin as well as high ascorbic acid levels (Kock and others 1992). Tannins may play a huge role in the metabolism of iron and the overall health of wild animals susceptible to ISD. Therefore, incorporating tannins into the diets of captive animals, prone to this disorder, is logical. As with human ISD patients, the addition of iron chelators to the diet is recommended. Pure tannins are most effective at binding iron but their cost does not make them an attractive candidate for dietary supplementation (Gaffney and others 2004). Therefore tannins sources, such as grape pomace should be tested (Clauss and others 2006).

2.3.2 Tannins & Prevention of Iron Storage Disease

Although iron is an essential element, it can be toxic when it accumulates excessively. The regulation of the intestinal absorption of iron is critical because humans, as well as many other species, have no physiological pathway for excretion (Andrews 1999). Iron overload may be due to excessive amounts of absorbable iron in the diet, or a genetic disorder of the control of iron absorption (Charlton and Bothwell 1983). Free iron is known to be a potent catalyzer of oxygen free radicals (Fujita and others 2007). The circulating ferric ions are reduced by superoxide and the ferrous product is reoxidized by peroxide to regenerate ferric ions and yield hydroxyl radicals (**Figure 6**), which attack all

classes of biological macromolecules (Weinberg 1999). Free iron readily damages tissues; hence very little free iron is found in the body (Sheppard and Dierenfeld 2002).

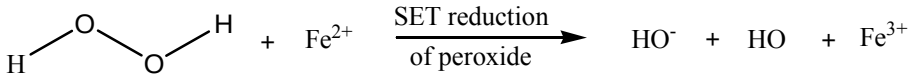


Figure 6: The Ferric ion, Fe^{2+}, produces a hydroxyl radical and hydroxide ion by Single Electron Transport (SET) reduction of peroxide.

Hemosiderosis has been reported in a wide range of species including mynahs, toucans, gorillas, lemurs, New World monkeys, marmosets, fruit bats, rock hyrax, rhinoceros, horses, tanagers, tapirs, fur seals, and pigs in zoos and private collections (Kaneko 1989; Smith and others 1995). In the 1970's it was noted that, in particular, birds of paradise, toucans, and mynahs died with livers marked by accumulation of iron (Sheppard and Dierenfeld 2002). Three publications since the 1980's have reported incidences of hemosiderosis ranging from 32%, to 69%, to 100% in lemurs over 1 year of age. Captive marmosets fed either a low (100 ppm) or high (350-500 ppm) iron diet had markedly different liver iron concentrations (Glenn and others 2006). Monogastric browsers appear to be particularly susceptible to excessive iron absorption when on captive diets, which generally contain high levels of iron and lack the natural iron-binding ingredients (such as tannins) found in wild diets (Clauss and others 2002; Bonar and others 2006).

Tannins, a group of phenolic compounds, can be used as a primary dietary additive to reduce risk of ISD in captive animals. Tannic acid, in an experiment involving starlings, prevented an increase of stored liver iron concentration, even though birds were fed an extremely high-iron diet (Olsen and others 2006). Finding in-expensive iron-binding agents for the diet would be a practical alternative to producing low-iron diets (Olsen and others 2006). Increased antioxidant status caused by the use of tannin substances could have a beneficial effect on animal health. However if tannins should be incorporated in designed diets, tannins sources, such as grape pomace should be tested (Clauss and others 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Grape Pomace Samples

Five wine grape varieties were selected for this research. The varieties included Chambourcin (a hybrid of unknown parentage), Grenache (*Vitis vinifera*), Michigan Concord (*Vitis labrusca*), Norton (*Vitis aestivalis*), and Vincent (*Vitis vinifera*) grapes. The Vincent variety matures early in the season and is typically used to make sweet red or rosé wines. The grape is lightly pressed to obtain light pigments from the skins and to avoid the astringent taste of tannins and other polyphenols. The Chambourcin, Grenache and Michigan varieties mature in mid-season and are used to make semi-dry, medium bodied, red wines. They are moderately pressed, using few rice husks to enhance extraction, to develop light tasting notes from the tannins and other polyphenols. The Norton variety matures late in the season and is heavily macerated, with the aid of many rice husks, to increase the tannin and polyphenol extraction for a wine that is very dry and full-bodied.

All grape pomace samples were pressed and fermented at Stone Hill Winery in Hermannn, Missouri, in September and October of 2007. All samples were immediately frozen after the vinification processing and transferred within one week to the University of Missouri-Columbia and stored at -35°C until further processing.

A portion of each grape pomace sample was freeze-dried to remove moisture. Each sample was then hand divided into three categories; skins, seeds, and whole pomace; all rice husks, often used during the mashing of grapes during fermentation,

were removed from the pomace samples. Each category sample from the pomace was ground to a fine powder using a UDY mill (Model 3010-030, Fort Collins, CO), and passed through a 0.1 mm mesh. Grinding was necessary to improve extraction efficiency. The powdered samples were then kept frozen in a -35°C freezer until used.

Ground high tannin sorghum flour, grown in Texas in 2006, was used as a control. This sorghum has a well documented phenolic composition (Awika and Rooney 2004).

3.2 Chemicals & Reagents

Chemicals purchased from Sigma-Aldrich (St. Louis, MO) were HPLC-grade hydrochloric acid, methanol, water. Folin-Ciocalteu Reagent, Sephadex, bead size 25-100μ, pure (±)catechin-hydrate, and (-)epicatechin.

Chemicals purchased from Acros Organics (Morris Plains, NJ) were ethanolamine, pure vanillin, dimethylformamide, trolox, tannic acid, and gallic acid.

Chemicals purchased from MP Biomedicals, LLC (Solon, OH) were ferric ammonium sulfate and gum arabic.

Chemicals purchased from Fisher Scientific (Rochester, NY; Fair Lawn, NY) were sodium acetate, sodium chloride, potassium persulfate, and sodium phosphate monobasic and dibasic.

AAPH was purchased from Wako Chemicals USA, Inc. (Richmond, VA) and sodium-fluorescence (SF) powder was purchased from Fluka (Steinheim, Germany).

3.3 Methods for Research Objective 1

3.3.1 Total Polyphenol Content Assay

3.3.1.1 Method Summary

The Folin-Ciocalteu method used in this study is not specific to proanthocyanidins, as it responds to many types of phenols with varying degrees of sensitivity (Broadhurst and Jones 1978). The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for a colorimetric assay of polyphenolic antioxidants. The method is an electron transfer based assay and measures the reducing capacity to estimate total phenolic content of biological materials using gallic acid as a standard compound.

3.3.1.2 Reagents

200 mL of the Folin-Ciocalteu Reagent was diluted with 100 mL of deionized water. 0.5 Ethanolamine was prepared by adding 30.9 mL to 100 mL deionized water.

3.3.1.3 Sample Extraction & Analysis

150 mg ground samples were extracted using 20 mL of 1% HCl in methanol in a shaker (Model SHKA 2506-1, Dubuque, IA), for 2 hours. The samples were then centrifuged at 3000 x g, at 13°C, for 10 minutes. The supernatant was poured off for analysis. The sample analysis was done using the Folin-Ciocalteu method (Obanda and Owuor 1997; Dev Choudhury and Goswami 1983; Broadhurst and Jones 1978). 0.1 mL of the sample was mixed with 1.1 mL of deionized water, 0.4 mL Folin-Ciocalteu reagent, and 0.9 mL of 0.5M ethanolamine. After 20 minutes each sample was read spectrophotometrically on a Shimadzu UV-1650PC spectrophotometer (10mm path-

length cell), at 600 nm. Pure gallic acid was used as the standard reference compound. Standard curves were made fresh daily; a standard curve example can be found in Appendix 1.1.

Total phenolic content of each sample (where GAE is gallic acid equivalents; c is the y-intercept) was calculated as:

$$\text{Phenol content GAE mg/g} = \frac{[(\text{Slope} * \text{absorbance})+c]}{\text{Sample Concentration}}$$

3.3.2 Proanthocyanidin Content Assay

3.3.2.1 Method Summary

The vanillin assay (Burns 1971) is widely used for quantitative measurement of condensed tannins. Proanthocyanidins react with vanillin in the presence of mineral acids to produce a red color. The A-ring and a single bond between C2 and C3 are required for positive vanillin reaction. The modified method (Maxson and Rooney 1972) was used in this study because it is more accurate. The advantage of this method is its specificity for a narrow range of flavanols that have free hydroxy groups in the B ring (Price and others 1978).

3.3.2.2 Reagents

Vanillin Reagent A was prepared by mixing 1 g of pure vanillin with 100 mL of methanol. Reagent B was prepared by mixing 92 mL of methanol with 8 mL of HCl. The vanillin reagent was mixed with 1:1 proportions of reagents A and B.

3.3.2.3 Sample Extraction & Analysis

Ground samples, 150 mg, were extracted using 8 mL of 1% HCl in methanol, vortexed, and placed in a 30°C water bath for 20 minutes. Each tube was then centrifuged at 3000 x g, at 13°C, for 10 minutes. Two 1 mL aliquots were taken from each sample and placed in separate tubes, for sample analysis and the blank, in a 30°C water bath for 20 minutes. While in the water bath 5 mL of the vanillin reagent was added to each sample tube and 5 mL of 4% HCl in methanol was added to the black tube. After 20 minutes their absorbencies were read on a Shimadzu UV-1650PC spectrophotometer (10mm path-length cell) at 500 nm. Pure (+)catechin-hydrate was used as the standard compound. Standard curves were made fresh daily; a standard curve example can be found in Appendix 1.2.

Proanthocyanidin content of each sample (where CE is catechin equivalents) was calculated as:

$$\text{CE mg/g} = \frac{\text{mL extract} * (\text{absorbance/slope})}{\text{sample weight (gm)}}$$

3.3.3 HPLC Analysis of Proanthocyanidins

3.3.3.1 Method Summary

HPLC is a popular method for the analysis of procyanidins and flavonols in beverages (Lunte and others 1988). Good separation, based on molecular mass, is easily achieved using a Silica column for normal-phase HPLC (Rigaud and others 1993). A modified version of the normal-phase HPLC method that Hammerstone and others (1999) developed was used (Prior and others 2001) in this study.

3.3.3.2 Solvents & Materials

The extraction solvent was a mixture of acetone/water/acetic acid (70:29.5:0.5). A lipophilic Sephadex column was prepared by packing 1 g into a 12 mL syringe with glass wool in the tip and equilibrated with 30% methanol overnight.

3.3.3.3 Sample Extraction & Purification

Sample extractions were carried out according to Gu and others (2003) with some modifications. Five grams of ground sample was defatted using 50 mL HPLC-grade hexane and dried in a hood overnight. Then 1 g of each dried, defatted sample was vortexed with 10 mL of the extraction solvent for 1 minute. The samples were then sonicated using a 500 watts, 20 kHz High Intensity Ultrasonic Liquid Processor (Model VC-505, Sonics and Materials, Inc., Newton, CT), with a 13 mm alloy probe, at 20% amplitude for 30 seconds. The samples were then allowed to sit at room temperature for 50 minutes; vortexing each after 25 minutes elapsed. Samples were then centrifuged at 3500 x g, at 13°C, for 15 minutes. 7.5 mL were removed from each sample and were concentrated under vacuum on a Brinkman Rotavapor-R. The residue was then frozen in the dark at -35°C. Once frozen, the samples were freeze dried in a Labconco Freeze Dryer-5 for 48 hours. The remaining residue was then reconstituted with 6 mL of 30% methanol.

Samples were then loaded onto the Sephadex column and washed with 30% methanol continuously until all pigments were removed. The proanthocyanidins were then eluted with 70% acetone and stored in a screw-cap tube in a -35°C freezer until HPLC analysis.

3.3.3.4 Normal-Phase HPLC Analysis

The standard compounds were (±)catechin-hydrate, (-)epicatechin and tannic acid; 1 mg of each was mixed with 10 mL of the extraction solvent. Analyses were preformed on a Perkin-Elmer (Waltham, MA), series 410 HPLC system equipped with a manual injector and column heater. Normal-phase separations of the procyanidins were carried out on a Supelco Lichrospher Silica-60 5µ column (250 mm x 4.6 mm) at 37°C. Fluorescence detection was recorded using a Waters (Milford, MA), 474 scanning Fluorescence Detector at an excitation of 276 nm and emission at 316 nm (Prior and others (2001). The ternary mobile phase is described by Hammerstone and others (1999), and consisted of (A) dichloromethane, (B) methanol, (C) acetic acid in water (1:1, v/v). Separations were effected by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/minute as follows: elution starting with 4% B in A; 28.4% B in A, 0-30 minutes; 50% B in A, 30-60 minutes; 86% B in A, 60-65 minutes; 65-70 minutes isocratic.

