BIOACTIVE CONSTITUENTS OF PECANS [*Carya illinoinensis* (Wangenh.) K. Koch]

by

KATHERINE SUZANNE ROBBINS

(Under the Direction of Robert L. Shewfelt)

ABSTRACT

Epidemiological studies have shown an inverse relationship between nut consumption and chronic diseases of humans. For this reason, commercially-viable U.S. cultivars were assessed for their antioxidant and anti-inflammatory constituents. Acetonic extracts of defatted pecans possessed marked *in vitro* antioxidant capacities as determined by a number of assays including total phenolics content (TPC), ferric reducing antioxidant potential (FRAP), hydrophilic-oxygen radical absorbance capacity (H-ORAC$_{FL}$), and total procyanidins content (DMAC). Depending on the cultivar, H-ORAC$_{FL}$ values ranged from 13.5 ± 3.5 to 25.5 ± 3.0 mmol Trolox eq/100 g. The procyanidins content of crude extracts (420 ± 20 to 655 ± 43 mg procyanidin B2 eq/100 g) correlated better with H-ORAC$_{FL}$ data than TPC values. The impact of roasting on the antioxidant activity and phenolics content was investigated. Pecans were roasted in an impingement oven at 175°C for 8.2 min. No significant differences were observed in FRAP quantities and only one cultivar showed a significant decrease in H-ORAC$_{FL}$. Significant decreases were observed for both TP (~10%) and PAC (~25%) contents. Fractionation of the crude acetonic extracts by Sephadex LH-20 column chromatography into low-molecular-weight (LMW) and tannin-rich phenolic compounds (HMW) was achieved.

Results indicated that the tannin-rich fraction, comprised of proanthocyanidins, possessed substantially more antioxidant activity than the LMW phenolic fraction. Phenolic acids identified in the crude acetonic extract and LMW phenolic fraction by RP-18 HPLC included gallic, ellagic, protocatachuic, and *p*-hydroxybenzoic acids as well as the proanthocyanidin monomers, catechin; these were tentatively identified, confirmed using ESI-LC-MS and quantified (both in their free, esterified, and bound forms) by HPLC. Normal phase-HPLC revealed that the tannin-rich fraction, which accounted for most of the antioxidant activity, comprised both hydrolyzable and condensed tannins. Degrees of polymerization for the tannin-rich samples were determined to contain monomers-pentamers with dimers representing the largest fraction (56.7%). Anti-inflammatory properties of the crude and acetonic extracts (both LMW and HMW fractions) were evaluated using lipopolysachharide-stimulated RAW 264.7 macrophage cells. Nitric oxide, an index of inflammation, was measured using the GRIESS assay. The LMW fraction proved to be most effective and showed a dose-dependent effect.

INDEX WORDS: Pecans, Antioxidant, Phenolics, Proanthocyanidins, Roasting, Inflammation

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DEDICATION

This dissertation is dedicated to my parents, Jeffrey and Susan Robbins, who have always encouraged me to follow my dreams and be the best I can be. I would never have been able to get through this degree without the constant reminder to "pull myself up by my bootstraps" and "keep my eye on the prize."

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CHAPTER 1

INTRODUCTION

Pecans [*Carya illinoinensis* (Wangenh.) K. Koch], native to the southern U.S, are an important crop to the Georgia and southern U.S. economy. In 2011, Georgia ranked 1st in the country with 102 million pounds of utilized pecans worth a total value of utilized production of $264 million (1). Compared to other agricultural commodities in the state, pecans ranked 10th in the 2010 Georgia Farm Gate report (2). This critical economic crop, along with other tree nuts, is garnering increased attention for its health benefits which are mainly attributed to a beneficial lipid profile and phenolic constituents (3-7). Pecans contain not only phenolic acids and flavonoids, such as the flavan-3-ol monomer (+)-catechin, they also contain oligomeric and polymeric proanthocyanidin (PAC) compounds (8). Pecans, when compared to other tree nuts, have elevated levels of these PACs (9). Only hazelnuts have comparable contents with the next highest nut type, pistachios, having only half the content of the other two nut types. It is believed that this somewhat unique phenolic profile could give pecans added antioxidant activity compared to other nut types. In a 2012 study by *Gentile* et al. (10), phenolic extracts from pistachios were discovered to demonstrate anti-inflammatory properties in cell culture studies utilizing RAW 264.7 macrophage cells.

Pecans have been shown to have elevated antioxidant activity (8, 11-12) *in vitro* and a recent study shows promising *in vivo* results (13). Recently, a feeding study was conducted using a pecan meal based diet (13). The researchers discovered that those participants fed a

pecan-based diet exhibited a decrease in postprandial lipid and cholesterol oxidation compared to control diets, as well as increases in antioxidant activity of plasma as measured by lipophilic and hydrophilic oxygen radical absorbance capacity (ORAC). The authors also reported that there was an increase in both the plasma γ-tocopherol and (+)-catechin concentrations indicating the absorption and bioavailablilty of these pecan compounds (13). Their research provides evidence that *in vitro* antioxidant activity and phenolic content may be predictive of positive *in vivo* activity.

The present study was performed to better characterize the phenolic constituents of U.S. pecan cultivars. There are very few recent publications that present data on U.S. pecans (8) as opposed to those found in other pecan producing regions across the world, such as Mexico (12). The present study not only incorporates data for pecans from three different major pecan producing states, Georgia, New Mexico, and Texas (1), it also employs eleven different cultivar types in order to investigate the effect of cultivar type and growing location on antioxidant activity and phenolic content. Specific objectives for the research study were as follows.

1. Establish the antioxidant capacity and phenolic content of pecan cultivars from different growing locations and cultivar types.
2. Investigate the effect of roasting on the antioxidant capacity and phenolic acid profile of key pecan cultivars.
3. Evaluate and characterize the individual components of low-molecular weight and high-molecular weight fractions as separated by column chromatography techniques.
4. Determine the potential of pecan phenolic extracts as an inflammatory modifier.

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CHAPTER 2

LITERATURE REVIEW

2.1 Pecans

The pecan [*Carya illinoinensis* (Wangenh.) K. Koch.] is a member of the Juglandaceae family (1). Other tree nuts in the family include walnut, hickory nut, heart nut, and butternut (2). Trees in this family are classified as heterodichogamous, monoecious, and deciduous nut trees (3). Determining the prehistoric existence of native pecan trees is difficult since differentiation past the *Carya* genus is not possible with current scientific dating methods. It is believed that the genus has been present for at least 34 million years (1). Traditionally, pecans were found along the sides of the Mississippi River and its tributaries (3). Texas, Oklahoma, and Louisiana are home to the oldest of the native species (1), but native speciments have also been found in other states across the Southeastern and Southwestern U.S. from Arkansas and New Mexico to Alabama and Georgia (4). Pecans are believed to have contributed significantly to the diets of Native Americans and early European explorers (1, 5). The word "pecan" most likely came from a word in the Algonquian language meaning "a general term for a hard shelled nut" (6) that was later adopted by the French settlers as "pecanes" or "pacanes" (7).

Pecan trees can be easily affected by their environment. Pecans should be grown in properly drained soil, a fairly neutral soil pH, and on flat land (5). When pecan tree roots are grown in too moist of a soil, root growth and oxygen delivery to the tree is limited. For this reason, it is recommended that the trees be planted in a soil that drains well, such as one with a sandy texture like those found near river beds (1). Trees should also be planted on fairly level

ground. This makes harvest easier and also helps maintain adequate water drainage. Land where water pools in some areas is not desirable for a pecan grove (1). As with most plants, pecan trees require a fairly fertile soil that can provide the necessary nutrients for growth. The optimal soil pH for growth is~6.4 (5).

Pecan trees are known for having remarkably long lives. There are two types of trees, native or seedling trees and cultivated/improved cultivars that have been developed from budding or grafting. Native trees have been found that are ~1,000 years old and cultivated trees can be as old as 120-150 years (5). More than 1000 pecan cultivars exist worldwide (8) and are mainly recognized based upon their original cultivation location. These are the northern (Illinois), southern (Georgia), and western (Texas) cultivars (5). One of the earliest cultivated and most popular cultivars is 'Stuart', which is still cultivated today in Georgia and across the southern portion of the U.S. (5). The cultivars currently recommended for Georgia in most growing situations are 'Caddo', 'Cape Fear', 'Creek', 'Desirable', 'Elliott', 'Forkert', 'Kanza', 'Kiowa', 'Oconee', 'Pawnee', 'Stuart','Sumner'(9). 'Desirable' and 'Stuart' are the most commercially important of the cultivars grown in Georgia (2).

Pecans and tree nuts have been shown to have important health benefits to humans due to their lipid constituents and phenolic compounds. In 2002, tree nuts received a qualified health claim from the FDA stating that, "Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease." (10) These benefits were again documented in the 2010 U.S. Dietary Guidelines. They state that, "moderate evidence indicates that eating peanuts and certain tree nuts (i.e., walnuts, almonds, and pistachios) reduces risk factors for cardiovascular disease when consumed as part of a diet that is nutritionally adequate and within calorie needs." (11)

2.1.1 Pecan Composition

The proximate composition of both raw and dry roasted unsalted pecans is given in Table 2.1 as reported by the USDA National Nutrient Database for Standard Reference, Release 25 (12). For both pecan types, lipids are the most abundant components with a content of 71.97 and 74.27 g/100 g for raw and roasted pecans, respectively. The moisture of the raw pecan samples was reported as 3.52%. As can be expected, with roasting the moisture level drops to 1.12% (12). The lipid and water contents were the only components to change significantly as a result of the dry roasting processing step. Carbohydrates, calculated by difference, were found to be 13.8 g/100 g nutmeat (raw) with total dietary fiber making up 69% of the carbohydrate portion. Proteins were found to be the next largest constituent with a content of 9.17 g/100 g nutmeat (raw).

Pecans and tree nuts are known for their high lipid content. Rudolph et al. (13) conducted a study that investigated the lipid content of 70 different pecan cultivars and breeding lines over four consecutive years. They discovered that not only did the oil content differ among the cultivars (67.1 -75.1 %), it also changed with harvest year. One cultivar analyzed had a difference of 13.6% between its highest and lowest years. These results were supported by those of Santerre (14) who found that pecan lipid content could vary from 65 to 75% based on the sample analyzed. The authors believed that the lipid content can depend on the method of production, age of the trees, type of cultivar, and the previous productivity of the trees (pecan trees commonly bear fruit on alternate years) (14). Wells *et al*. (15) have reported that in addition to these considerations, the level of irrigation/rainfall in an orchard can cause different

Table 2.1. Proximate Composition (g/100 g) Pecan Samples

Proximate Composition	Raw[a]	Dry Roasted
Water	3.52	1.12
Total lipid	71.97	74.27
Protein	9.17	9.50
Ash	1.49	1.56
Carbohydrate (by difference)	13.86	13.55
Fiber, total dietary	9.6	9.4

[a] Data from the USDA National Nutrient Database for Standard Reference, Release 25. (12)

results between trees. As reported by Heaton *et al*. (16), most of the lipid content of the nut (96 %) is found as triacylglycerides. Senter and Horvat (17) determined that the minor lipid constituents of the nut are more complex lipids including other acylglycerols, tocopherols and sterols.

One important class of complex lipids present in pecans is the tocopherols. Not only are the tocopherols important to our antioxidant defense system, they are thought to serve the same role for the nut during its growth and storage (2). Tocopherols are found in most plant tissues (18). There are eight different tocopherol isomers that can be found in edible oils: α, β, γ, and δ tocopherols and tocotrienols. The tocopherol isomers have saturated side chains while the tocotrienol side chains are unsaturated. The antioxidant activity increases from α-T to δ-T but it should be noted that these compounds may demonstrate pro-oxidant activity if consumed in high concentrations (18). It has been shown that as oxidation occurs in pecan oil, creating a rancid and darker product, the concentration of Ts decreases (19). Of the four main T isomers, γ-T is the most prevalent in pecan oil. In a study by Chun *et al*. (20), investigating three cultivars and a seedling sample over 2 years, the total T contents were reported to range from 21.3-32.0 mg/100 g nutmeat with γ-T representing > 90% of the total for all samples analyzed. The authors were unable to find any significant difference ($P > 0.05$) in the γ-T among the cultivars studied, and since the γ-T is by far the major T, they concluded that no significant differences existed in the total content of Ts.

The high unsaturated lipid content of pecans creates a concern for oxidative rancidity. Proper storage conditions must be maintained in order to prevent these detrimental effects. It has been reported that by storing pecans at 0°C and 70-75% RH, a shelf life of one year can be expected. By increasing storage temperature to 10°C, the shelf life is reduced to six months.

The shelf life can be considerably extended by decreasing storage temperature (21). Storing pecans in packaging with minimal O_2, such as vacuum packaging, can help to extend the shelf life of the product (5). Not only do these precautions prevent rancidity, they also maintain the level of the natural antioxidants during storage.

2.1.2 Pecans and Georgia Economy

In 2011, Georgia ranked 1st in the U.S. with102 million pounds of utilized pecans: 92 million pounds of improved cultivars and 10 million pounds of native trees and seedlings (4). The pecans were sold at an average price of $2.59/pound for a total value of utilized production of $264 million in 2011. For the same year, Georgia represented 37.8 % of the total U.S. production with New Mexico and Texas representing the next largest shares with 22 and 12%, respectively (Figure 2.1). As indicated in the 2010 Georgia Farm Gate Report, pecans ranked 10th in the Georgia Agricultural Commodity Rankings representing 1.9% of the total farm gate value. From 2002 to 2010, the GA farm gate value of pecans increased dramatically from $45.8 million in 2002 to $233.9 million in 2010. (22) This increase is due, in part, to the entry of China into the market causing a marked increase in price per pound. The USDA National Agricultural Statistics Service reports for the 2000-1, 2005-6, and 2010-11 seasons give the average price on a shelled basis for a pound of pecans as $2.57, $3.25, and $4.81, respectively (24).

Trends in pecan production can be difficult to monitor due to the alternate bearing nature of the crop (5). Pecan production in 2011 was estimated at 134,900 tons and represented an 8% decrease from the 2010 production level. However, in 2012 the production is expected to be 140,000 tons, a 3.8 % increase over 2011. The 2011 total utilized production for all tree nuts tracked by the USDA is 2.57 million tons measured on an in-shell basis which is an 8% increase

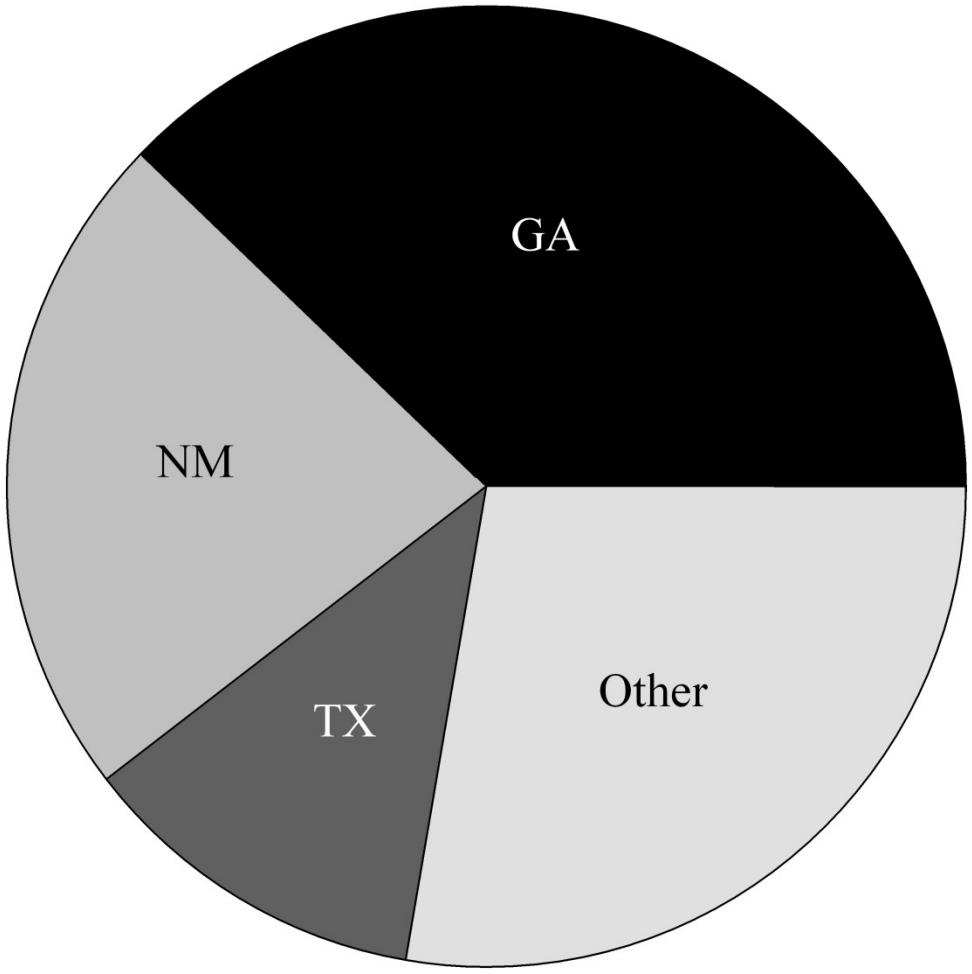


Figure 2.1.U.S. Production of Pecans by State. Data from USDA National Agricultural Statistics Service Nut and Non-Citrus Fruit Report (4).

from 2010. The 2011 utilized nut production was valued at 6.85 billion dollars which is a 17% increase from 2010 (4). Compared to other tree nut production in the U.S., pecans are 3rd in total production behind almonds and English walnuts (2, 4). Other countries historically producing pecans are Argentina, Australia, Brazil, Chile, Israel, Mexico, and South Africa (5). In 2010, Mexico was the largest importer of pecans into the U.S followed by China, Australia, and Thailand (24).

2.2 Phenolic Compounds

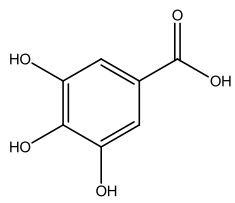
Phenolics are any compounds with a –OH group directly attached to a benzene ring (25). Numerous classes of phenolic compounds exist in natural products, some of which are highlighted in Figure 2.2. The simplest are the phenolic acids that are separated into two main families: the benzoic acids and the *trans*-cinnamic acids (26). The benzoic acid family (*i.e.*, gallic acid and protocatachuic acid) is comprised of acids with a benzoic acid backbone. The trans-cinnamic acid family is made up of phenolic acids with the cinnamic acid backbone. Examples are quinic and tartaric acids. These phenolic acids can be expanded to include more complex phenolic classes such as flavonoids, isoflavonoids, lignans, and stilbenes. The flavonoids are a class of 3-ring, 15-carbon compounds that can be further divided into flavones, flavonols, flavonones, flavanols, and anthocyanins. Variations of these compounds can exist as esters, glycosides, or in a bound form (18).

Even though different types of phenolics exist in nature, they all are synthesized in plants following the same basic mechanism. The pathways are activated both developmentally in specific tissues of the plant and also through environmental stress such as wounding or pathogen infection (27-29). Most phenolics in plants are synthesized from the amino acids L-

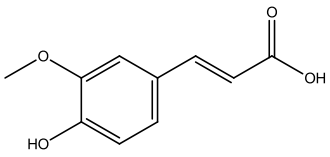
phenylalanine and L-tyrosine, formed via the pentose-phosphate and shikimic acid pathways, and by several cascading pathways evolving from phenylpropanoid metabolism (25, 29-31). The entire synthetic pathway is presented in Figure 2.3. Initially, 4-phosphate erythrose and phosphoenol pyruvic acid (PEP) react through condensation reactions to produce dihydroshikimate (25). Phenylalanine is formed in the shikimic acid pathway. In the initial step of the phenylpropanoid reaction, phenylalanine ammonia-lyase (PAL) removes an amine group from phenylalanine, producing cinnamic acid (30). The cinnamic acid is then converted in several steps by additional enzymes to hydroxycinnamic acid and 4-coumaric acid. Subsequent structural changes are completed by 4-coumarate-CoA ligase (4CL) to produce 4-coumaroyl-CoA. This compound is the basis for the formation of phenolic groups such as flavonoids, isoflavonoids, stilbenoids, and phenolic polymers through the action of additional enzymes (29). It should be noted that the benzoic acid family and gallic acid are produced through earlier steps in the process (Figure 2.3) (31).

The phenolic acids, from either the *trans*-cinnamic acid family (quinic, shikimic, and tartaric acids) or the benzoic acid family (gallic and protocatechuic acid), can be found in plants in either their monomeric form or as building blocks for larger polymeric compounds (32-33). The polymers formed from these phenolic acids, hydrolysable tannins, are divided into smaller groups based on their primary phenolic acid monomer (*i.e.*, ellagitannins and gallotannins). An additional type of phenolic polymer, the condensed tannins or proanthocyanidins (PACs) are made up of the flavan-3-ol monomers, (+)-catechin, and (-)-epicatechin (34).

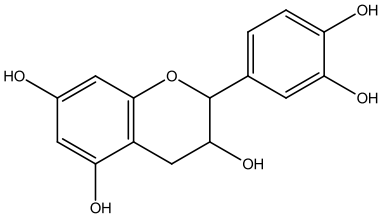
PACs are synthesized via the flavonoid pathway from flavanones (Figure 2.4). Slight structural differences in starting materials and enzymes along the pathway result in PACs that differ in the stereochemistry and substituents of their monomers, position of the linkages, and



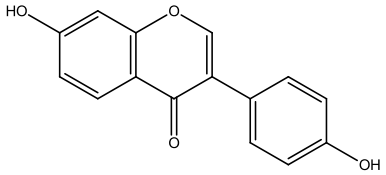
(a) Gallic Acid



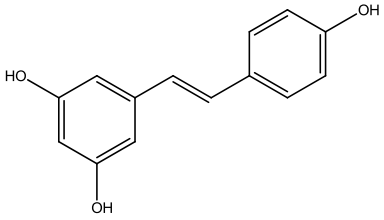
(b)Ferulic Acid



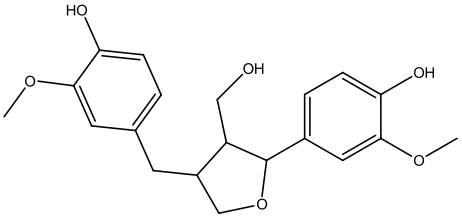
(c) (+)-Catechin



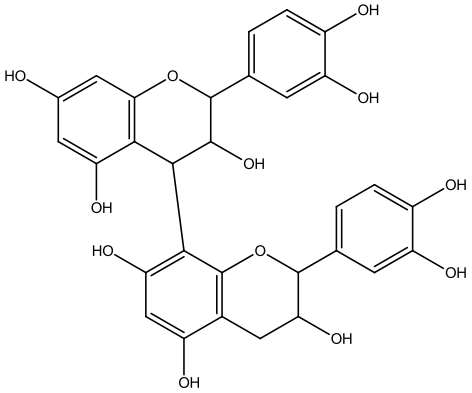
(d) Daidzein



(e) Resveratrol



(f) Lariciresinol



(g) Procyanidin B2

Figure 2.2 Examples of phenolic compound classes. Phenolic acids (a and b), flavonoids (c), isoflavonoids (d), stillbenes (e), lignans (f), and condensed tannins (g) are presented.

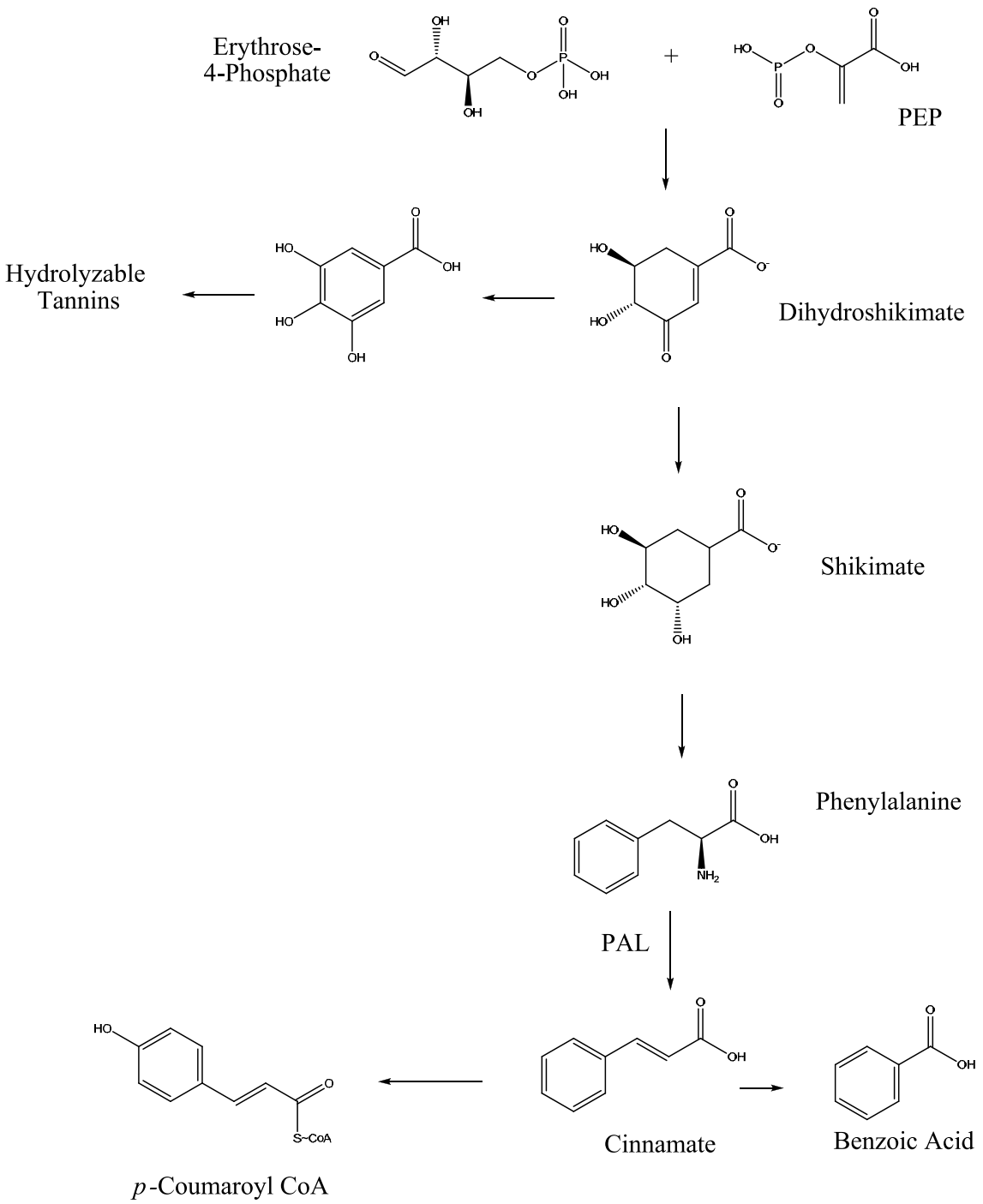


Figure 2.3 Phenylpropanoid synthesis mechanism adapted from Vogt, T. (31).

size (34). There are two main classes of PACs, procyanidins and prodelphinidins. Procyanidins, the most prevalent of the PACs found in foods, are composed of (-)-epicatechin monomers while the prodelphinidins are composed of epigallocatechin monomers. There are two main linkages present in PACs: A type linkages (C4-C8 or C4-C6, C2-O7) and B type linkages (C4-C8 or C4-C6) (34-35). PACs are usually classified based upon their degree of polymerization which can range anywhere from 2 (dimers) to 10+ (polymers). The properties of PACs, such as antioxidant and anti-mutagenic activity, can vary based upon structure and degree of polymerization (36).

One defining characteristic of PACs is their ability to bind metals, especially iron and zinc (34). Binding of metal is seen as a negative characteristic of the compounds because consuming large amounts of tannins may cause a reduction in the amount of certain bioavailable metals (37). One benefit of metal binding seems to be a reduction in bacterial growth and may explain the antibacterial nature of PACs (38). It has been hypothesized that plant samples with higher concentrations of PACs may be less susceptible to bacterial and fungal infections. Gonzalez de Colmenares *et al*. (39) investigated this theory in coffee plants and found that the plants with lower PACs content did in fact show a heightened susceptibility to fungal infection. PACs have also been shown to precipitate proteins which may be another concern when high levels of PACs are consumed (26).

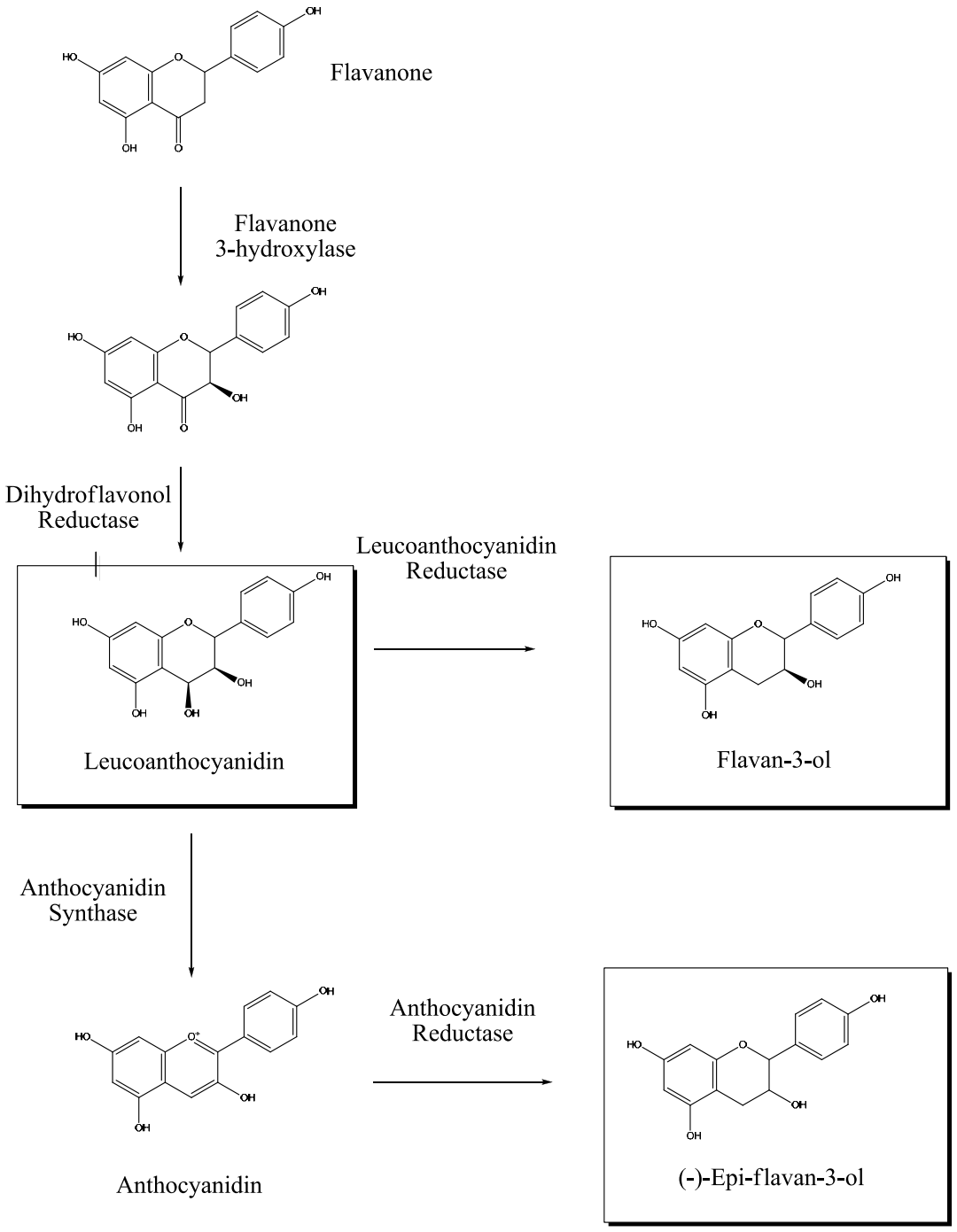


Figure 2.4.Flavonoid pathway for synthesis of PACs. Adapted from Dixon *et al.*(34).

2.2.1 Phenolic Compounds in Pecans

Phenolic compounds and tocopherols are the main compounds responsible for the antioxidant capacity of the pecan (2). Senter *et al*. (40) analyzed defatted pecan kernels using GC-MS and reported that the kernels contain six main phenolic acids: gallic, gentisic, vanillic, protocatechuic, *p*-hydroxybenzoic acid, and *p*-hydroxyphenylacetic acid as well as coumaric and syringic acid in small quantities. They determined that gallic acid accounted for almost 78% of the phenolic acid constituents with a reported content of 138 µg/g defatted kernel. In the same study, it was observed that as oxidative rancidity reactions progressed, phenolic acid compounds decreased (40). It was suggested that this decrease demonstrates that phenolic compounds may be acting as antioxidants inside the nut. Villareal-Lozoya *et al*. (41) conducted a similar study looking at the phenolic contents of six different pecan cultivars. The authors employed HPLC methods in order to determine some of the phenolic compounds present. They were able to account for gallic acid, ellagic acid, (+)-catechin, and (-)-epicatechin. There were no significant differences among the phenolic acid contents of the cultivars studied.

Pecans have been shown to have special health properties due to their tannin contents. Tannins are found in high concentrations in the shuck and the middle divider between the two nut halves and surrounding packing tissue. Smaller concentrations of tannins are present in the hull and the kernel of the nut (42-43). Tannins are a key colorant in pecans and also help the plant with pathogen and insect resistance (44). Due to the presence of gallic and ellagic acids and certain key ellagic acid derivatives in the hydrolyzed samples studied by Villareal-Lozoya *et al*. (41), it was concluded that pecans not only contain PACs (condensed tannins) but also hydrolyzable tannins. An additional study conducted by Polles *et al.* (42) tested 31 pecan cultivars for the presence of PACs. They determined that PACs are indeed present in the kernels

at levels of 0.70-1.71% depending on the cultivar investigated. Similar results have also been reported (2). Polles *et al*. (42) believe that the level of PAC present in a cultivar may give an indication of a cultivar's ability to resist insect and disease problems.

Gu *et al*. (45) reported in their 2002 study that pecans contained only B-type linkages in their PACs. Through HPLC analysis, it was identified that pecans contained epicatechins, epigallocatechins, and PACs with degrees of polymerization ranging from monomers through polymers. Interestingly enough, Gu *et al*. (45) were not able to find any detectable PACs in macadamia nuts, pine nuts, or Brazil nuts, demonstrating that the pecan PAC content may give it properties not found in these other tree nut types. In a 2004 follow-up study conducted by Gu *et al*. (46), pecans were found to have a total PAC content of 494.1 ± 86.2 mg/100 g pecan. The study reported that pecans contained monomers, dimers, trimers, tetramers-hexamers, heptamers-decamers, and polymers with varying amounts of17.2, 42.1, 26, 101, 84, and 223 mg/100g pecan, respectively.

2.3 Antioxidants and Free Radicals

Free radicals are highly reactive species due to the presence of an unpaired electron in their structure. Reactive oxygen species (ROS) are a special class of free radicals. The most common ROS are superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$), alkoxyl and peroxyl radical ($RO^{\cdot}$ & $ROO^{\cdot}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^{\cdot}O_2$), and ozone (O_3) (30). Also of note are the reactive nitrogen species (RNS), nitroxyl and peroxynitrite radicals. These ROS are formed naturally. Increase in ROS concentrations have been linked to environmental stresses, such as chemicals and pollution (47). Since these ROS are naturally generated in the body, there are defense systems in place to quench them. The most common

forms include endogenous antioxidant enzymes, endogenous factors, metal-ion sequestration systems, and endogenously generated primary and secondary antioxidants (48-50).

ROS attack almost all macromolecules in the body causing serious damage to cellular organelles and DNA and even cell death (51). The hydroxyl radical, produced in almost all plant cell organelles, is highly reactive and unscavangable in plant cells. When it accumulates, programmed cell death can occur (52). It has been suggested that ROS could play a role in development of chronic diseases (26).

Antioxidants are defined as compounds that, when present in a sample at lower concentrations than that of an oxidizable substrate, are able to postpone or inhibit oxidation (18). There are two types of antioxidants, primary and secondary (53). Primary antioxidants work directly to prevent oxidation reactions while secondary antioxidants prevent oxidation through an indirect method such as binding pro-oxidants or scavenging oxygen in a system. Antioxidant activity is a general measure of the ability of a compound to reduce oxidation events through free radical scavenging, singlet oxygen quenching, and metal chelation (18). There are two main mechanisms for antioxidant quenching, hydrogen atom transfer (HAT) and single electron transfer (SET). In most food systems and antioxidant action, both mechanisms are occurring at the same time and the predominant mechanism is determined by the chemical properties of the antioxidant, solubility of the antioxidant in the system, and the system solvent (54).

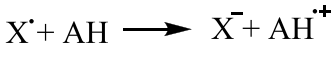
The HAT reaction occurs when an antioxidant transfers a hydrogen atom to a radical, quenching the radical activity. The basic equation for the reaction is highlighted by the following reaction:

$$X^{\cdot} + AH \longrightarrow XH + A^{\cdot}$$

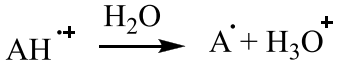
A radical is still present after the hydrogen atom transfer but the antioxidant forms a more stable species. In most cases, the radical is stabilized through resonance stabilization provided by the antioxidant's structure. More stable newly formed radical species, minimize the chance for the reactions to continue (55). An antioxidant's ability to perform a HAT reaction is judged by two defining characteristics, how likely it is to lose the hydrogen atom and how stable the resulting radical species will be. The ability of the compound to lose a hydrogen atom is dependent on the bond dissociation enthalpy (BDE) of that hydrogen. Hydrogens with lower BDE are more likely to be transferred, and there will be a higher antioxidant activity for the phenolic compound (55). The stability of the resulting radical species is affected by differences in its structure and the ring substituents (26). For example, if an electron withdrawing group is present on the ring in the meta-position, then the ring will be deactivated and the reaction will be hindered (56). It is also possible, however, to have electron donating groups which can activate the ring.

The SET reaction involves the transfer of a single electron from the antioxidant compound to the free radical species. After the initial electron transfer (equation 1), two additional reactions occur in order to stabilize the aromatic compound and form a stable group.

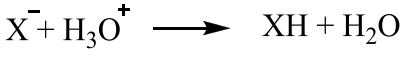
The reactions are highlighted below:



(1)



(2)



(3)

The SET reactions are highly dependent upon the ionization potential (IP) of the reactive groups (26, 54). The higher the ionization energy required for the electron transfer, the less likely it is for the antioxidant to part with its electron (55). In addition, pH and ionization energy are linked. In general, with higher pH's a decrease in IP will be observed. For this reason, SET reactions are generally favored in higher pH alkaline environments (26).