3.4 Methods for Research Objective 2

3.4.1 Determination of Iron-Binding Phenolic Capacity

3.4.1.1 Method Summary

Iron-binding phenolic capacity was measured using the method developed by Brune and others (1991) with slight modifications. The original method calls for the use of urea to enhance iron-binding among catechol groups. To obtain a natural iron-binding result, urea was not used in this study. Phenolic compounds, extracted by

dimethylformamide (DMF) in an acetate buffer, will react with a ferric ammonium sulfate reagent (FAS) to result in a color that can be read spectrophotometrically. The resulting color is read against a reagent blank at two wavelengths, 578 nm and 680 nm, corresponding to the absorbance maxima of iron-galloyl (blue color) and iron-catechol (green color).

3.4.1.2 Reagents

A 1% acetate buffer, pH4.4, was made by combing 630 mL of 0.1N acetic acid and 370 mL of 0.1N sodium acetate. The DMF-acetate solution was prepared by mixing equal volumes of DMF and 1% acetate buffer. A gum arabic solution was prepared by mixing 1 g in 100 mL deionized water. The FAS reagent was prepared by mixing 89 parts 1% sodium acetate buffer, 10 gum arabic solution, and 1 part ferric ammonium sulfate solution (5 g ferric ammonium sulfate in 100 mL 1% HCl). The food blank reagent was prepared by mixing 89 parts 1% sodium acetate buffer, 10 parts gum arabic solution, and 1 part 1% HCl.

3.4.1.3. Sample Extraction & Analysis

500 mg of each sample was extracted with 50 mL of the DMF-acetate solution. The samples were wrapped in foil, to block light, and shaken for 16 hours at room temperature. Each sample was then centrifuged at 3500 x g, at 13°C, for 15 minutes. The supernatant was poured off for analysis.

For spectrophotometric analysis, 2 mL of each diluted standard was mixed with 8 mL of the FAS reagent and read, after 15 minutes, at 578 nm (tannic acid standards), and 680 nm (catechin standards), against a reagent blank containing 2 mL of DMF-acetate

solution and 8 mL of the FAS reagent. A sample blank containing 2 mL of the sample and 8 mL of the food blank reagent, was read against a food blank containing 2 mL DMF-acetate solution and 8 mL of the food blank reagent.

The sample absorbance was determined by subtracting the sample blank absorbance from the sample absorbance, both at 578 nm and 680 nm. The content of the catechol and galloyl groups were calculated by using the linear regression equation from the standard curves of tannic acid and catechin-hydrate at the two respective wavelengths. Standard curves were made fresh daily; a standard curve example can be found in Appendix 1.3.1 and 1.3.2.

3.5 Methods for Research Objective 3

3.5.1 Oxygen Radical Absorbance Capacity

3.5.1.1 Method Summary

The oxygen radical absorbance capacity (ORAC) assay measures the free radical damage to a fluorescent probe through its change in fluorescence intensity (Cao and others 1993). In the presence of antioxidants, the inhibition of free radical damage is reflected in the protection against the change of probe fluorescence.

The reaction measured is driven to completion and the quantification is achieved using the area under the curve (AUC). The AUC technique allows the ORAC to combine both inhibition time and inhibition percentage of the free radical damage by the antioxidant into a single quantity (Ou and others 2001). The degree of antioxidant-

mediated protection is quantified using trolox as a standard. In this method, AAPH (2,2'-azobis(amidinopropane)dihydrochloride) is used as the free radical initiator.

3.5.1.2 Sample Extraction

For each sample, 300 mg went through a 3 step extraction process: 1) mixed with 10 mL of 70% acetone, shaken for 2 hours, centrifuged at 300 xg, at 13°C, for 10 minutes, and the supernatant poured off; 2) the residue was washed with 10 mL of 70% acetone, centrifuged, and the supernatant poured off; 3) the first step was repeated. Then all three aliquots were combined for analysis. The extracts were stored at -35°C.

3.5.1.3 Reagents

A phosphate buffer solution (PBS) was prepared by mixing 95 mL of sodium phosphate monobasic (2.98 g/100 mL) and 405 mL of sodium phosphate dibasic (15.6 g/500 mL), followed by 8.04 g of sodium chloride and filled to volume (1 L), lastly the pH was adjusted to 7.4 with 2M NaOH. The sodium-fluorescence reagent-A (SF-A) was prepared by dissolving 6 mg of SF powder in 20 mL of PBS. The sodium-fluorescence reagent-B (SF-B) was prepared by mixing 100µL SF-A with 19.9 mL of PBS. The sodium-fluorescence working solution was prepared by adding 10 µL of SF-B to 9.9 mL of PBS. The AAPH solution was prepared by dissolving 414 mg of AAPH in 10 mL PBS.

3.5.1.4 Plate Layout & Analysis

A 96-well micro-plate was used for analysis. 150 µL of the SF-working solution was added to each well followed by 25 µL of each sample, standard, or blank. The plate was incubated at 37°C for 30 minutes in the plate reader. Afterwards, 25 µL of AAPH

solution was added to each well and read over 2 hours. Trolox was used as the standard. Standard curves were made fresh daily; a standard curve example can be found in Appendix 1.4.

3.5.1.5 Calculations

The AUC is automatically calculated by the Gen5 software system, for each sample, standard, and blank. The Net-AUC was calculated as follows:

$$\text{Net-AUC} = \text{AUC sample well} - \text{AUC blank well}$$

The initial reading of the samples was not uniform with their blanks so a correction factor (CF) was determined as follows:

$$\text{Net-AUC sample} * \text{CF} = \text{corrected Net-AUC samples}$$

The trolox equivalents (TE) was calculated by interpolating the samples corrected Net-AUC against the trolox standard curve. The ORAC value of the sample was calculated as follows:

$$\mu\text{M TE/g sample} = \frac{\text{samples TE } \mu\text{M}}{\text{Sc (g/L)}}$$

3.5.2 ABTS Antioxidant Activity

3.5.2.1 Method Summary

ABTS or, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), antiradical decolorization test is a spectrophotometric method widely used for the assessment of antioxidant activity in a variety of substances (Wolfenden and Wilson 1982). The

improved technique (Miliauskas and others 2003), used in this assay, involves the direct production of the blue/green ABTS chromophore through the reaction between ABTS and potassium persulfate. The absorption maxima is 734 nm; the extent of decolorization as percentage inhibition of the ABTS radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts (Re and others 1999).

3.5.2.2 Reagents

A phosphate buffer solution (PBS) was prepared by mixing 95 mL of sodium phosphate monobasic (2.98 g/100 mL) and 405 mL of sodium phosphate dibasic (15.6 g/500 mL), followed by 8.04 g of sodium chloride and filled to volume (1 L), lastly the pH was adjusted to 7.4 with 2M NaOH. The ABTS mother solution was prepared by mixing 44.8 mg of ABTS, 8.12 mg potassium persulfate, and 20 mL of deionized water. The solution was allowed to react in the dark for 12 hours. The ABTS working solution was prepared by mixing 145 mL of PBS with 5 mL of the ABTS mother solution.

3.5.2.3 Sample Extraction & Analysis

The sample extracts used were those prepared by the ORAC assay.

To 2900 μL of the ABTS working solution, 100 μL of each extract was mixed. Each was allowed to react 15 minutes and then read spectrophotometrically at 734 nm. The absorbance of each dilution was subtracted from that of the blank. Trolox was used as the standard. Standard curves were made fresh daily; a standard curve example can be found in Appendix 1.5. Each sample absorbance was subtracted from the blank.

Total antioxidant activity of each sample (where TE is trolox equivalents; Δabs is change in absorbance; C is the y-intercept; and Sc is the sample concentration) was calculated as:

$$\mu M \text{ TE/g sample} = \frac{(\text{slope} * \Delta abs) + C}{Sc}$$

3.6 Statistical Analysis

The data were analyzed completely randomized. Treatment differences were determined using Fishers Protected Least Significant Difference, P = ≤ .05, and performed using the PROC GLM SAS 9.1 (Littell and others 2006). SAS tables, for significant differences among experimental treatments, are shown in Appendix 2.

CHAPTER 4

RESULTS & DISCUSSION

4.1 Phenolic & Proanthocyanidin Content

4.1.1 Total Phenolic Content

Comparison of total phenolic content in high tannin sorghum and each grape pomace variety is shown in **Figure 7**. Sorghum has been established as a grain containing high phenolic content (Awika and Rooney 2004) and was used as a comparison for the grape samples. All of the wine pomace samples contain higher total phenolic content than the sorghum (dry basis).

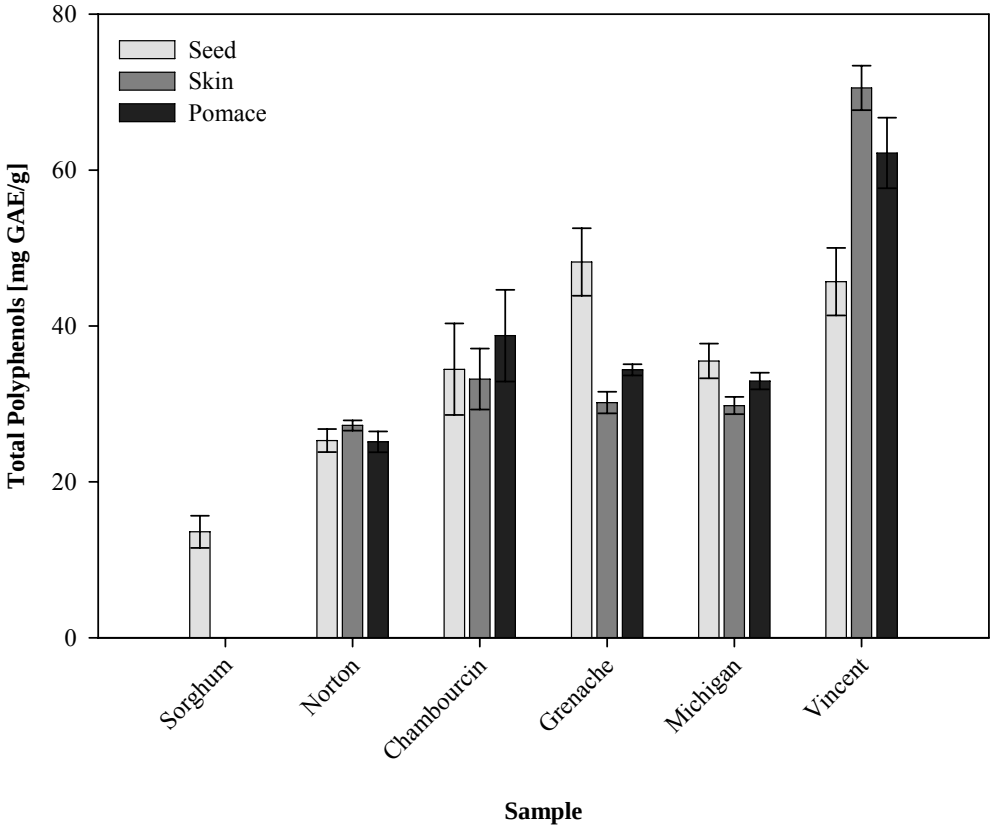


Figure 7: Total polyphenolic content of wine pomace samples and sorghum (dry basis) determined by the Folin-Ciocalteu assay, (GAE is gallic acid equivalents). Error bars represent ± standard deviations (n= 3). Coefficient of variability = 8.57%.

Except for Chambourcin, the total phenolic content of the pomace, from each grape variety (mg GAE/g) fell somewhere between the seed and skin phenol content. Makris and others (2007) also found that the red wine pomace phenol content fell in between the seed and skin extracts; with the seeds having higher phenol content than the skin.

The Norton variety had the lowest phenolic content (25.1 mg GAE/g) in the seeds and (27.3 mg GAE/g) in the skins. The Norton variety is heavily crushed during the wine

making process to intensely extract phenols from both the skins and the seeds of the grape. The phenols increase the deep purple color, the astringent taste, and round mouth-feel of the wine; this results in a pomace with little polyphenols. For dry red wines, the heavily crushed grapes are left together with the pressed juice for several days in order to let the anthocyanins penetrate into the juice, coloring it deeply red or purple (Baumgartel and others 2005).