Hydrophilic and lipophilic antioxidants are present in food samples. The phenolic compounds previously discussed (section 2.2) are the most prevalent hydrophilic antioxidants found in food and plant systems. Some researchers believe that lipophilic antioxidants have the potential to be more bioactive due to their ability to cross the plasma membrane of cells (57). The main lipophilic antioxidants found in food systems are the tocopherols.

2.4 Methods of Antioxidant Analysis

Numerous antioxidant capacity assays exist in the literature today. In choosing the proper method, one must consider the advantages and disadvantages of each method as well as the ideal conditions for each assay. Two main mechanisms of action exist for an antioxidant to quench a radical (54). These are hydrogen atom transfer and single electron transfer as described in the previous section (section 2.3). Assays exist to measure both mechanisms of action. When looking at the antioxidant capacity of a food or sample, one should measure the capacity for both mechanisms. Usually in a single system and even with a single compound of interest, both mechanisms of action can be employed. Methods based on the hydrogen atom transfer mechanism measure an antioxidant's capability to transfer a hydrogen atom to a radical. Methods based on the single electron transfer mechanism look at the capability of an antioxidant to transfer an electron to a compound in a redox-type reaction.

2.4.1 ORAC assay

Oxygen Radical Absorbance Capacity (ORAC) is an example of a hydrogen atom transfer assay (58). ORAC assays are commonly used to measure the total antioxidant activity of a food or substance. ORAC is seen as an improvement to previous antioxidant assays because its extended run time allows for the assay to follow the antioxidant activity through not only the lag time, as with previous assays, but also the initial rate, giving a better estimation of the total antioxidant activity (54). The assay operates by reacting a peroxyl radical with a fluorescent probe (equation 5) (54). When the radical reacts with the probe it produces a non-fluorescent compound. If an antioxidant is present in the sample, a reduced loss of fluorescence will be observed as the antioxidant reacts with the radical before it can react with the fluorescent probe (equation 6). Prior *et al.* (54) presented the following possible assay reaction mechanisms in their review:

$$ROO^{\bullet} + \text{probe (fluorescent)} \longrightarrow ROOH + \text{oxidized probe (loss of fluorescence)} \quad (1)$$

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet} \quad (2)$$

$$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA \quad (3)$$

The ORAC assay generally involves the use of fluorescein (FL), 3',6'-dihydroxyspiro[isobenzofuran-1-[3H],9'[9H]-xanthan]-3-one, as the fluorescent probe (58). Originally, B-phycoerythrin (B-PE) was used (58-59) but it has since been discontinued because its natural variability between lots makes good reproducibility difficult (60), and its inability to resist photobleaching during the assay negatively affects the results (54). Another issuewith B-PE occurred when measuring the antioxidant capacity of samples high in PACs. These

compounds have shown an ability to bind to the B-PE making it unavailable for the assay (54). FL does not seem to incur these same problems. It has not been shown to interact with any of the phenolic compounds usually found in the samples analyzed and fails to become photobleached (58). The only drawback with the use of FL is its sensitivity to pH. The assay is normally carried out at a pH of 7.4 and FL has a pk_a of 6.4. If the pH of the assay buffers drop below 7, a significant decrease in fluorescence intensity has been observed (58) and thus pH must be extremely well-controlled throughout the assay.

Criticisms for all *in vitro* antioxidant assays is that is difficult to know what the assay is truly measuring and whether the assay's results are transferable to biological systems (61). This same criticism has been made of the ORAC assay as well, and while its ability to predict *in vivo* outcomes has not been proven, experimental investigations into the mechanism of action has been performed. Ou *et al*. (58) used an LC-MS analysis to verify that the reaction mechanism is actually that of a classical HAT mechanism. Ou *et al.* (58) also proved that the assay was specific to antioxidants. The investigators conducted an experiment in which antioxidant-rich samples were incubated for 2 h with the radical generator AAPH as well as the Fenton reagent. All of the samples studied had no remaining activity at the end of the incubation period documenting the specificity of the assay.

The assay was automated in 1995 by Cao *et al.* (62) and can now be run using a multi-plate reader. The fluorescence of the FL probe is measured at an excitation wavelength of 493 nm and an emission wavelength of 515 nm for an extended period of time (*i.e.*> 30 minutes) (26). The fluorescence decay curves are determined for the blank, standards, and samples and an area under the curve (AUC) value is produced. The AUC for the standards and samples are determined by removing the AUC_{blank} value. Standard curves are prepared using various Trolox

concentration solutions and data is reported in Trolox equivalents (54). Previously, one was only able to measure antioxidant capacity of hydrophilic antioxidants but recent adaptations to the method have allowed for measure of lipophilic antioxidants (63).

While lipophilic antioxidants could play a role *in vivo* due to their ability to cross the cell membrane and be stored in the body (57), they are much more difficult to assess for antioxidant activity. The ORAC assay, as well as most other antioxidant assays, is conducted in an aqueous environment in which solubility plays a role. In order to alleviate this problem, Huang *et al.* (63) developed a method of improving the solubility of lipophilic antioxidants through the use of a molecular host, randomly methylated β-cyclodextrin (RMCD). Due to cyclodextrins cyclical structure containing (α-1,4)-linked oligosaccharides of α-D-glucopyranose, a hydrophobic inner side and hydrophobic outer side are created. In previous studies it was shown that by incorporating only 10-40% RMCD into one's methodology, there can be a 1000-fold improvement in a lipophilic compound's solubility in aqueous environments (63).

In addition to the assay limitation of pH sensitivity mentioned previously, an additional issue can come up with the ORAC assay. The assay is extremely temperature sensitive and must be carried out at 37˚C (54). In the plate reader, one must be careful to limit temperature difference across the plate and keep the temperature constant for the duration of the assay. Temperature effects can also be limited by ensuring that all assay reagents and buffers are brought to 37˚C prior to analysis. Prior *et al.* (64) found variation was reduced if the AAPH was added to the buffer after it had been incubated at 37 ˚C. The wells on the external edge of the plate can also be left empty in order to help limit the temperature variation across the plate (65).

Wu *et al.* (66) compared the lipophilic and hydrophobic ORAC scores for 28 different foods. They found that pecans had an H-ORAC$_{FL}$ score of 175.24 ± 10.36 μmol TE/g which was

higher than all other foods measured. The next highest food sampled was prune with an H-ORAC$_{FL}$ of 83.99 ± 16.56 μmol TE/g.

2.4.2 Total Phenolic Content Assay

The total phenolic content assay (TPC) was first developed based on a reagent, the Folin-Denis reagent, that was being used at the time to quantify tyrosine residues in protein samples (67-68). In 1927, Folin and Ciocalteu (69) improved the formulation of the reagent, Folin-Ciocalteu reagent (F-C reagent), incorporating additional molybdenum into the reagent in order to ensure improved sensitivity of the assay. In this method, the molybdenum compound changed from yellow to blue upon oxidation by a phenolic compound but it was a slow reaction that lacked specificity (54). The method was improved in 1965 by Singleton and Rossi (70) with an improved reagent, the molybdotunstophosphoricheteropoly anion reagent (54). Singleton and Rossi also included steps and considerations that must be utilized in order to get reliable results. The authors suggested that a certain volume ratio always be employed for the alkali reagent and the F-C reagent, an optimal reaction time and temperature for the color development, color monitoring at 765 nm, and gallic acid as the reference standard (54, 70). The overall mechanism of the reaction of the molybdenum ion formed from the F-C reagent, as presented by Craft *et al.* (26) is as follows:

$$Mo^{6+}\ (\text{yellow}) + ArOH \longrightarrow Mo^{5+}\ (\text{blue}) + [ArOH]^{\bullet +} \qquad (1)$$

As with any assay, drawbacks have been reported. One of the biggest complaints is a lack of standardization of conditions and reporting styles (54). Even though Singleton and Rossi (70) presented very specific conditions for the time, temperature, and optical densities of the measurements, a vast range of differences is seen in the literature. TPC values are also reported using various standard compounds. Singleton and Rossi (70) recommended the use of gallic acid as the standard compound, but authors have also reported values using *p*-coumaric acid (71), catechin (72) and chlorogenic acid (41) as standards. This recommendation is a controversial topic because while some authors believe that standardization of reporting methods is necessary (54), others believe that it is more important to use a standard that would best describe the type of phenolics found in one's sample.

The TPC assay has also displayed some interference with certain test substances. The interfering substances reported to date include: sugars, aromatic amines, sulfur dioxide, ascorbic acid, certain enediols and reductones, organic acids, and Fe(II) (54). If using a sample that contains one of these interfering compounds, background correction must be conducted in order to account for their presence. It should also be noted that false positives for some nonphenolic organic and inorganic compounds have been reported (54, 73). A final possible issue that can occur with the TPC assay is that the phenol reagent can precipitate out (26). The precipitation can occur if the assay reagents are subjected to high heat (>60˚C), high pH (>10), or if too much of the F-C reagent is used in the assay (> 5 ml/100 ml) (26, 74).

2.4.3 FRAP Assay

The Ferric Reducing Antioxidant Power (FRAP) assay is an example of a single electron transfer mechanism assay. The FRAP assay, originally named Ferric Reducing Ability of

Plasma, was first developed in 1996 by Benzie and Strain (75) to, as the name suggests, measure the reducing power in plasma samples. Since its original development, there have been numerous modifications to adapt the assay for use in quantifying antioxidant activity in other samples (54, 76-77). It was given its current name, the Ferric Reducing Antioxidant Power assay, by Benzie and Strain in 1999 (76). The most widely adopted method is that of Pulido *et al*. published in 2000 (78).

The FRAP assay is a colorimetric assay where the single electron transfer causes the reduction of a reagent, usually ferric 2,4,6-tripyridyl-*s*-triazine (Fe (III)-TPTZ), to a deep blue colored product, ferrous 2,4,6-tripyridyl-*s*-triazine (Fe(II)-$(TPTZ)_2$) (58, 75, 79). Since Fe^{3+}-TPTZ has a redox potential of 0.7 V, the FRAP assay is only usable for compounds with a redox potential of less than 0.7 V (54). The assay is usually conducted at a pH of 3.6 in order to ensure that the iron in the reagent remains stable and soluble (54). However, this is often noted as a drawback of the assay since this is much lower than physiological pH and thus may present results that are completely different than would be seen *in vivo* (58, 78). The pH can also affect the ionization potentials (IP) by decreasing the IP that is necessary for the electron transfer, causing an increase in the redox potential of the system (80, 81). The reaction of the FRAP assay is outlined in the equation below:

$$Fe^{3+}\text{-TPTZ (colorless)} + ArOH \longrightarrow Fe2+\text{-TPTZ (blue)} + [ArOH]^{\cdot +} \qquad (1)$$

The assay is not without its drawbacks. It has been stated that the assay may lack physiological purpose since there is no connection between a compound's ability to reduce iron and its ability to quench free radicals (54). Proponents of the assay have said that the ability to

reduce ferric compounds has its own place in antioxidant chemistry. They argue that a redox reaction of a radical can work to stop the destructive nature of radical chains (54). A more legitimate drawback is that the reducing capability of the antioxidants is only testing against the ferric ion. This situation does not carry over to biological systems very well, and thus the assay may not be a very good predictor of *in vivo* success (54, 58).

Another area of concern regarding the FRAP assay surrounds the time needed for the reaction. Originally, the assay was touted as a quick assay that could be completed in a matter of minutes. Pulido *et al.* (78) investigated what happened if the reaction was followed for a much longer time. They discovered that the absorbance values of some compounds (*i.e.*, caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin) did in fact increase slowly after the initial four minute reaction time and continued to increase for a matter of hours. When employing the assay, one must consider the possibility of underestimating FRAP values if too short of a time is allowed.

The FRAP assay, due to its unusual mechanism of action, is much different than other antioxidant assays, and for this reason can elucidate antioxidant capacity that other assays might miss. However, there are some compounds whose antioxidant power cannot be quantified by the FRAP assay. Any thiol antioxidants such as glutathione will be left out by this assay (82, 54), as well as carotenoid compounds (78).

2.4.4 DMAC Assay for Total Proanthocyanidin Content

The DMAC assay has been used for quite some time to measure the total flavanol content of beer and wine, but it is now beginning to be used in more widespread sample types. The assay was first utilized in 1978 by McMurrough and McDowell in an investigation of the

flavanolspresent in different beer ingredients (83). The main compound used in the assay is *p*-dimethylaminocinnamaldehyde which can also be written as 4-dimethylaminocinnamaldehyde (73, 84). This reagent can be seen abbreviated in several different ways and readers should be aware that DMAC, DMACA, and DAC all seem to be referring to the same compound (73, 83, 85).

This assay replaces the vanillin assay which is commonly used to quantify PACs and flavanols (83-85). The vanillin test needed to be replaced since the assay had several shortcomings. It cannot be used for any samples that contained anthocyanins because they interfered with the absorbance readings and often gave lower values for these samples (83-84). It was discovered that the DMAC assay provided a more specific and sensitive result than that which could be provided by the vanillin assay (86).

One of the initial complaints with the DMAC assay involved how quickly the reaction needed to be analyzed. Once all of the samples and reagents were combined in a sample well the reaction needed to be read within 2 minutes to avoid a possible decrease in color development (85). Nagel and Glories (85) began investigating ways of slowing the color development in order to extend the time available for reading the samples. They found that if the amount of hydrochloric acid utilized in the experiment was reduced to 0.4% HCl the color would remain developed for almost 20 minutes. The investigators also reported that if water is present in tested samples it can affect the rate and intensity of the color development of the samples (85). The DMAC assay has been shown to be quite specific and does have some structural requirements for its success (83). These requirements are as follows: the structures must have a meta-substituted dihydroxybenzene ring, a single bond between the C2 and C3 carbons, and must lack a carbonyl at C4 (87).

In 2010, Payne *et al.* (73) presented a thorough study of the DMAC analysis in quantifying the PACs present in cocoa samples. In order to discover more about the mechanisms involved in the assay the authors investigated the response of the assay to several different standards with varying chain length. They chose catechin, epicatechin, procyanidin B2, trimer and tetramer standards and found that the catechin and epicatechin produced much higher response values than the polymeric compounds. For this reason the authors have suggested that going forward with the assay, the oligomeric compounds are selected as standards in order to ensure that the PAC contents are properly estimated. The authors also investigated the two most widely debated aspects of the assay: the solvent selection and the concentration of HCl employed. Most researchers avoid the use of water as a solvent in the DMAC assay and have instead switched to methanol for the solvent system. Payne *et al*. (73) however suggest using ethanol since it is less likely to evaporate during the course of the assay. As far as the HCl concentration used in creating the DMAC reagent, the authors suggest using 10% HCl. This concentration ensures that the assay can be completed almost immediately and cuts down on the amount of time needed for the color development. While some authors were originally concerned about the speed of the assay, it is suggested that a faster assay would help to cut down on the run time and also reduce the risk of solvent loss to evaporation. With the development of speedy multi-plate readers, there should be little concern for not being able to read the plate immediately after addition of the reagent to the plate wells.

2.5 Effect of Roasting on Antioxidant Activity

Pecans can be enjoyed in the United States both raw and roasted. Roasting time, roasting temperature, nut type, and experimental conditions employed all have an effect on the

antioxidant activity of nuts. It is believed that two main mechanisms act during roasting to create this effect on the samples (88-91). These mechanisms include decomposition of antioxidants through the heating process and formation of new antioxidant compounds, either Maillard reaction products (MRPs) (89-90) or released previously-bound phenolic compounds (88). The result of this competition between antioxidant breakdown and new compound formation will determine the net effect on the change in the antioxidant activity of the samples. A third potential mechanism is the release of bound phenolics from the fibrous material in the nut meat through the high heat process. It is possible that with the high heat found during roasting, fibrous material is breaking down and bound phenolics are released from the meat (92). Through HPLC analysis, the mechanisms playing a role in antioxidant activity changes may be further elucidated and understood.

Chandrasekara *et al.* (92) investigated the effect of roasting cashews on the total phenolic content (TPC) and H-ORAC$_{FL}$ of the soluble phenolic extract. It was reported that the roasted cashew samples had significantly higher TPC values and the ORAC values for samples (kernels and testa) roasted under high temperature conditions showed an 8% increase in values from the raw samples. Through HPLC characterization of the cashew skins from raw and roasted samples, it has been shown that there was decreased PAC content and an increase in the individual monomers (92). The authors suggest that a part of the increase can be attributed not only to the Maillard reaction but also to a release of bound phenolic compounds from the fibrous material of the nut. A second study conducted with cashews investigated the effect of roast time and temperature by employing a low temperature roast (70°C for 6 h) and a high temperature roast (130°C for 33 min) (93). In both cases, an increase in TPC was observed. The low temperature nuts increased to 536 mg GAE/100 g nuts (dw) from 369 mg GAE/100 g nuts (dw)

raw cashew testa and the high temperature nuts increased to 1891 mg GAE/100 g nuts (dw). A third study involving cashews showed a significant increase in total antioxidant capacity with roasting (94). Besides cashews, peanuts were the only other nut type that showed a significant increase through roasting. Peanuts displayed a significant increase in both the TPC and lipophilic-ORAC (L-$ORAC_{FL}$) values of roasted skin and kernel samples, respectively (95). Interestingly, the TPC of peanut skin decreased with increasing roast temperature, a trend not seen in cashews. It is suspected that cashews were the only nut type that demonstrated an increase in TAC because of their relatively higher carbohydrate and protein profile (95) (Table 2.2). This observation may add evidence to the theory that the production of MRPs through roasting is limited in some nuts by a lack of reactive carbohydrates.

Table 2.2 Composition Data for 7 Nut Types[a]

	g/100 g nutmeat			
Nut Type	Moisture	Carbohydrate (Sugars)	Lipid	Crude Protein
Almonds	4.70	21.67 (3.89)	49.42	21.22
Cashews	5.20	30.19 (5.91)	43.85	18.22
Walnuts (English)	4.07	13.71 (2.61)	65.21	15.23
Hazelnuts	5.31	16.70 (4.34)	60.75	14.95
Pistachios	3.91	27.51 (7.66)	45.39	20.27
Pine Nuts	2.28	13.08 (3.59)	68.37	13.69
Pecans	3.52	13.86 (3.97)	71.97	9.17

[a] Taken from the USDA National Nutrient Database for Standard Reference, Release 25. (12)

While cashews did show favorable results upon roasting for the TPC and ORAC values, Chandrasekara *et al.* (92) observed a decrease in procyanidin content believed to be due to decomposition of the compounds at high temperatures. A marked decrease in PACs seemed to be a trend observed across all nut types. In almond skins, a 26% decrease in TPC and 34% decrease in FRAP values was attributed mainly to a decrease in PACs (88). Pistachios and walnuts showed a 90% and 14% reduction in PACs, respectively (96-97). Not only was a reduction in TPCs a trend but in a study conducted by Açar *et al.*(94) looking at the TAC values for raw and roasted pulses, nuts, and seeds, it was observed that for the samples analyzed, TAC decreased with short roasting times for all nut types. Furthermore, effects were observed for short (less than 30 minutes) versus long roasting time. The authors were able to compare those changes which occur due to thermally-labile antioxidants degrading (short time) and formation of new antioxidant compounds once moisture levels stabilized after 30 minutes. Only hazelnuts and pistachios displayed a significant increase in TAC with roasting times of greater than 30 minutes.

Several studies also reported no significant changes in antioxidant activity with roasting (88, 91, 95, 98-99). Three separate studies investigating the relationship between roasting and antioxidant activity showed that roasting did not significantly increase flavonoid content (98), H-$ORAC_{FL}$ (95), and TPC (91). Almond skins roasted at 295°F for 14 minutes showed no significant change in flavonoid and phenolic acid content (88).Roasted hazelnut skins also did not show any significant change in extractable polyphenols compared with their raw counterparts (99). It should be noted that no evidence of studies comparing antioxidant activities of raw and roasted pecans.

2.5.1 Maillard Reaction

Maillard browning, also referred to as a carbonyl-amino reaction, is a non-enzymatic reaction between an amine group, such as those found in amino acids, and a reducing sugar (100-102). Not only does this reaction not require an enzyme to proceed, it also can function without the presence of oxygen. As described in the review by Hodge (100), there are three main stages to the reaction: an initial stage, intermediate stage and final stage. The initial stage consists of the sugar-amine condensation and Amadori rearrangement steps while the intermediate stage consists of the sugar dehydration, sugar fragmentation, and amino acid degradation steps. The final stage is made up of the aldol condensation and aldehyde-amine polymerization steps. These steps are outlined in Figure 2.5 below which was adapted from the Hodge review (100) and Martins *et al*. (103) and will now be discussed in more detail.

The first step of the reaction is the sugar-amine condensation reaction. In this reversible reaction, an N-substituted glycosylamine (or other aldosylamine) is formed when the carbonyl group of the sugar compound is exposed and the amine is able to bind to it (100) producing an imine (101). The next step, the Amadori rearrangement, is seen as being a crucial step in the overall reaction. It has been proven that if this step is removed from the reaction, the characteristic browning does not develop (100). In this step, the N-substituted aldosylamines become α-aminoketones through a rearrangement of the structure (100-101). As with the previous step, the isomerization reaction of the Amadori rearrangement is reversible. However, this step appears to be more stable unless the products are subjected to a dry heating environment. Under these conditions additional changes to the structure and reactions can occur, forming reductones and other compounds (100). At this point, the reaction proceeds to the intermediate stage.

The first step of the intermediate stage, the dehydration of the sugar compound, can take different paths depending on the pH of the system. In an acidic system (pH below 4) furfurals are formed through a 1,2-enol tautomerization, and at higher pH's (about 6) reductones are formed through the 2,3-enol tautomerization (100-101). It is these reductones that have been shown to contain a fairly strong antioxidant and reducing powers (100). The resultant compounds are then further fragmented and undergo a Streker degradation reaction producing aldehydes (100). These aldehydes are believed to react with numerous different compounds that remain from previous reaction steps in order to produce compounds with brown pigments. The steps of the final stage, aldol condensation and aldehyde amine polymerization, are final reactions which result in the formation of melanoidin and other brown pigments (100).

2.5.2 Maillard Reaction Products and Antioxidant Activity

There is some debate as to where in the Maillard reaction the MRPs with the greatest antioxidant activity are formed. Some authors point to the more advanced stages of the reaction which produce large brown compounds (104) while others suggest that they are formed earlier in the reaction, during the intermediate stage (105-107). MRPs and melanoidin compounds have displayed high antioxidant activity due to their reductone-type structure (108), metal chelating properties (90), chain breaking properties (109-110), and oxygen consuming capabilities (111). It was shown by Hayase *et al*. (112) that both high-molecular weight and low-molecular weight compounds are successful as antioxidants but also lower-molecular weight compounds. The most successful MRPs seem to be enamines, pyrrole-like compounds, and reductones (112).

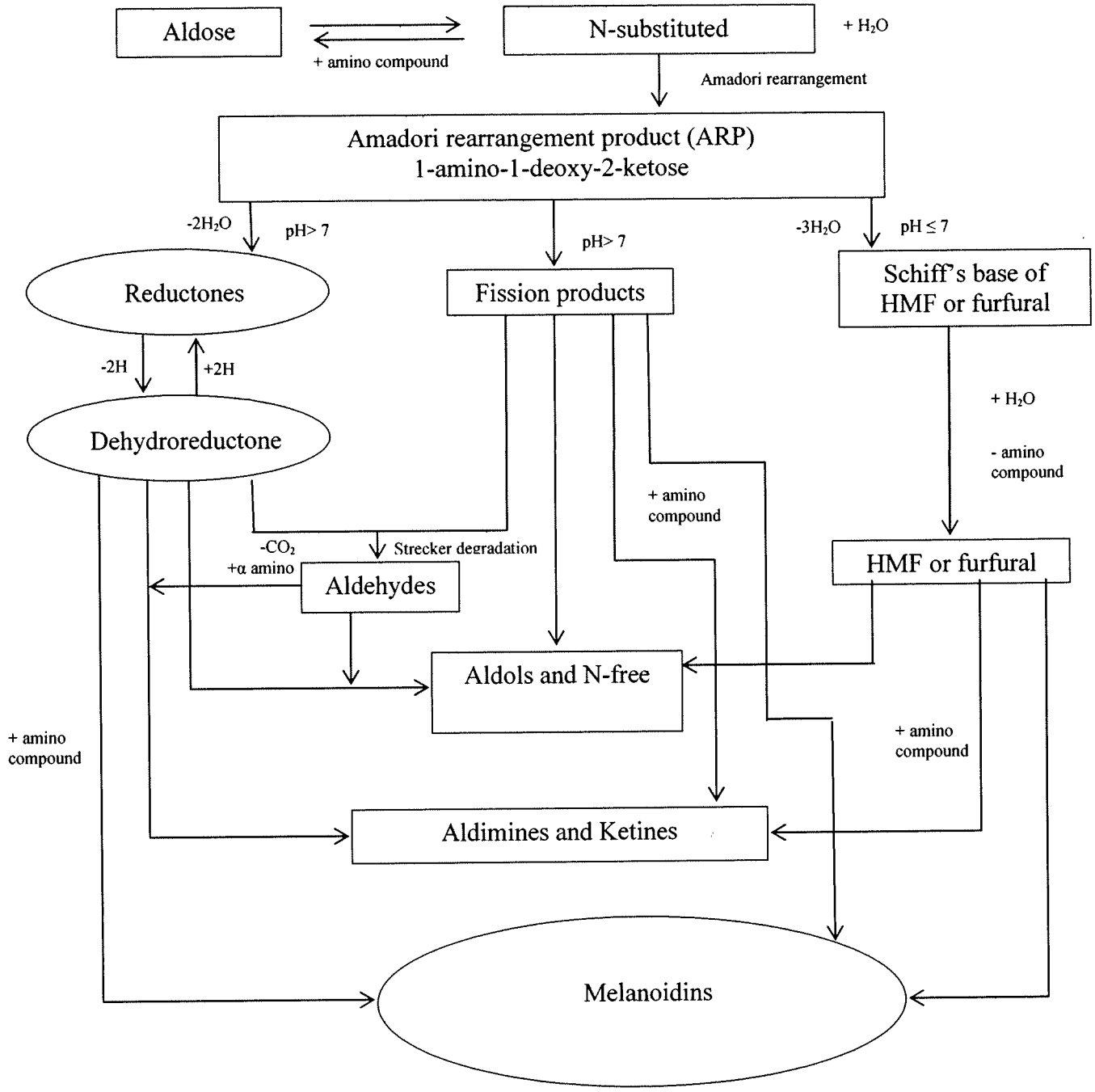


Figure 2.5.Maillard reaction scheme: Possible antioxidant compounds highlighted in oval shapes. Adapted from Hodge (100) and Martins *et al.* (103).

2.6 Inflammatory Processes

Inflammation has been linked to numerous chronic diseases and health issues including rheumatoid arthritis, osteoarthritis, spinal cord injury, stroke, diabetes and cardiovascular disease (113-115). There are several key players in the inflammatory process including the protein complex, nuclear factor-kappa beta (NF-κB), nitric oxide (NO), cytokines (TNF-α and IL-1β), and prostaglandins. Fortunately, laboratory assays have been developed in order to quantify the production of these inflammatory mediators in a cell culture environment. These assays will be covered in more detail in section 2.6.2.

2.6.1 Inflammatory Players and Mediators

NF-κB is a protein complex shown to play a key role in the inflammation process in macrophages and lymphocytes (116). During normal physiological conditions the protein complex is located in the cytosol of the cell as an inactive complex, IκB-NFκB. The inflammatory process begins when one of several possible stimuli activates the cell. These stimuli can be both exogenous (LPS) or endogenous (TFN-α or IFN-γ) (117-118). This stimulation then causes the enzyme IκB-kinase (IκK) to phosphorylate the inactive complex. Once the complex is inactivated, it can pass into the nucleus where it can stimulate gene expression for the inducible nitric oxide synthase (*i*NOS) gene (115, 118-121). Once the *i*NOS gene is activated, there is an increase in the production of cytokines and other inflammatory mediators (122). This hypothesis has been supported by the finding of an NF- κB binding site in the promoter region of (*i*NOS). It is important to note that NF- κB not only regulates *i*NOS but also about 200 other immune, growth and inflammation genes (123).

One interesting point regarding the NF- κB complex is the cyclical nature of its activation. Some of the same compounds that are produced by the complex once initiation occurs are also able to initiate the complex themselves. This is part of the reason that inflammation can be such a long-lasting and persistent process. Two compounds which have been shown to both activate and be activated by the NF-κB complex, are the cytokines IL-1β and TNF-α (122). Another activator, lipopolysachharide (LPS), is a major component of the cell wall of Gram-negative bacteria. LPS mainly targets macrophage cells and has been shown to be a powerful initiator of inflammation (124-127). In addition to activating NF- κB, LPS can also activate Toll-like receptor 4 (TLR4) which can then activate the MyD88-dependent and TRIF-dependent pathways (124).

Nitric oxide (NO) is a free radical that serves as a mediator of inflammation (128). As mentioned previously, NO is produced once the NF-κB complex has been activated by extracellular stimuli (117, 129), but NO is also produced during everyday activities in the body to help mediate other biological functions (128). NO has also been shown to serve a role as a neurotransmitter and a regulator for blood vessel tone (115). The amino acid L-Arginine is converted to NO by one of three NO synthases found in the body, constitutive synthases, endothelial (*e*NOS) and neuronal nitric oxide synthases (*n*NOS), and inducible nitric oxide synthase (*i*NOS). Constitutive synthases *e*NOS and *n*NOS, as the term would imply, are always found in cells and can be quickly activated when necessary by Ca^{2+}. Fairly different from the constitutive synthases, *i*NOS is not found in uninjured cells and functions independently of calcium (115). However, upon any form of injury or inflammation, the NF-κB complex will initiate cytokines in the cell to begin producing *i*NOS (130). *i*NOS is then signaled to begin producing NO from L-arginine. This biosynthesis occurs using NADPH as an electron donor

and heme, FMN, FAD, and tetrahydrobiopterin (H_4B) as cofactors. The process utilizes five electrons (115).

NO serves multiple roles in cells inflammation (93). One role is that NO causes the vascular smooth muscle to relax and encourages vasodilatation in the body. NO is also seen as a compound that helps control the inflammatory responses in the body. It serves to limit the amount of white blood cell participation, as well as interfere with the capability of platelets to form clots and adhere to the infection site. In general, NO prevents most events usually associated with inflammation. NO is a fairly small molecule and is able to easily diffuse from cell to cell. This enables the NO to easily maneuver from the site of formation to various sites of action (115, 132). Due to the free radical state of NO, it can be extremely reactive with other free radicals found in the cell. While the NO radical itself is not usually very damaging, it does form the much more reactive RNS, peroxynitrite anion ($ONOO^-$) (113). For this reason, the pro-oxidative NO has been shown to result in cellular oxidative damage (133-135). Damage in the cell has been shown in the form of modification to amino acids residues, inhibition of enzymes, induction of lipid peroxides, and depletion of normal cellular antioxidant levels (133). Therefore, it is common for investigators studying inflammation using cell culture models to test for the reactive oxygen species.

Most experts agree that there is some oxidative step involved with the activation of the NF-κB complex. This hypothesis has inspired numerous experiments looking at the role that antioxidants may be able to play in reduction of inflammation. While it has not been determined exactly which step in the process is controlled by the presence of ROS there have been several experimental observations that may lend support to this hypothesis. Under certain experimental conditions it has been shown that NF-κB can be activated by the presence of H_2O_2. It has also

been shown that antioxidants reduce the activation of NF-κB and that most of the compounds known to activate NF-κB also display the ability to produce ROS. Complicating the picture, however, is the fact that the H_2O_2 does not always reduce NF-κB activation. The activation results depend on the type of cell line tested. The ability for antioxidants to reduce activation also seems to be antioxidant dependent. Finally, even though the common activators of NF-κB may also be able to produce ROS, the actions of these compounds cannot be easily mimicked by H_2O_2. (136)

2.6.2 Inflammatory Mediator Assays

In order to quantify NO production by RAW 264.7 cells, most experimenters employ a Griess assay. The nitric oxide produced is spontaneously oxidized to form nitrite which is then quantified in the assay. The Griess reaction is a diazotization reaction that can be spectrophotometrically analyzed. The steps of the Griess assay are outlined in Figure 2.6. First, sulfanilic acid reacts with nitrite and forms a diazonium salt. The diazonium salt then reacts with N-(1-napthyl)ethylenediamine to form an azo dye which can then be quantified by measuring absorbance at 548 nm.

2.6.3 Phenolics and Inflammation

The effect of phenolics on inflammation has been studied using numerous food types including green tea, aged garlic, and pine bark extracts (124-125, 133). To date, an investigation into phenolics isolated from pecans has not been conducted, however a study analyzing the effects of a hydrophilic pistachio extract was recently published (137). Additional studies have explored the structure-activity relationship of various pure compounds differing in size, type, and

functional groups in order to better explain the mechanisms of anti-inflammatory compounds (138-139).

As mentioned, no study has been performed on the effect of pecan phenolics on NO production. The only tree nut investigation with macrophages used a hydrophilic pistachio extract (HPE) and was published by Gentile *et al*. (137). In this study, both a pre-incubation and a co-incubation with the HPE were used, but significant results were only found with the pretreatment studies. The authors were able to observe a decrease in both the *i*NOS and COX-2 enzymes during a 1 hour pretreatment with the pistachio extracts as well as a dose-dependent decrease in the NO production and TNF-α secretion. As previously mentioned, when the macrophage cells are exposed to LPS, IκB-α is phosphorylated and begins to degrade. This degradation causes an activation of the NF-κB complex and the p65/p50 portion of the complex is able to move from the cytosol into the nucleus (122). Through analysis of the NF-κB subunits, Gentile *et al.* were able to observe a significant dose-dependent decrease in the nuclear p65 subunit as well as the presence of an increased amount of the inactive subunit. These changes led the researchers to believe that the observed effects of *i*NOS and COX-2 reduction were due to a decrease in NF-κB activation. The authors believe that the significant effects of the pistachio extract are due to their PAC content. They believe that the PACs are able to bind with the LPS and reduce its effectiveness at initiating the inflammatory pathway. It is also reported that the amphiphilic nature of the PAC compounds allows them to better partition between both the membrane lipids as well as cellular proteins making them more effective at limiting expression and activation of inflammatory players (139).

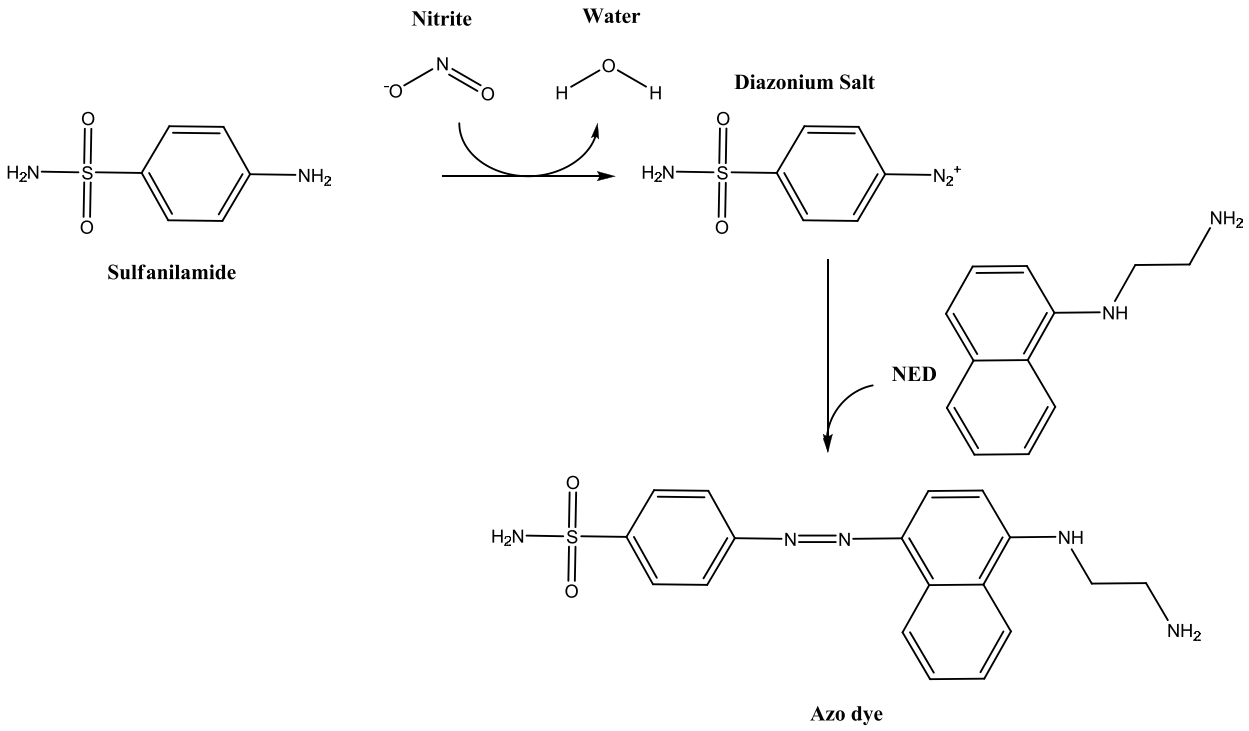


Figure 2.6.Reactions of the Griess Assay.

Terra *et al.* (117) conducted an experiment analyzing the effect of procyanidin extract (PE) on the production of NO and prostaglandins. The authors examined the effect with a preincubation of 4 hours and 10 hours, as well as a 19 hour co-incubation experiment. They showed that not only was the NO concentration reduced in a dose-dependent manner but also a time-dependent manner. When the PE was preincubated for 4 hours only a 20% reduction in NO production was seen but when the preincubation time was expanded to 10 hours, the authors were able to see a 50% reduction in NO production. A similar result was observed with the reduction in *i*NOS mRNA expression in the cell. This caused a reduction of *i*NOS present and may be responsible for the reduction in NO production levels. Interestingly, the same result was not seen with the PGE_2 production; an effect was only observed when treatment extracts were co-incubated and not with preincubation. These findings suggest that experimental design can have a significant effect on outcomes.

Another interesting and somewhat controversial conclusion that Terra *et al.* (117) drew was that NO production decreases with increasing length of the PACs. The authors reported that no inhibition of NO production was seen with the monomeric fraction, as well as with catechin and epicatechin standards. It is widely believed that the NF-κB transcription factor activation is regulated by some form of a redox step (140-144) and thus reduction in activation can be caused by antioxidant compounds. For this reason, the authors believe that procyanidins are able to act by radical scavenging of NO (145) as well as competition with LPS for receptor binding sites such as TLR-4. It is also believed that the procyanidins could directly bind with the LPS molecule itself.