The Chambourcin, Grenache, and Michigan grape seeds contained higher total phenols than their skins; a likely result from the vinification methods used for the wine. These three red wine varieties are moderately pressed during the wine making process leaving behind a fair amount of color pigments in the skin, but not more than the seeds, which are often not crushed during the processing.

The Vincent sample, on the other hand, had higher total polyphenolic content in the skin (70.78 ± 1.48 mg GAE/g) than the seed (45.8 ± 4.33 mg GAE/g), The Vincent variety is primarily used for sweet rosé wines which entail a very light pressing of the red skins to create a light pink wine; therefore resulting in a by-product that has many phenolics, such as anthocyanins, left in the skins that are typically extracted from other grapes for red wine. The Vincent skins left in the pomace are thus very deep purple in comparison with the other varieties due to high anthocyanin levels that are retained after pressing. This likely contributed to the high phenol content in the skin and the pomace.

In general the Vincent grape pomace has the highest total phenolic content (63.43 ± 1.48 mg GAE/g) when compared to the other pomace varieties. In a study by Makris and others (2007), results showed that extracts from red wine pomace contained

exceptionally high amounts of total polyphenols, 54.02 ± 266 mg GAE/g, on a dry weight basis, a great part of which is composed of flavanols; the Vincent variety had an even higher amount than the grapes previously tested. It has also been noted that red wine by-products contain a larger burden of phenolics as compared with other common food processing wastes (Makris and others 2007)

Grapes contain a large amount of polyphenols which include the phenolic acids, flavonoids, anthocyanins, and proanthocyanidins (Lou and Yeap Foo 1998). There is much interest in using not only phenolic anthocyanins found in the skins but also the grape seed polyphenols (Gomez-Plaza and others 2006). Due to its weak pressing, the Vincent pomace had the highest total polyphenolics in both the skins and seeds; the Vincent pomace, thus, has the greatest potential in the food industry as a functional ingredient. The results show that pressing has a large impact on phenolic content of pomace.

4.1.2 Total Condensed Tannin Content

None of the samples used in this study reached levels beyond 62.7 mg $\pm$ 2.5 mg catechin equivalent (CE) per gram of the Grenache seed. The trend expected was that the seed would contain a higher condensed tannin content than the skin and that the pomace would fall somewhere in between the two; this trend is confirmed in **Figure 8**. A recent study showed that the seeds are the storage sites of condensed tannins, with lower levels in the skins (Shrikhande 2000). Gonzalez-Paramas and others (2004) also confirmed that grape seeds contained more condensed tannins, starting at 136 mg catechin equivalents

per gram (dry basis), than the whole pomace starting at 29 mg CE/g which give ranges to show variability.

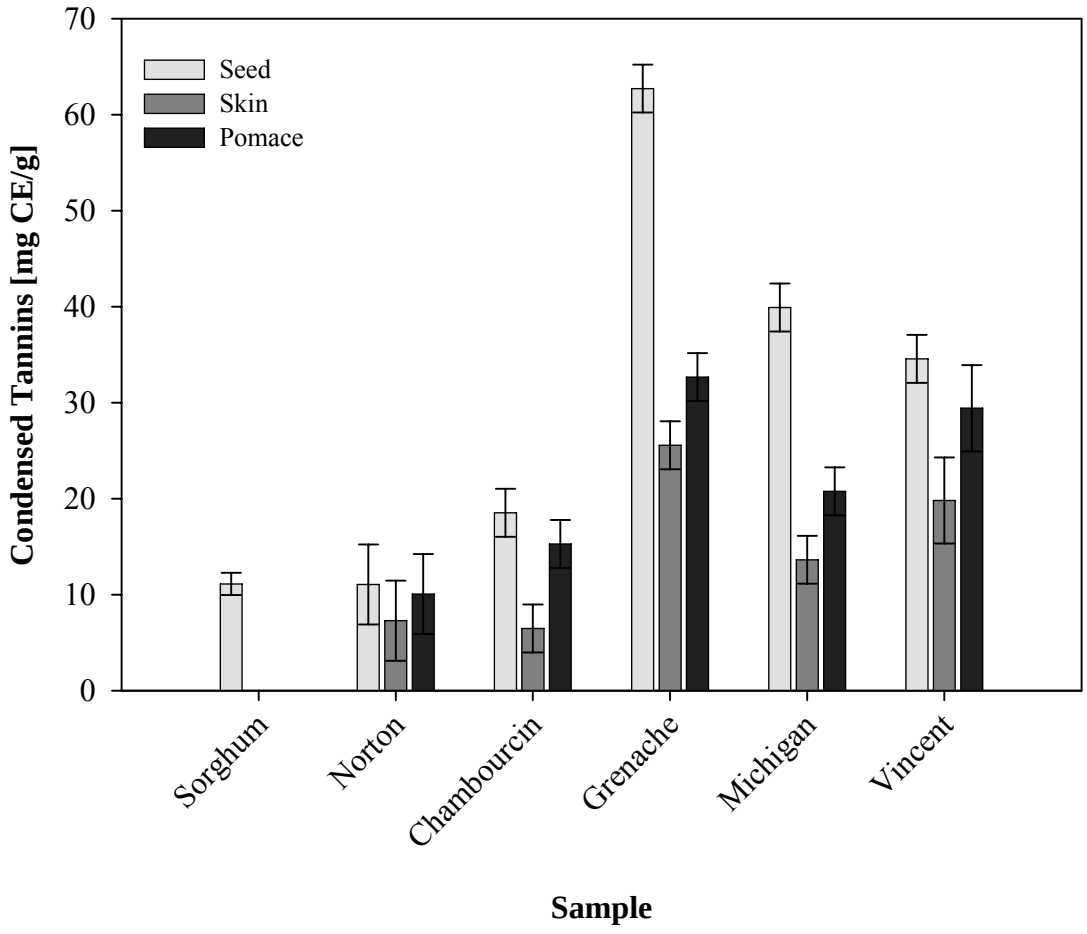


Figure 8: Total condensed tannin content of wine pomace samples and sorghum (dry basis) determined by the Vanillin-HCl assay; represented in catechin equivalents (CE). Error bars represent ± standard deviations (n= 3). Coefficient of variability = 9.81%.

As previously discussed, the Norton is a grape that is heavily crushed during the wine-making process for tannin extraction desired in the wine and had the lowest content

overall in the skins (7.3 mg CE/g) and in the seeds (11.10 mg CE/g). The Grenache seed had the highest condensed tannin content of 62.70 $\pm$ 2.50 mg CE/g. In regard to the pomace samples, the Vincent contained 30.04 ± 0.78 CE per gram; which was similar to that of the Grenache pomace (32.93 ± 7.853 mg CE/g). The results show that the Vincent and Grenache varieties had the highest condensed tannin content. The Grenache pressing method is similar to the Chambourcin and the Michigan but it had significantly higher condensed tannin content. This was mostly due to the fact that its seed had the higher condensed tannin content, likely a result of varietal difference. The Vincent was expected and confirmed to also have high condensed tannin content in the pomace.

Although the Vanillin-HCl method underestimates total proanthocyanidin content (Price and others 1978) it still provides a qualitative analysis of the primary location of proanthocyanidins within the sample as well as comparing grape pomace varieties. Gu and others (2004), reported that dry grape seeds to contain an average of 35.3 mg of total proanthocyanidins per gram of sample. The results from this study show that several of the grape seed varieties are close in comparison, however, due to grape variety and wine production methods, there is variability among them.

Condensed tannins are among the most abundant phenolics in grapes (Gu and others 2003); therefore it is reasonable to assume that the total polyphenolic content and the condensed tannin content would highly correlate. **Figure 9** displays the correlation between total phenolic content and condensed tannin content for sorghum and each grape sample variety. The correlation was rather weak ($R^2 = 0.19$); showing that approximately 19% of the total polyphenols are attributable to condensed tannins. The other phenols

were present in higher quantities probably due to more of the tannins being extracted during vinification.

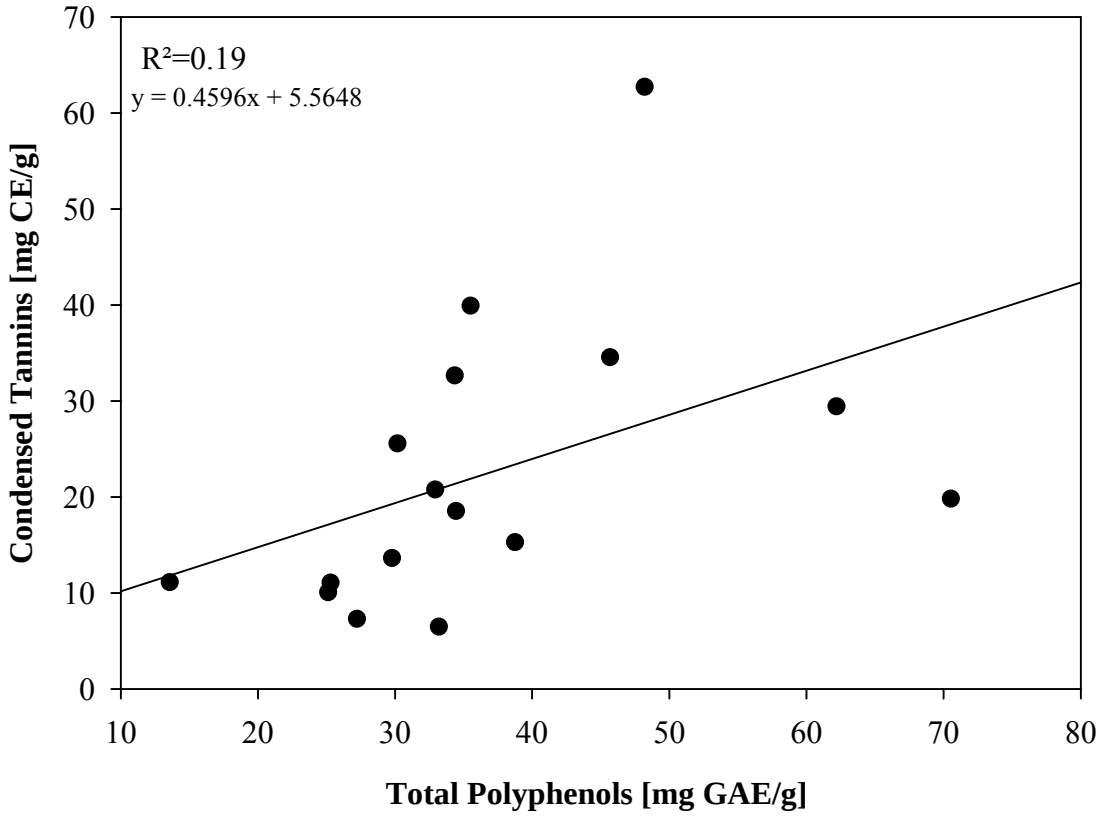


Figure 9: Correlation between the Folin-Ciocalteu (Gallic Acic Equivalents; GAE) and Vanillin-HCl (Catechin Equivalents; CE) assays for the sorghum and grape pomace samples.

The assay confirmed that the Grenache and Vincent pomaces had the highest tannin content; thus presenting the strongest potential as a source of compounds that may benefit health by inhibiting human low-density lipoprotein, reducing heart disease, preventing cancer, and chelating metal irons (Ozkan and others 2004).

4.1.3 HPLC Analysis of Procyanidins

All samples, excluding sorghum, depicted large peaks for (+)catechin and (-)epicatechin monomers eluting at 12 and 15 minutes, respectively (**Figure 10**).