Hou *et al.* (125) investigated proanthocyanidins isolated from green tea extract concentrating on prodelphinidin B2 3,3'-di-O-gallate and determined that the compounds were

able to reduce the expression of COX-2 and prostaglandins (PGE_2) in a dose-dependent manner when used in a concentration range of 12.5-50.0 μM. Not only were the PACs analyzed as an extract but individual compounds were also isolated and investigated separately. The researchers looked at 5 different compounds [prodelphinidin B2 (PD), prodelphinidin B2 3′ *O*-gallate (PDG), prodelphinidin B2 3,3′ di-*O*-gallate (PDGG), epigallocatechin (4β-8) epicatechin-3-gallate (EGC (4B-8)ECG), and epigallocatechin-3-gallate (4β-8) epicatechin-3-gallate (EGCG (4B-8)ECG)] and were able to determine that PD was unable to repress the expression of the enzymes and prostaglandins. It was determined by the authors that this is most likely due to PG's lack of a galloyl moiety, which could impart an increased affinity for the lipid bilayer of the cell making it more effective in the cellular environment (125).

Virgili *et al.* (133) investigated the role of Pycnogenol, a commercial product made from the flavonoids extracted from the bark of Pinus *maritimus*, in the reduction of *i*NOS expression in macrophages activated by both LPS and IFN-γ. The researchers found that this concentrated mixture of flavonoids was able to cause a dose-dependent inhibition of *i*NOS activity at levels above 50 μg/ml. It was demonstrated that no effects on the DNA binding activity occurred after pretreatment with the extracts but that once the macrophages were activated a dose-dependent effect was observed in *i*NOS mRNA. The authors believe the expression of the gene was affected at the posttranscriptional translation level (146).

In an attempt to learn more about the structure-activity relationship of these phenolic compounds, Park *et al.* (138) investigated the effect of increasing chain lengths of purified flavonoids on the activation of NF-κB, NO production and TNF-α secretion. With the monomeric compounds catechin, epicatechin, and taxifolin, a significant decrease in NO production was observed. With the dimeric procyanidin B1 and B2 a moderate decrease was

observed. With the trimeric procyanidins an actual increase in NO productions was observed. It is believed that the monomeric compounds were able to reduce the NO production levels by affecting the NO scavenging activity, inhibiting the *i*NOS enzyme and reducing the level of *i*NOS mRNA expression (147). The results of this study contradicted those reported by Terra *et al.* (117). The differences could be due to the fact that Park *et al.* (138) used IFN-γ as opposed to LPS used in the other study (117). Activation of the cells by IFN-γ instead of LPS was chosen because of reports showing a negative interaction between flavonoid extracts and LPS (148) which may falsely affect the results of the experiment. Similar results were also observed with the TNF-α secretion levels. A further experiment was conducted which demonstrated the ability of the trimer procyanidins to induce TNF-α secretion in the absence of IFN-γ. This lends to the idea that the mechanism of action of the reduction in inflammatory mediators must be in some way connected to the change in activation or inhibition of the NF-κB complex (138).

Kim *et al.* (139) also conducted an investigation into the structure-activity relationship of flavonoids and NO production but concentrated more on the chemical composition of the compounds as opposed to their size. The researchers were able to conclude that the most inhibitory flavonoids contained a C-2,3 double bond and a 5,7-dihydroxyl group in the A ring. NO inhibition may be related to the presence of an 8-methoxyl group in the A-ring and a 4' or 3',4'-vicinal substitution in the B-ring. Additionally, a lack of effect on NO inhibition was observed with the presence of 2',4'-(meta)-hydroxyl substitution in the B-ring or a 3-hyrdroxyl moiety in the C-ring. On a more general note, the authors found that if the flavonoids were glycoside derivatives NO inhibition was markedly decreased. It is believed that the glycoside residues inhibited the flavonoids from diffusing through the cell membrane due to their hydrophilicity or bulky size and steric hindrance (149). This was supported by a study

conducted by Lyu *et al.* (146) who also demonstrated that glycoside molecules did not have much effect on NO production in an investigation of flavonoid compounds.

Kim *et al*. (139) also provided several possible mechanisms of action for affecting the NO production through a down-regulation of the *i*NOS enzyme. The authors believe that the down-regulation could occur at the translational level of the enzyme, further upstream at the signal transduction level, or by affecting the enzyme activity itself. In addition, protein kinase C and tyrosine kinase are key players in the activation of *i*NOS by LPS (150). Some flavonoids have been shown to experimentally inhibit the action of those kinases which could potentially affect the activity of *i*NOS (151). A final possible mechanism of action outlined by Kim *et al.* (139) is the reduction in the *i*NOS control by changing the production of eicosanoids via the cyclooxygenase/lipoxygenase pathway.

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CHAPTER 3

ANTIOXIDANT ACTIVITY OF U.S. PECANS

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ABSTRACT

Epidemiological studies have shown an inverse relationship between tree nut intakes and chronic diseases. Commercially-viable U.S. pecan cultivars were assessed for their antioxidant activity and bioactive constituents. Eighteen different cultivars from three states were analyzed using the hydrophilic-oxygen radical absorbance capacity (H-ORAC$_{FL}$), total phenolics content (TPC), 4-dimethylaminocinnamaldehyde (DMAC), and ferric reducing antioxidant power (FRAP) assays. Crude phenolic extracts were also analyzed by RP-HPLC and LC-ESI-MS to determine the constituents of these samples, which may be responsible for their antioxidant activity. The H-ORAC$_{FL}$ values ranged from 13.5-25.5 mmol trolox equivalents (TE)/100 g nutmeat and the FRAP values ranged from 14.0-20.7 mmol Fe^{2+} eq./100 g nutmeat. The total phenolics content (TPC) was found to be 1.11-1.60 g CE/100 g nutmeat and the total procyanidins content (TPrC) was 420-655 mg procyanidin B2 eq./100 g nutmeat. The TPrC values correlated much better with the H-ORAC$_{FL}$ values than the TPC values. Dominant phenolic acids identified in the crude acetonic extract by RP-18 HPLC included gallic and ellagic acids as well as the proanthocyanidin monomer, catechin.

3.1 INTRODUCTION

Pecans [*Carya illinoinensis* (Wangenh.) K. Koch] have recently garnered increased attention for their numerous health benefits. Nut consumption was highlighted in the 2010 Dietary guidelines: “Moderate evidence indicates that eating peanuts and certain tree nuts (*i.e.*, walnuts, almonds, and pistachios) reduces risk factors for cardiovascular disease when consumed as part of a diet that is nutritionally adequate and within calorie needs (1)”. While the increased attention is due partially to the beneficial lipid components of the pecan (*i.e.*, fatty acid profile,

tocopherol isomers and phytosterols) (2), it can also be associated with their beneficial phenolics and proanthocyanidins (PAC) content (3).

Reactive oxygen species (ROS) are free radicals found naturally in the human body. The most common ROS are superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), alkoxyl and peroxyl radicals ($RO^{\bullet}$ and $ROO^{\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^{\bullet}O_2$), and ozone (O_3) (4). Humans have natural biochemical defense systems that appear to control these ROS such as endogenous antioxidant enzymes, endogenous factors, metal-ion sequestration systems, and endogenously-generated primary and secondary antioxidants (5-7). However, when environmental conditions or stress cause the content of ROS to become higher than normal, the defense system can no longer handle these highly reactive species. It is at this point that damage to cellular systems and DNA can occur. The damage caused by ROS has been linked to several chronic diseases and conditions such as autoimmune diseases (8).

With the rising incidence of these chronic diseases, the importance of dietary antioxidants is beginning to gain public awareness. Antioxidants can function via two main mechanisms: a hydrogen atom transfer (HAT) and/or a single electron transfer (SET) (9). In the HAT reaction, an antioxidant transfers/donates a hydrogen atom to a peroxyl radical, quenching the radical and forming a more stable radical through resonance stabilization. The SET reaction is more similar to the classical redox reactions; that is, an electron is transferred from the antioxidant to quench the radical. Because two significantly different mechanisms are involved with antioxidant action, it is essential to employ more than one type of antioxidant assay to help to develop a complete picture of a sample's true activity (10).

In addition to hydrophilic antioxidants, pecan lipids have been shown to possess significant quantities of tocopherol (T) isomers, which are believed to contribute to lipophilic antioxidant capacity (11). Pecans are unique in that a significant portion of theirTs (89.4 %)are represented by the γ-T isomer (2). γ-Tocopherol is important from a health standpoint because it has been shown to be a better free radical scavenger than α-T (12). Efficacy of pecan bioactive compounds, such as γ-T and flavan-3-ol monomers, against LDL oxidation has been shown in both *in vitro* and *in vivo* studies. For instance, in a study by Hudthagosol *et al*. (13), the participants were fed meals consisting of 90 g of pecans and postprandial plasma antioxidants and oxidized-LDL cholesterol levels were measured. It was found that the pecan meals resulted in an increase in plasma antioxidant activity as assessed by both hydrophilic and lipophilic ORAC levels, as well as by a decrease in oxidized LDL over a time course. A characterization of the phenolic contents and antioxidant activity of pecans from various geographic production locations, as well as among cultivar types is therefore warranted.

While there are studies on the antioxidant activity of a few cultivars and growing locations within a state or country, there are no studies that look at the various growing locations throughout the Southern United States (14-17). Cultivar and growing location should be an important consideration, because it is well known that cultivar type and growing location can have a significant effect on the level of phenolics in plants (18, 19). In the present study, eighteen pecan cultivars from across the Southern U.S. were assessed for their antioxidant properties. Four different antioxidant-based assays (*i.e.*, TPC, DMAC, FRAP, and H-ORAC$_{FL}$) were employed to determine the mechanisms responsible for the pecan's positive health benefits. The tocopherol contents of the cultivars were also determined, assessing both the hydrophilic and

lipophilic portions of the nut kernel. Finally, the phenolic acids present were analyzed by HPLC methodology.

3.2 METHODS

Chemicals and Glassware. Sea sand, sodium carbonate, glass wool, ethanol (95%) and ACS-grade methanol, hexanes, and acetone as well as HPLC-grade water, methanol, hexanes, isopropanol and acetonitrile were acquired from Fisher Scientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid, hydrochloric acid, potassium phosphate monobasic, potassium phosphate dibasic, sodium acetate, and iron sulfate were purchased from VWR International, LLC (Suwanee, GA, USA). Fluorescein (3′6′-dihydroxy-spiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one), AAPH (2,2′-azobis[2-amidinopropane] dihydrochloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu's phenol reagent, (+)-catechin hydrate, TPTZ (2,4,6-tripyridyl-*s*-triazine), and iron chloride were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Collection of Samples. Eighteen pecan cultivars were collected from test plots and pecan orchards in Georgia (11), New Mexico (2), and Texas (5) over a 3-month period. The nuts were raw and remained in-shell through transport, packaging and storage. After arrival to the Department of Food Science & Technology, Athens, GA, the pecan samples were placed in labeled pouches (Sealed Air Corporation, Elmwood Park, NJ, US), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to prevent any oxidative degradation, and stored at -80°C until analyzed. On the day of analysis, pecan samples were removed from the freezer, shelled, and allowed to warm to room temperature to ensure a proper mass reading. Approximately 12 g of nutmeat was placed into a -20 °C freezer and allowed to partially refreeze to facilitate

grinding. Each nut sample was then combined with ~60 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ), to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

Lipid Extraction. A Soxhlet apparatus was employed to extract the lipids from all samples. A known quantity of the ground matter (pecan and sand) was placed into a cellulose extraction thimble (single thickness, 43 mm I.D. and 123 mm external length, Whatman International Ltd., Maidstone, England) and the mass recorded. Glass wool was placed in the top of the thimble to ensure that the contents would remain in place during extraction which was performed using ~350 mL of hexanes for 20 h. Upon completion, the thimbles were removed and allowed to dry overnight. The hexanes were removed from the lipid extract using a BüchiRotavapor R-210 (Büchi Corporation, New Castle, DE). The lipid portion was weighed for gravimetric analysis. Extracted lipids were transferred to amber-colored vials, flushed with N_2, capped, and held at -80°C until analyzed.

Extraction of Phenolic Compounds. The defatted pecan kernel was removed from the dried thimble and placed in an Erlenmeyer flask. As described by Wu *et al.*(18), a 100-mL portion of extraction solvent ($(CH_3)_2CO/H_2O/CH_3COOH$ solvent mixture, 70:29.5:0.5 v/v/v) at a ratio of ~6:10 thimble contents:extraction solvent was used to extract the phenolic compounds. Extraction was carried out according to Craft *et al.* (20). Briefly, the contents in the flasks were heated at 50°C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed three times and the supernates pooled. Acetone was evaporated from the pooled supernate using the Rotavapor. The aqueous portion was poured into crystallization glasses, covered with filter paper, and placed in a -80 °C freezer until

completely frozen. The samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MO). The dried extract was placed in amber-colored vials, capped and stored at 4 °C until ready to be used. The lipid and phenolic extractions were completed in triplicate for each cultivar.

Antioxidant Assays. *Hydrophilic-ORAC$_{FL}$ Assay***.** A hydrophilic-ORAC$_{FL}$ assay was completed on the acetonic crude extract powders according to Prior *et al.* (21). Phosphate buffer, pH 7.4, was used as the blank and the diluent. Fluorescein (FL), 0.1 μM, was employed as the reaction probe and AAPH, 80 μM in phosphate buffer, was used as the radical initiator. Both working solutions were held at 37 °C for the duration of the experiment. The phenolic extract was diluted to 0.5 mg/mL with ethanol. The ethanolic solution was further diluted with phosphate buffer, pH 7.4, to a final concentration of 0.025 mg/mL.

A BMG FLUOstarOmega (Ω) microplate reader (BMG Laboratories, Cary, NC) equipped with two internal 500-μL reagent pumps, an external lead system, temperature control set at 37 °C, fluorescent detection set at an excitation/emission pair of 485/520 nm, and a 3-h run time was employed for analysis. Black, clear bottom 96-well plates were used for the analysis and 20 μL of each sample, blank, or standard were pipetted into individual wells. During the analysis, 200 μL of FL and 20 μL of AAPH were added using an automated addition waiting one cycle between each reagent. A standard curve based on five different Trolox concentrations (12.5, 25, 50, 80, and 100 μM in phosphate buffer) was constructed. The area under the curve (AUC) was determined and following blank correction, samples and standards were compared. Final values were reported as mmol Trolox eq./100 g pecan nutmeat from triplicate samples.

TPC assay. The total phenolics content (TPC) was determined using a method adapted from Swain and Hillis (22) employing the Folin-Ciocalteu phenol reagent. Each acetonic crude

extract was diluted to 0.20 mg/mL in methanol. The assay was performed using 1 mL of methanolic extract, 7.5 mL deionized water, 0.5 mL Folin-Ciocalteu phenol reagent, and 1 mL of saturated sodium carbonate. The solution was vortexed for 30 s. A quiescent period of 1 h was employed to allow for optimal color development. The absorbance of the resulting solution was measured at $\lambda = 750$ nm using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc.). A standard curve was created from solutions of (+)-catechin (1.6-8.0 μg/mL). TPC values were reported as g (+)-catechin eq./100 g pecan nutmeat.

DMAC Assay. The method described by Payne *et al.* (23) was employed to quantify the total procyanidins in the acetonic crude extract. Briefly, a 30-mL volume of DMAC solution was prepared with 10% (w/v) HCl in reagent alcohol. DMAC (0.03 g) was then added to this solution and swirled until dissolved. The same 0.20 mg/mL methanolic extract solution, as employed to determine the TPC content, was used in this assay. Standard solutions of procyanidin B2 were prepared at concentrations of 1, 10, 50, and 100 ppm. The microplate reader and 96-well plates were used for analysis. All wells contained 250 μL DMAC solution and 50 μL of alcohol (blank), standard solutions, or sample solution. The plate was read at 640 nm and the maximum absorbance for each well recorded. The data was expressed as mg procyanidin B2 eq./100 g pecan nutmeat.

FRAP assay. The reducing capacity of the acetonic crude extracts was determined using the ferric reducing antioxidant power (FRAP) assay employing the method of Pulido *et al*. (24). The same methanolic extract was used in this assay as that used in the TPC and DMAC assays. The FRAP reagent was prepared freshly using 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$ and 25 mL of a 0.3 M acetate buffer at pH 3.6. The FRAP reagent was brought to 37°C prior to analysis. A standard curve was conducted with aqueous

iron sulfate solutions ($FeSO_4 \bullet 7H_2O$) at five different concentrations ranging from 250 to 1600 μM. The FRAP reagent (200 μL), deionized water (20 μL), and blank/sample/standard (6.6 μL) were pipetted into a 96-well microtitre plate and read using the microplate reader with temperature control set at 37 °C. The maximum absorbance readings of the samples were recorded at λ = 595 nm. The antioxidant activity was reported as mmol Fe^{2+} eq./100 g pecan nutmeat.

Tocopherol (T) and Tocotrienol (T3) Analysis. *Sample Preparation.* Briefly, 1 g of lipid was dissolved in 5 mL of the mobile phase (0.85% isopropanol in hexanes) prior to injection into the HPLC. Yellow light (devoid of UV and blue light by a filter; λ range 460 to 800 nm) was employed throughout the extraction and BHT (0.01 %) was added to the mobile phase to prevent photo oxidation of the Ts. Analyses of the triplicate extractions were performed.

HPLC Quantitation. HPLC analysis of the lipid extracts was accomplished as described by Shin *et al*. (25) with slight modifications. Lipid extracts were injected into an HPLC system comprising a Shimadzu LC-10AT controller/pump, CBM-20A Prominence communications bus module, DG-14A degasser, RF-$10A_{XL}$ fluorescence detector and EZStart chromatography software (Shimadzu Corp., Columbia, MD, USA). A LiChrosorb Si 60 column (4 mm × 250 mm, 5-μm particle size; HibarFertigsäule RT, Merck, Darmstadt, Germany) was connected to a LiChroCART 4-4 guard column packed with LiChrospher Si 60 (5 μm), and an isocratic mobile phase comprising 0.85% (v/v) isopropanol in hexanes at a flow rate of 0.8 mL/min was employed. Before use, the mobile phase was vacuum filtered through a 0.45-μm nylon membrane filter (MSI, Westboro, MA, USA) and degassed by stirring under vacuum. Before injection, the samples were filtered through a 0.45-μm nylon membrane filter (MSI, Westboro, MA, USA). The excitation and emission wavelengths for fluorescent determination of the T and

T3 isomers were 290 and 330 nm, respectively. Twenty microliters of sample extract and of T standard solution were injected per run with a sampling frequency of 2 Hz. A palm oil sample was used to identify the retention times of the T3 isomers based on the analysis conditions employed.

Tocopherol standards were prepared as described by Lee *et al.*(26). Extinction coefficients of the T homologues, first published by Scott (27) and as presented by Eitenmiller *et al.* (28), were used to establish the percent purity. After purity determination, stock solutions of the standards were prepared with concentrations of 1.98, 2.07, 2.01, and 2.26mg/mL for α-, β-, γ- and δ-T, respectively. Concentrations of the Ts were calculated from each peak area determined by the EZStart software based on the fluorescence response of each T isomer in a 20-μL injection of the working standard stock solution.

Reversed-Phase HPLC Characterization (RP-HPLC). The RP-HPLC method reported by Srivastava *et al.* (29) was followed to characterize the crude extracts of the pecan cultivars. Briefly, an Agilent 1200 series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA) was employed for the chromatography. A reversed-phase Luna C_{18}(2) column (4.6 x 250 mm, 5 μm; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A (H_2O/CH_3CN/CH_3COOH, 93:5:2, v/v/v) and mobile phase B (H_2O/CH_3CN/CH_3COOH, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 mL/min was employed. A volume of 100 μL was injected for each of the acetonic extracts (4.0 mg/mL methanol). Detection wavelengths were λ = 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids, notably of the *trans*-

cinnamicacid family), and 360 nm (flavonols). Tentative identification of separated components was made by matching UV-VIS spectra and retention time mapping with standard compounds.

Acid and Base Hydrolysis of Phenolic Acids. To facilitate phenolic identification and confirmation by LC-ESI-MS, an acid and base hydrolysis was performed on the crude extract. A method previously reported by Amarowicz and Weidner (30) separated the phenolic acids into three classes which could be more easily analyzed by MS. Briefly, 800 mg of crude extract was dissolved in 20 mL of acidified water (pH 2.0, 6M HCl). Using a separatory funnel, the phenolic acids were extracted with diethyl ether (5 x 20 mL). The organic phase was removed and then redissolved in methanol and injected into the HPLC. To the remaining aqueous portion 2M NaOH (20 mL) was added, the vials were flushed with N_2, and the mixture was hydrolyzed for 4 h. After hydrolysis, the pH was reduced to 2 using 6 M HCl and then the phenolic esters were recovered with diethyl ether (5 x 30 mL). The organic portion was once again evaporated and redissolved in methanol, prior to injection. The aqueous portion was combined with 15 mL of 6 M HCl, flushed with N_2, and placed in a 100 °C oven for 1 h. The liberated glycosides were then extracted using 5 x 45 mL portions of diethyl ether. The organic phase was once again evaporated and redissolved in methanol prior to HPLC injection.

Reversed-Phase HPLC-ESI-MS Confirmation (LC-ESI-MS). Tentative identification of separated compounds was confirmed using LC-ESI-MS techniques. Briefly, a Thermo Scientific Accela series HPLC system consisting of a binary pump with degasser, autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and Xcalibur software (Thermo Scientific, Waltham, MA) was employed for the chromatography. A perfluorophenyl column (150 mm x 2.1 mm, 3-µm particle size, ThermoScientificAccucore, Waltham, MA) was utilized. A gradient elution consisting of mobile

phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 100 min period at a flow rate of 0.4 mL/min was employed. A volume of 10 μL was injected for each of the samples. For the MS analysis, N_2 was the carrier gas and a cone voltage of 55 V was employed. A total scan in negative-ion mode was conducted from 100-1000 Daltons. The probe tip temperature was set to 350 °C. Comparison of parent molecular ions ($[M-H]^-$) was employed to assist with elucidation of the identities of the phenolic acids. When necessary, comparison of relative retention times (RRTs), $[M-H]^-$ values, and fragmentation patterns of phenolic acids to those reported in the literature for phenolics was employed.

Method Validation and Quality Control. A study of precision and accuracy was carried out for each of the assays using the standard compound employed in each assay. The precision was measured through the relative standard deviation achieved on each of the trials for the compound of interest at all tested concentrations. The accuracy was a measure of how close the predicted concentration from the developed standard curve was to the actual concentration of the standard compound and was modeled after that presented in Huang *et al.*(31). The interday RSD and percent recovery were determined using the following equations:

$$\% \text{ RSD}_r = \frac{\text{Standard Deviation}}{\text{Mean}} * 100\ \%$$

$$\% \text{ REC} = \frac{\text{Theoretical value-Analytical value}}{\text{Theoretical value}} * 100\%$$

where theoretical value is determined based on the concentration of the standard solution and the analytical value is determined from the standard curve calculations.

The linearity of the T standard curves was determined. LOD and LOQ were determined for each T analyzed. The following equations were utilized to determine the LOD and LOQ for the standards:

$$\text{LOD} = \frac{3*\sigma}{S}$$

$$\text{LOQ} = \frac{10*\sigma}{S}$$

where σ is the standard deviation of the y-intercept and S is the slope of the calibration curves.

Statistical Analysis. The data was analyzed using an analysis of variance with subsamples. The lipid extraction was performed in triplicate on each cultivar. In addition, each resulting acetonic phenolic extract was solubilized and assayed in triplicate. Differences in the means of each cultivar were determined for each of the measured quantities with the PROC MIXED procedure allowing for a random analysis. Differences were analyzed between all eighteen cultivars, as well as between the three states (Georgia, New Mexico, and Texas) and each cultivar type (*i.e.*, 'Desirable' and 'Stuart') used in the analysis. Differences in means were determined with the LS means procedure and a Tukey's mean separation test with $P < 0.05$ using the Statistical Analysis System software, Version 9.2 (SAS Institute, Cary, NC). Correlation values between H-ORAC$_{FL}$, FRAP, DMAC, and TPC was accomplished using a PROC CORR analysis with the SAS statistical software.

3.3 RESULTS AND DISCUSSION

Method Validation. The precision and accuracy of the H-ORAC$_{FL}$, TPC, and FRAP assays was calculated using the percent relative standard deviation (% RSD) as an indication of precision and the percent recovery (% REC) as an indication of accuracy (Table 3.1). The % RSD for the H-ORAC$_{FL}$ ranged from 1.9 to 23.9 % across the five concentrations of Trolox investigated. It should be noted that the smallest concentration, 12.5 μM, had a significantly higher % RSD. As the concentrations increased, there was a marked decrease in the % RSD. All experimental values of this study fell significantly above the smallest concentration tested; thus, this large % RSD is not a concern. A similar trend was observed with the % REC for the H-ORAC$_{FL}$ assay. The % REC ranged from 72.5 to 106.1%, but the lowest concentration studied had a significantly lower % REC than the other concentrations. The authors recommend that any experimental response values fall between 25 to 100 μM Trolox for the H-ORAC$_{FL}$ assay.

The TPC assay had % RSD values ranging from 1.0 to 1.5% and % REC values ranging from 98.1 to 103.7% for the five concentrations studied (1.6-8.0 μM (+)-catechin, n=8). There was little variation in these values across the five concentrations. The FRAP assay was evaluated at five concentrations ranging from 250 to 1600 μM $FeSO_4$. The % RSD values ranged from 2.3 to 4.7 with a significantly higher value of 10.5 for the lowest concentration of 250 μM. The % REC ranged from 95.3 to 102.2. Based on these results, the researchers conducted experimental dilutions to keep all concentrations higher than 250 μM.

Antioxidant Assays. The TPC values were reported as g (+)-catechin eq. (CE)/100 g nutmeat (Table 3.2). The values across all cultivars ranged from 1.11 ± 0.12 to 1.60 ± 0.04 g CE/ 100 g nutmeat for ‘Desirable’ harvested in Tift Co., GA and ‘Wichita’ harvested in Tift Co.,

GA, respectively. Significant differences ($P<0.05$) were determined between means for the 11 pooled cultivars investigated (Table 3.3). The 'Wichita' cultivar, 1.49 g CE/ 100 g nutmeat, was found to be significantly higher ($P<0.05$) than the 'Desirable' and 'Sumner' cultivars, both having a value of 1.20 g CE/100 g nutmeat. There were no significant differences ($P > 0.05$) found between the three growing locations, Georgia, New Mexico, and Texas. It is believed that the lack of significant differences is due to natural variation between the cultivars studied, as well as a difference in the number of cultivars selected from each growing state (i.e., Georgia (11), New Mexico (2), and Texas (5)). None of the four assays investigated showed differences at the location level.

Most values reported in the literature are as mg gallic acid equivalents (GAE)/g fresh weight. In this study, (+)-catechin was believed to be a more representative standard due to the classes of phenolics most frequently found in pecan samples. For this reason, comparisons with data in the literature are difficult due to a difference in reporting. Wu *et al.* (18) determined the total phenolics of numerous different food samples including pecans. The pecan samples were reported to have values of 2016 ± 103 mg GAE/100 g nutmeat. The mean values of our study were slightly lower than those of Wu *et al.*(18). It is believed that these differences may be due to a variation in the type of cultivar and harvest year. In a review article published by Bolling *et al.* (32), a pooled total phenolics content value of 1588 mg GAE/100 g nutmeat was reported. This value is much more consistent with our results and other results currently published in the literature (14, 33). The consistency is to be expected due to the pooled nature of their value.A pooled value should better account for natural variation, different cultivar types, growing location, and harvest year.

Total proanthocyanidins (PACs) were also measured using the DMAC assay (Table 3.2). Values ranged from 420 ± 20 mg procyanidin B2 eq./100 g nutmeat to 655 ± 43 mg procyanidin B2 eq./100 g nutmeat for the 'Desirable' pecans and the 'Wichita' pecans both harvested in Tift Co., GA, respectively. As seen in the TPC assay, significant differences were determined between means for the 11 pooled cultivars investigated (Table 3.3). 'Cheyenne', 'Choctaw', and 'Wichita' pecans were found to be significantly higher ($P<0.05$) than the 'Desirable' and 'Pawnee' cultivars. The DMAC assay has not previously been used with pecans to determine total (PACs) in the literature. It has shown success, however, in studies analyzing cocoa PAC contents (23), and also in wine (34), and beer (35).

When comparing our values to those reported in the USDA Database for the Proanthocyanidin Content of Selected Foods (36), comparable results were noted even though there was a difference in method used. The database separates the content by size and reports that pecans have a monomer content of 17.22 ± 2.55 mg/100 g, dimer content of 42.13 ± 5.42 mg/100 g, trimer content of 26.03 ± 1.98 mg/100 g, 4-6mers content of 101.43 ± 10.45 mg/100g, 7-10mers content of 84.23 ± 12.90 mg/100 g, and a polymer content of 223.01 ± 59.05 mg/100 g for a total PAC content of 494.05 mg/100g. An additional database, Phenol Explorer, reports a PAC content of 476.7 mg/100 g fresh weight (37). An average value of PACs in pecans from all the literature values was reported as 493.9 mg/100 g in the Bolling *et al.* review (32). This value falls into the range of the present study values. Some of the current study cultivars had values 100 mg higher than these reported averages. Our results add validity to the DMAC method for quantifying total procyanidins in nut samples.

The H-ORAC$_{FL}$ values displayed quite a broad range across the eighteen samples studied (Table 3.2). The data ranged from 13.5 ± 3.5 mmol TE/100 g nutmeat with the McWilliams 'Stuart' cultivar grown in Crisp Co., GA to 25.5 ± 3.0 mmol TE/100 g nutmeat for the 'Stuart' cultivar grown in Tift Co., GA. It should be noted that two-thirds of the samples had capacities above 16.0 mmol TE/100 g nutmeat. Significant differences were noted between the means of the 11 cultivars investigated (Table 3.3). 'Cheyenne' samples had a significantly higher ($P<0.05$) H-ORAC$_{FL}$ value than that of the 'Desirable' samples.

Our data is similar to that reported by Wu *et al.* (18) for the H-ORAC$_{FL}$ of pecan samples. They reported an average capacity of 17.5 ± 1.0 mmol TE/100 g nutmeat. It should be noted that the range was found to be 3.1 mmol TE/100 g. Our average value of 18.0 ± 3.3 mmol TE/100 g nutmeat is comparable and slightly higher than those reported in the Wu *et al.* study (18). Two additional studies reported ORAC$_{FL}$ numbers higher than those in our study. An investigation presented by de la Rosa *et al.* (14) reported ORAC values of 23.1 ± 1.5 mmol TE/100g, 26.2 ± 3.8 mmol TE/100g, and 22.7 ± 5.0 mmol TE/100 g nutmeat for cultivars from various locations around Mexico. While these capacities are higher than those reported here, certain cultivars performed at similar levels providing more evidence that ORAC data can be dependent on cultivar type studied.

The FRAP data showed somewhat of a different trend when comparing the eighteen samples examined (Table 3.2). Numbers ranged from 14.0 ± 1.2 mmol Fe^{2+} eq./100 g nutmeat for the 'Elliott' sample from Tift Co., GA to 20.7 ± 0.7 mmol Fe^{2+} eq./100 g nutmeat for the 'Stuart' sample, also from Tift Co., GA. It should be noted that with the FRAP assay there were three cultivars performing above 20.0 mmol Fe^{2+} eq./100 g nutmeat, two 'Stuart' samples harvested from Crisp and Tift counties and 'Wichita' samples also from Tift Co., GA. The

FRAP assay was the only assay that did not show any significant differences ($P > 0.05$) among cultivars (Table 3.3). Blomhoff *et al.* presented a study investigating the FRAP contents of pecan cultivars from USA, Norway, and Mexico (19). They reported much lower values of 8.33 ± 1.50 mmol/100g with a range of 6.32 to 11.05 mmol/100 g.

Correlation coefficients were determined between all four different antioxidant assays for the pooled cultivar mean data (Figure 3.1a-f). Of the correlations analyzed, all were found to be significant except those which included the FRAP means (Figure 3.1c, e, f). The significance is to be expected since FRAP is a SET assay while the H-$ORAC_{FL}$ assay is a HAT assay; each assay measures and accounts for different mechanisms (9). The strongest correlation of the six analyzed was TPC vs. DMAC with a correlation coefficient of $r = 0.834$ and $P = 0.014$ (Figure 3.1a). The authors believe that the higher correlation demonstrates that most of the phenolics in pecan samples can be represented by the procyanidins. The hypothesis is further supported by the correlation coefficients of the DMAC vs. H-$ORAC_{FL}$($r = 0.898$, Figure 3.1d) compared to those of the TPC vs. H-$ORAC_{FL}$($r = 0.606$, Figure 3.1b). It is thought that because the DMAC vs. H-$ORAC_{FL}$ relationship is stronger, one can conclude that the procyanidins have a stronger contribution to the antioxidant activity than the lower-molecular weight phenolics as measured by the HAT mechanism.

Tocopherol (T) and Tocotrienol (T3) Analysis. All eighteen cultivars were analyzed for α, β, δ, and γ-T and -T3 contents (Table 3.4). Quantities were determined based on T standard curves developed from commercial standards. The linearity was determined for all four isomers and was determined to be 0.99 for all established curves. Since the T and T3 isomers have the capacity to function as lipophilic antioxidants, it is important to determine their contents in pecans from numerous cultivar types and growing locations. All samples were devoid of T3s.

The α-T contents ranged from 0.59 mg/100 g nutmeat for 'Pawnee' from Tift Co., GA to 1.89 mg/100 g nutmeat for the McWilliams 'Stuart' harvested in Crisp Co., GA.The average value for α-T for the eighteen cultivars was 1.01 ± 0.32 mg/100 g nutmeat which compares well with that presented in the USDA Database for Standard Reference, Release 25 (39), 1.40 ± 0.08 mg/100 g nutmeat. While our contents are slightly lower than the database, the present study has a sample size double the database, which could increase the variability seen from multiple cultivars and growing location. Our data is more similar to a previous study investigating the T contents of commercially-available pecans. In that study (2), Robbins *et al*. reported α-T contents of 1.21 ± 0.30 mg/100 g nutmeat.

The β-T contents ranged from 0.02 mg/100 g nutmeat for the 'Wichita' samples from Tift Co., GA to 0.74 mg/100 g nutmeat for the 'Desirable' nuts grown in Lowndes Co., GA. The β-T samples showed the greatest variation of the four T isomers analyzed. The database reports a β-T content of 0.39 ± 0.11 mg/100 g nutmeat (39) which compares to our average value of 0.28 ± 0.18 mg/100 g nutmeat. It should be noted that two of the cultivars analyzed had negligible quantities of β-T. If these values are removed and the average is recalculated, the resulting average is 0.31 ± 0.16 mg/100 g nutmeat. The values for β-T seem to be quite variable in the literature indicated by elevated standard deviations. The previous study of commercially-available pecans reports a β-T value of 0.78 ± 0.29 (2).

The predominant T for all cultivars analyzed was γ-T with the other three Ts having much smaller contents, comparatively. The γ-T values ranged from 15.98 mg/100 g nutmeat for the 'Choctaw' from Texas to 27.73 mg/100 g nutmeat for the New Mexico 'Westerns' with an average value of 22.23 ± 3.28 mg/100 g nutmeat across all samples. The database (39) reports an average value of 24.44 ± 1.50 mg/100 g nutmeat and the study investigating the

commercially-available pecan samples reports a value of 21.94 ± 1.79 mg/100 g nutmeat (2). These numbers are quite similar to those reported in the present study. Because there is an increased awareness that the γ-T isomer may play a role in quenching reactive nitrogen species, it is critical that values reported in the literature and databases are as accurate as possible.

The final isomer identified in the pecan oil samples was δ-T. It ranged from 0.02 mg/100 g nutmeat to 0.17 mg/100 g nutmeat for the New Mexican 'Wichitas' and Lowndes Co., GA 'Desirable', respectively. The average for the present study was determined to be 0.08 ± 0.04 mg/100 g nutmeat which is significantly less than the database reported value of 0.47 ± 0.09 mg/100 g nutmeat (39). The commercially-available pecans were noted to have a content of 0.60 ± 0.23 mg/100 g nutmeat (2). These differences could be due to the increased sample size in our study, as well as the inclusion of multiple growing locations and cultivar types. As with the β-T values, it seems that the Ts with the smallest contribution to the total are those that are exhibiting the greatest variation both between replicates and cultivars.

HPLC Characterization of Phenolics. RP-HPLC and LC-ESI-MS were employed to characterize the phenolics present in the crude extracts of the eighteen pecan cultivar samples. To find the best characterization using LC-ESI-MS, the samples were subjected to acid and base hydrolysis steps prior to extraction with diethyl ether. The pecan samples contained several phenolic acids including gallic, ellagic, methylellagic, protocatachuic, and *p*-hydroxybenzoic acid. Also included were catechin and a valoneic acid dilactone. These are highlighted in Figure 3.2c & f. The catechin, gallic acid, ellagic acid, protocatachuic acid, and *p*-hydroxybenzoic acids were positively confirmed by comparing their retention times to those of commercial standards. Additional peaks were present but could not be identified based on RT or MS ions.

In addition, molecular ion confirmation was made from the LC-MS analysis. Peak 1 was confirmed to be gallic acid with a retention time of 5.2 min and a parent ion at *m/z* of 169. Peak 2 was confirmed to be (+)-catechin with a retention time of 12.8 and a parent ion at *m/z*of 289. The protocatachuic and *p*-hydroxybenzoic acid peaks are not displayed in Figure 2 due to their small peak area, but they were identified and confirmed through MS. The peaks were found to have retention times of 9.0 and 13.8 min and parent ions at *m/z*of 153 and 137 for protocatachuic and *p*-hydroxybenzoic acids, respectively. Ellagic acid was identified as peak 3 and had a retention time of 23.7 min and a parent ionat *m/z* of 301.