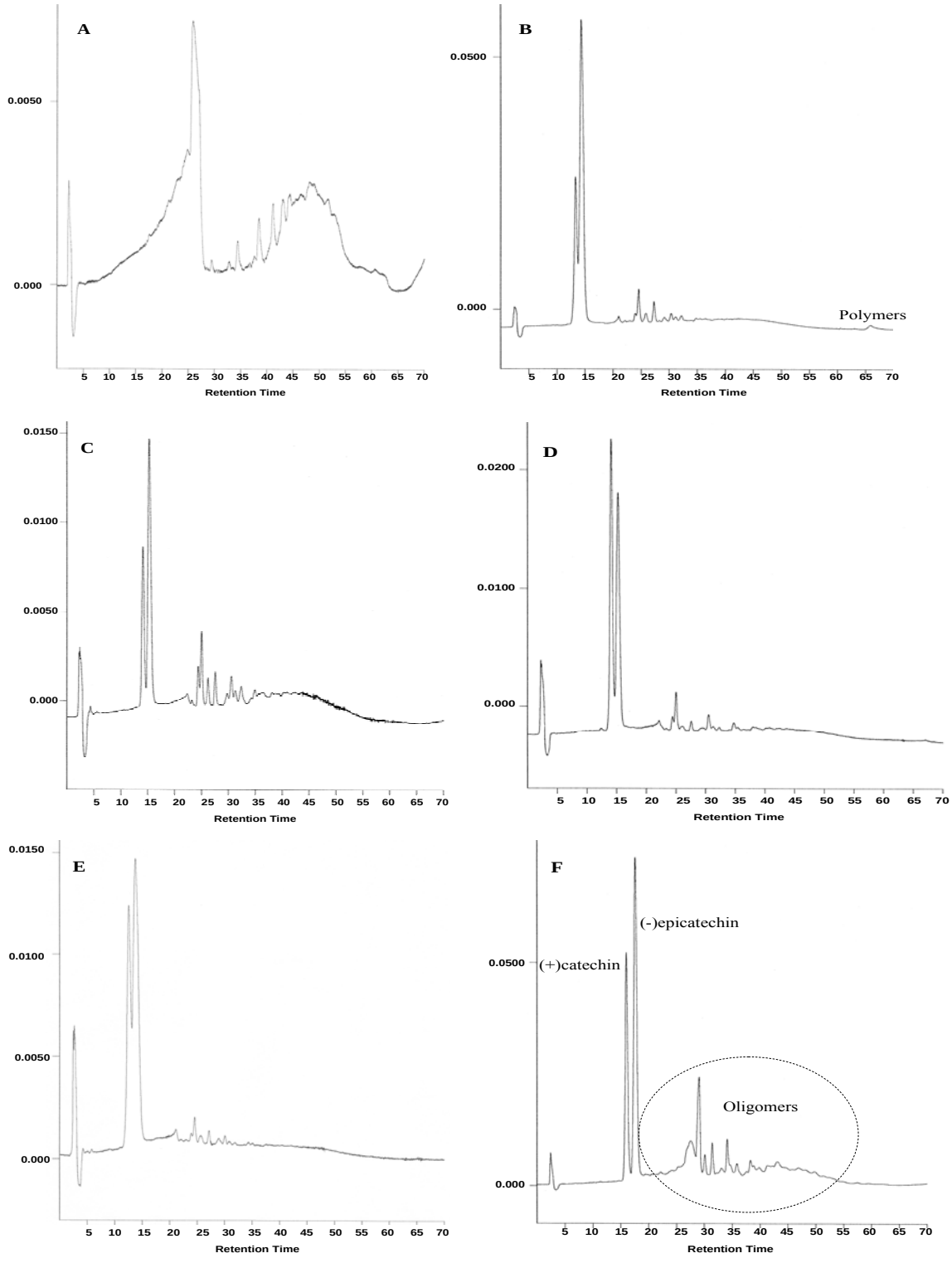


Figure 10: Normal-phase HPLC chromatograms of procyanidin extracts. **A**) sorghum; **B**) Chambourcin; **C**) Grenache; **D**) Michigan; **E**) Norton; **F**) Vincent.

The Norton pomace, as well as the sorghum extract, show the smallest procyanidin peaks; monomer and oligomers. The low values, also seen in the total phenols and condensed tannins, are once again a result of the strong pressing methods used during the wine production. The vinification process leaves little procyanidins in the pomace by-products; which is confirmed through HPLC.

As for the Grenache and Michigan varieties, they fell in between the other varieties with medium-sized peaks from monomers to oligomers.

The Vincent pomace extract contained the highest peaks for monomers as well as all other oligomers. The Vincent variety is lightly pressed during the wine making process leaving a high level of procyanidins in the pomace. These data suggest that the method of pressing has the largest impact on procyanidin content of red wine pomace.

For the sorghum and the Grenache pomace extractions the chromatogram separations eventually became a broad hump, typically after the trimers or tetramers. A study completed by Rigaud and others (1993), indicated similar problems using normal-phase HPLC. They observed that the tetramers gradually fused into two broad, unresolved humps; probably due to the large diversity of structures as the degree of polymerization increased.

It is well established that grape seeds contain flavan-3-ols and its repeat unit including dimer, trimers, etc., and much larger polymers, perhaps with a degree of polymerization from 7 to 16 (Haslam 1980). Procyanidins up to dodecamers have been resolved on normal-phase HPLC in previous studies (Lazarus and others 1999). Although the method used was chosen for its separation of polymers, only the Chambourcin

pomace sample gave a small peak for the polymers after 65 minutes. A recent study detected polymers in low-bush blueberry extracts, using normal-phase HPLC, with a single peak with a retention time of 50.6 minutes. Detection of polymers was expected as research confirms they exist in large quantities in grape constituents (Gu and others 2002). However, due to older lab equipment, the sensitivity required for appropriate separations was not obtained.

The results from the normal-phase HPLC analysis confirm, once again, that the Vincent pomace has the greatest potential as a source of tannins for animal and human applications whereas the Norton, again, has the least. The HPLC analysis supports the data from the other assays in that vinification methods have an impact on tannin content.

4.2 *In Vitro* Iron-Binding Phenolic Capacity

The iron-binding capacity of the grape pomaces was tested to determine whether or not the samples could be potentially used to prevent iron storage disease in captive zoo species. Tannic acid and catechin were used as the standards as proposed by Brune and others (1991).

Among all samples, there were large differences between tannic acid equivalent (TAE) and catechin equivalent (CE) (**Figure 11**). This indicates the samples primarily contained condensed tannins; this CE values were a better indicator of iron-binding capacity of the pomace samples.

The Norton pomace had the lowest iron-binding capacity by both TAE and CE (19.62 ±.0.25 mg TAE/g). As the Norton variety is heavily macerated during the wine-

making process, few proanthocyanidins were left in the pomace; explaining its poor iron-binding potential in comparison with the other samples.

The Chambourcin, Grenache, and Michigan samples had very little difference among them. These three varieties are so similar because they have very similar pressing methods during the vinification processing.

The Vincent pomace variety showed the highest iron-biding capacity (120.23 ± 0.25 mg CE/g). The high iron-binding capacity expected as this variety is used to make sweeter wines and many of the proanthocyanidin constituents are left in the by-product.

A study completed by Brune and others (1991), showed that, on a dry basis, red sorghum contained 5.5 mg TAE/g and 16.4 mg CE/g and green tea leaves contained 84 mg TAE/g and 76.6 mg CE/g. Green tea is considered a good source of proanthocyanidins. Our data shows that the Vincent pomace had higher condensed tannin iron-binding capacity than both red sorghum and green tea; indicating that it is a good, if not better, source of tannins. The Vincent pomace showed the strongest potential as a source of dietary phenolics that can interfere with the bioavailability of iron in the diet for species with hemosiderosis or iron storage disease. Thus it seems that targeting grape pomace from rosé-type wine pressings would provide very high levels of iron-chelating phenols.

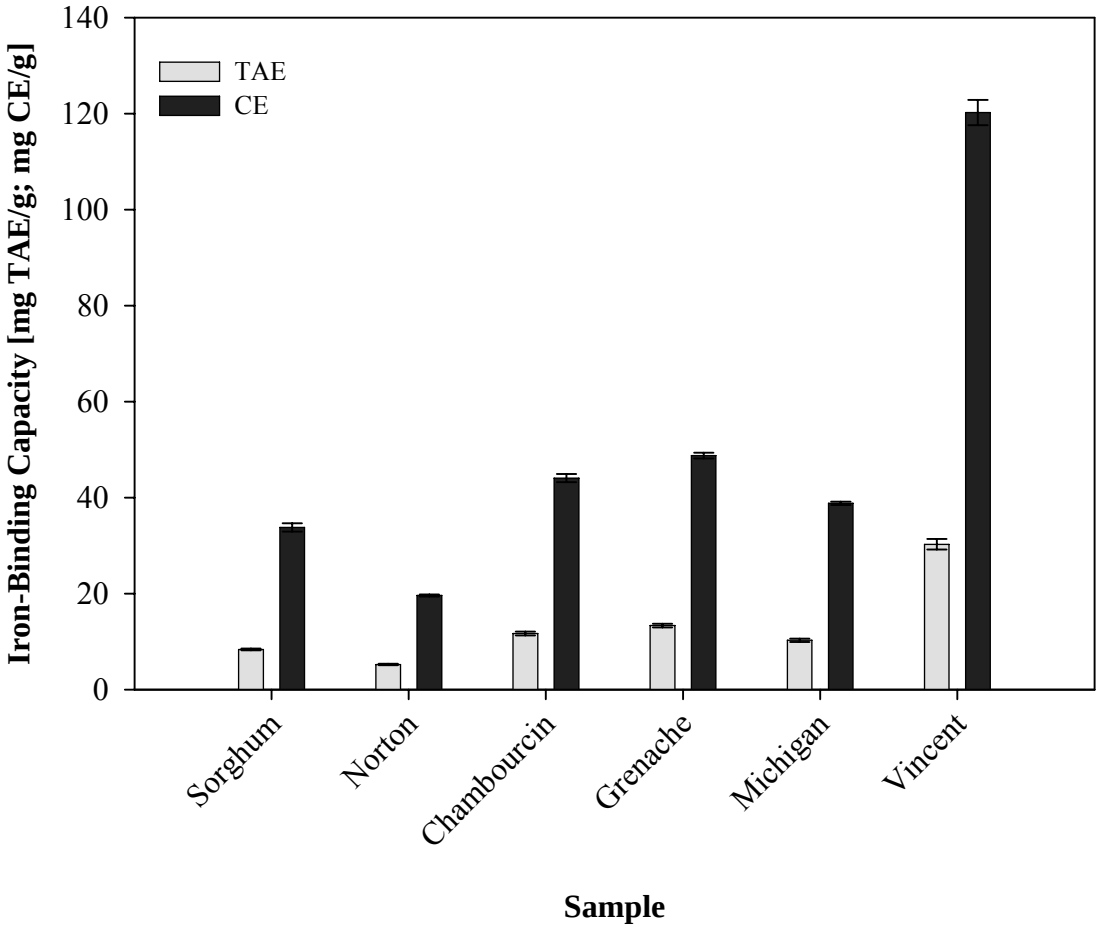


Figure 11: Tannin iron-binding capacity of wine pomace samples and sorghum (dry basis), determined by the Phenolic Iron-Binding assay. Error bars represent ± standard deviations (n= 3). Coefficient of variability for tannic acid equivalents (TAE) = 2.91% and for catechin equivalents (CE) = 2.50%.

The iron-binding method can be an alternative quantitative method for condensed tannins estimation and is relatively rapid and precise (Brune and others 1991). The Vanillin-HCl is known to underestimate tannin content (Price and others 1978). **Table 1** summarizes the catechin equivalents [mg/g] measured by both the Vanillin-HCl assay

and the iron-binding phenolic assays. In general, the iron-binding assay gave CE values that were 2 to 4 times higher than the Vanillin-HCl assay.

	Catechin Eq. [mg/g]			
Sample	**Vanillin-HCl**		**Iron-Binding**	
	Mean	***Std. Dev.***	***Mean***	***St. Dev.***
Sorghum	11.09	± 1.39	33.79	± 0.88
Chambourcin Pomace	15.28	± 1.01	44.06	± 0.83
Grenache Pomace	32.93	± 7.85	48.74	± 0.58
Michigan Pomace	20.83	± 2.00	38.81	± 0.33
Norton Pomace	10.18	± 0.40	19.62	± 0.22
Vincent Pomace	30.04	± 0.78	120.23	± 2.63

Table 1: Comparison of the Vanillin-HCl assay and iron-binding phenolic assays for quantifying tannins. Results are presented on a dry basis.

Although the methods differ in quantitative values, they showed similar trends. In fact, the methods correlated fairly well ($R^2 = 0.44$) (**Figure 12**).