The two additional identified compounds, methylellagic acid and valoneic acid dilactones were tentatively identified based on their retention times, parent molecular ions and fragment ions; these are labeled on Figure 3.2 as peaks 3 and 5, respectively. De la Rosa *et al*. (14) reported on the phenolic compounds in the kernels of pecans and reported the presence of both methylellagic acid and valoneic acid dilactone in their pecan samples. They reported that the methylellagic acid had a $[M-H]^-$ of 315.0 and λ_{max} values of 256.0 and 363.0. These values compare identically with our experimental values for peak 5. The same authors reported the presence of valoneic acid dilactone with a $[M-H]^-$ of 469.0 and fragment ions at *m/z*of 425.0 and 301.0. The valoneic acid dilactone also has a very similar UV spectrum as that found with ellagic acid. These results are once again identical to those found in this study for peak 3. Both of these compounds have shown up in literature articles investigating phenolics in pecans prior to the de la Rosa study but lacked identification and were simply referred to as ellagic acid derivatives (15). The valoneic acid derivative was also identified in walnuts by Li *et al.*(38). It should be noted that the valoneic acid dilactone increases greatly under basic and acidic hydrolysis. The presence of valoneic acid dilactone has been known to indicate the presence of

ellagitannins in a sample (14). Thus, it is believed that the crude extracts contained not only condensed tannins but also hydrolyzable tannins.

Two representative chromatograms are pictured in Figure 3.2a-b and 3.2d-e. Chromatograms 3.2a and 3.2d represent one of the top performing cultivars in this study, 'Wichita', while chromatograms 3.2b and 3.2e represent the lowest performing cultivars in this study, 'Desirable'. Comparisons between the chromatograms were made to determine if noticeable differences were present which may account for the observed differences in antioxidant assay values. When comparing the two sets of chromatograms, there did not appear to be any individual peaks or compounds that were reduced dramatically. Instead, it appears that there is an overall reduced peak area for all peaks in the chromatogram. The chromatograms show that there seems to be a set list of expected compounds for all eighteen cultivar values (*i.e.*, gallic acid, catechin, and ellagic acid) and that the differences observed in the experimental values of the assay can be more attributed to a reduction in compound concentration. Since most phenolic compounds are secondary plant metabolites (40, 41), it can be expected that differences would merely exist in the amount of compound produced and not in the compounds presence in the sample.

3.4 SUMMARY AND CONCLUSION

Pecans have a significant level of phenolic acids and PACs. These compounds, especially the PACs, are most likely responsible for the antioxidant activity that was observed in the crude pecan extracts. No significant differences were observed among the states in which the pecans were produced, but there were some significant differences observed between different cultivar types. It is believed that any observed differences are due to quantity of phenolic

compounds (i.e., gallic acid, catechin, and ellagic acid) and not in the types of phenolic compounds present.

3.5 ACKNOWLEDGEMENTS

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Table 3.1 Interday precision (% RSD_r) and accuracy of the antioxidant assays determined by analysis of standard compounds.

	QC1	QC2	QC3	QC4	QC5
H-$ORAC_{FL}$					
Trolox (μM)[a]	12.5	25	50	80	100
% RSD_r[b]	23.9	8.1	6.4	2.3	1.9
% REC[c]	72.6	106.1	105.9	102.6	96.9
N[d]	18	18	18	18	18
TPC					
(+)-catechin(μM)	1.6	3.2	4.8	6.4	8.0
% RSD_r	1.2	1.5	1.3	1.0	1.1
% REC	103.7	98.1	100.4	100.6	100.7
N	8	8	8	8	8
FRAP					
$FeSO_4$ (μM)	250	500	1000	1250	1600
% RSD_r	10.5	4.7	3.5	4.0	2.3
% REC	95.3	102.2	100.0	100.9	99.4
N	17	17	17	17	17

[a] Concentration at which QC conducted in μM.
[b]Interday relative standard deviation (SD x 100/mean).
[c] Percent Recovery= Accepted value – Analytical value x 100% where the accepted value is determined based on the concentration of the standard stock solution and the analytical value is determined from the standard curve calculations.
[d] N=Number of trials that the calculations are based upon.

Table 3.2. Four antioxidant capacities of pecan cultivars (n=3).[a]

Cultivar	TPC (g CE/ 100 g nutmeat)	DMAC (mg procyanidin B2 eq./100 g nutmeat)	H-ORAC$_{FL}$ (mmol TE/ 100 g nutmeat)	FRAP (mmol Fe^{2+} eq./ 100 g nutmeat)
'Stuart' Clarke Co. (GA)	1.27 ± 0.06	454 ± 20	15.7 ± 1.0	16.9 ± 2.5
Cordele 'Stuart' Crisp Co. (GA)	1.56 ± 0.05	627 ± 87	19.2 ± 1.3	20.1 ± 1.9
'Desirable' Lowndes Co. (GA)	1.23 ± 0.31	452 ± 105	14.4 ± 3.6	19.1 ± 3.0
'Stuart' Tift Co. (GA)	1.51 ± 0.08	593 ± 42	25.5 ± 3.0	20.7 ± 0.7
'Wichita' Tift Co. (GA)	1.60 ± 0.04	655 ± 43	23.6 ± 2.2	20.0 ± 3.0
'Desirable' Crisp Co. (GA)	1.20 ± 0.08	471 ± 70	14.2 ± 2.6	18.0 ± 3.1
'Desirable' Tift Co. (GA)	1.11 ± 0.12	420 ± 20	15.2 ± 1.3	18.8 ± 0.1
'Sumner' Tift Co. (GA)	1.20 ± 0.20	453 ± 15	19.7 ± 3.6	15.2 ± 2.2
'Pawnee' Tift Co. (GA)	1.21 ± 0.06	430 ± 51	14.3 ± 2.2	15.5 ± 2.1
McWilliams 'Stuart' Crisp Co. (GA)	1.20 ± 0.12	438 ± 31	13.5 ± 3.5	15.3 ± 1.9
'Elliott' Tift Co. (GA)	1.23 ± 0.06	516 ± 49	18.1 ± 0.7	14.0 ± 1.2
'Wichita' Pinal Co. (NM)	1.38 ± 0.04	583 ± 87	17.5 ± 0.6	14.7 ± 1.3
'Western' Doña Ana Co. (NM)	1.33 ± 0.06	517 ± 36	17.9 ± 0.1	17.0 ± 0.4
'Desirable' (TX)	1.23 ± 0.07	428 ± 51	17.9 ± 1.3	18.4 ± 0.9
'Cheyenne' (TX)	1.32 ± 0.06	560 ± 19	21.6 ± 1.4	16.1 ± 0.3
'Choctaw' (TX)	1.42 ± 0.10	630 ± 107	20.0 ± 2.9	17.6 ± 2.0
'Kiowa' (TX)	1.28 ± 0.01	447 ± 63	16.8 ± 1.6	17.9 ± 0.6
'Gracross' (TX)	1.35 ± 0.19	482 ± 55	18.0 ± 2.1	17.3 ± 3.0

[a] Number of samples, data represents the mean ± SD of triplicate extractions for each sample.

Table 3.3. Four antioxidant capacity assays for 11 pooled pecan cultivars.[a]

Cultivar	TPC (g catechineq./ 100 g nutmeat)[b]	DMAC (mg procyanidin B2 eq./ 100 g nutmeat)[b]	H-ORAC$_{FL}$ (mmol TE/ 100 g nutmeat)[b]	FRAP (mmol Fe2+ eq/ 100 g nutmeat)[b]
'Cheyenne' (n=1)	1.32ab	563 a	21.6 a	16.1 a
'Choctaw' (n=1)	1.42ab	562 a	20.0 ab	17.6 a
'Desirable' (n= 4)	1.20b	420 b	16.3 b	18.6 a
'Elliott' (n=1)	1.23ab	491 ab	18.1 ab	14.0 a
'Gracross' (n=1)	1.35ab	495 ab	18.0 ab	17.3 a
'Kiowa' (n=1)	1.28ab	425 ab	16.8 ab	17.9 a
'Pawnee' (n=1)	1.21ab	400 b	17.0 ab	15.5 a
'Stuart' (n=4)	1.39ab	515 ab	18.8 ab	18.3 a
'Sumner' (n=1)	1.20b	452 ab	18.6 ab	15.2 a
'Western' (n=1)	1.33ab	487 ab	17.9 ab	17.0 a
'Wichita' (n=2)	1.49a	583 a	20.1 ab	17.3 a

[a] n= Number of samples for each pooled cultivar, all samples were analyzed in triplicate.
[b] Means in the same column with the same letter are not significantly different by Tukey's multiple range test (P>0.05).

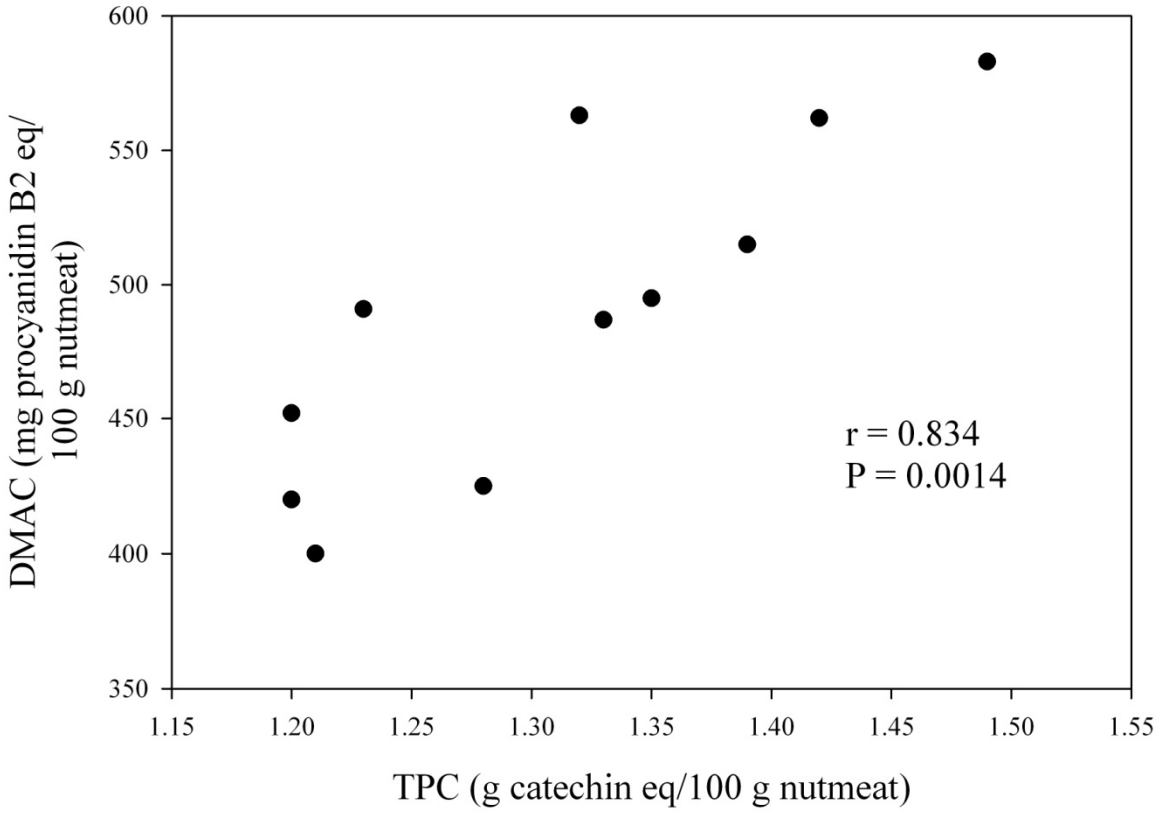
Table 3.4. Tocopherol (T) content of 18 different pecan cultivars (mg/100 g nutmeat, n=3).

Cultivar	α-T[a]	β-T	γ-T	δ-T
'Stuart' Clarke Co.(GA)	0.90 b	0.33 ab	21.22 ab	0.08 abc
Cordele 'Stuart' Crisp Co.(GA)	1.47 ab	0.55 ab	25.16 ab	0.11 abc
'Desirable' Lowndes Co.(GA)	0.72 b	0.74 a	25.93 ab	0.17 a
'Stuart' Tift Co. (GA)	1.10 ab	0.26 ab	21.68 ab	0.10 abc
'Wichita' Tift Co. (GA)	0.86 b	0.02 b	22.53 ab	0.06 abc
'Desirable' Crisp Co. (GA)	1.09 ab	0.31 ab	19.37 ab	0.11 abc
'Desirable' Tift Co. (GA)	1.43 ab	0.45 ab	20.74 ab	0.11 abc
'Sumner' Tift Co. (GA)	0.68 b	0.20 ab	16.74 b	0.07 abc
'Pawnee' Tift Co. (GA)	0.59 b	0.19 ab	20.44 ab	0.09 abc
McWilliams 'Stuart' Crisp Co. (GA)	1.89 a	0.37 ab	23.80 ab	0.13 bc
'Elliott' Tift Co. (GA)	0.85 b	0.26 ab	22.57 ab	0.13 b
'Wichita' Pinal Co. (NM)	1.03 ab	0.11 ab	25.83 ab	0.02 c
'Western' Doña Ana Co. (NM)	1.00 ab	0.10 ab	27.73 a	0.02 bc
'Desirable' (TX)	0.85 b	0.29 ab	18.75 ab	0.14 a
'Cheyenne' (TX)	0.82 b	0.06 b	26.48 ab	0.04 abc
'Choctaw' (TX)	0.74 b	0.28 ab	15.98 b	0.03 bc
'Kiowa' (TX)	1.15 ab	0.35 ab	23.06 ab	0.07 abc
'Gracross' (TX)	0.96 b	0.21 ab	22.19 ab	0.03 bc

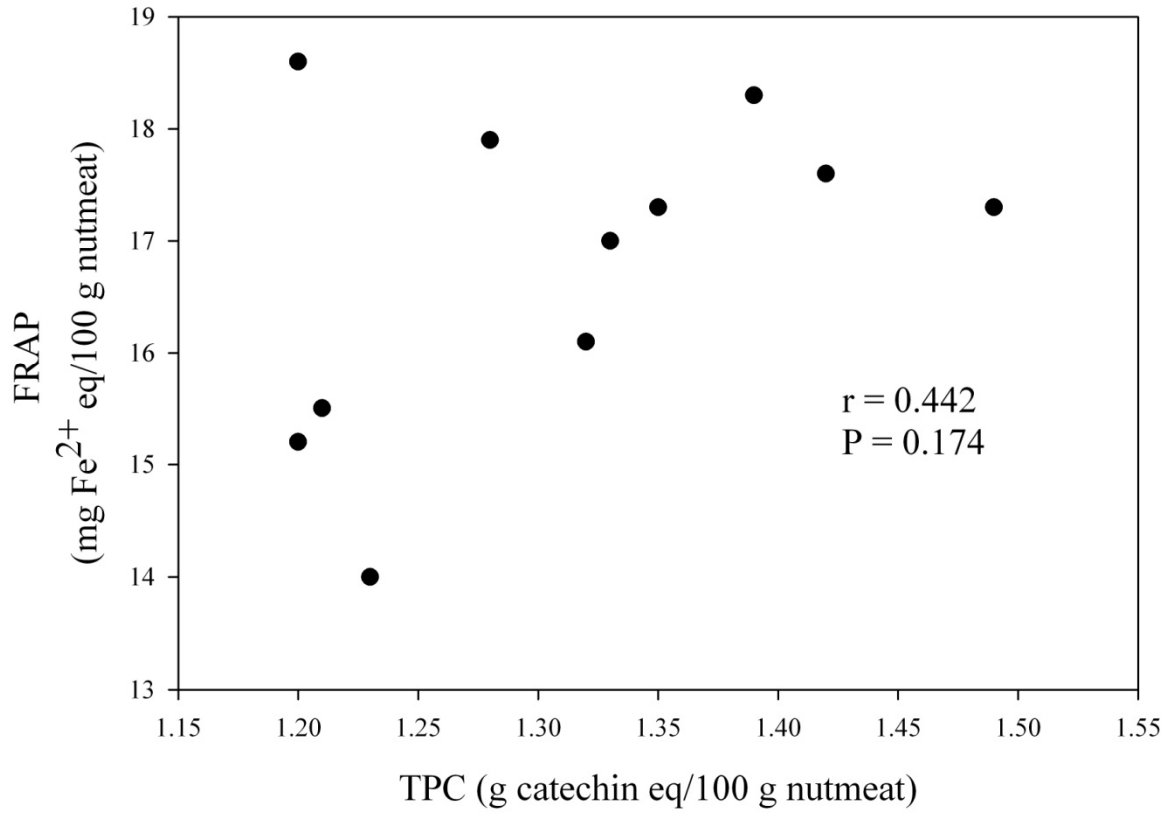
[a]Means in the same column with the same letter are not significantly different by Tukey's multiple range test ($P > 0.05$).

Figure 3.1.Correlation comparisons of all four antioxidant assays for pooled cultivar means (n=11). Pearson Correlation Coefficients (r) and p-values determined using PROC CORR (SAS 9.2).

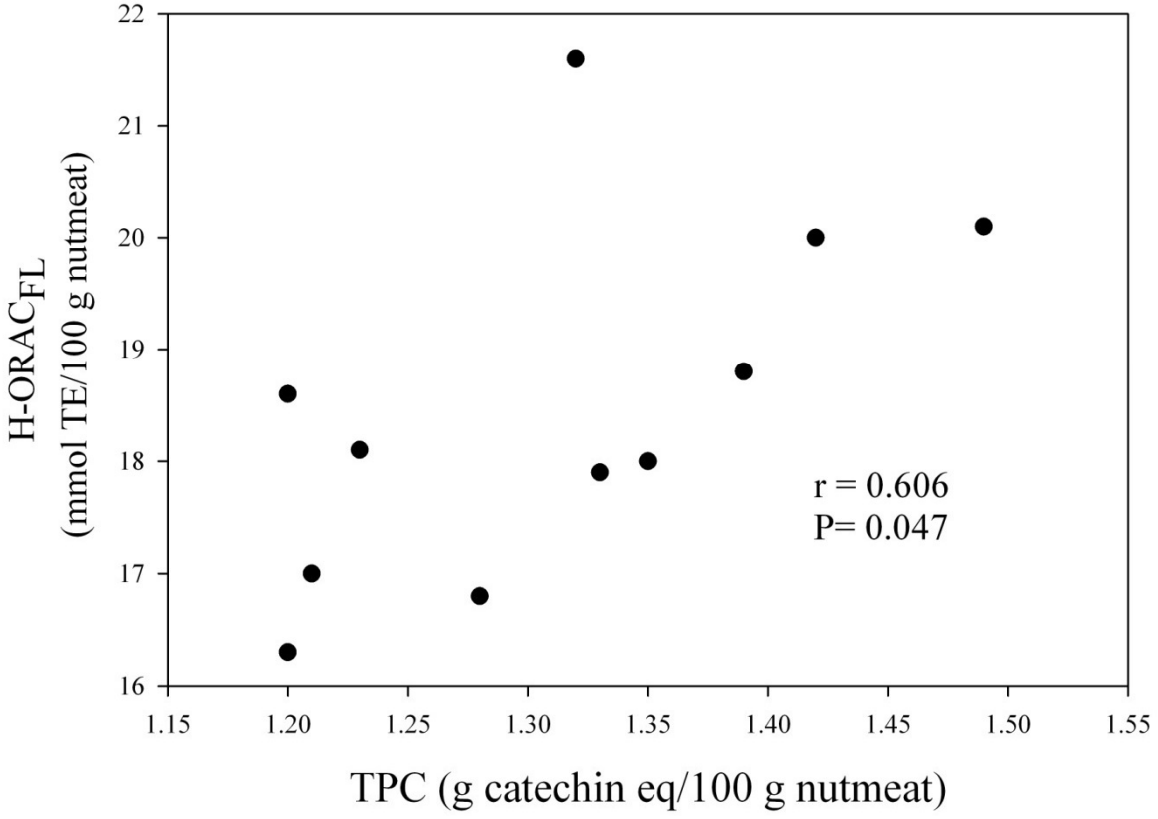
1a.



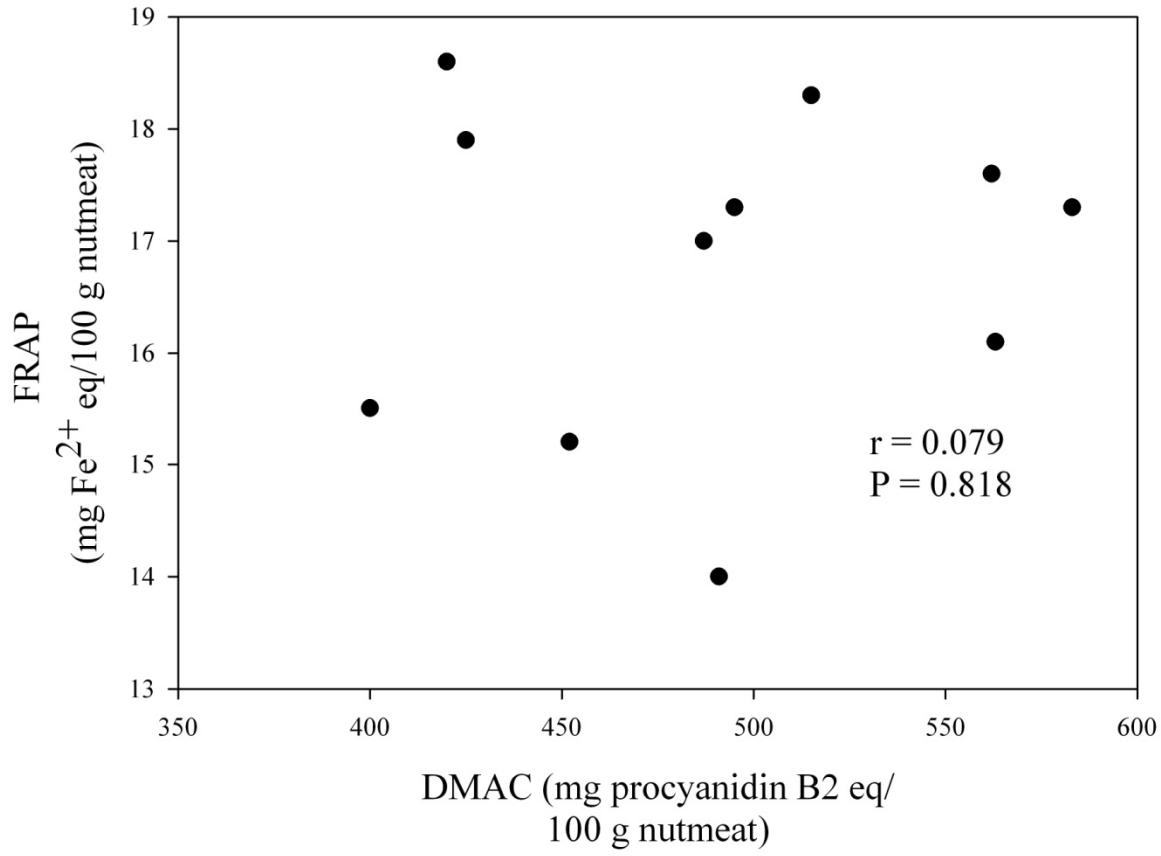
1b.


FRAP
(mg Fe^{2+} eq/100 g nutmeat)
TPC (g catechin eq/100 g nutmeat)
r = 0.442
P = 0.174
19
18
17
16
15
14
13
1.15
1.20
1.25
1.30
1.35
1.40
1.45
1.50
1.55

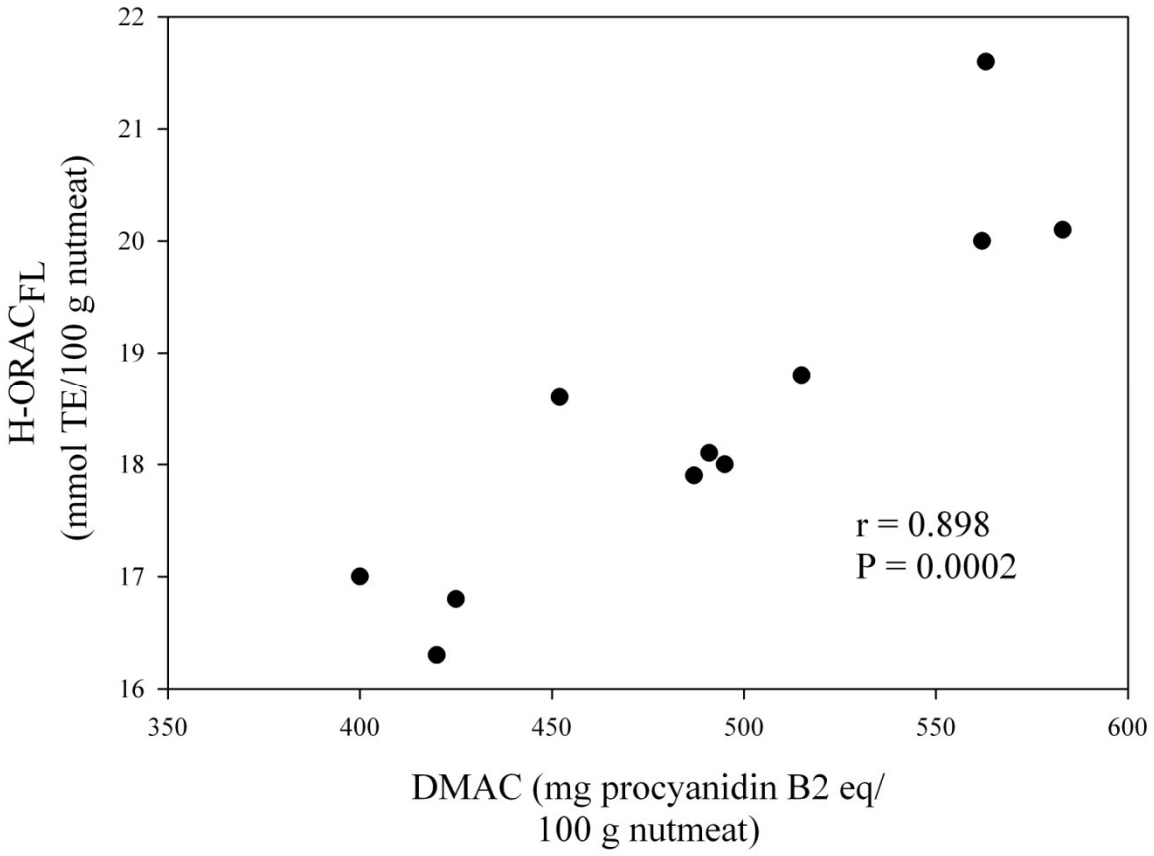
1c.


H-ORAC$_{FL}$
(mmol TE/100 g nutmeat)
22
21
20
19
18
17
16
1.15
1.20
1.25
1.30
1.35
1.40
1.45
1.50
1.55
r = 0.606
P= 0.047
TPC (g catechin eq/100 g nutmeat)

1d.


FRAP
(mg Fe^{2+} eq/100 g nutmeat)
19
18
17
16
15
14
13
350
400
450
500
550
600
r = 0.079
P = 0.818
DMAC (mg procyanidin B2 eq/
100 g nutmeat)

1e.


H-ORAC$_{FL}$
(mmol TE/100 g nutmeat)
22
21
20
19
18
17
16
350
400
450
500
550
600
DMAC (mg procyanidin B2 eq/
100 g nutmeat)
r = 0.898
P = 0.0002

1f.

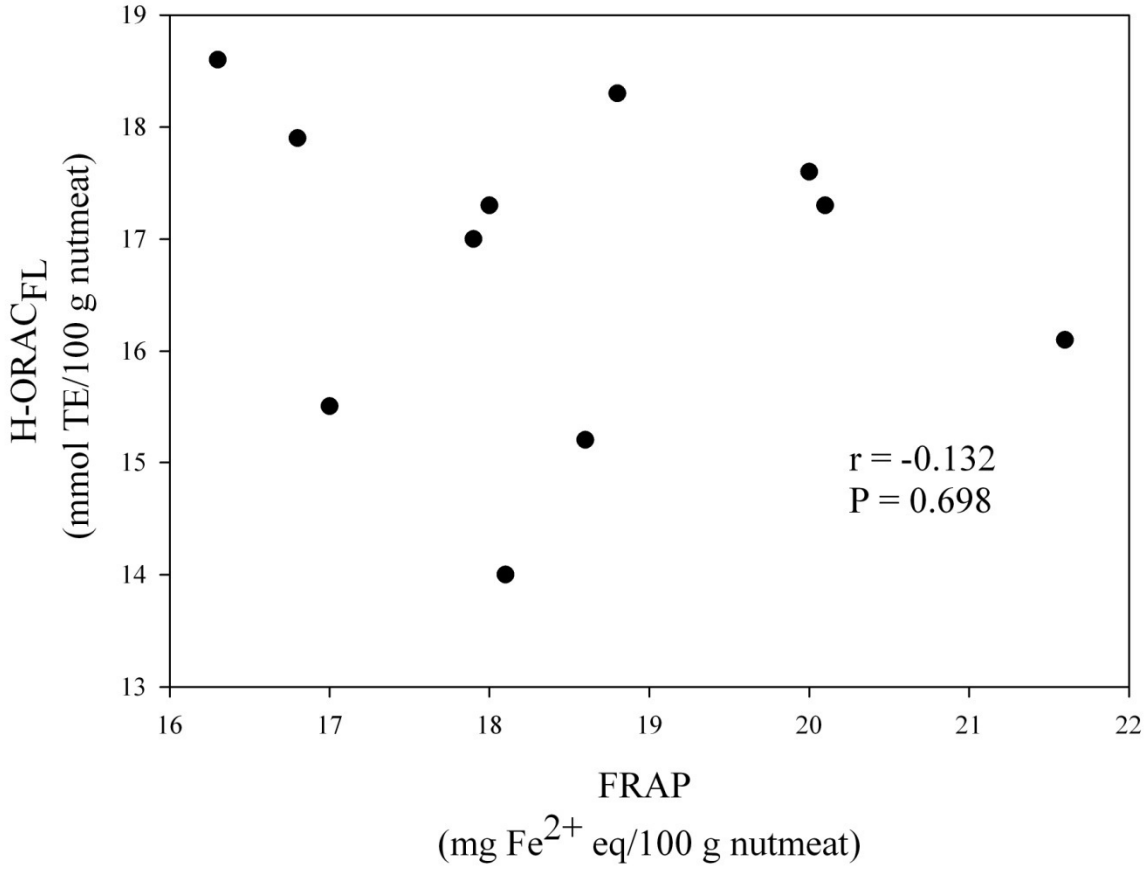
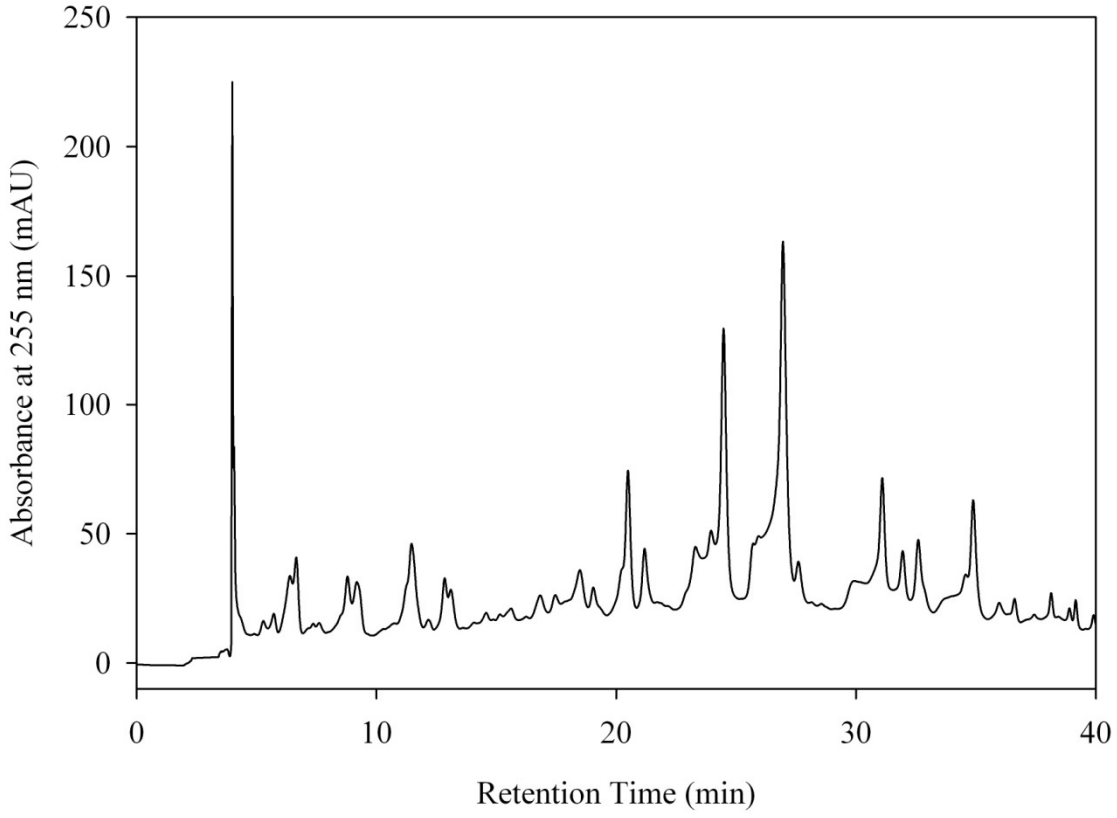
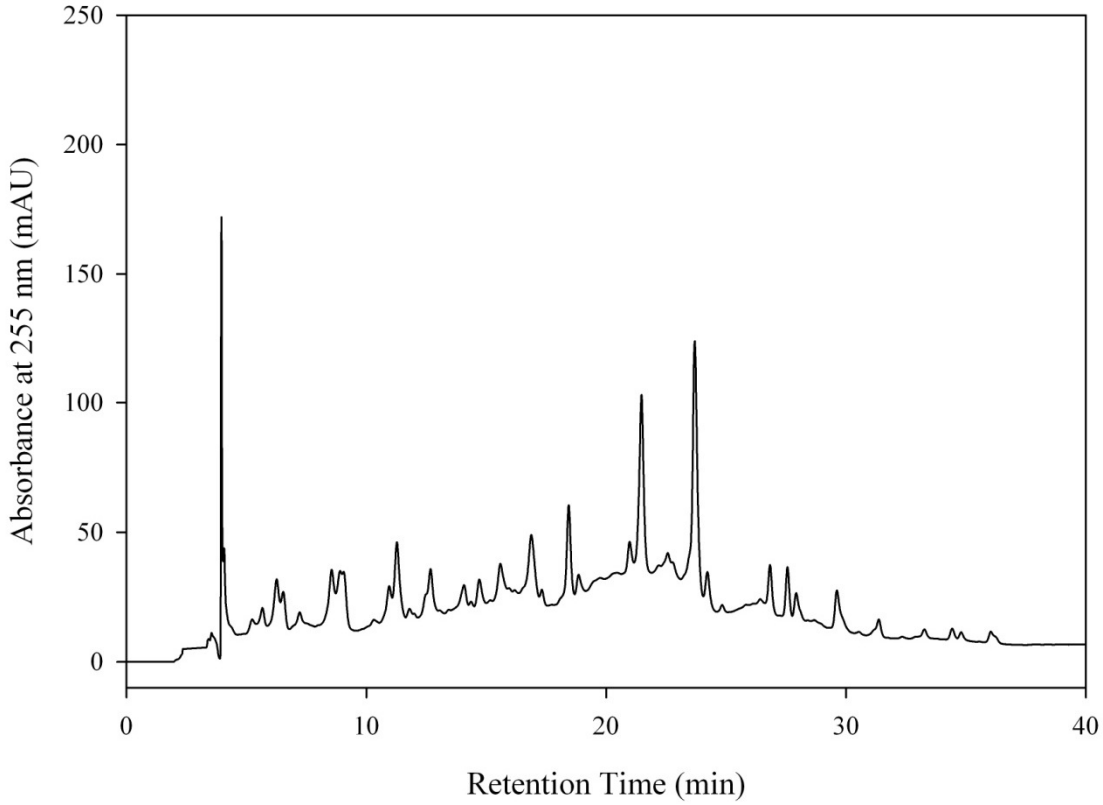

H-ORAC$_{FL}$
(mmol TE/100 g nutmeat)
19
18
17
16
15
14
13
16
17
18
19
20
21
22
r = -0.132
P = 0.698
FRAP
(mg Fe^{2+} eq/100 g nutmeat)

Figure 3.2.RP-HPLC Chromatograms of Pecan Extract. (a). Crude 'Wichita' extract at 255 nm. (b) Crude 'Desirable' extract at 255 nm. (c) Free phenolic acids extracted with diethyl ether from crude 'Desirable' extract at 255 nm. (d) Crude 'Wichita' extract at 280 nm. (e) Crude 'Desirable' extract at 280 nm. (f) Free phenolic acids extracted with diethyl ether from crude 'Desirable' extract at 280 nm. Compounds identified using LC-ESI-MS to be (1) gallic acid, (2) (+)-catechin, (3) valoneic acid dilactone (ellagic acid derivative), (4) ellagic acid. Protocatachuic and *p*-hydroxybenzoic acid were also identified using the LC-ESI-MS but are not shown on the above chromatograms.

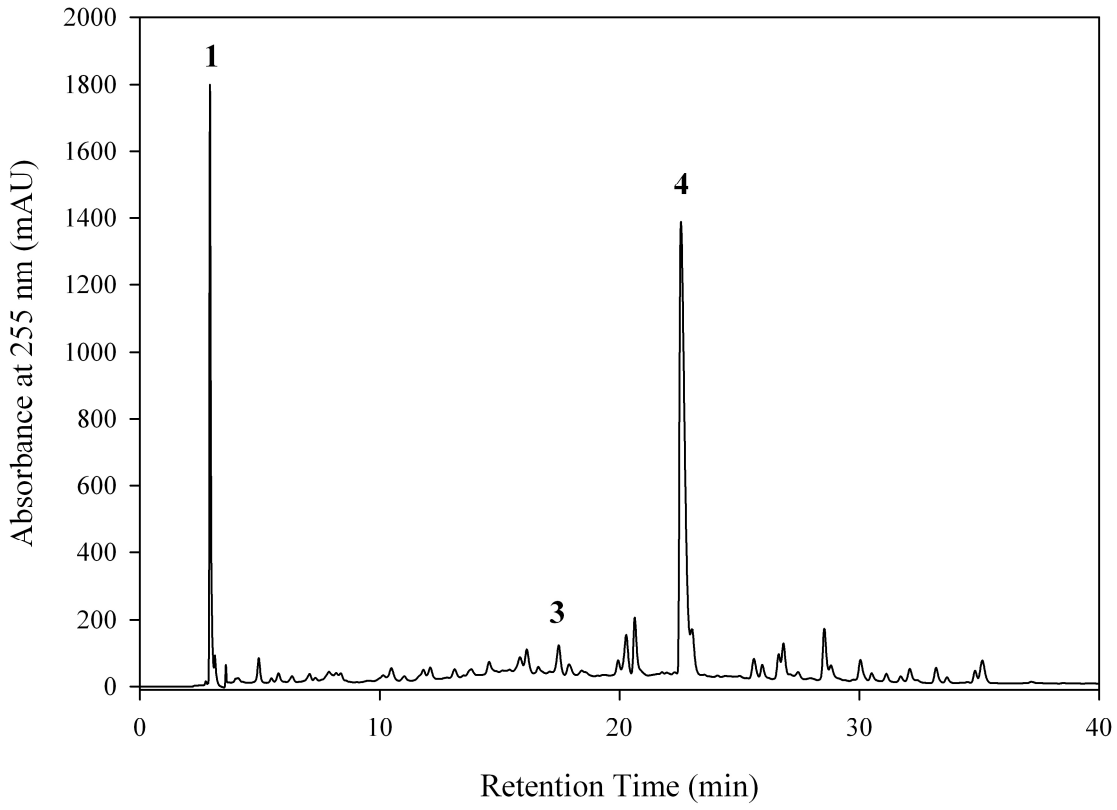
2a.