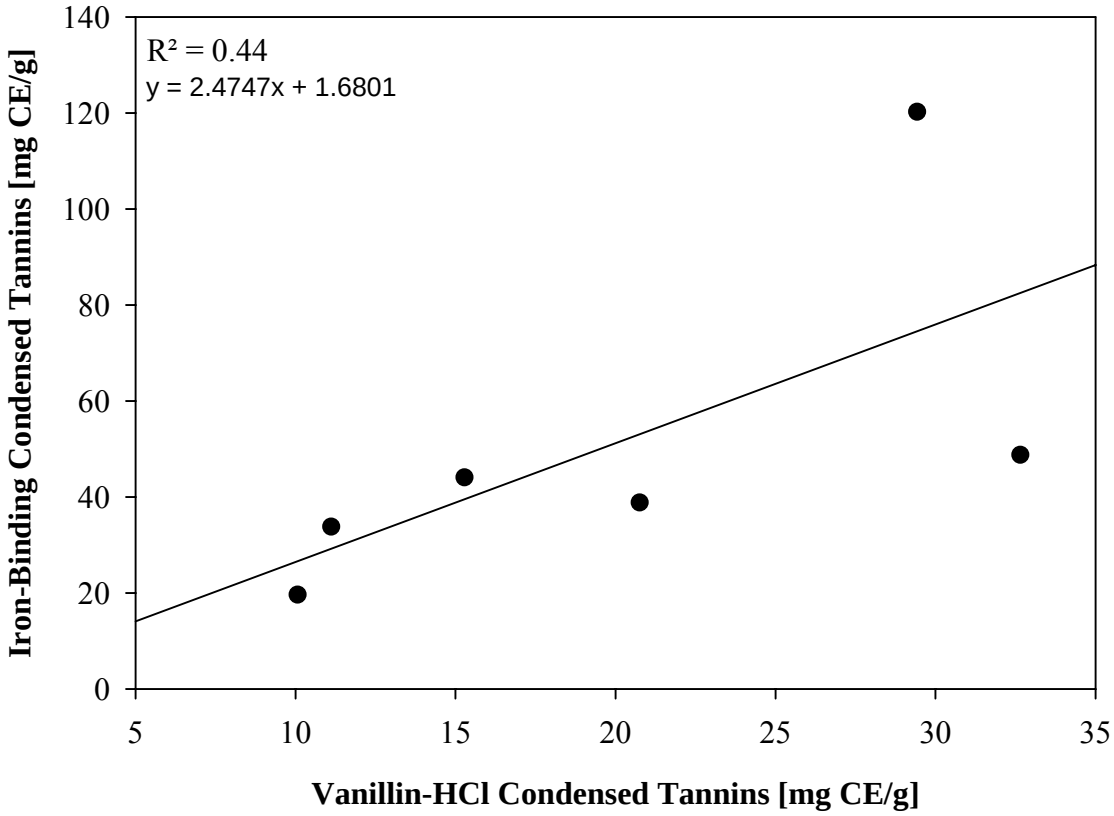


Figure 12: Correlation between the Vanillin-HCl and iron-binding phenols catechin equivalents (CE) assays for the sorghum and grape pomace samples.

The iron-binding phenolic assays may be a more accurate quantitative method for condensed tannins than the Vanillin-HCl assay. However, its major limitation is that it is nonspecific to condensed tannins. Correlation between total polyphenols by the Folin-Ciocalteu method and iron-binding phenolic assay was much higher ($R^2 = 0.80$), (**Figure**

13), compared to the correlation between Vanillin-HCl and the Folin-Ciocalteu method ($R^2 = 0.45$). This may be due to the nonspecific nature of the both the Folin-Ciocalteu and iron-binding assays.

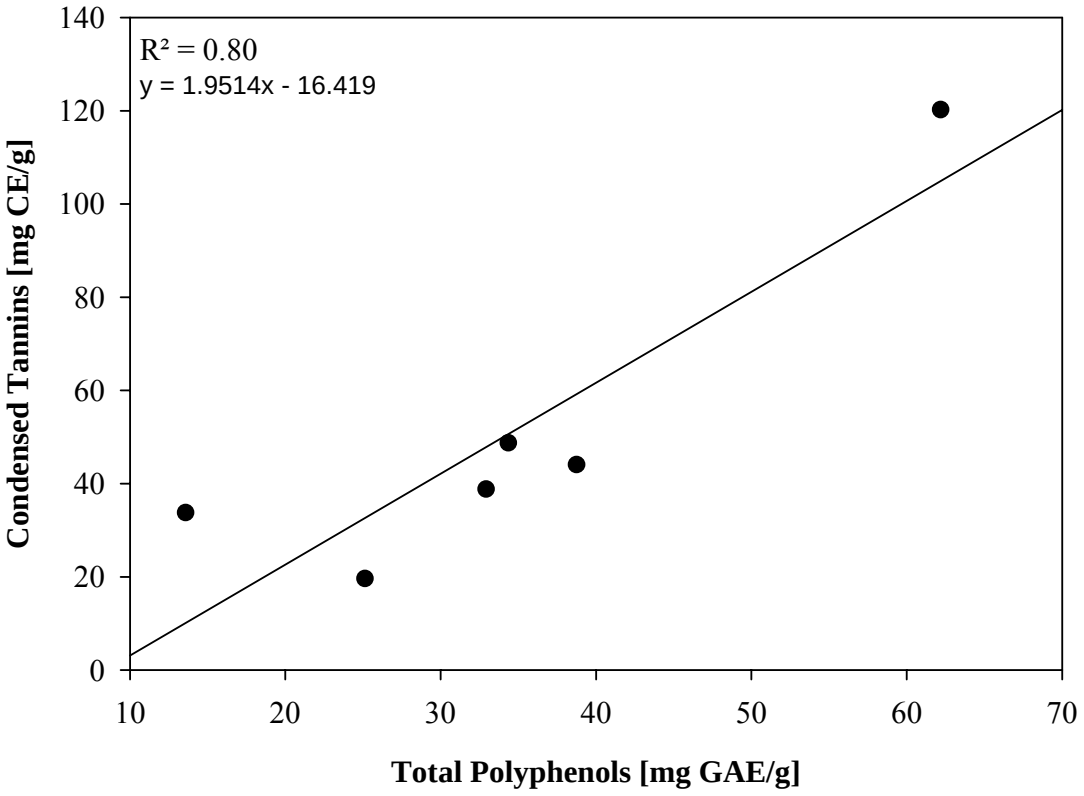


Figure 13: Correlation between the Folin-Ciocalteu and Iron-binding phenolic assays.

In hemosiderosis, excess iron is deposited in the liver, pancreas, heart, joints, and endocrine glands, resulting in tissue damage that can lead to ISD (Whitlock and others 2006). Currently deferiprone, administered orally, and deferoxamine, administered parenterally, are the only compounds available for treating and preventing ISD. Potential complications include ocular, auditory, and cerebral neurotoxicity, abnormalities in

cartilage formation, stunted linear growth, acute respiratory hypersensitivity, local skin reactions, and increased bacterial and fungal infections (Whiteside and others 2004). For captive animal species, the stress of frequent handling for daily injections and the lack of oral efficacy would preclude the use of the drugs in susceptible animal species. Dietary sources of natural iron-chelators are a better therapy.

Tannins can function as antioxidants with many positive health benefits including direct OH^- scavenging and iron chelation (Lopes and others 1999). The iron-binding assay has confirmed that grape pomace has strong capability to chelate iron *in vitro*; primarily due to the condensed tannins. Adding natural chelators, such as tannins, to diets for ISD susceptible species has been a frequent suggestion (Sheppard and Dierenfeld 2002) and wine pomace, particularly a low-pressed variety, such as the Vincent, would be a logical choice.

4.3 Antioxidant Activity

4.3.1 ORAC Antioxidant Assay

Oxygen radical absorbance capacity (ORAC) for all samples is shown in **Figure 14.** The Vincent pomace had the strongest antioxidant capacity in its skin, (1116.28 ± 186.22 μM trolox equivalents (TE) per gram) which was likely due to the high anthocyanin content left in them. The Vincent, being so lightly pressed, retained a deep purple color in the skin which contributed to its antioxidant activity. The Vincent pomace also had a high antioxidant capacity (914.42 ± 75.86 μM TE/g), when compared with the other pomace varieties; with the exception of the Grenache (1322.26 ± 378.32 μM TE/g).

The Grenache pomace had the highest antioxidant capacity when compared to all other pomace samples. Often pomace contains stems leftover from the harvest and vinification method. Grape stems contain significant amounts of antioxidative and polyphenolic compounds, especially phenolic acids, and flavonols (Souquet and others 2007). These stems can contribute to antioxidant activity and is the likely cause of the high ORAC values.

The Norton pomace had the lowest overall antioxidant capacity (365.29 ± 72.57 μM TE/g), when compared to other pomace samples. The Chambourcin and Michigan generally had similar ORAC values.

Grape skin extracts were reported to have ORAC values of 1567 μM TE/g while grape seed extracts had values of 1188 μM TE/g (Ou and others 2001). Their values are comparable to those obtained in this assay. Lowbush blueberries, known for their high antioxidative properties, had ORAC values of 264 μM TE/g, dry basis (Prior and others 2001). All grape pomace samples used in this study had values higher than that of the lowbush blueberry indicating that they are a good potential source of antioxidants for animals and humans.

The major differences seen are primarily an effect of the wine making process. The vinification process can extract high quantities of anthocyanins and tannins in order to give structure to wine and depends greatly on the pressing methods applied (Alvarez and others 2006).

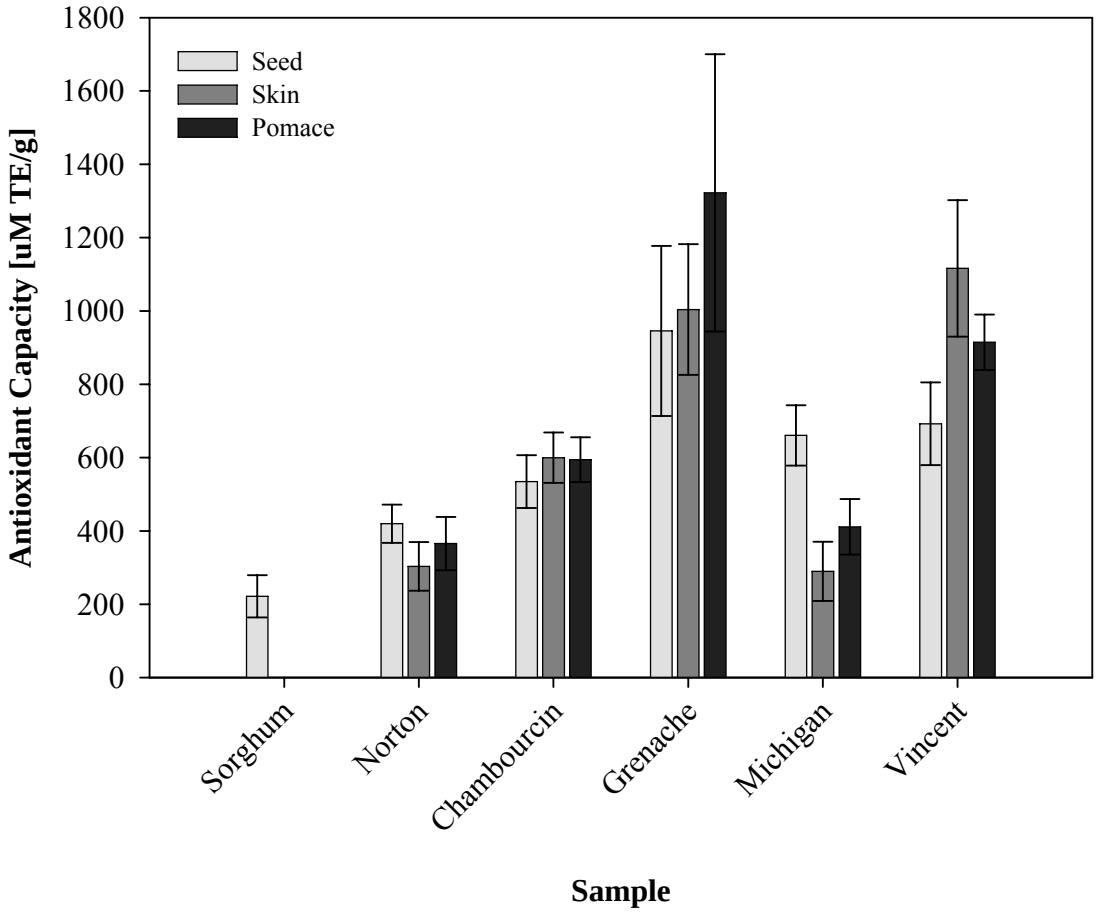


Figure 14: Antioxidant capacity of wine pomace samples and sorghum (dry basis), determined by the ORAC assay. Error bars represent ± standard deviations (n=3). Coefficient of variability = 19.43%.

4.3.2 ABTS Antioxidant Activity Assay

The ABTS results (**Figure 15**) showed that the sorghum and the Norton variety pomace had the lowest antioxidant activity, similar to the ORAC results. The Chambourcin, Grenache, and Michigan varieties fell in the middle for mean antioxidant activity in the seeds, skins, and pomace. The Vincent variety had the highest antioxidant activity in the skins, seeds, and pomace compared to all other samples.

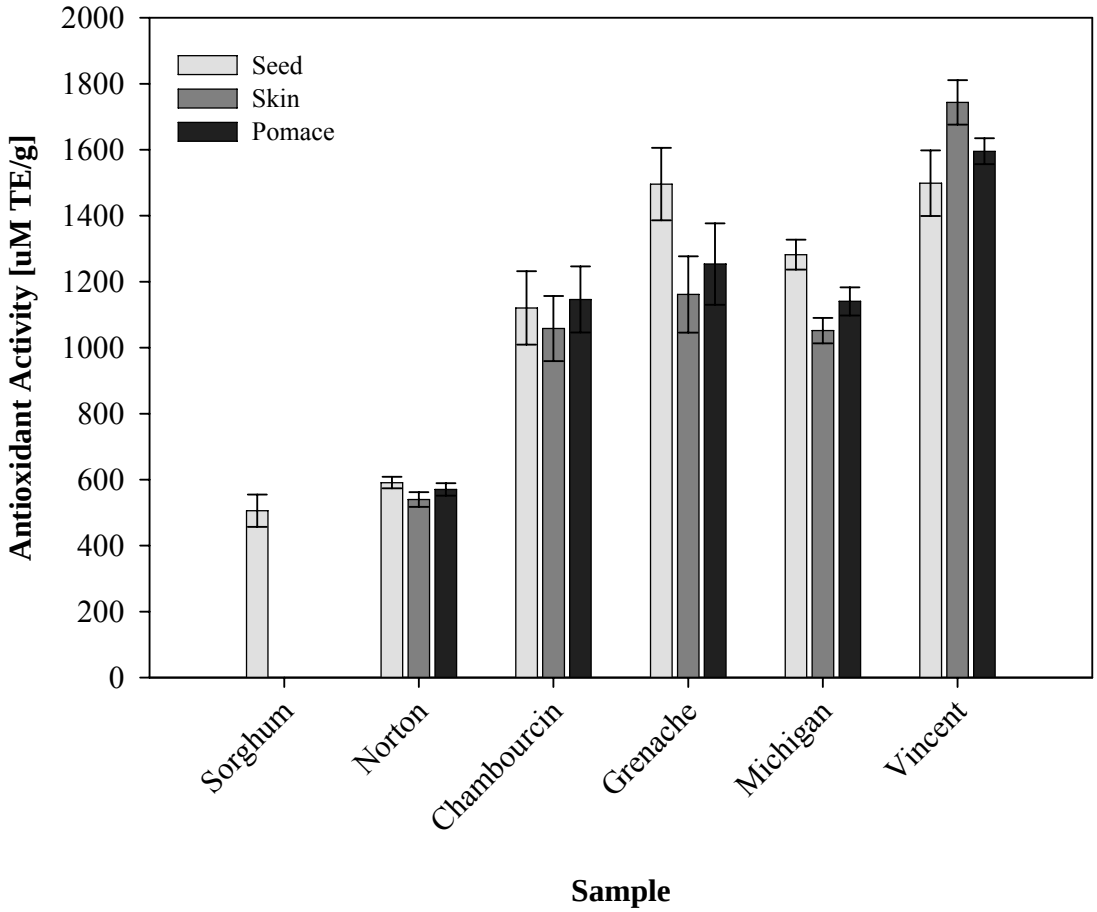


Figure 15: Antioxidant activity for wine pomace samples and sorghum (dry basis), determined by the ABTS assay. Error bars represent ± standard deviations (n=3). Coefficient of variability = 4.21%.

Both the ORAC and ABTS method confirmed that the Vincent grape pomace had the highest antioxidant activity in the skins; 1116.20 μM TE/g by the ORAC assay and 1743.20 μM TE/g by the ABTS method. The Norton, again, had the lowest antioxidant activities by both the ORAC and the ABTS assays. The methods showed similar trends and correlate well (R^2 = 0.56) (**Figure 16)**. Although both methods are using the same standard equivalents, it is important to remember that the methods are testing different

reactions. The ORAC assesses the antioxidant capacity using an area-decay curve; whereas the ABTS method is testing antioxidant activity after a specific amount of time. It is difficult to determine when the reaction is complete in the ABTS method; and where that specific end point is relative to the end of the reaction in the ORAC assay.

Recently, the ability of phenolics to serve as antioxidants has been recognized (Decker 1995), leading to the speculation about the potential benefits of ingesting plant proanthocyanidins. The phenolics present in plants can either prevent free radical formation (i.e. by chelating redox active metal ions) or inhibit free radical chain-propagation reactions (i.e. by reacting with peroxyl radical to form stable free species) (Simic and Jovanovic 1994).

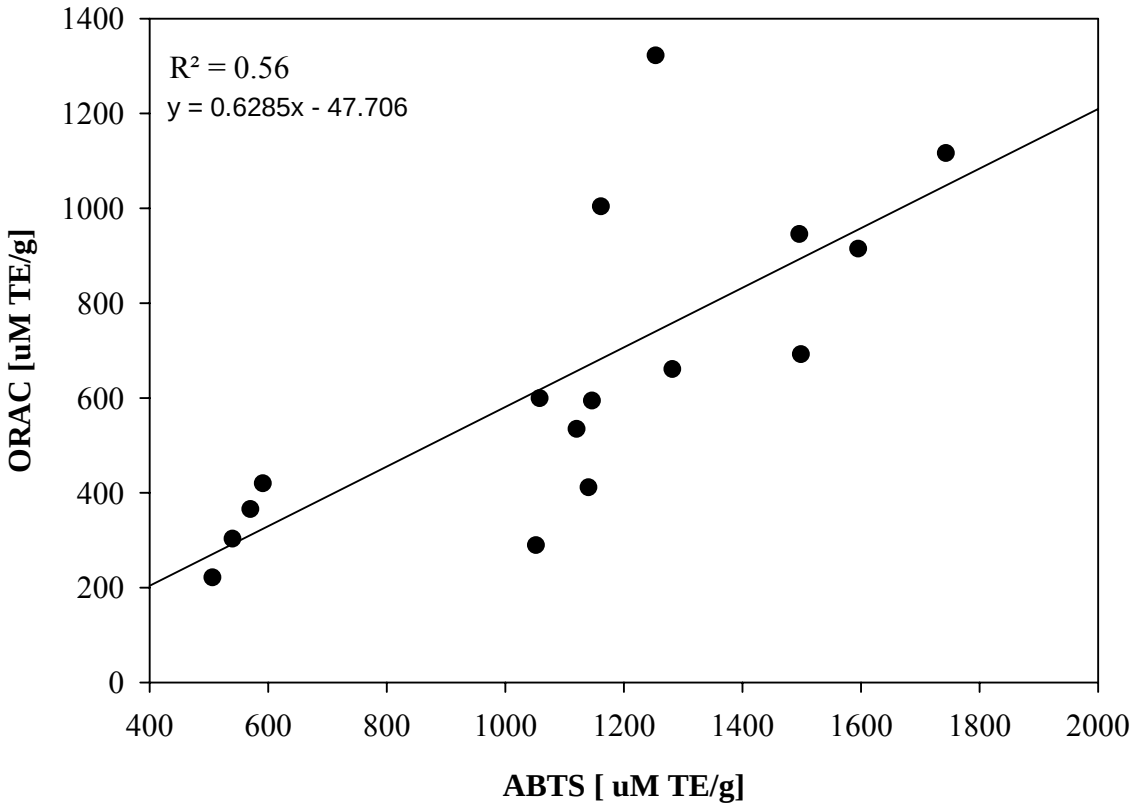


Figure 16: Correlation between ABTS and ORAC assays for the wine pomace and sorghum samples.

The correlation between antioxidant activity and total phenolics was determined (**Figure 17**) to look at the relationship that exists between the two. The correlation was quite high (R^2 = 0.78), for the Folin-Ciocalteu and ABTS assays, suggesting that antioxidant activity was strongly contributed by the polyphenols in the grape varieties. The correlation between the Folin-Ciocalteu and ORAC assays was lower (R^2 = 0.43); but still significant.

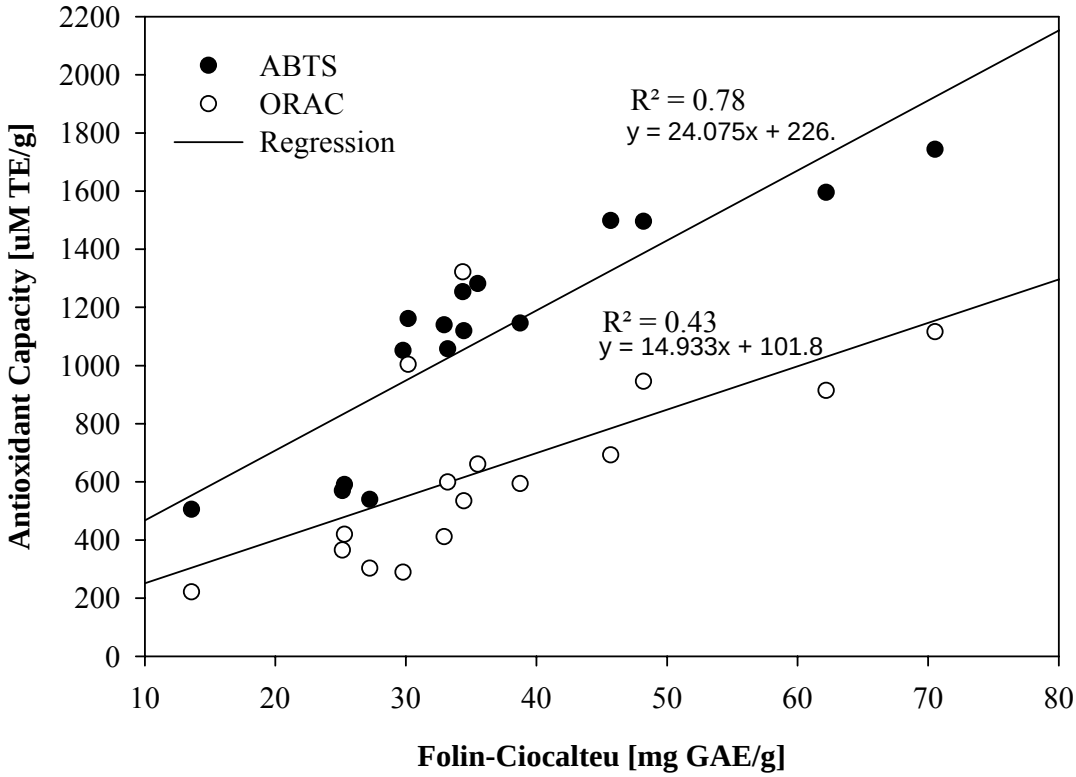


Figure 17: Correlation between Folin-Ciocalteu and antioxidant capacity assays for wine pomace and sorghum samples.

Prior and others (2001) reported that up to 32 and 54% of the antioxidant capacity in blueberries and cranberries can be accounted for by condensed tannins. In this experiment, the data suggest that the tannins, accounted for 65-72% of the antioxidant activity by the ABTS method (**Figure 18**) and 25-82% of the antioxidant activity by the ORAC method (**Figure 19**). The lower correlation between the ORAC and the iron-binding assay was because the ORAC results showed the Grenache pomace to have the highest antioxidant capacity whereas the iron-binding showed the Vincent pomace to

have the highest condensed tannin content. These results confirm that the tannins were a major antioxidant in the grape pomace samples tested.