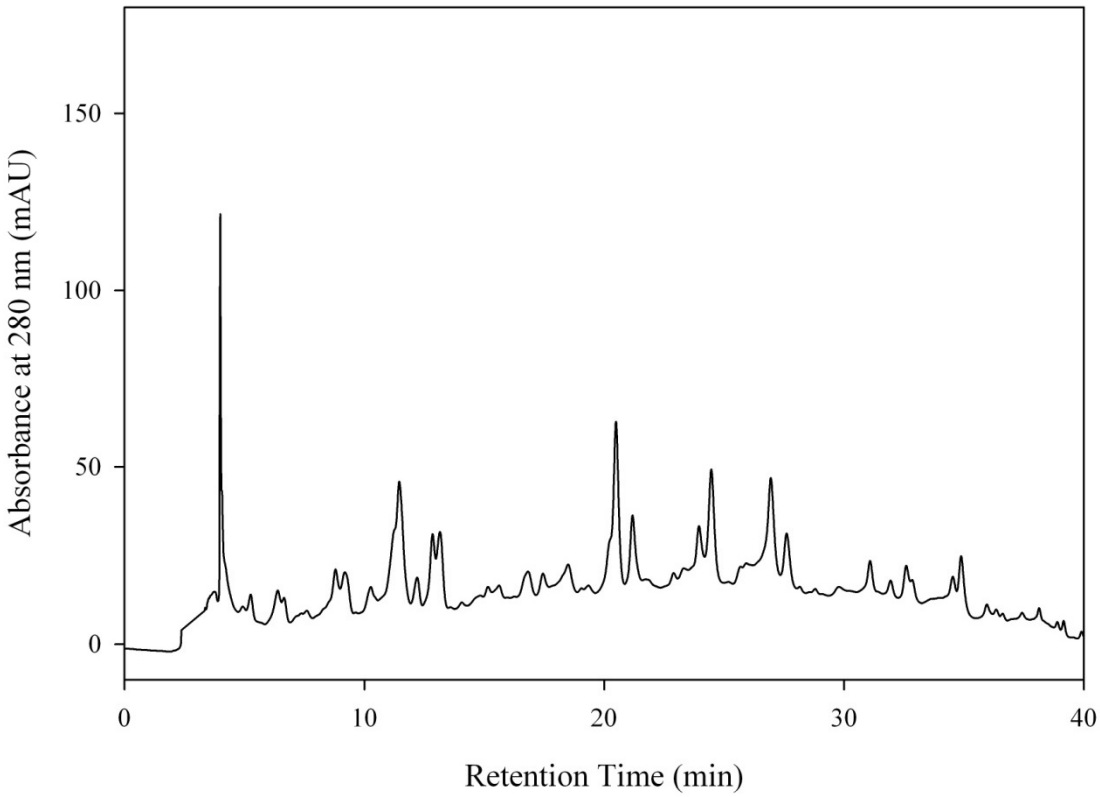
2b.


250
200
150
100
50
0
Absorbance at 255 nm (mAU)
0
10
20
30
40
Retention Time (min)

2c.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
Absorbance at 255 nm (mAU)
1
4
3
0
10
20
30
40
Retention Time (min)

2d.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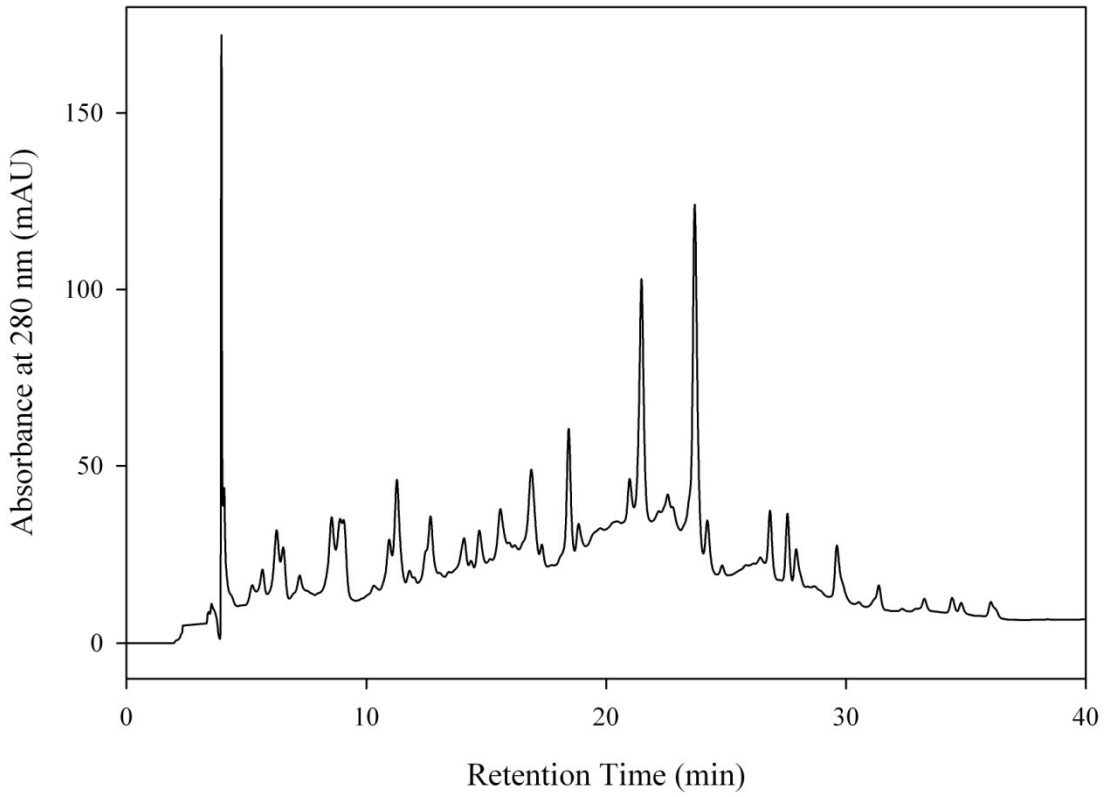
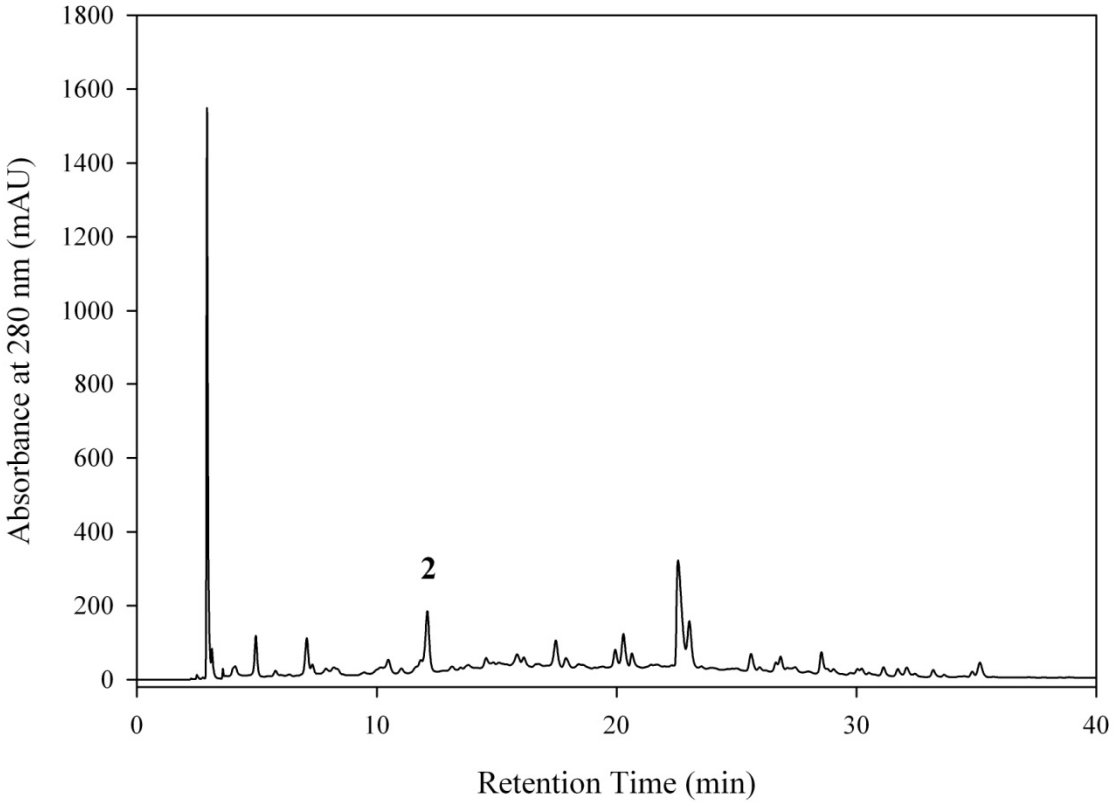
100
50
0
0
10
20
30
40
Retention Time (min)

2e.


Absorbance at 280 nm (mAU)
150
100
50
0
0
10
20
30
40
Retention Time (min)

2f.


1800
1600
1400
1200
1000
800
600
400
200
0
Absorbance at 280 nm (mAU)
2
0
10
20
30
40
Retention Time (min)

CHAPTER 4

IMPACT OF ROASTING ON THE PHENOLIC COMPOUNDS FOUND IN U.S. PECANS

[1] Robbins, K. S.; Wells, M. L.; Shewfelt, R. L.; Pegg, R. B. To be submitted to *Journal of Agricultural and Food Chemistry.*

ABSTRACT

Roasting has been shown to affect the antioxidant activity of several different nut types but has not been investigated with pecans. Four different commercially-viable cultivars from two different U.S. states were analyzed for their phenolics content and antioxidant activity. The cultivars were then roasted and analyzed again. Comparisons of H-ORAC$_{FL}$, TPC, PAC content, and FRAP values were made between the raw and roasted samples for each cultivar investigated. Significant decreases ($P < 0.05$) were found for all cultivars in regards to the total phenolics and PACs with roasting. Three out of the four cultivars showed no significant changes in H-ORAC$_{FL}$ values and all cultivars showed no significant changes in FRAP values. Gallic acid, catechin, and ellagic acid were found to be the prominent phenolic acids in all samples. Changes in these compounds among cultivars and processing steps were investigated and reported.

4.1 INTRODUCTION

Pecans [*Carya illinoinensis* (Wangenh.) K. Koch] are grown across the Southern U.S. and are consumed as a healthy snack across the globe. Pecans have been found to be a rich source of phenolic and polyphenolic compounds which have been shown to possess marked *in vitro* antioxidant activity (1). Phenolic compounds determined in plants are usually synthesized from the amino acids L-phenylalanine and L-tyrosine by phenylpropanoid metabolism (2-3). The phenolic acids, from either the *trans*-cinnamic acid family (quinic, shikimic, and tartaric acids) or the benzoic acid family (gallic and protocatechuic acid) can be found in plants in their monomeric form or as building blocks for larger polymeric compounds, tannins (4-5). Polyphenols in pecans are found as hydrolyzable tannins, gallotannins and ellagitannins, as well as the condensed tannins, proanthocyanidins (6-7).

Phenolic contents may be underestimated due to the presence of phenolic compounds as soluble esters and soluble glycosides. Even though these compounds can be extracted using an aqueous solvent system, they are often lost during chromatographic analysis and are unable to be properly quantified (7). By subjecting crude phenolic extracts to basic and acidic hydrolysis, phenolicscan be liberated from the soluble esters and glycosides, and then quantified (8). Hydrolysis of these compounds can occur *in vivo* making them bioactive (9-10) and thus they should be properly determined and reported in the literature.

Raw and roasted pecans are enjoyed by consumers in the United States. The effect of roasting nuts on their antioxidant activity can be affected by roasting time, roasting temperature, nut type, and experimental conditions employed. It is believed that two main mechanisms act during roasting to affect the antioxidant activity of the samples (11-14). These include decomposition of antioxidants through the heating process and formation of new antioxidant compounds, either Maillard reaction products (MRPs) (12-13) or released previously-bound phenolic compounds (11). The result of this competition of mechanisms will determine the net effect on the change in the antioxidant activity of the samples. Significant increases, decreases, and no change in antioxidant activity have been shown in hazelnuts, peanuts, cashews, walnuts, almonds and pistachios, but no studies exist investigating the effect on pecan samples (11, 14-22).

Due to the conflicting observations reported in the literature concerning other nut types and the lack of analysis of pecan cultivars, the impact of roasting on the antioxidant activity of four different pecan cultivars were analyzed. The cultivars were selected to represent a range of antioxidant activities in the raw samples and represent different growing locations across two different U.S. states. HPLC characterization was employed in order to assess differences that

might exist between the roasted and raw samples in order to determine the mechanisms of action for changes in antioxidant activity and phenolic contents.

4.2 MATERIALS AND METHODS

Chemicals and Glassware. Sea sand, sodium carbonate, glass wool, ethanol (95%) and ACS-grade methanol, hexanes, and acetone as well as HPLC-grade water, methanol, hexanes, isopropanol and acetonitrile were acquired from Fisher Scientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid, hydrochloric acid, potassium phosphate monobasic, potassium phosphate dibasic, sodium acetate, and iron sulfate were purchased from VWR International, LLC (Suwanee, GA, USA). Fluorescein (3′6′-dihydroxy-spiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one), AAPH (2,2′-azobis[2-amidinopropane] dihydrochloride), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin-Ciocalteu's phenol reagent, (+)-catechin hydrate, TPTZ (2,4,6-tripyridyl-*s*-triazine), and iron chloride were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Sample Selection. Four cultivars were selected from the thirteen Georgia cultivars studied previously. Cultivars were chosen based upon the results of the antioxidant capacity assays presented in the previous study (Chapter 3). Selection was conducted to ensure that each of the four quartiles was represented in the present study. The nuts were raw and remained in-shell through transport, packaging and storage. After arrival to the Department of Food Science & Technology, Athens, GA, the pecan samples were placed in labeled pouches (Sealed Air Corporation, Elmwood Park, NJ), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to prevent any oxidative degradation, and stored at -80 °C until analyzed.

Roasting. Nuts were roasted in a Lincoln Impinger (Model 1450) impingement oven (Lincoln Foodservice Products, Fort Wayne, IN) at 175 ± 10 °C for 8.17 minutes. The roasting profile was developed based on that presented by Erickson *et al.* (23). Pecans were initially roasted at 150°C for 10 min and then allowed to cool. A colorimeter with a 2° observer angle (Model CR-410, Konica Minolta Sensing Americas, Inc., Ramsey, NJ) was employed to assess colorfor a subsample (n=10 pecan halves) using the Commission Internationale de l'Éclairage (CIE) *L**C* h system, which represents a color space expressed in cylindrical coordinates. The findings were compared to commercial roasted pecans, as well as those values reported by Erickson *et al.*(23).Once the roasting profile was finalized, color measurements were also continued for each roasted sample to ensure that a comparable roast was delivered to all nuts. Prior to measurement, however, the colorimeter was calibrated with a white D_{65} standard (Y = 94.7, x = 0.3156, and y = 0.3319) provided by the manufacturer. *L** indicates lightness (0 to 100) and is the same as the *L** of the *L*a*b** color space, C* is chroma {0 to 100; $C^* = [(a^*)^2 + (b^*)^2)^{½}]$} which measures the color saturation or purity, and h is hue angle {h = arctan (*b*/a**)} that indicates the primary color (0 to 360°).Color was measured four times for each sample and mean values are reported. Prior to each replication, the colorimeter was recalibrated against a white standard tile provided by the manufacturer. Differences between the raw and roasted values was determined and reported.

Lipid Extraction. On the day of analysis and roasting, each pecan cultivar was allowed to properly cool after roasting, and then the mass recorded. The pecan halves were selected at random from all roasted halves. Approximately 12 g of nuts were placed into a -20 °C freezer and allowed to partially refreeze to facilitate grinding. Each nut sample was then combined with ~60 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee

Grinder, Cuisinart, East Windsor, NJ), to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

A Soxhlet apparatus was employed to extract the lipids from all pecan samples. Briefly, a known quantity of the ground matter (pecan and sand) was placed into a cellulose extraction thimble (single thickness, 43 mm I.D. and 123 mm external length, Whatman International Ltd., Maidstone, England) and the mass recorded. Glass wool was placed in the top of the thimble to ensure that the contents would remain in place during extraction. Extraction was performed using ~350 mL of hexanes for 20 h. Upon completion, the thimbles were removed and allowed to dry overnight. The hexanes were removed from the lipid extract using a BüchiRotavapor R-210 (Büchi Corporation, New Castle, DE). The lipid portion was weighed for gravimetric analysis. Extracted lipids were transferred to amber-colored vials, flushed with N_2, capped, and placed in the -80°C freezer until analyzed.

Extraction of Phenolic Compounds. The defatted pecan kernel was removed from the dried thimble and placed into an Erlenmeyer flask. As employed by Wu *et al.*(18), a 100-mL portion of extraction solvent ($(CH_3)_2CO/H_2O/CH_3COOH$ solvent mixture, 70:29.5:0.5 v/v/v) at a ratio of ~6:10 thimble contents:extraction solvent was used to extract phenolic compounds. Extraction was carried out according to Craft *et al.* (20). Briefly, the contents in the flasks were heated at 50°C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed three times and the supernates pooled. Acetone was evaporated from the pooled supernate using the Rotavapor. The aqueous portion was poured into crystallization glasses, covered with filter paper, and placed in a -80 °C freezer until completely frozen. The samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer,

Labconco Corp., Kansas City, MS). The dried extract was placed in amber-colored vials, capped and stored at 4 °C until ready to be used. A lipid and phenolic extraction was completed in triplicate for each cultivar.

Antioxidant Assays. *Hydrophilic-ORAC$_{FL}$ Assay***.** A hydrophilic-ORAC$_{FL}$ assay was completed on the acetonic crude extract powders according to Prior *et al.* (21). Phosphate buffer, pH 7.4, was used as the blank and the diluent. Fluorescein (FL), 0.1 μM, was employed as the reaction probe and AAPH, 80 μM in phosphate buffer, was used as the radical initiator. Both working solutions were held at 37 °C for the length of the experiment. The phenolic extract was diluted to 0.5 mg/mL with ethanol. The ethanolic solution was further diluted with phosphate buffer, pH 7.4, to a final concentration of 0.025 mg/mL.

A BMG FLUOstarOmega (Ω) microplate reader (BMG Laboratories, Cary, NC) equipped with two internal 500-μL reagent pumps, an external lead system, temperature control set at 37 °C, fluorescent detection set at an excitation/emission pair of 485/520 nm, and a 3-h run time was employed for analysis. Black, clear bottom 96-well plates were used for the analysis and 20 μL of each sample, blank, or standard were pipetted into each well. During the analysis, 200 μL of FL and 20 μL of AAPH were added using an automated addition waiting one cycle between each reagent. A standard curve based on five different Trolox concentrations (12.5, 25, 50, 80, and 100 μM in phosphate buffer) was constructed. The area under the curve (AUC) was determined and following blank correction, samples and standards were compared. Final values were reported as mmol Trolox eq./100 g pecan nutmeat from triplicate samples.

TPC assay. The total phenolics content (TPC) was determined using a method adapted from Swain and Hillis (22) employing the Folin-Ciocalteu phenol reagent. Each acetonic crude extract was diluted to 0.20 mg/mL in methanol. The assay was performed using 1 mL of

methanolic extract, 7.5 mL deionized water, 0.5 mL Folin-Ciocalteu phenol reagent, and 1 mL of saturated sodium carbonate. The solution was vortexed for 30 s. A quiescent period of 1 h was employed to allow for optimal color development. The absorbance of the resulting solution was measured at $\lambda = 750$ nm using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc.). A standard curve was created from solutions of (+)-catechin (1.6-8.0 μg/mL). TPC values were reported as g (+)-catechin eq./100 g pecan nutmeat.

DMAC Assay. The method described by Payne *et al.* (23) was employed to quantify the total procyanidins in the acetonic crude extract. Briefly, a 30-mL volume of DMAC solution was prepared with 10% (w/v) HCl in reagent alcohol. DMAC (0.03 g) was then added to this solution and swirled until dissolved. The same 0.20 mg/mL methanolic extract solution, as employed to determine the TPC content, was used in this assay. Standard solutions of procyanidin B2 were prepared at concentrations of 1, 10, 50, and 100 ppm. The microplate reader and 96-well plates were used for analysis. All wells contained 250 μL DMAC solution and 50 μL of alcohol (blank), standard solutions, or sample solution. The plate was read at 640 nm and the maximum absorbance for each well recorded. The data was expressed as mg procyanidin B2 eq./100 g pecan nutmeat.

FRAP assay. The reducing capacity of the acetonic crude extracts was determined using the ferric reducing antioxidant power (FRAP) assay employing the method of Pulido *et al*. (24). The same methanolic extract was used in this assay as that used in the TPC and DMAC assays. The FRAP reagent was prepared freshly using 2.5 mL of a 10 mM TPTZ solution in 40 mMHCl with 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$ and 25 mL of a 0.3 M acetate buffer at pH 3.6. The FRAP reagent was brought to 37°C prior to analysis. A standard curve was conducted with aqueous iron sulfate solutions ($FeSO_4 \bullet 7H_2O$) at five different concentrations ranging from 250 to 1600

μM. The FRAP reagent (200 μL), deionized water (20 μL), and blank/sample/standard (6.6 μL) were pipetted into a 96-well microtitre plate and read using the microplate reader with temperature control set at 37 °C. The maximum absorbance readings of the samples were recorded at λ = 595 nm. The antioxidant activity was reported as mmol Fe^{2+} eq./100 g pecan nutmeat.

Extraction and Separation of Bound Phenolics. Free phenolic acids, phenolic acids released from soluble esters and those liberated from soluble glycosides were extracted and separated according to Amarowicz and Weidner (29). Briefly, ~800 mg of crude extract was suspended in 20 mL of acidified water (pH 2, 6M HCl); the free phenolic acids were then extracted using diethyl ether (5 x 20 mL). The ether was evaporated to dryness using the Rotavapor. The residual aqueous solution was neutralized with NaOH to a pH of 7, flushed with N_2, capped and hydrolyzed for 4 h at room temperature. After acidification to pH 2 using 6M HCl, phenolic acids released from soluble esters were extracted from the hydrolyzate with diethyl ether (5 x 30 mL). The extract was evaporated to dryness. A 15 mL aliquot of 6M HCl was added to the aqueous phase, the vial flushed with N_2, and then placed in a forced-air convention oven (Precision Mechanical Convention Oven, GCA/Precision Scientific Corp., Chicago, IL) at 100°C for 1 h. Phenolic acids released from soluble glycosides were extracted with diethyl ether (5 x 45 mL). The ether was evaporated to dryness. Dry residues were dissolved in 2 mL anhydrous methanol and passed through a 0.45-μm nylon filter. The samples were then injected onto an HPLC column.

Reversed-Phase HPLC Characterization (RP-HPLC). The RP-HPLC method reported by Srivastava *et al.* (29) was followed to characterize the crude extracts of the pecan cultivars. Briefly, an Agilent 1200 series HPLC system consisting of a quaternary pump with degasser,

autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA) was employed for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 x 250 mm, 5 μm; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 mL/min was employed. A volume of 100 μL was injected for each of the acetonic extracts (4.0 mg/mL methanol). Detection wavelengths were λ = 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids, notably of the *trans*-cinnamic acid family), and 360 nm (flavonols). Tentative identification of separated components was made by matching UV-VIS spectra and retention time mapping with standard compounds.

Reversed-Phase HPLC-ESI-MS Confirmation (LC-ESI-MS). Tentative identification of separated compounds was confirmed using LC-ESI-MS techniques. Briefly, a Thermo Scientific Accela series HPLC system consisting of a binary pump with degasser, autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and Xcalibur software (Thermo Scientific, Waltham, MA) was employed for the chromatography. A perfluorophenyl column (150 mm x 2.1 mm, 3-μm particle size, ThermoScientific Accucore, Waltham, MA) was utilized. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 100 min period at a flow rate of 0.4 mL/min was employed. A volume of 10μL was injected for each of the samples. For the MS analysis, N_2 was the carrier gas and a cone voltage of 55 V was employed. A total scan in negative-ion mode was conducted from 100-1000 Daltons. The probe tip temperature was set to

350 °C. Comparison of parent molecular ions ($[M-H]^-$) was employed to assist with elucidation of the identities of the phenolic acids. When necessary, comparison of relative retention times (RRTs), $[M-H]^-$ values, and fragmentation patterns of phenolic acids to those reported in the literature for phenolics was employed.

Method Validation and Quality Control. A study of precision and accuracy was carried out for each of the assays using the standard compound employed in each assay. The precision was measured through the relative standard deviation achieved on each of the trials for the compound of interest at all tested concentrations. The accuracy was a measure of how close the predicted concentration from the developed standard curve was to the actual concentration of the standard compound and was modeled after that presented in Huang *et al.*(31). The interday RSD and percent recovery were determined using the following equations:

$$\% \text{ RSD}_r = \frac{\text{Standard Deviation}}{\text{Mean}} * 100\ \%$$

$$\% \text{ REC} = \frac{\text{Theoretical value-Analytical value}}{\text{Theoretical value}} * 100\%$$

where theoretical value is determined based on the concentration of the standard solution and the analytical value is determined from the standard curve calculations.

The linearity of the phenolic standard curves was determined. LOD and LOQ were determined for each standard analyzed. The following equations were utilized to determine the LOD and LOQ for the standards:

$$LOD = \frac{3*\sigma}{S}$$

$$LOQ = \frac{10*\sigma}{S}$$

where σ is the standard deviation of the y-intercept and S is the slope of the calibration curves.

Statistical Analysis. Differences in the means of each cultivar were determined for each of the measured quantities with the PROC MIXED procedure allowing for a random analysis. Differences were analyzed between all 4 cultivars studied, in particular looking at patterns elucidated through the HPLC characterization of roasted vs. unroasted nuts. Differences in means were determined with the LS means procedure and a Tukey's mean separation test with $P<0.05$ using the Statistical Analysis System software, Version 9.2 (SAS Institute, Cary, NC). Differences between the roasted vs. unroasted nut means were analyzed using a student's t-test $P<0.05$ with the SAS software. Correlation values between H-ORAC$_{FL}$, DMAC, and TPC values were analyzed using a PROC CORR analysis with the SAS statistical software.

4.3 RESULTS

Method Validation. The precision and accuracy values for each of the assays employed in this study were determined. The H-ORAC$_{FL}$ assay for the roasted samples was found to have a RSD of 3.1 to 7.3% when the standard values greater than 25 μM were included in the analysis. The % REC values were determined to be between 96.50 and 107.6% for all Trolox concentrations greater than 25 μM. Both the RSD and % REC values for the 12.5 μM Trolox standard level were found to be less accurate and precise (*i.e.,* RSD of 26.31 and % REC of

73.65 %) than those calculated for the other standard values. For this reason, all experimental concentrations were made to have values higher than 25 μM Trolox equivalents.

The TPC values for the roasted samples had percent recovery values of 98.1 to 103.7 % and relative standard deviations of 0.9 to 3.3%. These were found to be comparable to those values previously reported for the unroasted samples. The unroasted samples had % REC values ranging from 98.1 to 103.7 % and % RSD_r values of 1.0 to 1.5. These values were also determined for the $FeSO_4$ concentrations of the FRAP assay for the roasted samples. The values for the roasted samples were comparable if not slightly better than the unroasted samples. The % REC values ranged from 98.26 to 100.8 % and the % RSD_r values ranged from 1.13 to 2.26 for the values greater than 500 μM. As with the unroasted samples, the lowest concentration, 250 μM, had a larger % RSD_r than the other concentrations. All samples were made to have response values between 500 and 1600 μM to assure accurate values.

The color characteristics (lightness, hue angle, and chroma) of 10 pecan halves were determined before and after roasting and are presented in Table 4.1.In the *L** C* h system used, *L** varies from 0 to 100 (black to white) and C* from 0 to 100 (neutral grey to color purity). A hue angle (h) of 0° would correspond to a red color while 90° would correspond to yellow. Δ*L** showed the greatest differences across the cultivar types, ranging from -7.93 ± 1.13 for the 'Pawnee' cultivar to -14.56 ± 1.88 for the 'Western' cultivar sample. Hue angle differences were close to -0.40 for all cultivars, ranging from -0.33 ± 0.06 to -0.45 ± 0.02 for the 'Pawnee' and 'Western'samples, respectively. Chroma ranged from -8.74 ± 0.28 for the 'Pawnee' cultivar to -11.34 ± 0.48 for the 'Stuart' sample. The negative direction for each color characteristic represents a darkening (Δ*L**), increased red character (h) and lower intensity (C*) during roasting. No roasting trial seemed to be an outlier for all of the cultivar samples studied.

Antioxidant Assays. The H-ORAC$_{FL}$ values of the four roasted cultivars ranged from 14.7 mmol TE/100 g nutmeat for the 'Desirable' sample to 17.5 mmol TE/100 g nutmeat for the 'Stuart' sample. The unroasted samples ranged from 15.5 mmol TE/100 g nutmeat to 20.7 mmol TE/100 g nutmeat for the same samples. No significant differences ($P > 0.05$) were found between the roasted values and the values of the unroasted samples for all cultivar samples except for 'Stuart' (Table 4.2). Higher H-ORAC$_{FL}$ values are an indication that one sample has a greater potential over another to serve as an antioxidant via the hydrogen atom transfer mechanism.

The TPC values were found to be significantly different ($P < 0.05$) between the roasted and unroasted samples for all cultivars examined. The values for the raw samples ranged from 1.21 to 1.56 g (+)-catechin eq./100 g nutmeat while the roasted samples ranged from 1.06 to 1.27 g (+)-catechin eq./100 g nutmeat.TPC values are used as a means of quantifying the presence of phenolics in a sample. Due to the resonance stabilizing characteristics of phenolic structures, a greater TPC value may be indicative of a higher antioxidant activity. No significant differences ($P < 0.05$) were found between the raw and roasted samples for the FRAP values. The FRAP values of the raw samples ranged from 15.5 to 20.1 mmol Fe^{2+}eq./100 g nutmeat and the roasted samples ranged from 15.2 to 18.8 mmol Fe^{2+}eq./100 g nutmeat. The FRAP values of a sample are unique from the other three assays utilized in this study. Instead of the hydrogen atom transfer mechanism, FRAP measures antioxidant activity in regards to a single electron transfer mechanism. A higher FRAP measurement could be an indication that a sample has a greater potential than another sample to serve as an antioxidant through the single electron transfer mechanism.

The DMAC assay is a measure of the procyanidin content of a sample. The total procyanidin values of the raw pecans, as reported by the DMAC assay, were found to range from 400 mg procyanidin B2 eq./100 g nutmeat for 'Pawnee' to 567 mg procyanidin B2 eq./100 g nutmeat for 'Stuart'. The roasted DMAC values ranged from 304 mg procyanidin B2 eq./100 g nutmeat for 'Pawnee' and 'Desirable' to 383 mg procyanidin B2 eq./100 g nutmeat for 'Western'. All roasted values were significantly lower ($P < 0.05$) than the raw values.

The four cultivars analyzed were chosen to properly represent each quartile of the data range from the raw data values of the TPC, H-ORAC$_{FL}$, DMAC, and FRAP assays previously reported (Chapter 3). The researchers wanted to determine if the trends observed for a high performing cultivar would be consistent with those observed for lower performing cultivars. Interesting to note, the 'Stuart' cultivar which was the highest performing cultivar from the previous study exhibited the largest decrease for the H-ORAC$_{FL}$, TPC, and DMAC assays. This cultivar showed decreases of 15.5 %, 18.6 %, and 33.0% for the H-ORAC$_{FL}$, TPC, and DMAC assays, respectively. The second highest performing cultivar, the 'Western' samples, showed the smallest decreases for the H-ORAC$_{FL}$ and DMAC assays with decreases from the raw samples of 2.8% and 21.4%, respectively. The previously lowest performing cultivar, 'Desirable', had the second highest decrease of all cultivars for the TPC and DMAC investigation and the highest decrease for the FRAP assay with values of 13.8%, 28.1%, and 12.0% for the TPC, DMAC, and FRAP assays, respectively. It should be noted that the FRAP assay exhibited some interesting trends compared to the other assays investigated. The FRAP assay was the only one studied where there was actually an increase in values between the roasted and raw samples. This increase was found with the 'Western' cultivar which exhibited an increase of 8.8% with roasting.

HPLC Characterization and Quantification. The crude extracts were hydrolyzed under acidic and basic conditions to yield three different fractions: free phenolic acids, phenolic acids released from soluble esters, and phenolic acids liberated from soluble glycosides. These fractions were initially characterized by HPLC-DAD and then potential identities were confirmed using LC-ESI-MS. A chromatogram captured for the raw 'Desirable' sample is depicted in Figure 1. Peak 1 was determined to be gallic acid due to its retention time of 5.2 min and a parent molecular ion, $[M-H]^-$, at an *m/z* of 169. The peak identity was confirmed using commercial standards and the peak was quantified at $\lambda = 280$ nm. (+)-Catechin was identified as peak number 2 with a retention time of 12.8 min and a parent ionat an *m/z* of 289. The peak identity was also confirmed using a commercial (+)-catechin hydrate standard. The identity of peak 4, ellagic acid, was also determined based on the retention time and UV spectrum as compared to that of a commercial standard. Protocatachuic acid and *p*-hydroxybenzoic acid were also identified in the samples but are not pictured in the chromatograms in Figure 1. The protocatachuic acid had a retention time of 9.0 min and had a molecular ionat an *m/z* of 153 while the *p*-hydroxybenzoic acid had a retention time of 13.8 min and a molecular ionat an *m/z* of 137. These tentative identifications were confirmed by comparison with commercial standards and UV spectral patterns.

Peak 3 was tentatively identified as valoneic acid dilactone. This identification was based upon previous findings by de la Rosa *et al*. (6) in pecans and Li *et al*. in walnuts (32). The peak was found to have a retention time of 16.8 min and a molecular ion at an *m/z* of 469. Additional fragment ions at *m/z* of 301 and 425 add to the strength of the identification. It is believed that this compound is an ellagic acid derivative due to its 301 fragment ion and extreme similarities between the UV spectral patterns of the two compounds as seen in Figure 2.

Standard curves were prepared for all five compounds in which commercial standards are available: gallic acid, (+)-catechin, protocatachuic acid, *p*-hydroxybenzoic acid, and ellagic acid. Because no commercial standard was available, the valoneic acid dilactone peaks were quantified using the ellagic acid curve, due to their spectral and structural similarity. Purity values were taken into consideration during curve construction. All curves were prepared with either five or ten different concentrations, ranging in values similar to those expected from the samples. Linearity was found for all five curves. The ellagic, catechin, and protocatachuic acid curves had R^2 values of 0.99 and the *p*-hydroxybenzoic acid and gallic acid curves had R^2 values of 0.98. All samples were quantified based on their free phenolic acids, acids released from soluble esters, acids liberated from soluble glycosides, and the combined total value for all phenolics analyzed (Table 4.3).

None of the unroasted samples contained quantifiable free gallic acid but there were small amounts (*i.e.*, less than 2.2 mg/100 g nutmeat) present in the roasted samples. For all of the cultivars studied except the 'Stuart', the highest percentage of gallic acid was found in the fraction liberated from the soluble esters. Both of the 'Stuart' samples had the highest phenolic percentage represented by the fraction liberated from soluble glycosides. Total gallic acid contents ranged from 33.51 mg/100 g nutmeat in the unroasted 'Western' samples to 79.57 mg/100 g nutmeat in the unroasted 'Desirable' samples. A trend between the roasted and unroasted samples was not present for the esters, glycosides and total values. Certain cultivars demonstrated an increase with roasting and some exhibited a decrease with roasting.

All samples had detectable and quantifiable catechin peaks for all three fractions evaluated. All of the unroasted samples had higher free catechin values than the roasted samples with values for the unroasted samples ranging from 6.03 to 7.27 mg/100 g nutmeat for the

unroasted samples and 4.76 to 6.32 mg/100 g nutmeat for the roasted samples. The catechin released from soluble esters contributed various amounts to the total catechin values. Four samples (i.e., both 'Stuart' samples, the unroasted 'Western', and the roasted 'Pawnee') had contributions from the esters of ~30% while the remaining four samples had much higher contributions of ~53%. The trends in contributions were reversed for the glycosides. The unroasted 'Stuart' sample had the highest total catechin value of 57.89 mg/100 g nutmeat and the unroasted 'Western' had the lowest total catechin content of 26.42 mg/100 g nutmeat.

No free protocatachuic acid was found in four of the samples analyzed. The four samples which did, however, contain the acid possessed very low levels (i.e., less than 0.35 mg/100 g nutmeat); these did not contribute greatly to the total value determined between the three fractions. All samples analyzed contained detectable and quantifiable amounts of the protocatachuic acid liberated from soluble esters. Except for the 'Stuart' samples, the roasted samples contained a higher amount of the acid than their unroasted counterparts. Three of the samples did not have any detectable levels of protocatachuic acid liberated from soluble glycosides. All five of the samples that did have detectable levels had contents which were higher than those released from soluble esters. The total content of protocatachuic acid in the nuts ranged from 0.04 to 4.06 mg/100 g nutmeat. Interestingly, it should be noted that the unroasted 'Stuart' sample had a total protocatachuic content that was almost three times higher than any of the other samples. This observation is even more poignant because all of the other cultivars showed higher total contents for the roasted samples than the unroasted samples.

Numerous samples did not contain any detectable levels of *p*-hydroxybenzoic acid in all three fractions analyzed. Of the eight cultivars investigated, the *p*-hydroxybenzoic acid liberated from soluble glycosides contributed the highest percentage of the three fractions to the total

content. The unroasted ‘Stuart’ sample gave the highest total *p*-hydroxybenzoic acid content with 2.73 mg/100 g nutmeat and the unroasted ‘Desirable’ sample had the lowest total content with no quantifiable acid present. The ‘Stuart’ samples were also the only samples in which the unroasted analog had a higher total content than the roasted samples.

For all eight cultivars studied, there was very little of the total valoneic acid dilactone present in the free form, with only one cultivar having a free acid contribution greater than 1%. The rest of the valoneic acid dilactone concentrations are pretty evenly split between the esters and the glycosides for the cultivars analyzed. The unroasted ‘Desirable’ showed the highest total valoneic acid dilactone content of 47.99 mg/100 g nutmeat and the roasted ‘Stuart’ sample had the lowest total content with 11.92 mg/100 g nutmeat.

All eight samples had detectable and quantifiable values for all three ellagic acid fractions. The majority of the ellagic acid content was present as acids released from soluble glycosides. For all samples, except the ‘Desirable’, the unroasted nuts had higher values than those roasted. The ellagic acid released from soluble esters ranged from 7.60 to 22.70 mg/100 g nutmeat and those liberated from soluble glycosides ranged from 14.23 to 65.61 mg/100 g nutmeat. The unroasted ‘Western’ possessed the lowest total ellagic acid content with 28.87 mg/100 g nutmeat and the roasted ‘Desirable’ samples had the highest total with 89.97 mg/100 g nutmeat. Of all compounds quantified, gallic acid yielded the highest mean total content across all samples with 55.23 ± 14.53 mg/100 g nutmeat. Nonetheless, ellagic acid also had elevated mean total contents of 48.26 ± 23.97 mg/100 g nutmeat.

4.4 DISCUSSION

The roasting profile was developed based on that presented by Erickson *et al.* (23), in which pecans were roasted at 150 °C for 10 min in a batch rotating oven. In order to account for

variations in initial color, differences in lightness, hue angle and chroma were calculated. Erickson *et al*. reported color change of -3.90, -4.23, and -0.05 for Δ*L**, ΔC*, and Δh, respectively. When the samples from this study were roasted with a similar profile, the pecans were much lighter and lacked the taste profile often associated with roasted pecans. For this reason, the temperature of the impingement oven was increased to ~175 °C. To make a complete comparison, roasted pecans were purchased from a local grocery store in Athens, GA. Unfortunately no comparison of color change from pre-roast to post-roast was available; so, only the roasted color was recorded. The experimental pecans gave *L**, C*, and h values of 38.10, 22.70, and 0.90, respectively. These were compared to 33.05, 18.84, and 0.69 for the *L**, C* and h values of the commercial pecans. The purchased pecans seemed to be coated in oil and were salted, suggesting that the visual color differences and taste profile were similar with the developed roasting profile. The profile was continued for all pecan cultivars with close attention given to any changes in color parameters (Table 4.1).

The H-ORAC$_{FL}$ values showed a significant decrease for the 'Stuart' cultivar ($P < 0.05$) and the remaining cultivars were the same ($P > 0.05$) with roasting. Even with the decrease observed for the 'Stuart' cultivar, the H-ORAC$_{FL}$ values are still elevated compared to other nut types and plant products. No previous studies in the literature have investigated the effects of roasting pecans on their antioxidant capacities. However, other nut types have been examined with different results reported. In a study by Chandrasekara *et al*. (15), cashews that were roasted at 130 °C for 33 min showed an increase in the H-ORAC$_{FL}$ values for soluble extracts of cashew kernels and testa. It was expected that this increase might be attributed to the formation of Maillard reaction products (MRPs) that have been shown to possess antioxidant activity. It is expected that this increase was not also seen in pecans due to the compositional differences

between cashews and pecans. Cashews have a much higher starch and protein content than that found in pecans. Starch and protein content are important in determining the level of browning that can occur. Specifically, cashews contain 30.19 % carbohydrates and 18.22% crude protein compared to only 13.86% carbohydrates and 9.17% protein for pecans (33).

Açar *et al.* (17) investigated the effect of roasting on antioxidant activity in several different nuts, seeds and pulses. They discovered that two main mechanisms are competing during roasting. Initially, a sharp decrease in antioxidant activity can be observed due to degradation in thermal-labile antioxidants. However, there appears to be a point in the roasting of certain products where the production of MRPs becomes the dominant mechanism and antioxidant activity increases. It is suspected that the initial degradation of the thermal-labile antioxidants occurred in the pecan samples and MRPs were beginning to form but not predominate. This hypothesis explains why three out of the four cultivars displayed no significant decreases ($P > 0.05$) in our study. Longer times may enable for an increased production of MRPs but caution must be given to protect against burning. With the high lipid content of pecans, a small window of opportunity exists to properly develop the roasting color and flavor without burning and decreasing the palatability of the samples.

One trend that appears to be consistent across all nut types is a dramatic decrease in procyanidin content with roasting. In this study, a significant decrease ($P < 0.05$) was observed for all four cultivars with reductions ranging from 21.4% for the 'Western' cultivar to 33.0% for the 'Stuart' cultivar. This trend was also observed in cashews (15), almond skins (11), pistachios (18), and walnuts (19). These decreases ranged from minimal to dramatic depending on the heat treatment and nut type. Walnuts roasted at 204 °C for only 5 min displayed a 14% reduction in tannin content (19), but pistachios subjected to a toasting treatment had PAC contents 90% lower

than the raw counterparts (18). It is suspected that these tannin compounds represent a large proportion of those antioxidant compounds which are highly susceptible to degradation upon heating at high temperatures (15, 17). While decreases were observed in our study, it should be noted that only one time and temperature combination was utilized. Future experiments should be conducted looking at additional time and temperature conditions. It may be possible that with a longer time, lower temperature roast fewer PACs would be degraded.

Significant decreases ($P < 0.05$) in the total phenolics content (TPC) of the pecans were observed with roasting. These decreases ranged from only 4.95% in the 'Pawnee' cultivar to 18.6% in the 'Stuart' cultivar. Similar trends were observed with the TPC of pistachios. Upon toasting, the pistachios displayed ~60% reduction in their TPC (18). However, roasting did not cause any change in the TPC of peanuts (14) and caused a significant increase ($P < 0.05$) of roughly 20 to 344% in cashews with testa, depending on the level of heat treatment employed (16). It is believed that these differences may also be tied in to the production of MRPs through the roasting process. Another possible mechanism suggested in the literature is that the heat treatment breaks down fibrous material in the nut matrix thereby increasing the ease of extraction of phenolic compounds (11). It should be noted that in a study conducted by Locatelli *et al*. (22), a prolonged roasting treatment did not result in a significant increase ($P > 0.05$) in the extractable polyphenols from hazelnut skin, possibly signifying that the type of nut matrix involved may affect ease of extraction.

The FRAP assay was the only assay demonstrating no significant decreases ($P > 0.05$) for any of the cultivars investigated. One cultivar actually showed a slight, yet not significant increase, in FRAP value. This increase can possibly be attributed to the fact that the FRAP assay measures a different type of antioxidant mechanism, known as the SET mechanism. Most of the

other antioxidant assays, present in the literature, all function by the HAT mechanism. It is suspected that the new antioxidants created through the Maillard browning process may be more likely to function under redox-type conditions. The class of MRPs known as the melanoidins have been reported to demonstrate metal ion-chelating properties (13) which would be quantified using the FRAP assay. There is little reported on the effect of roasting on FRAP contents but it was reported that roasting of almond skins caused a decrease in the FRAP values of 34% compared to the raw skins (11). It is suspected that this significant decrease may be related to the high content of PACs found in nut skins. A significant decrease in PACs could easily overshadow the production of any melanoidins by the Maillard reaction. As mentioned previously, additional time and temperature combinations should be investigated. With different roasting profiles it is possible that a greater amount of melanoidins could be produced.

The main phenolic compounds determined in the present study were gallic acid, (+)-catechin, and ellagic acid. These were also found to be the main contributing acids in previously reported studies by Villareal-Lozoya *et al*. (7), Malik *et al*. (34), and de la Rosa *et al*. (6). In addition to these, an additional compound, valoneic acid dilactone, was also found to be present in large amounts in the fractions liberated from soluble esters and glycosides. Historically, this peak in chromatograms was identified simply as an ellagic acid derivative that eluted prior to the ellagic acid peak and possessed a similar UV spectrum to that of ellagic acid (7,34). This compound was originally identified in walnuts through MS fragmentation patterns and UV spectra as being valoneic acid dilactone by Li *et al*. (32) and was also identified in pecans by de la Rosa *et al*. (6). Valoneic acid dilactone is not present in high quantities in the free form but appears in substantial quantities once subjected to acid or base hydrolysis. While this increase post hydrolysis may be indicative of the samples being present as soluble esters or glycosides, it

also may indicate the presence of hydrolyzable tannins (32, 6). Hydrolyzable tannins, when subjected to the acid and base hydrolysis, could degrade further into both the valoneic acid dilactone and ellagic acid. The presence of gallic acid in the hydrolyzed samples might also be indicative of the presence of hydrolyzable tannins in the crude extracts (6).

Comparisons between the contents given in the present study and those reported in the literature are difficult. No previous study utilizes separate acid and base hydrolysis steps in order to quantify all forms of the constituents present in the samples. Villareal-Lozoya*et al*. (7) did employ both basic hydrolysis and acid hydrolysis steps, but there was no extraction between the two steps. It is not clear whether the lack of two separate steps would affect the quantification of the compounds present. In addition, there are discrepancies in reporting styles. In the present study, all of the constituent data is reported per 100 g nutmeat in order to represent the form of consumption. Other investigators have reported their values in terms of defatted pecan kernels (7). To assist with comparisons, our data was converted to defatted pecan kernels assuming a 75% lipid content which is an average estimate for all of the samples analyzed. Moreover, it should be noted that in the present study, compounds were only quantified from the three different fractions produced via hydrolysis. Crude extracts were not quantified due to the difficulty in acquiring accurate data from the chromatograms, where there was a prevalence of overlapping peaks and a drifting baseline.

The pecan samples in the current study had total gallic acid contents ranging from 33.51 to 79.57 mg/100 g nutmeat. De la Rosa *et al*. (6) report lower values of 19.6 to 27.4 mg/100 g nutmeat. Based on the work presented here, it is suspected that the differences compared to the de la Rosa *et al.* (6) study might be caused by the lack of accounting for the bound compounds that were released through hydrolysis.These authors indicate that the compounds were not

quantified. It should be noted however that the free phenolic acids published in their investigation were much higher than those reported in this study. It is believed that these comparisons are due to the quantification of the crude extracts, in their study, which was not completed in the present work. Villarreal-Lozoya*et al*. (7) also reported the values of gallic acid from Texas cultivars as 651 to 1300 µg/ g defatted kernel. The present study had values of 1340 to 3180 µg/g defatted kernels. These differences could be due to the variations in harvest year, cultivar type, and growing location, which have been shown to cause differences in phenolic content.

Ellagic acid was also quantified by both of these authors. De la Rosa *et al.*(6) citedvalues ranging from 460 to 550 mg/100 g nutmeat, which are much higher than those reported in the present study, 28.87 to 89.97 mg/100 g nutmeat. It is believed that these differences can be attributed to the fact that the present study did not attempt to quantify these compounds in crude extracts but instead hydrolyzed samples. Moreover, the employment of the basic and acidic hydrolysis conditions could have prompted some of the ellagic acid compounds to break down and form other compounds or ellagic acid derivatives. None of the published studies on pecans include quantities for the valoneic acid dilactones. It might be possible that these contents are instead being grouped with ellagic acid itself.

The content of selected phenolic compounds was compared with the antioxidant capacities of the raw and roasted samples for each cultivar in order to elucidate possible mechanisms for the observed reduction in antioxidant activity. One key observation is that all four cultivars showed a reduction in total valoneic acid dilactone with roasting. Because valoneic acid dilactones are indicative of the presence of ellagitannins in the crude sample (6), this suggests that these tannin constituents are part of the thermally-labile compounds which are

degraded in the early stages of the roasting process. It should also be noted that the two cultivars with the largest reductions in antioxidant activity, 'Stuart' and 'Desirable', were also the only cultivars that showed a decrease in total gallic acid content. This trend could denote that the reduction in gallic acid or gallotannins is responsible for a loss in antioxidant activity and key compounds. As no clear trends are identifiable between the roasted and unroasted samples, it is suspected that there are numerous mechanisms coexisting and affecting the antioxidant activity of the samples.

4.5 CONCLUSION

Roasting has a negative effect on the *in vitro* antioxidant activity of pecans. Raw pecans had significantly higher total phenolic and PAC contents ($P < 0.05$). These differences do not seem to cause an effect on the FRAP activity of the samples as no significant differences were observed between raw and roasted samples. It should be noted that while decreases do exist the values are only *in vitro* measures of antioxidant activity and do not necessarily predict the effect that will be observed in a biological system. In addition, due to the limited production of MRPs, which have been shown to have anti-nutritive properties, roasted pecans may have a better nutritional profile than other roasted nut products. It is suspected that two main mechanisms are playing a role. Initially, there is a degradation of thermal-labile antioxidants which is then countered by the production of MRPs which have shown antioxidant activity. It is believed that MRPs do not develop as strongly as in other nut types due to the relatively lower carbohydrate and protein content of pecans. The high temperature and short time combination of the selected roasting profile may have been a factor in the observed differences. It is suggested that further

work developing a roasting profile be performed in order to study the mechanisms of these effects and determine the optimal roasting conditions for antioxidant activity.

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Table 4.1. Color values for roasted pecan halves (n=10).

Cultivar	Δ Lightness[a]	ΔHue Angle[b]	ΔChroma[c]
Cordele 'Stuart' Crisp Co. (GA)	-10.26 ± 0.54	-0.39 ± 0.02	-11.34 ± 0.48
'Western' Doña Ana Co. (NM)	-14.56 ± 1.88	-0.45 ± 0.02	-10.44 ± 1.40
'Pawnee' Tift Co. (GA)	-7.93 ± 1.13	-0.33 ± 0.06	-8.74 ± 0.28
'Desirable' Lowndes Co. (GA)	-10.19 ± 0.25	-0.35 ± 0.06	-9.86 ± 0.96

[a] Change in *L** values between post- and pre-roasting color measurements.

[b] Change in h values between post- and pre-roasting color measurements.

[c] Change in C* values between post- and pre-roasting color measurements.

Table 4.2. Antioxidant assay values for raw and roasted samples of four different cultivars (n=3)[a].

Cultivar	H-ORAC$_{FL}$ (mmol TE/ 100 g nutmeat)		TPC (g (+)-catechineq./ 100 g nutmeat)		DMAC (mg procyanidin B2 eq./ 100 g nutmeat)		FRAP (mmol Fe^{2+} eq./ 100 g nutmeat)	
	Raw	Roasted	Raw	Roasted	Raw	Roasted	Raw	Roasted
Cordele 'Stuart' Crisp Co. (GA)	20.7a	17.5b	1.56 a	1.27 b	567 a	380 b	20.1 a	18.8 a
'Western' Doña Ana Co. (NM)	17.9a	17.4a	1.33 a	1.23 b	487 a	383 b	17.0 a	18.5 a
'Pawnee' Tift Co. (GA)	17.0a	16.0a	1.21 a	1.15 b	400 a	304 b	15.5 a	15.2 a
'Desirable' Lowndes Co. (GA)	15.5a	14.7a	1.23 a	1.06 b	423 a	304 b	19.1 a	16.8 a

[a]Means in the same row within the same assay with the same letter are not significantly different by Tukey's multiple range test ($P > 0.05$) for each assay.

Table 4.3. Content of Phenolic Acids in Hydrolyzed Crude Pecan Samples

	Gallic Acid (mg/100 g nutmeat)				(+)-Catechin (mg/100 g nutmeat)			
	Free	Esters	Glycosides	Total[a]	Free	Esters	Glycosides	Total
Unroasted 'Stuart'	0[b]	22.37	48.44	70.81	7.27	16.60	34.02	57.89
Roasted 'Stuart'	0.58	18.86	37.56	57.00	4.76	11.74	25.64	42.14
Unroasted 'Western'	0	24.86	8.65	33.51	6.04	7.60	12.78	26.42
Roasted 'Western'	2.18	42.10	11.00	55.28	5.81	19.96	9.35	35.12
Unroasted 'Pawnee'	0	37.58	6.57	44.15	6.03	22.70	13.51	42.24
Roasted 'Pawnee'	1.20	35.04	16.14	52.38	4.79	13.80	22.09	40.68
Unroasted 'Desirable'	0	45.47	34.10	79.57	7.09	13.03	8.46	28.58
Roasted 'Desirable'	1.34	41.59	6.23	49.16	6.32	19.84	9.64	35.80

	Protocatachuic Acid (mg/100 g nutmeat)				*p*-Hydroxybenzoic Acid (mg/100 g nutmeat)			
	Free	Esters	Glycosides	Total	Free	Esters	Glycosides	Total
Unroasted 'Stuart'	nd[c]	0.22	3.84	4.06	0.11	nd	2.62	2.73
Roasted 'Stuart'	0.22	0.10	1.17	1.49	0	0.03	1.75	1.78
Unroasted 'Western'	0.02	0.09	0.13	0.24	nd	0	0.43	0.43
Roasted 'Western'	nd	0.52	0.59	1.11	nd	0.19	0.66	0.85
Unroasted 'Pawnee'	nd	0.33	nd	0.33	nd	0.18	0.68	0.86
Roasted 'Pawnee'	0.34	0.56	0.59	1.49	0.21	0.21	1.76	2.18
Unroasted 'Desirable'	nd	0.04	nd	0.04	nd	0	nd	0
Roasted 'Desirable'	0.35	0.40	nd	0.75	0.22	0.12	0.91	1.25

	Valoneic Acid Dilactone (mg/100 g nutmeat)				Ellagic Acid (mg/100 g nutmeat)			
	Free	Esters	Glycosides	Total	Free	Esters	Glycosides	Total
Unroasted 'Stuart'	0.21	6.11	6.70	13.02	3.25	16.60	17.53	37.38
Roasted 'Stuart'	0.05	6.72	5.15	11.92	4.33	11.74	14.23	30.30
Unroasted 'Western'	0.02	16.48	14.04	30.54	2.27	7.60	19.00	28.87
Roasted 'Western'	0.16	11.06	14.85	26.07	3.94	19.96	14.48	38.38
Unroasted 'Pawnee'	0.11	18.56	20.14	38.81	4.19	22.70	53.40	80.29
Roasted 'Pawnee'	0.18	12.12	17.43	29.73	0.04	13.80	16.11	29.95
Unroasted 'Desirable'	0.19	24.97	22.83	47.99	4.22	13.03	33.67	50.92
Roasted 'Desirable'	0.22	15.60	21.01	36.83	4.52	19.84	65.61	89.97

[a] Total calculated by determining the sum of the free, liberated from soluble esters, and liberated from soluble glycosides.
[b] Peak was detectable but was not quantifiable. LOQ = 12.7, 2.5, 4.8, 30.6, 17.6 ng/ml for catechin (C), protocatachuic (PR), p-hydroxybenzoic (PHB), gallic (G), and ellagic acid (E), resp. [c] No detectable peak. LOD = 4.0, 0.74, 1.4, 9.2, 5.3 ng/ml for CAT, PR, PHB, G, E, respectively.

Figure 4.1.RP-HPLC Chromatograms of Pecan Extract. (a). Free phenolic acids extracted with diethyl ether from crude 'Desirable' raw extract at 255 nm. (b) Free phenolic acids extracted with diethyl ether from crude 'Desirable' raw extract at 280 nm. (c) Free phenolic acids extracted with diethyl ether from crude 'Desirable' roasted extract at 255 nm. (d) Free phenolic acids extracted with diethyl ether from crude 'Desirable' roasted extract at 255 nm. (e) Phenolic acids liberated from soluble esters with diethyl ether from crude 'Desirable' raw extract at 255 nm. (f) Phenolic acids liberated from soluble esters with diethyl ether from crude 'Desirable' raw extract at 280 nm. (g) Phenolic acids liberated from soluble esters with diethyl ether from crude 'Desirable' roasted extract at 255 nm. (h) Phenolic acids liberated from soluble esters with diethyl ether from crude 'Desirable' roasted extract at 280 nm. (i) Phenolic acids liberated from soluble glycosides with diethyl ether from crude 'Desirable' raw extract at 255 nm. (j) Phenolic acids liberated from soluble glycosides with diethyl ether from crude 'Desirable' raw extract at 280 nm. (k) Phenolic acids liberated from soluble glycosides with diethyl ether from crude 'Desirable' roasted extract at 255 nm. (l) Phenolic acids liberated from soluble glycosides with diethyl ether from crude 'Desirable' roasted extract at 280 nm. Compounds identified using LC-ESI-MS to be (1) gallic acid, (2) (+)-catechin, (3) valoneic acid dilactone (ellagic acid derivative), and (4) ellagic acid. Protocatachuic and *p*-hydroxybenzoic acid were also identified using the LC-ESI-MS but are not shown on the above chromatograms.

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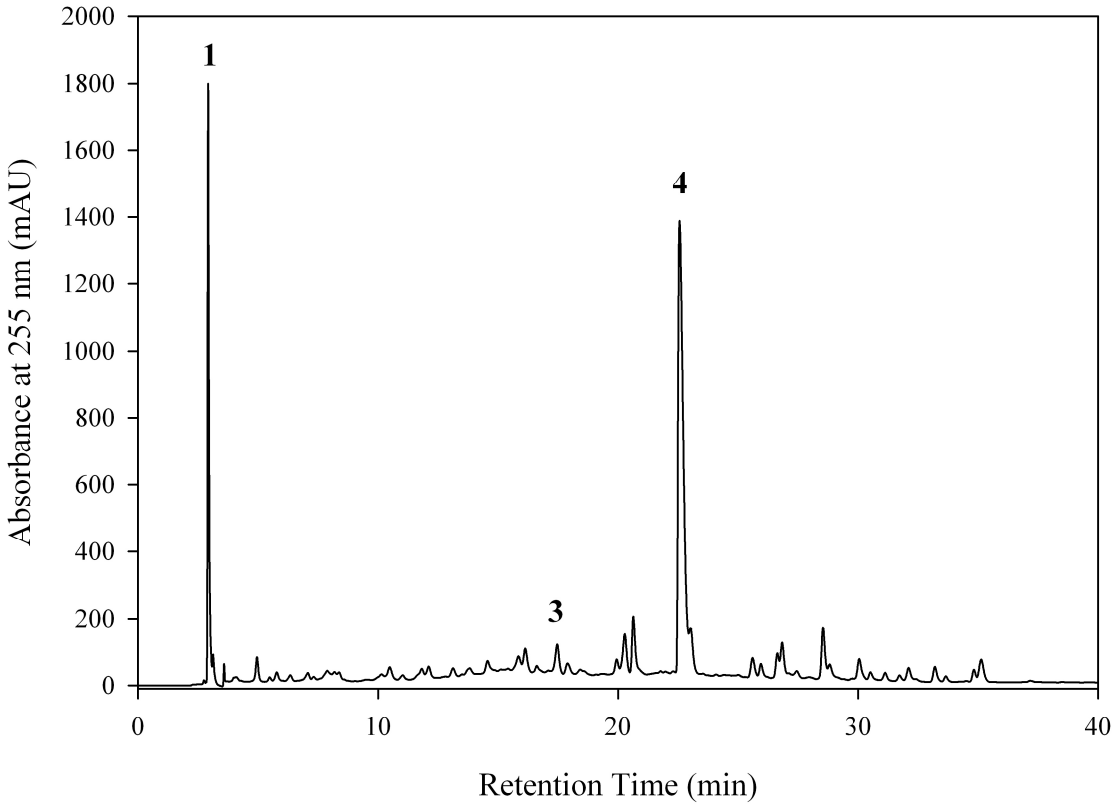
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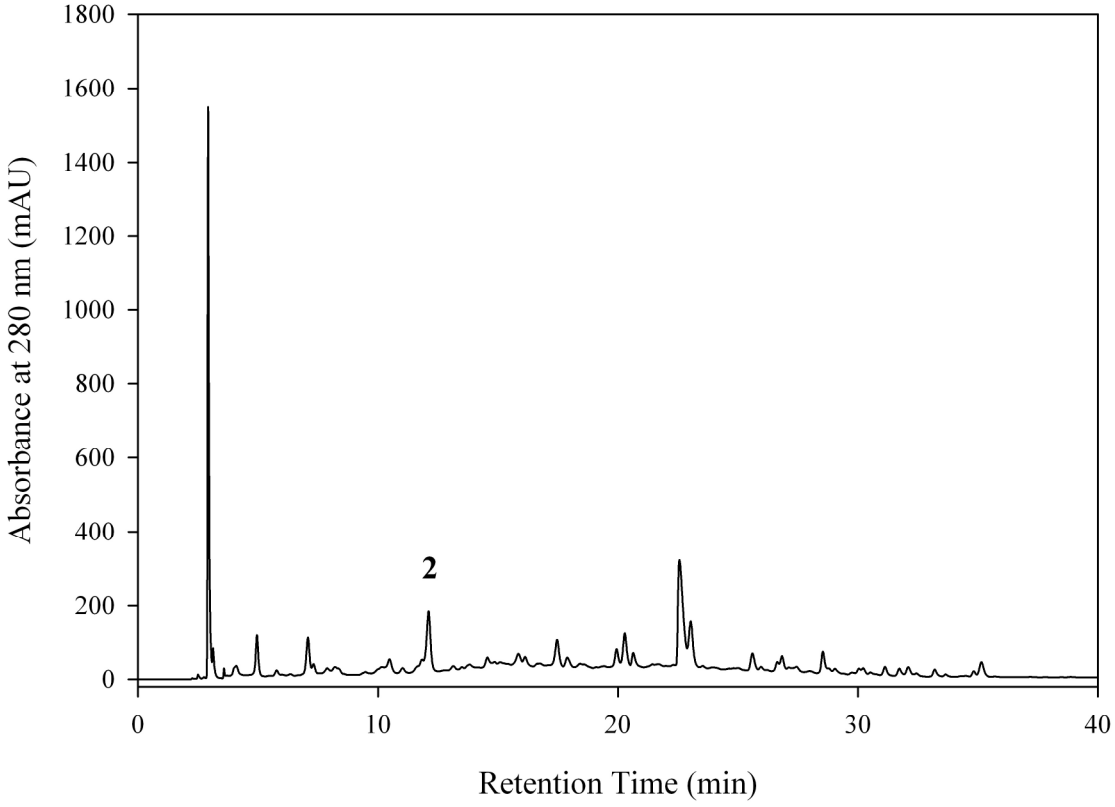
1b.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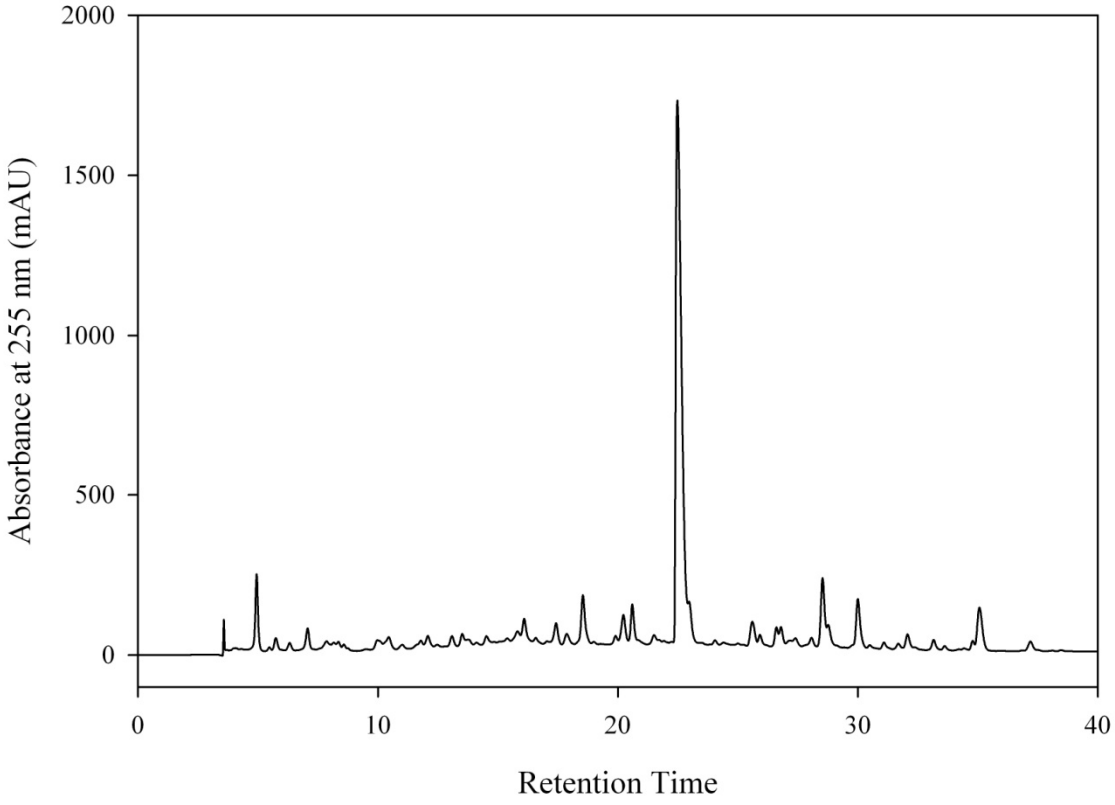
Retention Time (min)

1c.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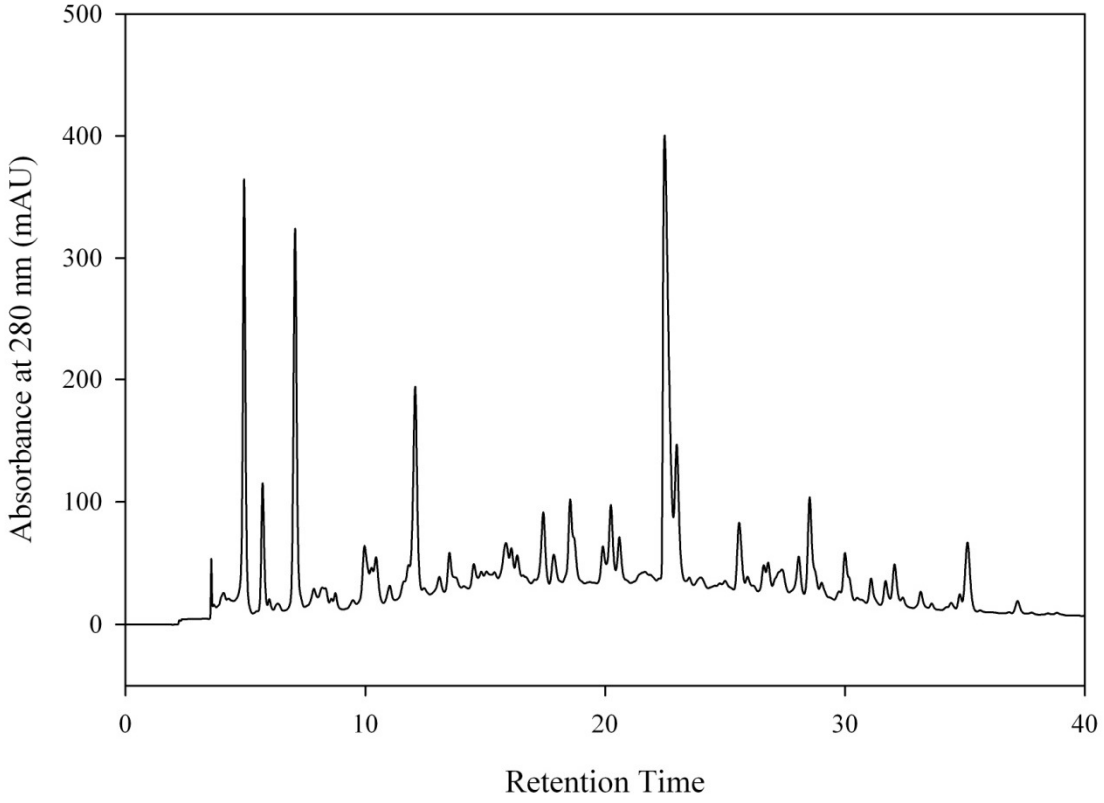
Retention Time

1d.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Retention Time

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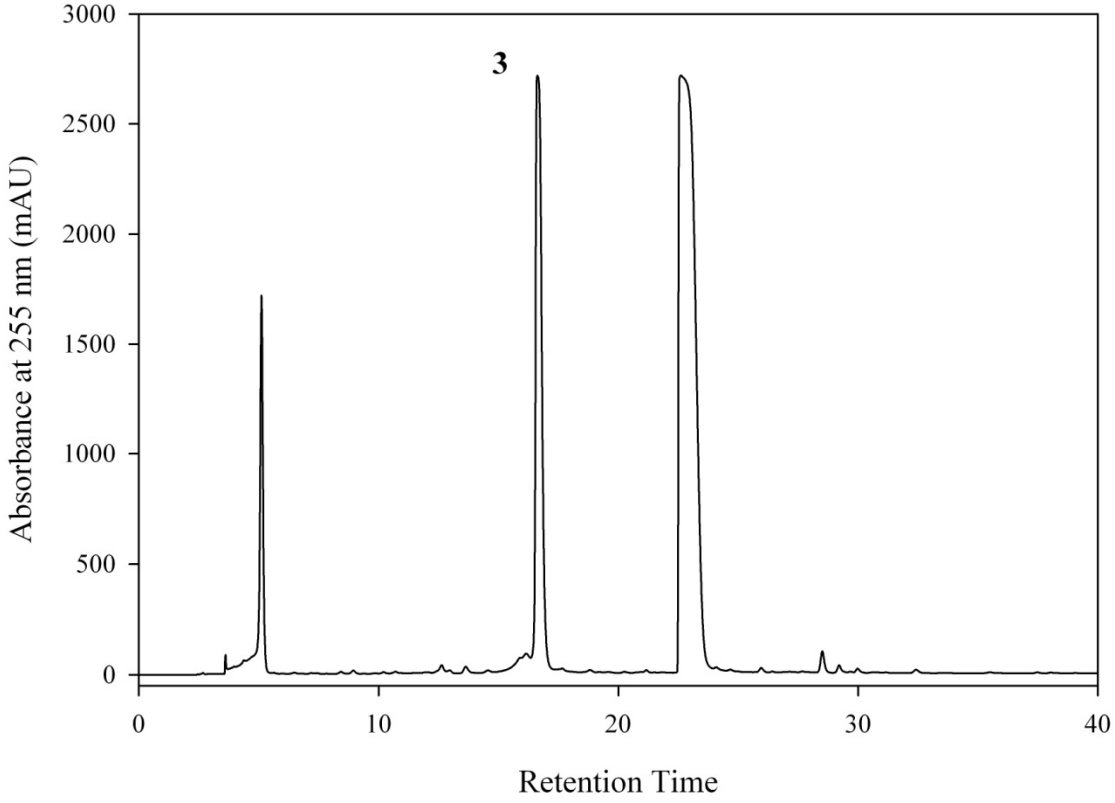
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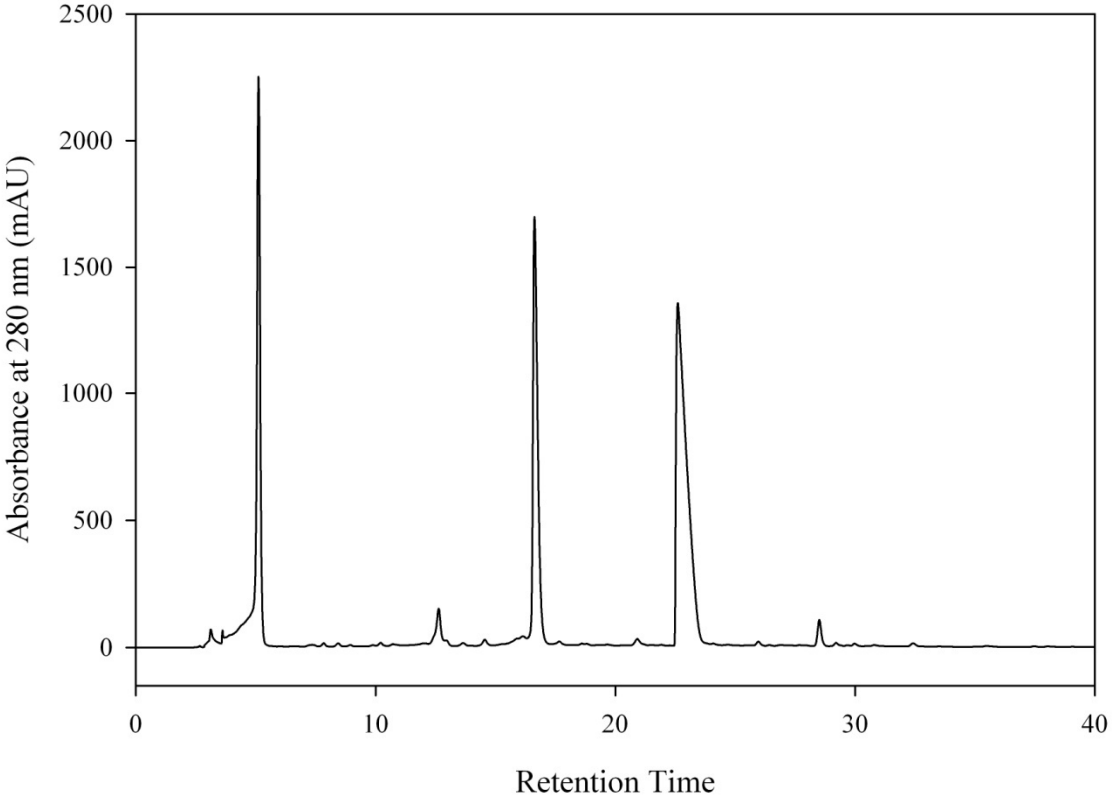
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Retention Time