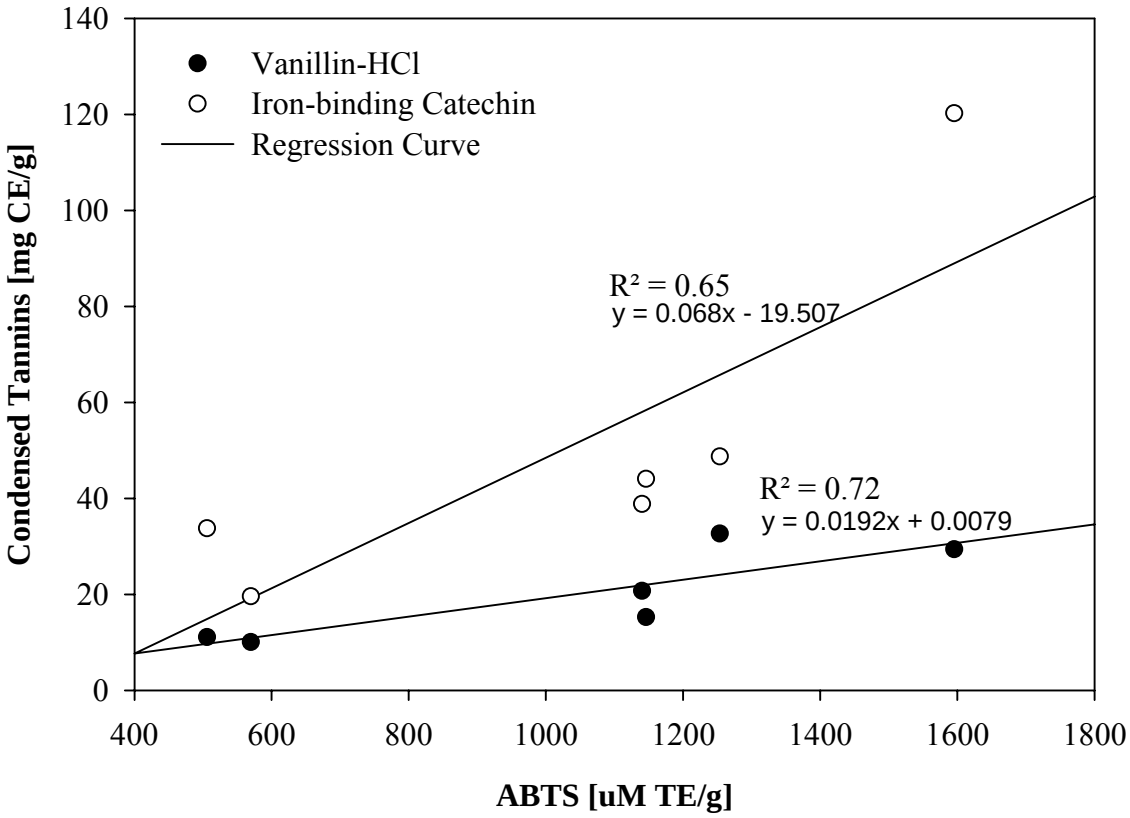


Figure 18: Correlation between ABTS, Vanillin-HCl and iron-binding assays, for the wine pomace and sorghum samples.

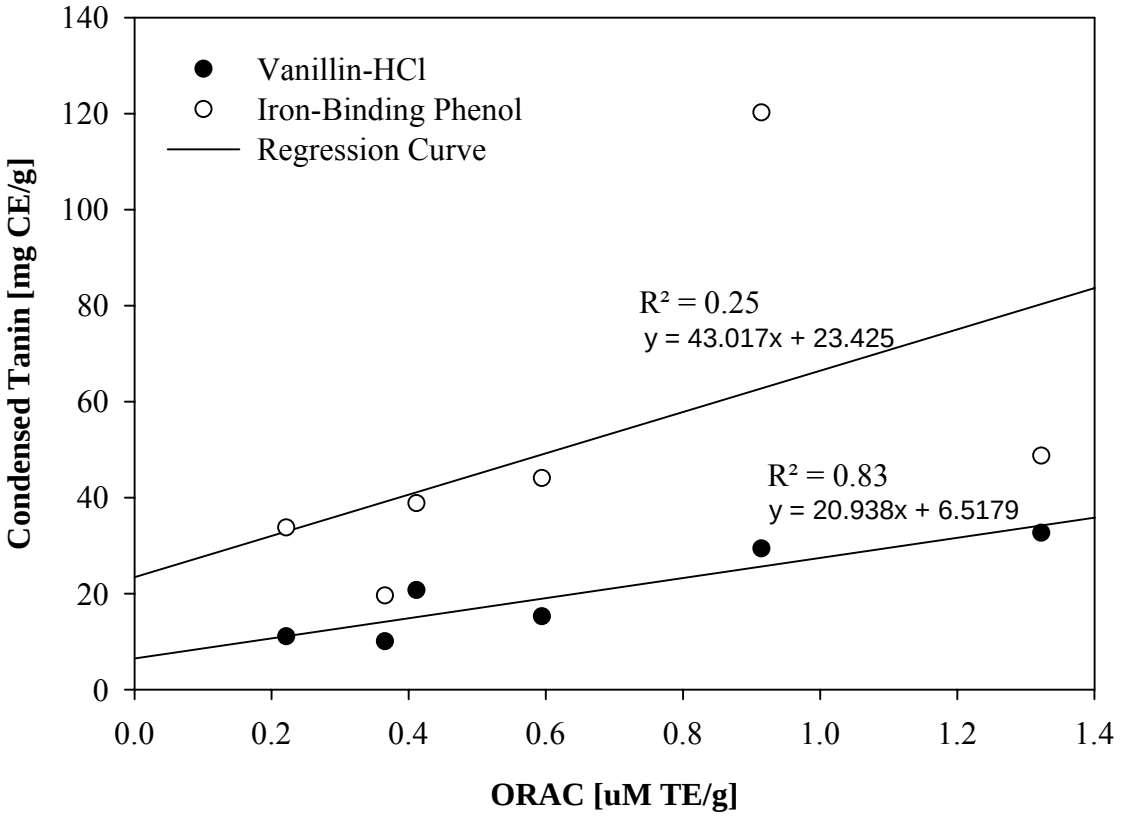


Figure 19: Correlation between ORAC, Vanillin-HCl and iron-binding assays, for the wine pomace and sorghum samples.

CHAPTER 5

CONCLUSION

5.1 Summary & Conclusion

This research showed that wine pomace varieties contain high quantities of polyphenols, condensed tannins, and antioxidant activities. The major difference within the grape varieties was primarily dependent on the vinification method applied during the wine making process; however some varietal differences were shown. The vinification effects showed that grapes, such as the Norton, which are pressed heavily to make deeply colored, astringent, full-bodied wines, resulted in a pomace low in phenols, tannins, and antioxidants. On the other hand, varieties such as the Vincent, used for sweeter, rosé wines will result in a by-product with higher antioxidant and polyphenolic activity primarily as a result of the light pressing methods used during wine production. Varieties such as the Chambourcin, Grenache, and Michigan are typically pressed into semi-dry, medium-bodied, wines and result in a pomace that falls in the middle of the two extremes.

The Vincent variety of grape pomace had the highest total phenolic content while the Norton pomace the lowest. Polyphenols exhibit a wide range of biological, pharmacological and chemoprotective properties as free radical scavengers preventing oxidation and cancer initiation. They have chemoprotective and antiviral properties as well. The Vincent pomace could be very beneficial to the food and pharmaceutical industries.

High condensed tannin content and the highest, most defined separation of procyanidins by degree of polymerization, was found in the Vincent variety. Condensed tannins are of extreme interest in nutrition and medicine because of their potent antioxidant and iron chelating capacity. Hemosiderosis is a common problem among several captive animal species and can eventually lead to ISD if not treated properly. These animals consume high levels of tannins in their natural diets however; there is a lack of these natural iron-chelators in captive diets. The grape pomace, particularly the Vincent grape variety, may provide an inexpensive dietary additive with high levels of natural tannins to help them excrete excess iron.

The antioxidant capacities for each grape variety were quite high, particularly for the Vincent variety. The Vincent variety, being lightly pressed, has a great deal of color pigments residing in the pomace and can greatly contribute to the food industries, as well as the cosmetic industries, that are searching for inexpensive natural colorants that have antioxidative properties.

As a result of the wine making process, the Vincent variety grapes are not heavily crushed and the skins and seeds retain many of the antioxidative compounds; resulting in a pomace with great potential for health benefits. The Norton variety is heavily crushed in the wine making process leaving behind less antioxidative compounds; therefore it was the variety with the least potential. The Chambourcin, Grenache, and Michigan varieties are moderately pressed during wine making and therefore results in a pomace by-product that has potential however; not as much as the Vincent variety. The Vincent, as well as other varieties pressed similarly, produce a by-product that has great potential

applications in animal and human health. With more in-depth research, this underused waste product could become an inexpensive resource of health promoting compounds such as antioxidants and tannins.

5.2 Limitations

The biggest limitation in this study is the amount of grape pomace samples. It would have been extremely beneficial to have obtained several other varieties that undergo light and heavy pressings during vinification. This would help establish better understanding of how the vinification processes affect the by-product.

Most of the methods used are fairly simple and rapid methods but do contain some weaknesses. The Vanillin-HCl method does underestimate condensed tannin levels and although the iron-binding method is better for quantification, it too slightly underestimates condensed tannins.

The normal-phase HPLC method used was not able to detect polymers in the wine pomace samples due to older lab equipment. This also resulted in an underestimation of condensed tannins.

5.3 Recommendations

The efficacy of the iron-binding capacity of grape pomace phenols must be tested *in vivo* before considering it as a routine dietary additive. Tannins have the ability to bind other trace minerals as well as some macronutrients; therefore it is necessary to understand their full impact before using them on a routine basis. Preventing ISD is an

important goal, but it is necessary to understand any side effects, if there are any, to preventative treatments.

It would also be beneficial to run further HPLC analyses on the grape varieties to determine which contain the highest amount of polymers as they are extremely common in plants. Polymeric condensed tannins are quite abundant in grape constituents and are quite underestimated or disregarded without proper analysis.

Continued research on the antioxidants and color pigments present in the grape pomace would be beneficial to the food industry as well as other industries such as pharmaceuticals or cosmetics. Polyphenols are not the only antioxidative compounds present in grape by-products. The anthocyanins could be a huge source of natural dyes that contain powerful antioxidant activity.

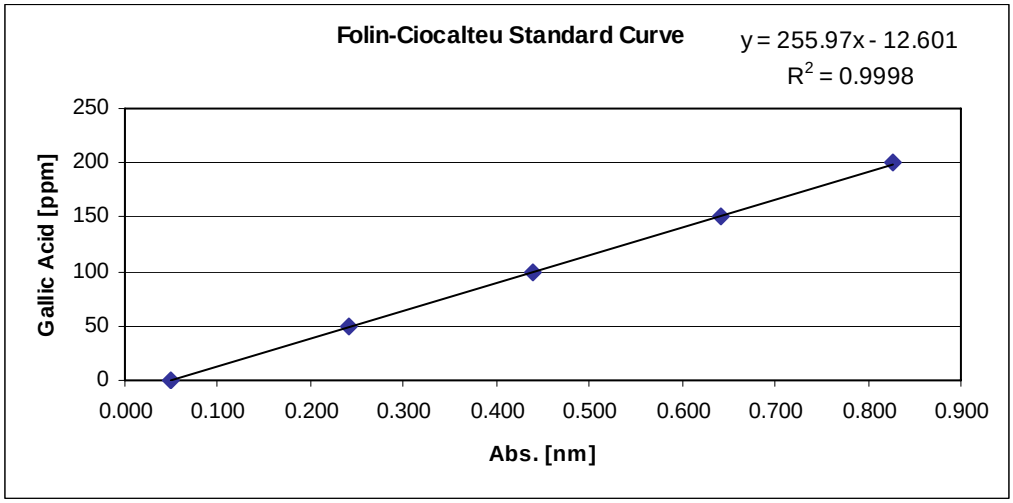
Before choosing a grape pomace variety to work with, it is necessary to do further research on the vinification process and how it affects the pomace. Taking one specific grape variety and pressing it several different ways would allow for more distinction between vinification methods and varietal differences.

Though further research is needed, this study provided a basis to understanding which varieties may have the greatest potential for health benefits associated with antioxidants, and preventative therapy for captive animals susceptible to iron storage disease. The findings of this research, more studies can be completed to aid both animal and human.