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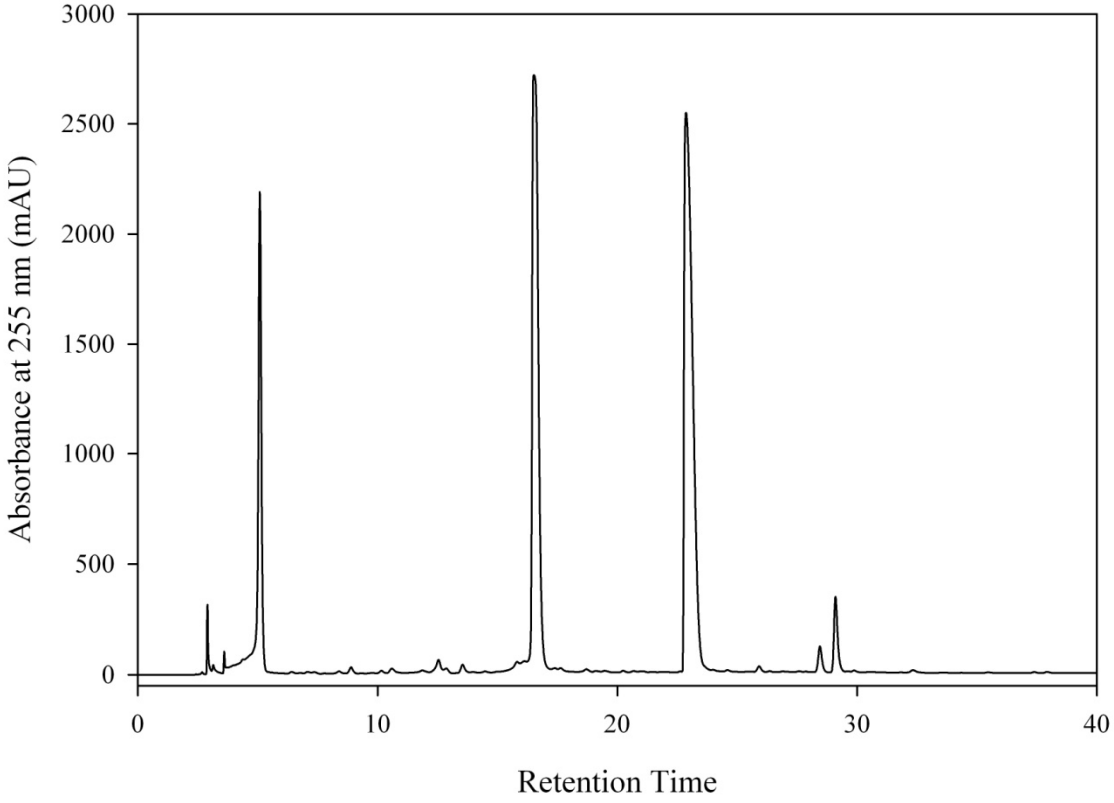
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Retention Time

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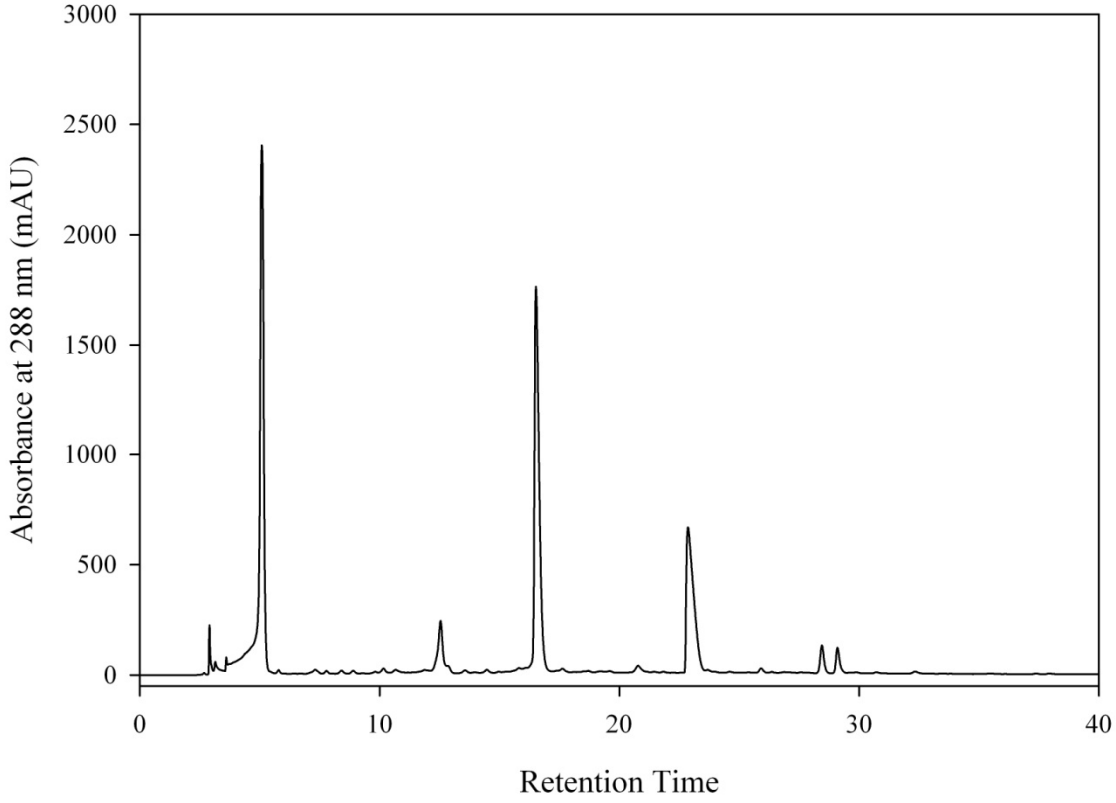
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Retention Time

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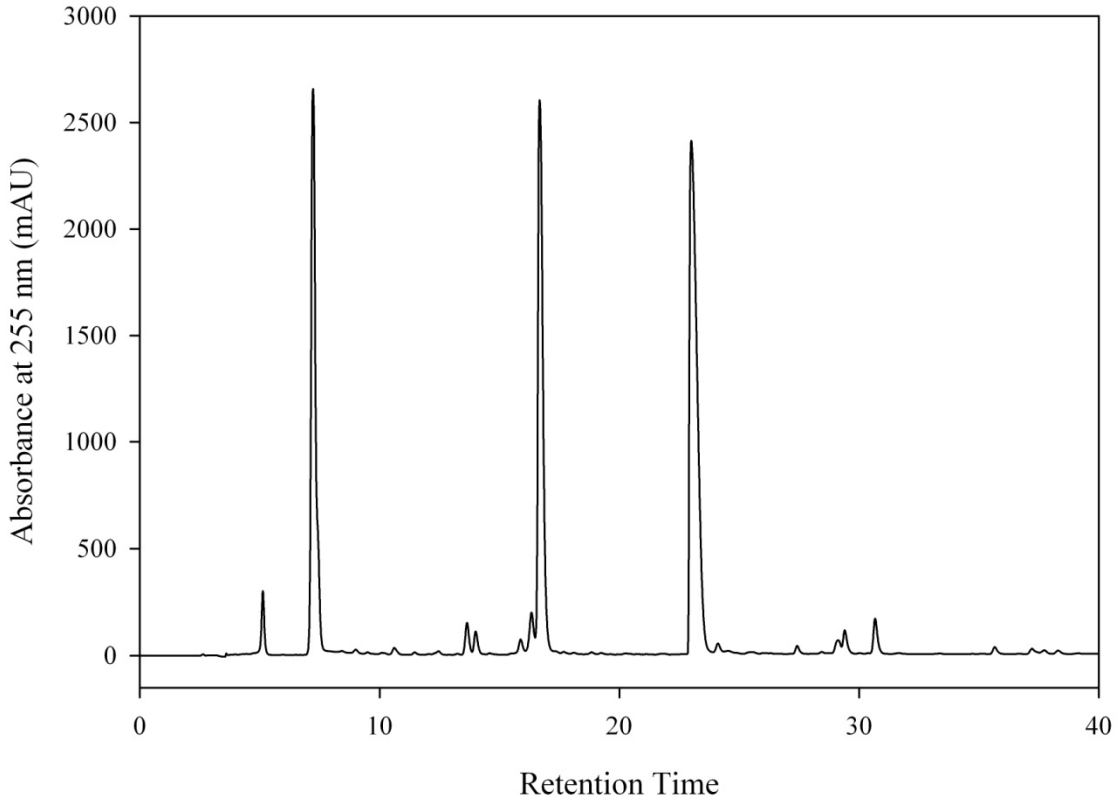
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Retention Time

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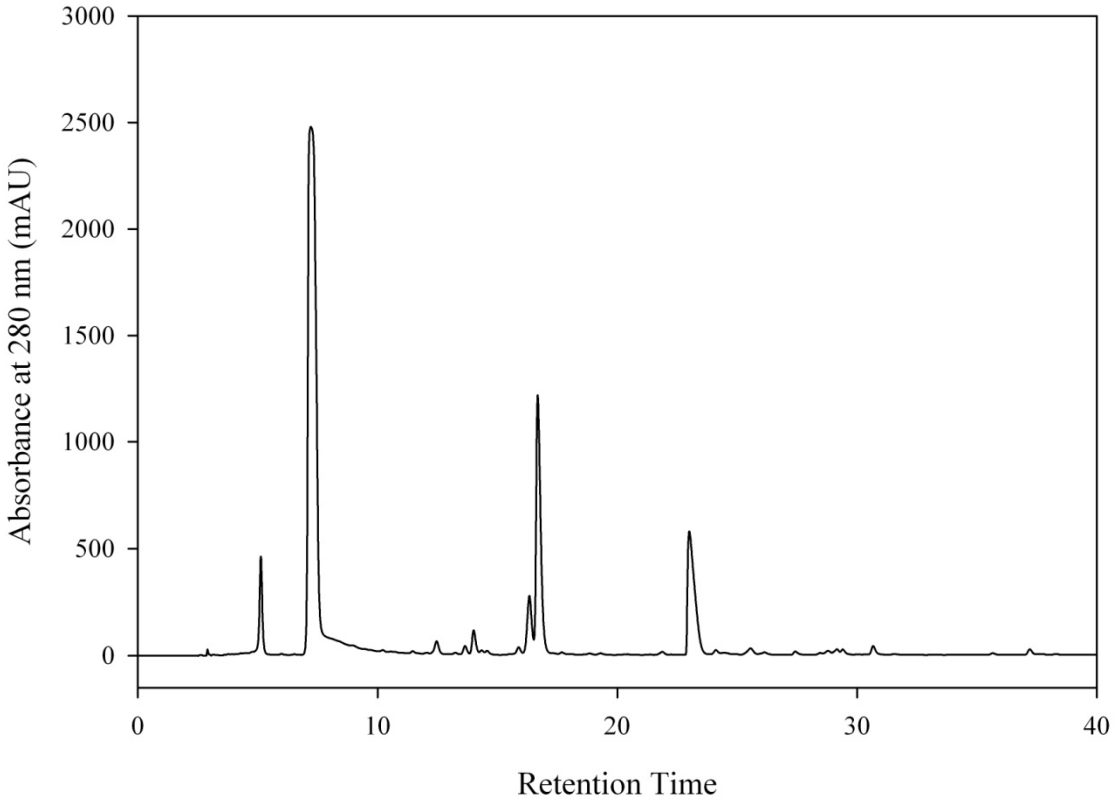
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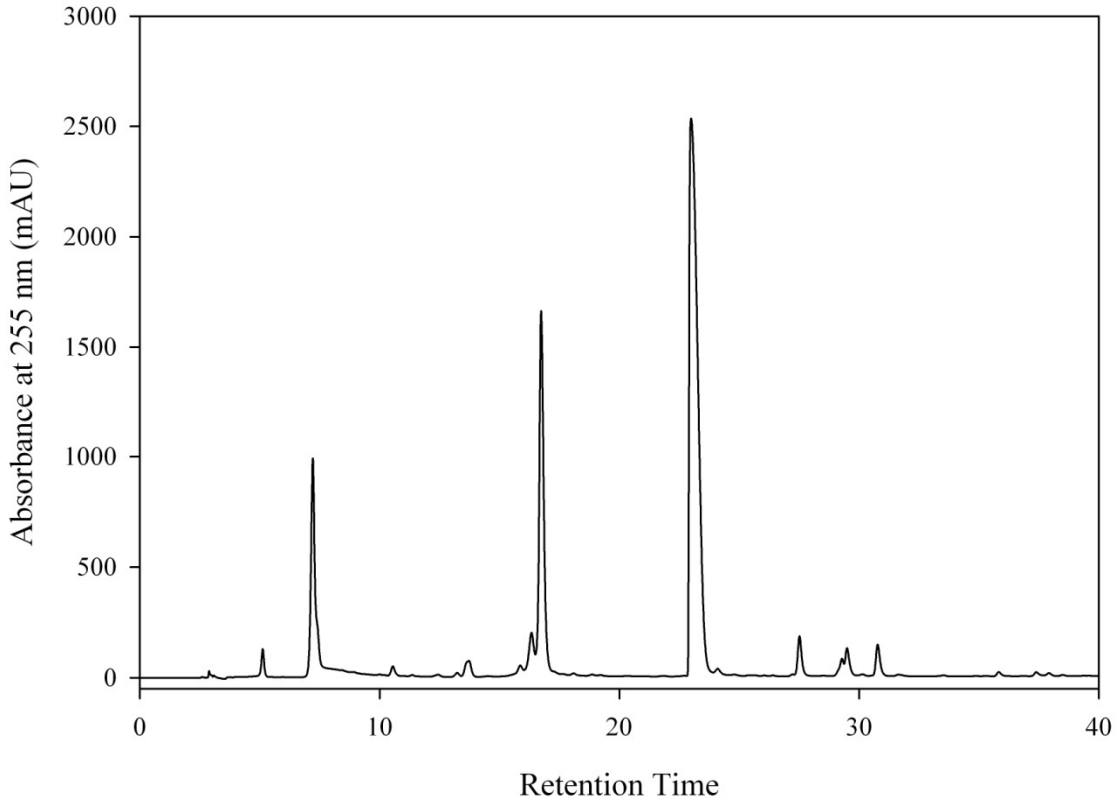
Retention Time

1k.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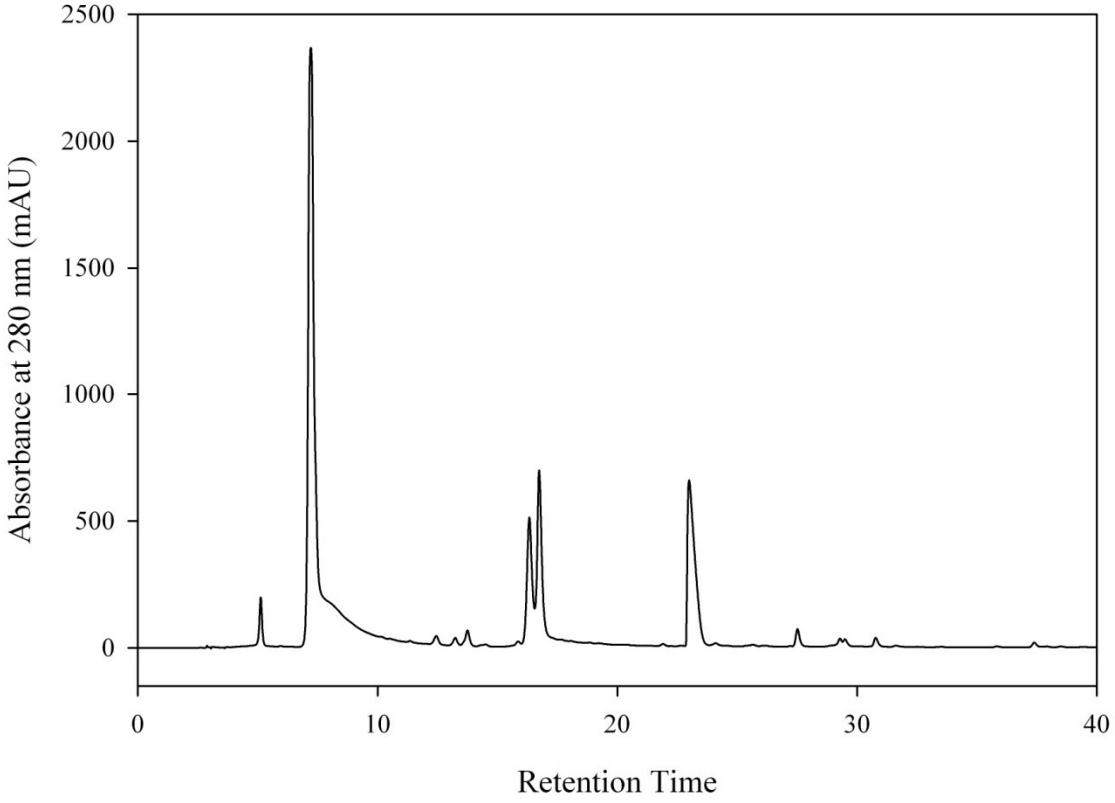
Retention Time

11.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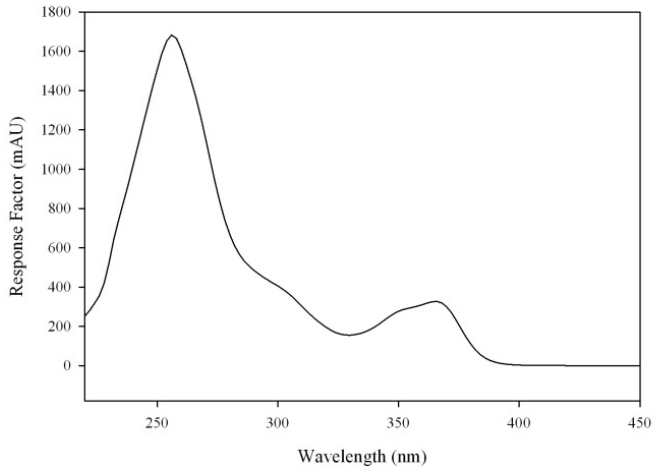
Retention Time

Figure 4.2.RP-HPLC-DAD UV Spectrum for (a) valoneic acid dilactone and (b) ellagic acid.

a.



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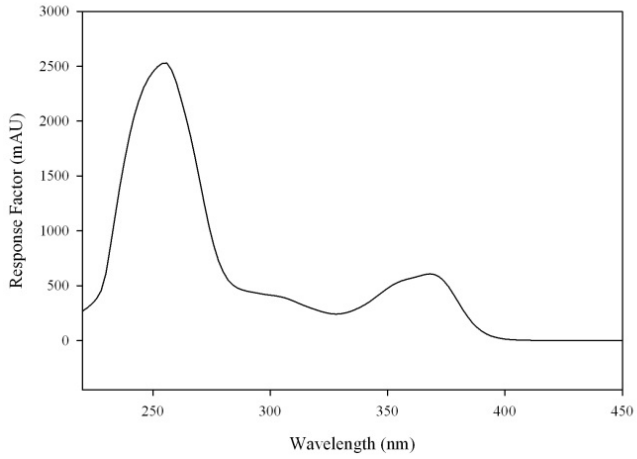

CHAPTER 5

FRACTIONATION AND CHARACTERIZATION OF U.S. PECAN PHENOLICS[1]

[1] Robbins, K. S.; Greenspan, P.; Pegg, R. B. To be submitted to *Journal of Agricultural and Food Chemistry.*

ABSTRACT

The phenolic acids and proanthocyanidins (PACs) of pecans possess bioactive properties which might be useful in retarding the onset of and ameliorating the status of certain chronic disease states. There is a general lack of informationin the literatureregarding such compounds, especially the PACs. Crude phenolic extracts pooled from eight commercially-significant cultivarswere selected based on their relatively high antioxidant capacities. The pooled extracts were separated via Sephadex LH-20 column chromatography into five ethanolic low-molecular weight (LMW) fractions and one acetonic high-molecular weight (HMW) fraction. Thepreparations were then characterized using RP-HPLC and NP-HPLC in order to determine the key constituents present in the LMW and HMW fractions, respectively. As previously observed in pecan nutmeat,ellagic acid and (+)-catechin were found to be the major phenolics in the LMW fractions. The last eluting LMW fraction was shown to contain few phenolic acids but instead possessed PAC monomers and dimers. The HMW fraction comprised a majority of its PACs as dimers; yet, monomers, trimers, tetramers, and pentamerswere also present.

5.1 INTRODUCTION

Pecans [*Carya illinoinensis* (Wangenh.) K. Koch] and other tree nuts are receiving increased attention in the literature due to their purported health benefits (1-4) and a qualified health claim approved by the U.S. Food and Drug Administration. Most reports associate these benefits with healthful lipid profiles (*i.e*. MUFAs, PUFAs, and γ-tocopherol), but more recently attention has turned toward the favorable phenolic profiles which tree nuts possess. Endogenous pecan phenolics can be segregated into two main classes, namely phenolic acids (*e.g*.ellagic and

gallic acids) and condensed tannins/PACs with varying degrees of polymerization (5). Characterization of these important classes, especially the PACs, is lacking in the literature (6).

PACs, the condensed tannins, are a type of phenolic polymer. There are two main types; that is, procyanidins and prodelphinidins. Procyanidins, the most prevalent PAC found in foods, are composed of (-)-epicatechin monomers whereas the prodelphinidins comprise (-)-epigallocatechin monomers (7). PACs are usually not classified based on the individual compound type, rather they are based on the degrees of polymerization which exist (8).The degrees of polymerization can range anywhere from 2 (dimers) to 10+ (polymers). The antioxidant properties and antimutagenic activityof PACs can vary based upon structure and degree of polymerization (7).

Pecan PACs and theirflavan-3-ol monomers have shown potential for important health-related benefits *in vivo* (6). In a feeding study, conducted by Hudthagosol *et al*. (6), panelists consumed meals of pecans or pecans blended with water. Afterwards, the plasma antioxidant capacities and the level of (+)-catechin and EGCG in the panelists' plasma were determined; both the concentrations of plasma catechin and EGCG increased! The authors found that the postprandial antioxidant capacity (assessed by both FRAP and $ORAC_{FL}$ assays) increased significantlyfollowing pecan ingestion. While the flavan-3-ol monomers can be absorbed in the small intestine, there is evidence that all PACs with a degree of polymerization of 3 and higher may not be able to be properly absorbed (9). Nevertheless, larger PACs may still participate in the health benefits of these compounds due to their breakdown into smaller secondary metabolites by colon microflora (10).

In this study, we employed Sephadex LH-20 column chromatography to fractionate pecan phenolic acids and PACs. Once separated, these fractions were characterized by TPC

analyses, UV spectral analysis, and RP-HPLC characterization for the LMW fractions and NP-HPLC and *n*-butanol/HCl analyses for the HMW fraction.

5.2 MATERIALS AND METHODS

Chemicals and Glassware. Sea sand, sodium carbonate, glass wool, ethanol (95%) and ACS-grade methanol, hexanes, *n*-butanol and acetone as well as HPLC-grade water, methanol, hexanes, isopropanol and acetonitrile were acquired from FisherScientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid and hydrochloric acid were purchased from VWR International, LLC (Suwanee, GA, USA). Sephadex LH-20, Folin&Ciocalteu's phenol reagent, ferric ammonium sulfate, (+)-catechin hydrate, gallic acid, ellagic acid and protocatechuic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Collection of Samples. Eighteen pecan cultivars were collected from test plots and pecan orchards in Georgia (11), New Mexico (2), and Texas (5) over a 3-month period.The nuts were raw and remained in-shell through transport, packaging and storage. After arrival to the Department of Food Science & Technology, Athens, GA, the pecan samples were placed in labeled pouches (Sealed Air Corporation, Elmwood Park, NJ), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to prevent any oxidative degradation, and stored at -80 °C until analyzed. On the day of analysis, pecan samples were removed from the freezer, shelled, and allowed to warm to room temperature to ensure a proper mass reading. Approximately 12 g of nutmeat was placed into a -20 °C freezer and allowed to partially refreeze to facilitate grinding. Each nut sample was then combined with ~60 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ), to a very

fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

Lipid Extraction. A Soxhlet apparatus was employed to extract the lipids from all pecan samples. Briefly, a known quantity of the ground matter (pecan and sand) was placed into a cellulose extraction thimble (single thickness, 43 mm I.D. and 123 mm external length, Whatman International Ltd., Maidstone, England) and the mass recorded. Glass wool was placed in the top of the thimble to ensure that the contents would remain in place during extraction. Extraction was performed using ~350 mL of hexanes for 20 h. Upon completion, the thimbles were removed and allowed to dry overnight. The hexaneswereremoved from the lipid extract using a BüchiRotavapor R-210 (Büchi Corporation, New Castle, DE). The lipid portion was weighed for gravimetric analysis. Extracted lipids were transferred to amber-colored vials, flushed with N_2, capped, and placed in the -80°C freezer until analyzed.

Phenolic Compound Extraction. The defatted pecan kernel was removed from the dried thimble and placed into an Erlenmeyer flask. As employed by Wu *et al.*(11), an $(CH_3)_2CO/H_2O/CH_3COOH$ solvent mixture (70:29.5:0.5 v/v/v, 100 mL) was used to extract phenolic compounds. Extraction was carried out according to Craft *et al.* (12). Briefly, the contents in the flasks were heated at 50°C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed three times and the supernates pooled. Acetone was evaporated from the pooled supernate using the Rotavapor.The aqueous portion was poured into crystallization glasses, covered with filter paper, and placed in a -80 °C freezer until completely frozen. The samples were then lyophilized (LabconcoFreezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS). The dried extract was placed in amber-

colored vials, capped and stored at 4 °C until ready to be used. A lipid and phenolic extraction was completed in triplicate for each cultivar.

Sample Selection and Extract Separation. Based on preliminary data, the eight cultivars with the best performance overall in H-ORAC$_{FL}$ assays were selected for use in this study. A 250-mg sample of each crude pecan extract was pooled. The resultant extract (2 g) was mixed in a small volume of ~75% (v/v) ethanol and sonicated to ensure that it was completely dissolved. The sample was then applied via a Pasteur pipette to the top of a chromatographic column packed with Sephadex LH-20(bead size: 25-100 μm; Chromaflex column, 30 x 400 mm [I.D. x length], Kontes, Vineland, NJ, USA). The LMW phenolic compounds were eluted with ~1.5 L of 95% (v/v) ethanol. Fractions (15 mL) were collected using a Beckman Coulter SC100 collector (Fullerton, CA, USA). The eluent was then switched to 50% (v/v) aqueous acetone and a HMW phenolic fractionwas eluted from the column with ~600 mL of the second mobile phase.

Fraction Determination. LMW phenolic fractions were analyzed using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) scanning across a wavelength range of 200 to 400 nm. Spectral differences were used as a guide to group the collected fractions into five pooled lots. After combining, the ethanol was evaporated using a BüchiRotavapor R-210 (Büchi Corporation, New Castle, DE) with the water bath set at 45°C. To ensure complete removal of solvent and moisture, the pooled fractions were lyophilized(LabconcoFreezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS)and then stored in amber-glass bottles at 4°C until analysis. Apropos the HMW fraction: acetone was also removed *in vacuo* and the aqueous residue lyophilized and stored as described above.

TPC Assay. The total phenolics contents (TPC) of the ethanolic fractions were determined based on a method adapted from Swain and Hillis (13) employing Folin-Ciocalteu's

phenol reagent. Each fraction was diluted to 0.20 mg/mL in anhydrous methanol. The assay was performed using 1 mL of methanolic extract, 7.5 mL deionized water, 0.5 mL Folin-Ciocalteu's phenol reagent, and 1 mL of saturated sodium carbonate.The resultant solution was vortexed for 30 s. The tubes sat for 1 h to allow for optimal color development. The absorbance of the resulting chromophore from this colorimetric reaction was measured at $\lambda = 750$ nm using the Agilent spectrophotometer.A standard curve was prepared using known concentrations of working solutions of (+)-catechin(1.6 to 8.0 μg/mL). TPC values are reported as g catechin eq./g ethanolic extract.

***n*-Butanol/HCl Hydrolysis.** The method of Porter *et al*. (14) was employed to gain a better understanding of the PAC content of the acetonic tannin fraction separated on the Sephadex LH-20 column. Briefly, an *n*-butanol/HCl solution was prepared by mixing 475 mL of *n*-butanol with 25 mL of concentrated HCl.In a 10-mL capped tube, 6 mL of the *n*-butanol/HCl reagent, 1 mL of the tannin fraction (1 mg/mL CH_3OH), and 0.2 mL of an iron reagent (2 % (w/v) ferric ammonium sulfate in 2 M HCl) were combined. The contents in the tubes were thoroughly mixed, loosely capped, and placed in a boiling water bath for 50 min. The tubes were then cooled, contents transferred to 25-mL volumetric flasks, and brought to volume with the *n*-butanol/HCl reagent. The absorbance of the solutions was measured using the Agilent spectrophotometer at $\lambda = 550$ nm. The results or triplicate determinations were reported as mean absorbance values at 550 nm/mg tannin fraction.

HPLC Characterization. *Extraction and Separation of Bound Phenolics.* To facilitate phenolic identification, an acid and base hydrolysis was performed on the fractions according to Amarowicz and Weidner (29). Briefly, ~600 mg of the pooled LMW fractions were suspended in 20 mL of acidified water (pH 2.0, 6 M HCl). Using a separatory funnel, the free phenolic

acids were extracted with 5 x 20-mL portions of diethyl ether. The pooled ether layer was then evaporated to dryness. The remaining aqueous solution was neutralized with 2 M NaOH to a pH of 7, the vial flushed with N_2, capped, and the mixture allowed to hydrolyze at room temperature for 4 h. After hydrolysis, the pH was reduced to 2 using 6 M HCl; the phenolic acids released from soluble esters were then extracted with diethyl ether (5 x 30 mL). The organic portion was once again evaporated. The aqueous solution was combined with 15 mL of 6 M HCl, flushed with N_2, and placed in a 100 °C oven for 1 h. Phenolic acids liberated from soluble glycosides were then extracted using 5 x 45 mL portions of diethyl ether. The pooled ether layers were once again evaporated. The recovered products were dissolved in 2 mL of anhydrous methanol, filtered through a 0.45-µm nylon filter and then injected onto an HPLC column.

Reversed-Phase HPLC Characterization (RP-HPLC). The RP-HPLC method reported by Srivastava*et al.* (15) was followed to characterize the crude extracts of the pecan cultivars. An Agilent 1200 series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA) was employed for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 x 250 mm, 5 µm; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 mL/min was employed. A volume of 100 µL was injected for each of the acetonic extracts (4.0 mg/mL methanol). Detection wavelengths were λ = 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids, notably of the *trans*-cinnamic

acid family), and 360 nm (flavonols). Tentative identification of separated components was made by matching UV-VIS spectra and retention time mapping with standard compounds.

Normal-Phase HPLC Characterization (NP-HPLC). PACs were separated based on their degree of polymerization according to Kelm*et al.* (16). The same Agilent 1200 Series HPLC for the reversed-phase analyses was used, but with an AstecDiol HPLC column (250 mm × 4.6 mm, 5 µm particle size; Supelco, Bellefonte, PA)and a fluorescence detector. A gradient elution consisting of mobile phase A [(A) CH_3CN:CH_3COOH (98:2, v/v) and(B) CH_3OH:H_2O:CH_3COOH (95:3:2, v/v/v) was utilized. The linear gradient was completed at 1.0 mL/min as follows: 0-35 min, 0-40% B; held for 5 min; 40-45 min, 40-0% B and then an additional 5 min hold in order to equilibrate the system. A volume of 20 µL was injected for each sample; they were first dissolved in anhydrous CH_3OH (20 mg/mL) and then further diluted 1:9 (v/v) with mobile phase A to a final concentration of 2 mg/mL. Samples were passed through a 0.45-µm PTFE syringe filter prior to injection. Fluorescence detection was utilized with excitation/ emission at 276/316 nm, respectively. Commercial purified standards were analyzed to determine the retention times and area values for two monomers [(+)-catechin and (-)-epicatechin], three dimers (B2, 2a, 2b), and a set ofpolymers of varying degrees ranging from three to ten. Quantification of samples was based on standard retention times and area values.

Statistical Analysis. Mean concentrations of the TPC and phenolic acids for the five fractions are reported.

5.3 RESULTS AND DISCUSSION

Fraction Determination. Fractions eluting a Sephadex LH-20 column with ethanol were analyzed spectrophotometrically. Based on similarities and observed differences in the spectra

of the samples, pooling yielded five fractions (Figure 5.1). Fraction I can be characterized by a large peak at ~240 nm and a smaller peak at ~350 nm. As fraction I transitioned to fraction II, there appeared to be a shift to the left of the peak maximum. Fraction II can be characterized by a maximum absorbance at 208 nm and then a gradual decrease in absorbance with increasing wavelength. There is a slight rise in the absorbance at ~260 nm. The transition from fraction II to fraction III was characterized by an increase in peaks at 255 nm and 278 nm. Final development of fraction III was represented by a large peak at ~210 nm and a secondary peak with a maximum height at 278 nm. The transition from fraction III to IV was identified by a drastic increase in absorbance at ~250 nm. Fraction IV contained peak maxima at ~255 nm with minor peak maxima at 208 nm and 354 nm. Fraction V was highlighted by a reduction in all peaks to almost zero producing only a slight peak at 210 nm. After freeze drying, the mass of each fraction was recorded. Fraction I comprised 77.4 % of the total mass fractionated. The acetonic tannin fraction, referred to as VI, represented 16.5 % of the mass. Fractions II, III, IV, and V each made up less than 5% of the total mass with mass percentages of 3.6, 0.6, 1.0, and 0.7%, respectively.

Total Phenolics Content. The TPC was determined for each of the pooled fractions (Table 5.1). The TPC value is an indication of the quantity of phenolic compounds in a sample, and it can potentially provide information as to the antioxidant capacity of a sample. Fractions I and II had significantly lower TPC values than fractions III-V. Fraction III gave the highest TPC value of 197 ± 4 mg CE/g fraction. Similarly, fractions IV and V exhibited high values. This indicates that most of the phenolic compounds within the LMW fraction are in III-V.

RP-HPLC Characterization. RP-HPLC was employed to profile the phenolics in the pooled ethanolic fractions. Four main groups of phenolic acids were identified across the five

fractions: gallic acid, protocatachuic acid, catechin, and ellagic acid. These compounds were quantified in each sample and reported as mg phenolic/g fraction (Table 5.2). Fraction V did not appear to contain any phenolic acids identifiable by RP-HPLC. It was suspected that the ethanol may have started to elute some of the LMW tannin compounds. This was confirmed through the use of NP-HPLC and by observing the spectral pattern of fraction V (Figure 5.1). All peaks in the wavelength range of 250 to 350 nm disappeared from fraction IV to V. Both monomers and dimers were identifiable in fraction V. For this reason, Table 5.2 only contains data for fractions I-IV.

Fraction I is believed to contain a high amount of 'filler' isolated from the pecan crude extract. Even though it possessed the greatest mass content of the fractions, it displayed the lowest TPC. In fact, there were practically no identifiable phenolic acids present in the chromatogram for fraction I. The only acid identified was protocatachuic acid, which was identified based on its retention time and UV spectrum as compared to commercial standards. Fraction II also displayed a comparatively lower TPC than that of fractions III-V. Three phenolic acids were identified in fraction II: gallic, catechin, and ellagic acid. While gallic acid was identified based on its retention time and UV spectrum, it was below the limit of quantification. Catechin and ellagic acid contents of 16.1 and 17.2 mg/ g fraction, respectively, were quantifiable but represented the lowest proportion of those acids in any of the fractions. This pattern can be observed in the spectra of fraction II (Figure 5.1). There is a large peak below what could be expected for any of these phenolic acids, and then there is a slight rise around 255 nm signifying the presence of ellagic acid.

Fraction III is comprised of ellagic acid and its derivatives. It should be noted that there were several peaks present in the RP-HPLC chromatogram for fraction III that contained spectral

patterns similar to those of ellagic acid and for this reason were quantified as ellagic acid derivatives, adding to the total content values (Table 2). Of all the fractions analyzed, fraction III possesses the greatest content of ellagic acid. Based on the spectral pattern for fraction III, it was assumed that a high proportion of its fraction consisted of ellagic acid due to its peak maximum near 255 nm (Figure 5.1).

Fraction IV had almost three times more (+)-catechin, 47.7 mg/g fraction extract, than any of the other fractions (Table 5.2). Furthermore, (-)-epicatechin was found to be present in fraction IV based on the presence of a peak with a later retention time than catechin and with a UV spectrum similar to that of both catechin and an epicatechin commercial standard. Due to the small contribution of this peak, it was not quantified and therefore is not represented in the total. There was a small quantity of ellagic acid present in this fraction, 4.97 mg/ g fraction but nothing compared to its content in fraction II or III. It is not surprising that fraction IV is composed mainly of catechin and epicatechinbecause the spectral pattern for fraction IV shows a large peak at ~280 nm (Figure 5.1).

While there is no other study in the literature that attempted to examine individual fractions of pecans separated by SephadexLH-20 column chromatography, the main phenolic acids identified here match those reported in pecans (5, 17, 18) as well as our investigations into the phenolic acids present in pecan crude extracts (Chapter 4). Comparison of the actual mass is difficult due to differences in how the data was reported. A gravimetric analysis was conducted through the whole process, but it is believed that presenting the data as mg/g fraction is the most accurate. With the use of the spectrophotometer and all of the steps involved in the process, it is believed that accuracy would be lost if contents were taken all the way out to the pecan nutmeat level.

The pooled LMW fractions were hydrolyzedto release phenolic acids from soluble esters and glycosides. These compounds are highlighted in the chromatograms in Figure 5.2a-f. The most prominent compounds are outlined in 5.2a-2b as (1) gallic acid, (2) protocatachuic acid, (3) catechin, (4) *p*-hydroxybenzoic acid, (5) valoneic acid dilactone, and (6) ellagic acid. The main phenolic compounds present across all three categories were catechin and ellagic acid with contents of 1.35 and 1.37 mg/g LMW fraction, respectively (Table 5.3). Present in the fractions but in smaller amounts were gallic, protocatachuic, and p-hydroxybenzoic acids. Notably, the *p*-hydroxybenzoic acid and ellagic acid had their highest contents present as phenolic acids liberated from soluble glycosides. Compared to other samples previously analyzed, there was not a significant contribution to the phenolic total of compounds bound as soluble glycosides besides these two compounds (Figure 5.2e-f). The catechin total was split between the free phenolic acid form and the phenolics present as soluble esters.

Valoneic acid dilactone, an ellagic acid derivative found in large quantities in crude pecan samples (Chapter 4), was not present in any quantifiable form in these samples. It has been hypothesized that its occurrence can be indicative of the presence of ellagitannins (17). It can be assumed that in a sample separated by Sephadex LH-20 column chromatography, the LMW fraction should contain little to no tannin constituents. The lack of valoneic acid dilactone compared to the crude extract samples seems to suggest that there was an overall lack of hydrolyzable tannins in the sample and that the valoneic acid dilactone appears in samples only through the breakdown of these tannin compounds. Other potential ellagic acid derivatives were observed in the free phenolic acid chromatogram (Figure 5.2) based on their UV spectral patterns. A positive identification of these compounds is not possible without the use of LC-MS

technology, but it is assumed that these compounds may be building blocks of ellagic acid and ellagitannins.