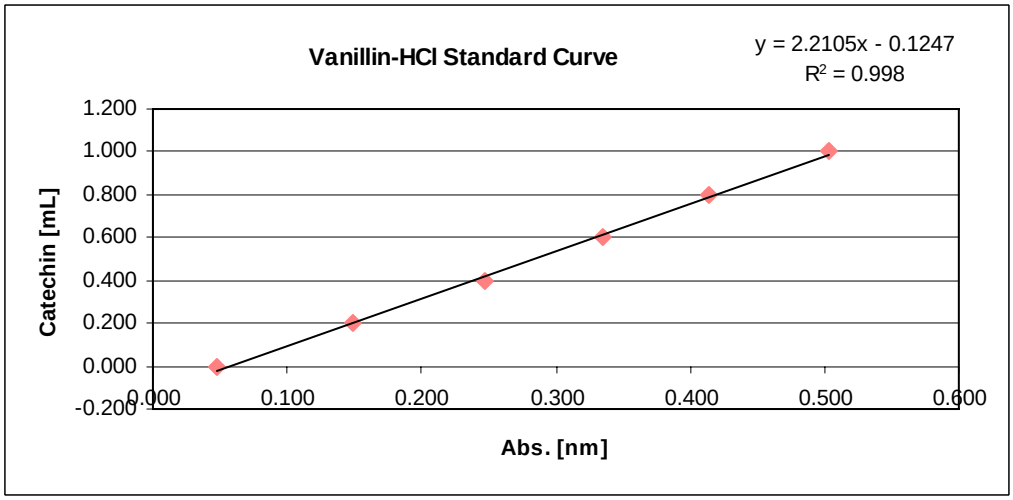
APPENDIX

1. METHOD STANDARD CURVES

1.1 Folin-Ciocalteu Assay

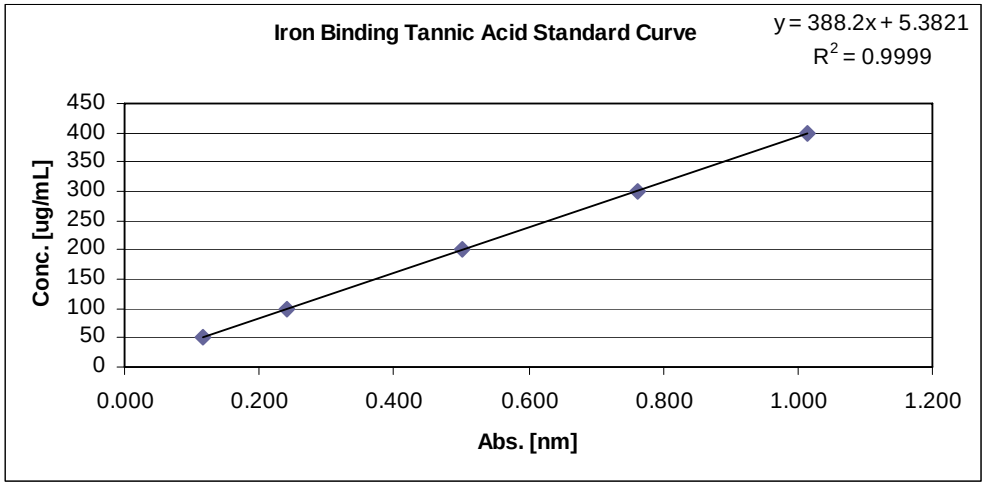


1.2 Vanillin-HCl Assay

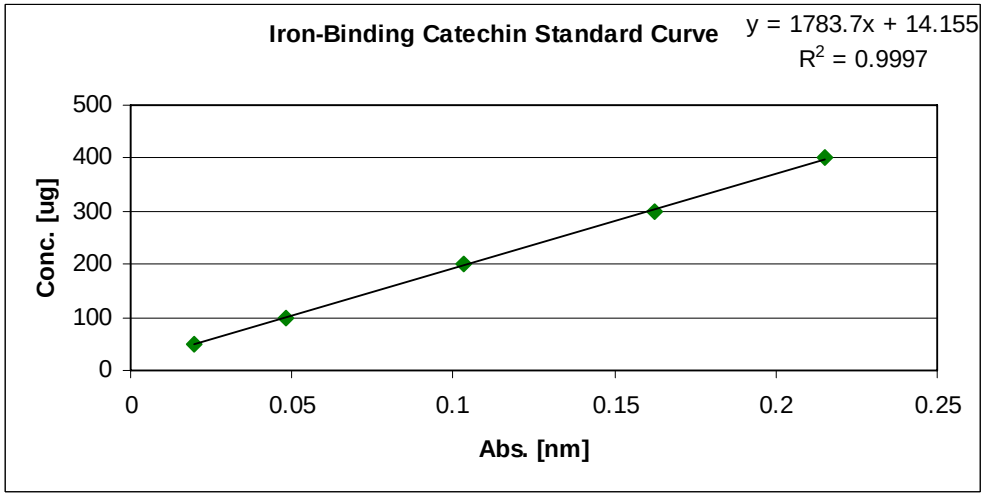


1.3 Iron-Binding Phenolic Capacity Assay

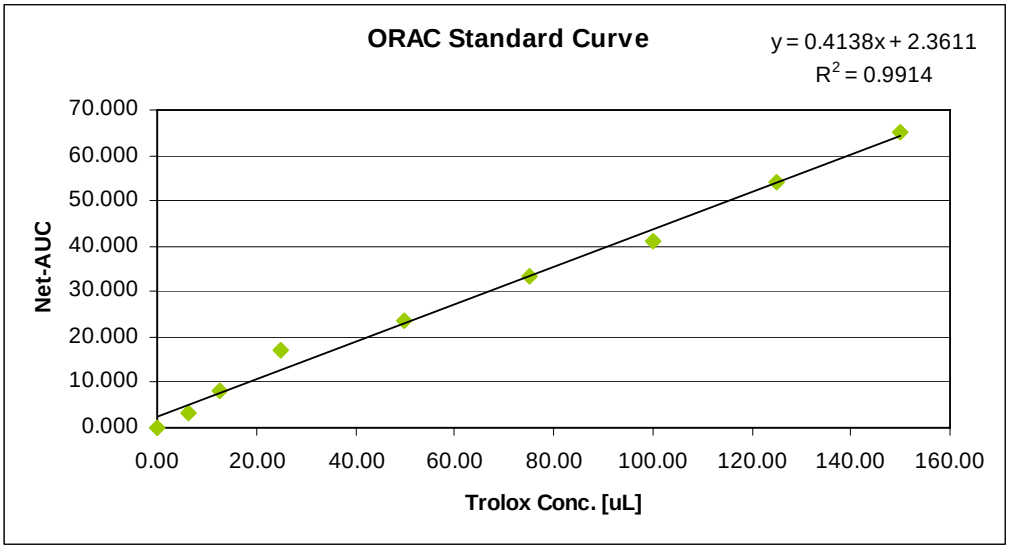
1.3.1 Tannic Acid Equivalents



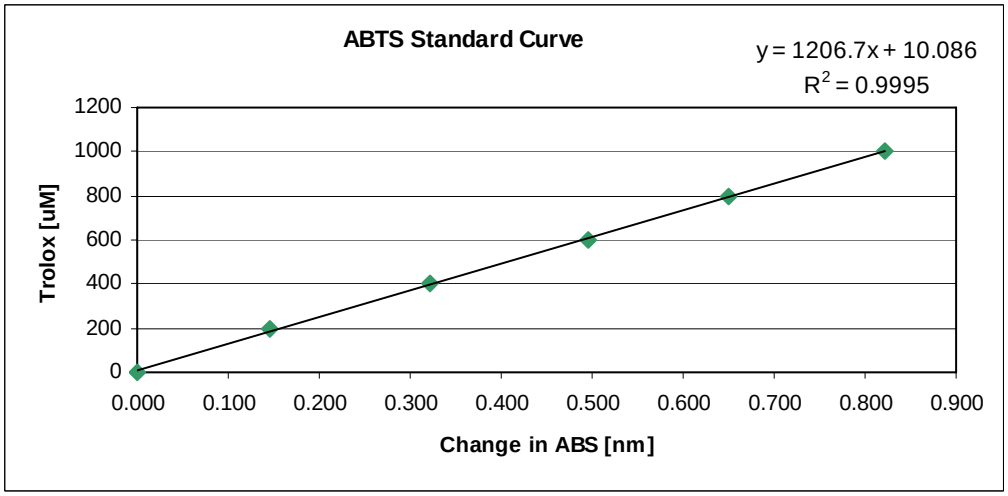
1.3.2 Catechin Equivalents



1.4 ORAC Assay



1.5 ABTS Assay



2. SAS DATA

Abbreviations in the following SAS tables are as follows:

- HTS High Tannin Sorghum
- CSD Chambourcin seed
- CSK Chambourcin skin
- CP Chambourcin pomace
- GSD Grenache seed
- GSK Grenache skin
- GP Grenache pomace
- MSD Michigan seed
- MSK Michigan skin
- MP Michigan pomace
- NSD Norton seed
- NSK Norton skin
- NP Norton pomace
- VSD Vincent seed
- VSK Vincent skin
- VP Vincent pomace

2.1 SAS Table for the Folin-Ciocalteu Assay

Means with the same letter are not significantly different.

t Grouping				Mean	N	trt
		A		70.536	12	VSK
		B		62.181	12	VP
		C		48.202	9	GSD
		C				
		C		45.675	12	VSD
		D		38.744	9	CP
		D				
E		D		35.497	9	MSD
E		D				
E		D	F	34.440	9	CSD
E		D	F			
E	G	D	F	34.359	9	GP
E	G		F			
E	G		F	33.187	9	CSK
E	G		F			
E	G		F	32.926	9	MP
	G		F			
	G	H	F	30.167	9	GSK
	G	H				
I	G	H		29.780	9	MSK
I		H				
I		H	J	27.227	9	NSK
I			J			
I			J	25.294	9	NSD
			J			
			J	25.120	9	NP
		K		13.581	48	HTS

2.2 SAS Table for the Vanillin-HCl Assay

Means with the same letter are not significantly different.

t Grouping	Mean	N	trt
A	62.690	9	GSD
B	40.054	9	MSD
C	34.755	9	VSD
C			
C	32.932	9	GP
D	30.041	6	VP
E	25.775	9	GSK
F	20.826	9	MP
F			
G F	19.770	6	VSK
G			
G	18.511	9	CSD
H	15.284	9	CP
H			
H	13.623	9	MSK
I	11.087	42	HTS
I			
I	10.926	6	NSD
I			
I	10.178	6	NP
J	7.074	6	NSK
J			
J	6.454	9	CSK

2.3 SAS Table for the Iron-Binding Phenolic Assay

Means with the same letter are not significantly different

2.3.1 Tannic Acid Equivalents

t Grouping	Mean	N	trt
A	30.2716	9	VP
B	13.3247	9	GP
C	11.6484	9	CP
D	10.2577	9	MP
E	8.3494	9	HTS
F	5.2272	9	NP

2.3.2 Catechin Equivalents

t Grouping	Mean	N	trt
A	120.2328	9	VP
B	48.7406	9	GP
C	44.0618	9	CP
D	38.8071	9	MP
E	33.7906	9	HTS

			F	19.6159	9	NP

2.4 SAS Table for the ORAC Assay

Means with the same letter are not significantly different

t	Grouping			Mean	N	trt
		A		1322.3	12	GP
		A				
B		A		1116.3	6	VSK
B						
B				1003.9	11	GSK
B						
B				945.5	12	GSD
B						
B		C		914.4	6	VP
		C				
D		C		692.1	6	VSD
D						
D		E		660.7	12	MSD
D		E				
D		E	F	599.5	18	CSK
D		E	F			
D		E	F	594.1	18	CP
D		E	F			
D	G	E	F	534.6	18	CSD
	G	E	F			
H	G	E	F	419.7	18	NSD
H	G		F			
H	G		F	411.2	12	MP
H	G		F			
H	G		F	365.3	18	NP
H	G					
H	G			303.2	18	NSK
H						
H				289.6	12	MSK
H						
H				221.4	60	HTS

2.5 SAS Table for the ABTS Assay

Means with the same letter are not significantly different

t	Grouping		Mean	N	trt
		A	1743.20	6	VSK
		A			
	B	A	1595.41	6	VP
	B				
	B		1498.58	6	VSD
	B				
	B		1495.77	9	GSD
		C	1281.69	9	MSD
		C			
		C	1253.53	9	GP
		C			
	D	C	1161.15	9	GSK
	D	C			
	D	C	1146.03	9	CP
	D	C			
	D	C	1140.19	9	MP
	D	C			
	D	C	1120.07	9	CSD
	D				

D		1057.79	9	CSK
D				
D		1051.69	9	MSK
	E	590.78	9	NSD
	E			
	E	569.76	9	NP
	E			
	E	539.68	9	NSK
	E			
	E	505.65	42	HTS

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