***n*-Butanol/HCl Assay for Procyanidins.** The *n*-butanol/HCl assay has been shown to be a good indicator of the procyanidin content of a sample (14). The values are reported as the absorbance at 550 nm/mg of sample analyzed. While this analysis is a relative measure (*i.e.*, there is no standard comparison or equivalence reporting), the assay is able to give some prediction as to what one can expect for the PAC content of a sample. In this study, the *n*-butanol/HCl assay was performed on the tannin fractions (Table 5.4). Interestingly, the pooled cultivar sample showed a value of 0.711 ± 0.042 whereas the single cultivar gave 0.752 ± 0.006. Though the single cultivar sample had a higher value than the pooled one in the three repetitions analyzed, with a larger sample size there may not be any significant differences between the samples.

NP-HPLC Characterization. The tannin fraction of two samples fractionated by Sephadex LH-20 was analyzed via NP-HPLC. The two samples were chosen for analysis to compare a pooled cultivar sample with a single cultivar sample. The chromatograms of these samples were similar in terms of their monomers, dimers, and trimers (Figure 5.3). The pooled cultivar possessed degrees of polymerization from monomers to pentamers, whereas the single cultivar sample only had monomers to tetramers. The observed differences in degrees of polymerization may be due to the fact that the pooled cultivars are made up of the top eight performing cultivars from preliminary antioxidant assays. The single cultivar chosen had an antioxidant activity that was more in the mid-range of the cultivars analyzed. These results show that tannin fractions, in particular those with the higher degrees of polymerization, may play a role in determining the observed *in vitro* antioxidant activity of a sample. The pooled sample

had 16.7 % of its tannin fraction present as tetramers or larger while the single cultivar had only 4.3 % tetramers or larger.

For both of the samples, the greatest proportion of the tannin fraction was present as dimers. Dimers were present in 56.7% of the tannin fraction of the pooled cultivars, whereas the single cultivar had 62.1%. The next largest degrees of polymerization were the trimers with 25.3 and 33.2% for the pooled and single cultivar sample, respectively. Both samples had similar quantities for the catechin and epicatechin monomers with contents of 0.289 and 0.212 mg/g tannin extract for the pooled and single cultivar samples, respectively. The similarity in monomer content can most likely be attributed to the similarity in the method of separation by the Sephadex LH-20. It is to be expected that the majority of the monomers are present in the ethanolic fraction and are only found in small quantities in the acetonic HMW fraction.

Gu *et al*. (8) reported the PACsare present in numerous different food types including pecans. They reported that the monomers, dimers, trimers, 4-6mers, 7-10mers, and >10mers constituted 3.4, 8.5, 5.3, 20.5, 17.0, and 45.2 %. It should be noted that in the present study, we did not find any PACs larger than pentamers present. With the removal of the 7-10mers and >10 mers, from the Gu *et al*. data (8), the monomers, dimers, trimers, and 4-6mers now account for 9.2, 22.5, 13.9, 54.3 %, respectively. In comparison to the prevalence of tetramers-hexamers in the former study, the present one found dimers to be most abundant.

Earlier methods of PAC analysis (*i.e*., TLC analysis, RP-HPLC, and early NP-HPLC methods) were discredited for failing to separate compounds with degrees of polymerization greater than 4 (19). However, in preparing for this research, standards were run with degrees of polymerization of 8, 9, and even 10 with fine resolution and detection (Figure 5.4). In a study by Kelm*et al*. (16), in which the NP-HPLC methodology was first introduced, there was also

excellent resolution up to a degree of polymerization of 14. For this reason, it is suspected that these higher degrees of polymerization would have resolved properly if present in the samples.

It has also been noted that when PACs are separated on Sephadex LH-20 via isocratic elution, the first compounds to elute will be those PACs with smaller molecular weights and the heavier PACs will elute last (20). A possible reason for a reduction in the quantity of the larger PACs in our study could be that enough of the solvent system was not employed to properly elute these PACs from the column. Based on the mass balance performed in this work, it is not believed that this could account for the extreme differences observed. A total of 1.92 g of crude extract was loaded onto the LH-20 column and 1.90 g of the various fractions were recovered after lyophilization. This signifies that 99% of the crude extract was recovered through the experiment and that very small quantities of higher degrees of polymerization, if they existed, could remain on the column. Unfortunately, the Gu *et al*. (8) study is the only literature source that quantifies and characterizes PAC components of pecan nutmeat. Further and more extensive characterization of these compounds is necessary to determine more information about the contents of pecans, in particular the HMW PACs. By utilizing LC-MS/MS technology, one may be able to further elucidate the structure of these compounds in pecans from various growing locations and cultivar types. LC-MS/MS analysis has previously been used to characterize the PAC profile of peanut skins (21).

Researchers aim to quantify and characterize phenolic compounds in natural products due to their beneficial health benefits. PAC compounds have been shown to remain intact in the low pH environment of the stomach and arrive to the small intestine intact (22). In thisform, the PACs are unable to be absorbed in large quantities (10). Colon microflora is able to breakdown PAC polymers to smaller metabolites which can be absorbed and utilized (10). There is

additional evidence that these small metabolites may be beneficial in reducing the efficacy of cancer cell lines (23) and should not be overlooked. However, the full benefit of these metabolites has not yet been fully understood makingpredictions of biological activity of the higher degree polymers difficult. Hudthagosol *et al*. (6) demonstrated that concentrations of the main PAC monomers, catechin and epicatechin, increased in the plasma of individuals after pecan consumption. These results are encouraging for the consumption of these flavan-3-ol monomers and other lower molecular weight PACs. Further research into the bioavailability and digestion of PACs needs to be investigated in order to gain a better perspective of the role of these compounds in disease prevention.

5.4 ACKNOWLEDGEMENTS

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Figure 5.1. Spectral characteristics of five ethanolic fractions isolated from crude pecan extract using Sephadex LH-20 chromatography.

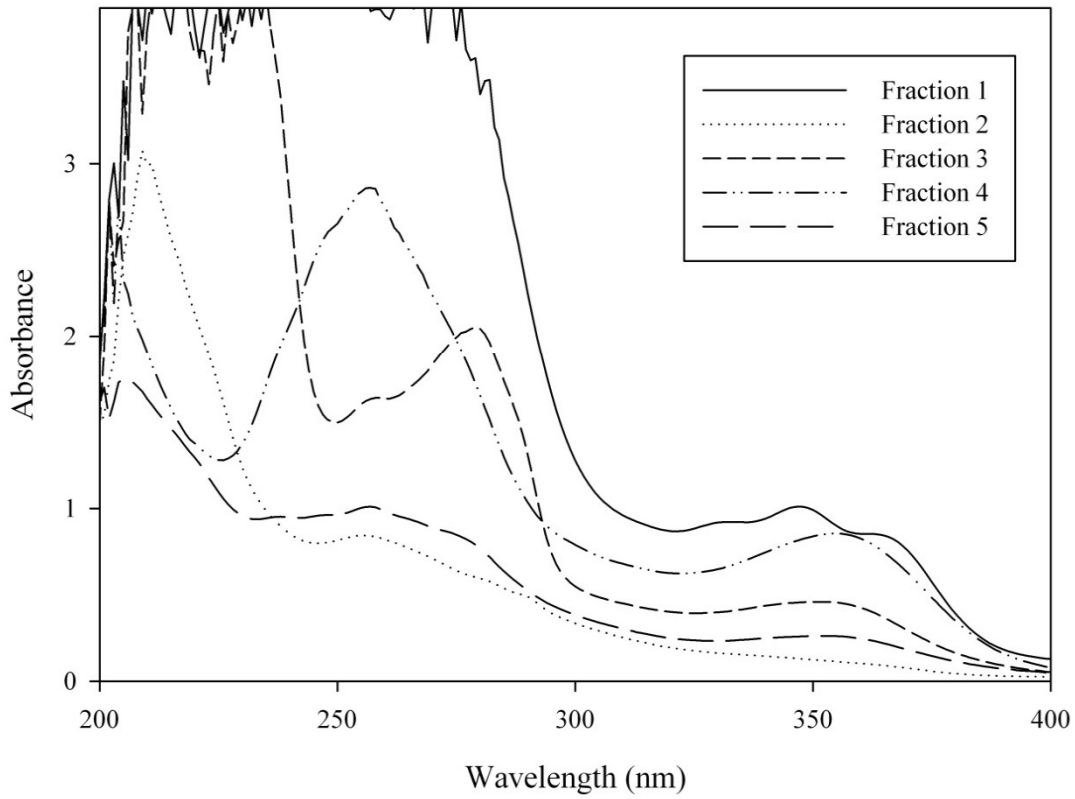
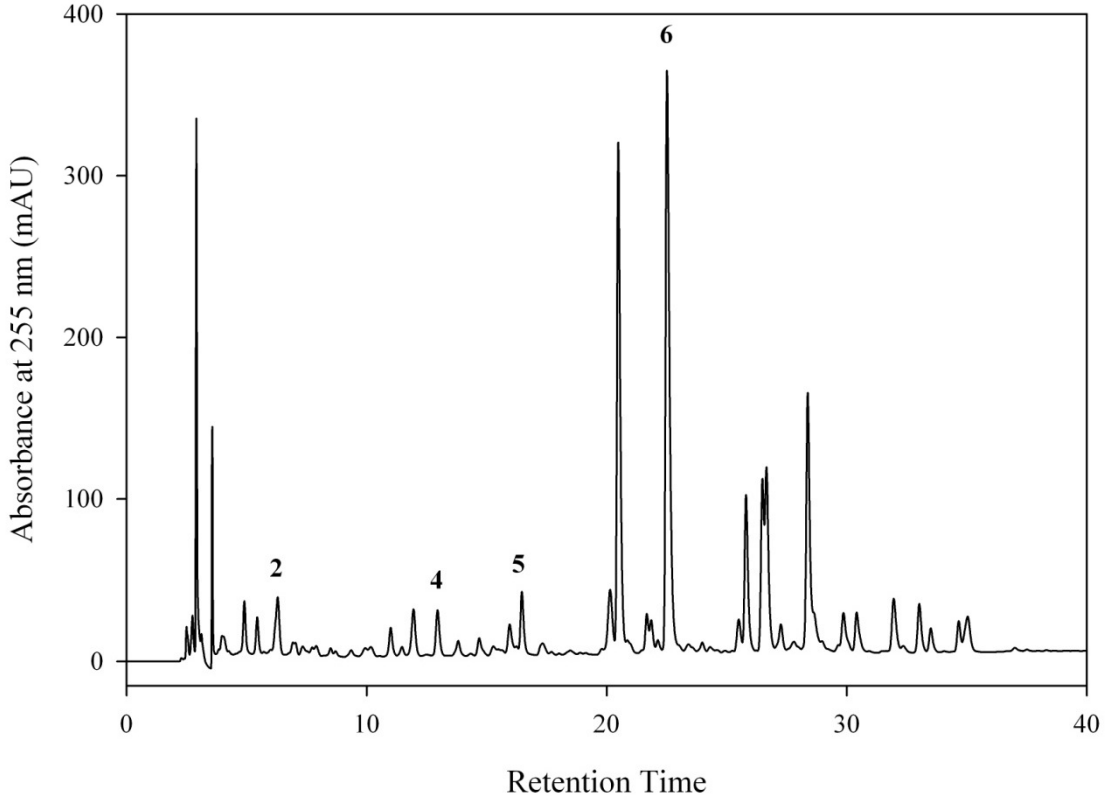
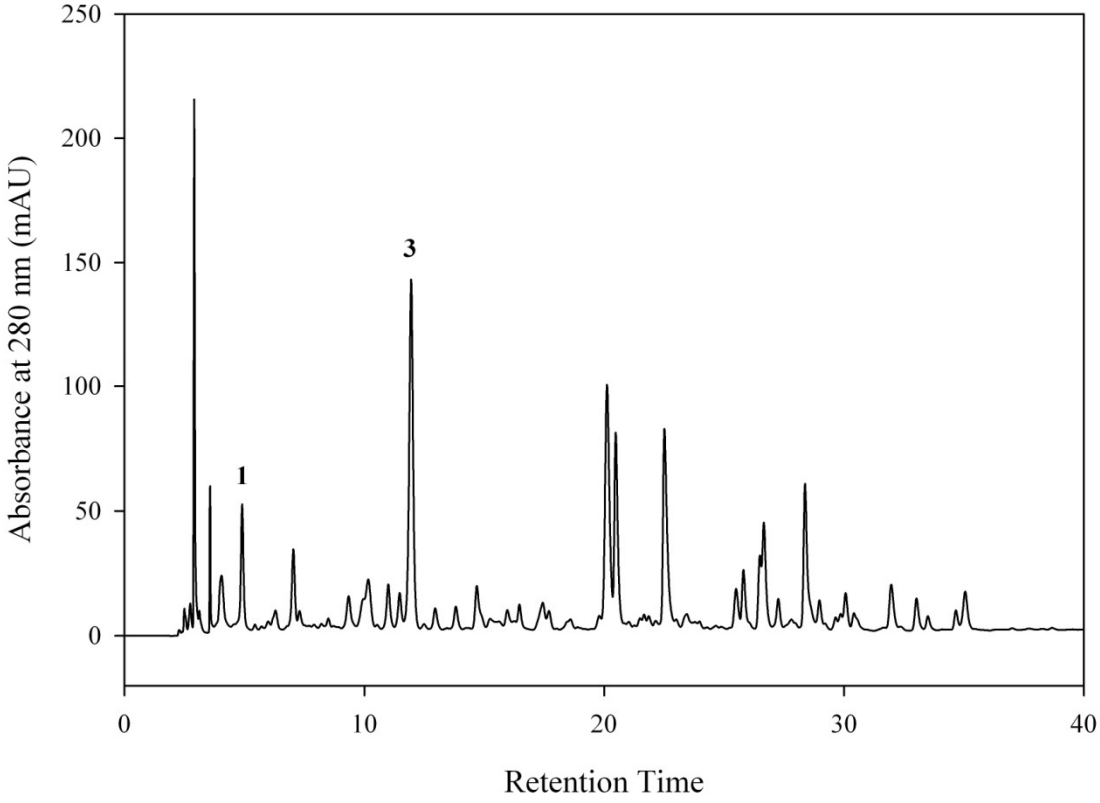


Figure 5.2. RP-HPLC Chromatograms of a pooled LMW extract (fractions I-V) (a) Free phenolic acids at 255 nm. (b) Free phenolic acids at 280 nm. (c) Phenolics isolated from soluble esters at 255 nm. (d) Phenolics isolated from soluble esters at 280 nm. (e) Phenolics isolated from soluble glycosides at 255 nm. (f) Phenolics isolated from soluble glycosides at 280 nm. Phenolic acids identified as follows: (1) gallic acid, (2) protocatachuic acid, (3) catechin, (4) *p*-hydroxybenzoic acid, (5) valoneic acid dilactone, and (6) ellagic acid.

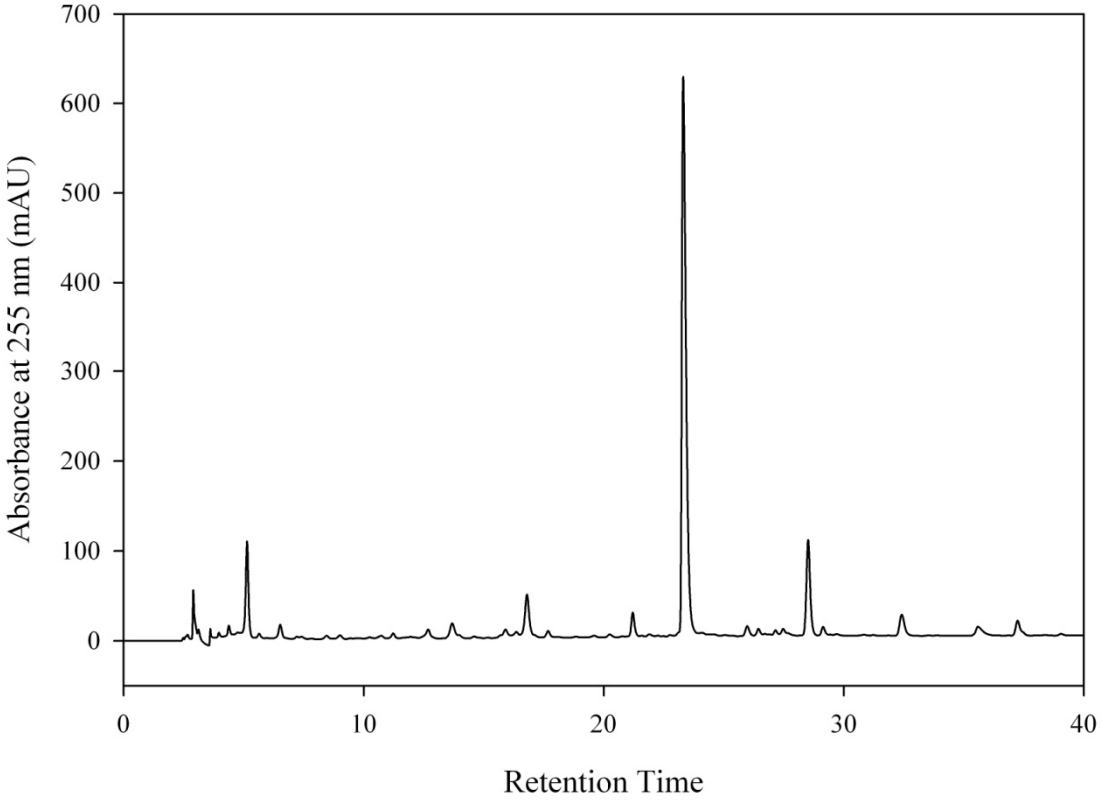
2a.


400
300
200
100
0
Absorbance at 255 nm (mAU)
2
4
5
6
0
10
20
30
40
Retention Time

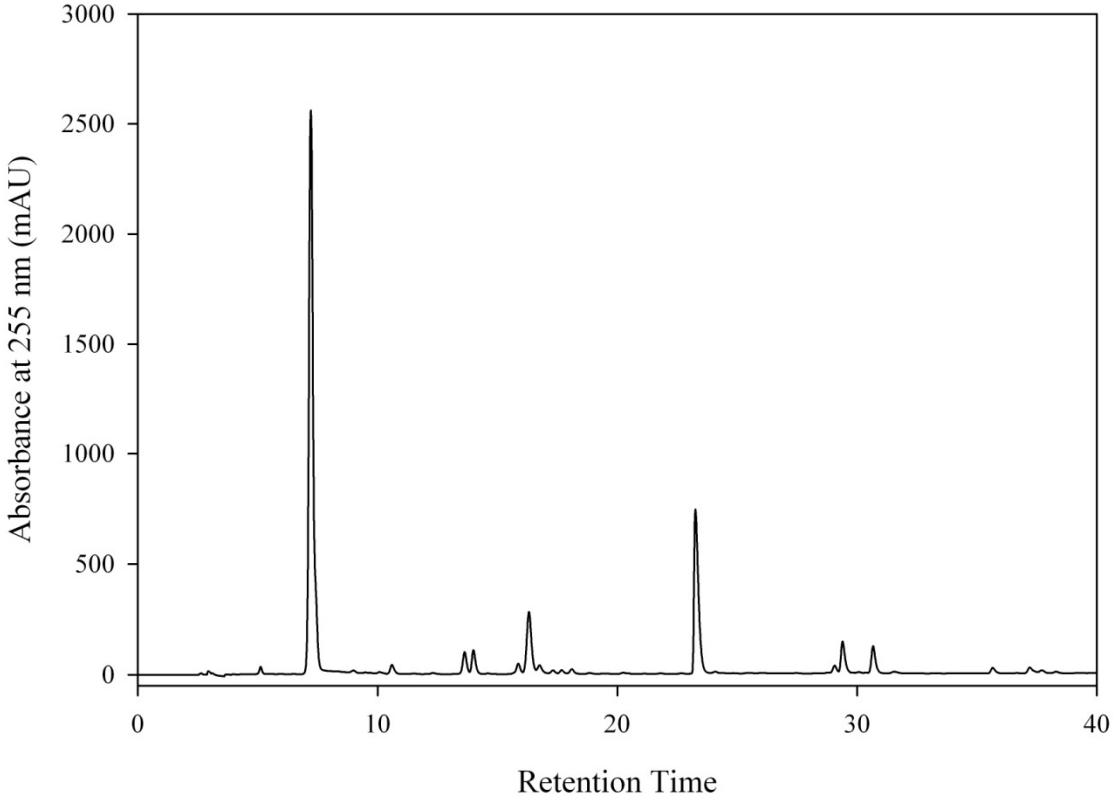
2b.


250
200
150
100
50
0
Absorbance at 280 nm (mAU)
1
3
0
10
20
30
40
Retention Time

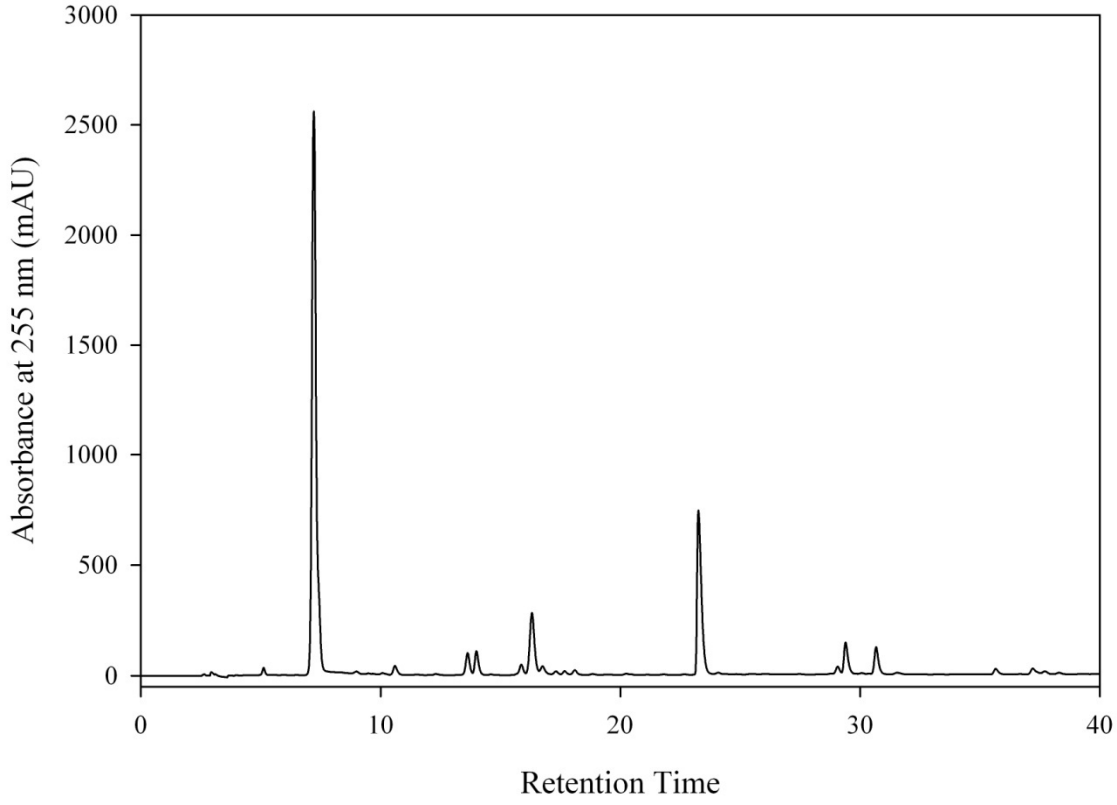
2c.


700
600
500
400
300
200
100
0
Absorbance at 255 nm (mAU)
0
10
20
30
40
Retention Time

2d.


3000
2500
2000
1500
1000
500
0
Absorbance at 255 nm (mAU)
0
10
20
30
40
Retention Time

2e.


3000
2500
2000
1500
1000
500
0
Absorbance at 255 nm (mAU)
0
10
20
30
40
Retention Time

2f.

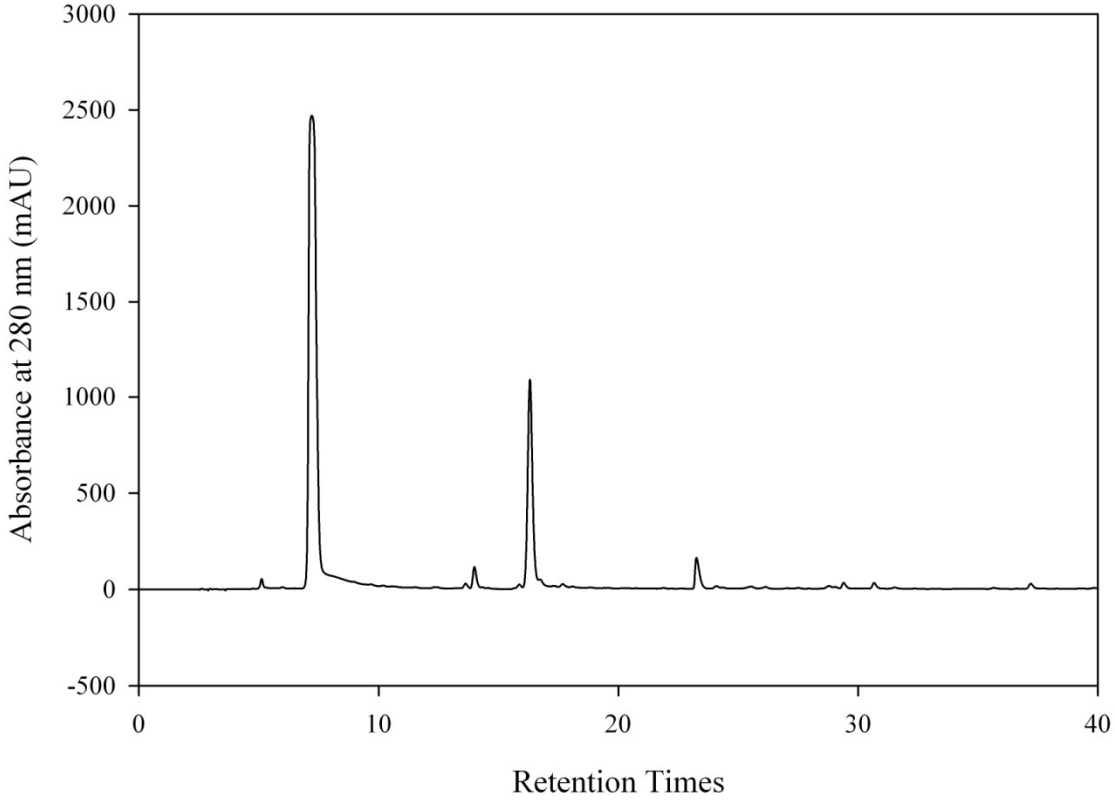

3000
2500
2000
1500
1000
500
0
-500
Absorbance at 280 nm (mAU)
0
10
20
30
40
Retention Times

Figure 5.3. NP-HPLC chromatograms for two acetonic tannin fractions isolated from (a) 8 pooled cultivars and (b) a single cultivar sample.

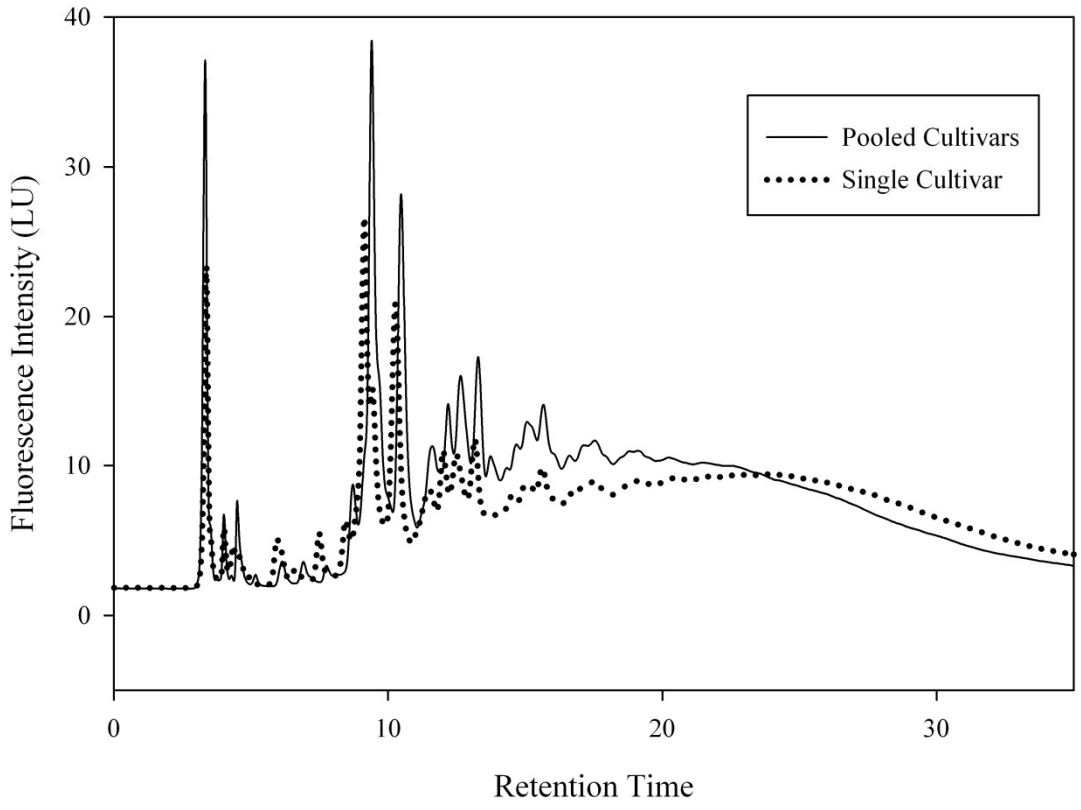
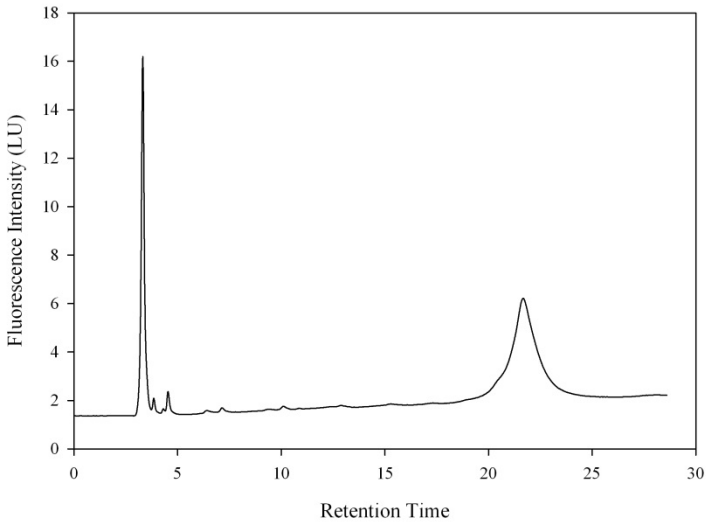
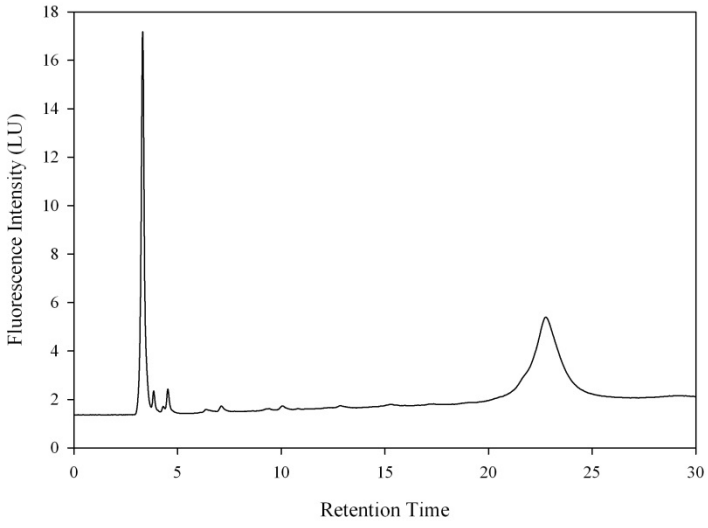


Figure 5.4. NP-HPLC Chromatograms for 3 different PAC standards. (a) Degree of polymerization of 8, (b) degree of polymerization of 9, (c) degree of polymerization of 10.

(a)



(b)



(c)

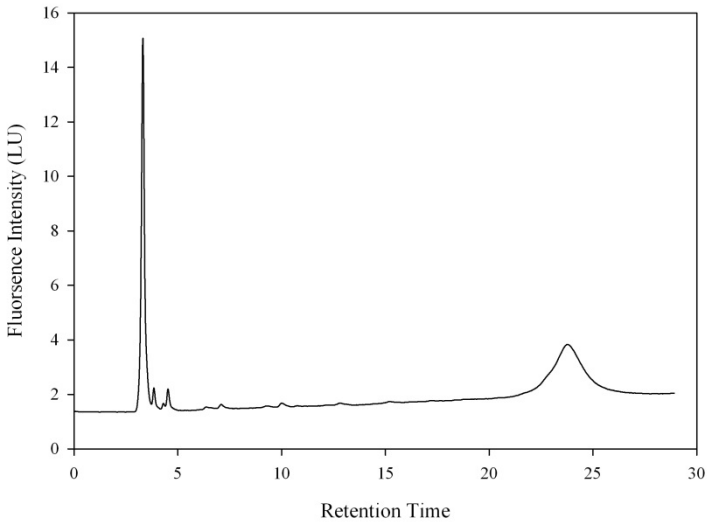

Fluorsence Intensity (LU)
Retention Time
0
2
4
6
8
10
12
14
16
0
5
10
15
20
25
30

Table 5.1. Total phenolic content for 5 ethanolic fractions separated by LH-20 chromatography (mean ± SD).

Fraction	TPC (mg CE/ g fraction)
I	48.2 ± 1.0
II	66.8 ± 2.6
III	198 ± 4
IV	169 ± 2
V	180 ± 4

Table 5.2. Phenolic acid contents for four ethanolic fractions isolated by LH-20 Chromatography. [a]

Fraction	Gallic acid	Protocatachuic Acid	Catechin	Ellagic Acid
I	nd[b]	0.343	nd	nd
II	Tr[c]	nd	16.1	17.2
III	nd	nd	nd	22.2
IV	nd	nd	47.7	4.97

[a] All values expressed as mg/ g fraction extract.

[b]nd = not detected, LOD = 4.0, 0.74, 1.4, 9.2, 5.3 ng/ml for catechin (C), protocatachuic (PR), p-hydroxybenzoic (PHB), gallic (G), and ellagic acid (E), respectively.

[c] Peak was detected but was not quantifiable. LOQ = 12.7, 2.5, 4.8, 30.6, 17.6 ng/ml for C, PR, PHB, G, E, respectively.

Table 5.3. Content of free phenolic acids and phenolic acids from soluble esters and glycosides for a LMW fraction sample (mg/g fraction)

Compound	Free	Esters	Glycosides
Gallic Acid	Tr[a]	25.0	Tr
Protocatachuic Acid	65.7	27.4	nd[b]
p-Hydroxybenzoic Acid	0.49	nd	112
Catechin	738	620	nd
Ellagic Acid	114	431	829
Valoneic Acid Dilactone	Tr	0.00	nd

[a]Peak was detectable but was not quantifiable. LOQ = 12.7, 2.5, 4.8, 30.6, 17.6 ng/ml for catechin (C), protocatachuic (PR), p-hydroxybenzoic (PHB), gallic (G), and ellagic acid (E), respectively. [b] No detectable peak. LOD = 4.0, 0.74, 1.4, 9.2, 5.3 ng/ml for CAT, PR, PHB, G, E, respectively.

Table 5.4. Characterization and quantification of 2 acetonic tannin fractions separated by LH-20 chromatography.[a]

Degree of Polymerization	Pooled Cultivars[b]	Single Cultivar
Monomer	0.29	0.21
Dimer	47.3	40.7
Trimer	21.1	21.8
Tetramer	9.19	2.82
Pentamer	5.56	0.00
Total	83.5	65.5
A_{550}/mg[c](mean ± SD)	0.711 ± 0.042	0.752 ± 0.006

[a] Characterization by NP-HPLC and quantification completed using commercial standards with varying degrees of polymerization.

[b] All quantities except A_{550}/mg are listed as mg compound/g tannin extract.

[c] A_{550}/mg results of *n*-butanol/HCl hydrolysis assay.

CHAPTER 6

THE EFFECT OF PECAN PHENOLICS ON THE RELEASE OF NITRIC OXIDE IN RAW 264.7 MACROPHAGE CELLS[1]

[1] Robbins, K. S.; Greenspan, P.; Pegg, R. B. To be submitted to *Journal of Agricultural and Food Chemistry.*

ABSTRACT

Inflammation has been linked to numerous chronic disease states such as cancer and autoimmune diseases. Recent attention has turned toward phenolic compoundsbecause a number possess anti-inflammatory properties. In this study, a phenolic crude extract was prepared from pecan nutmeat using a Soxhlet extraction followed by a phenolic extraction with acetone:water:acetic acid (70:29.5:0.5, v/v/v). The crude phenolic-rich extract was separated via Sephadex LH-20 column chromatography to yield low-molecular-weight (LMW) and high-molecular-weight (HMW) fractions. Anti-inflammatory properties of the fractions were assessed in LPS-stimulated RAW 264.7 murine macrophage cells. Nitric oxide (NO) and reactive oxygen species (ROS) production was monitored after three different experimental protocols: (1) pre-treatment with *E. coli* O111:B4 lipopolysaccharide (LPS); (2) pre-treatment with pecan extracts; and (3) co-incubation of LPS and pecan extracts. The LMW fraction displayed a dose-dependent decrease in NO production and a significant decrease from LPS control in ROS production when cells are either co-incubated with or pre-treated with LPS. The fractions were analyzed using reversed phase- high performance liquid chromatography (RP-HPLC) and normal phase-high performance liquid chromatography (NP-HPLC) in order to characterize the constituents which may be responsible for the observed effect.

6.1 INTRODUCTION

NF-κB is a protein complex shown to play a key role in the inflammation process in macrophages and lymphocytes (1). During normal physiological conditions the protein complex is located in the cytosol of the cell as an inactive complex, IκB-NFκB. The inflammatory process begins when one of several possible stimuli activates the cell. These stimuli can be

exogenous (LPS) or endogenous (TFN-α or IFN-γ) (2, 3). The stimulation then causes the enzyme IκB-kinase (IκK) to phosphorylate the inactive complex. Once the complex is activated, it can pass into the nucleus where it can stimulate gene expression for the inducible nitric oxide synthase (*i*NOS) genes (3-7).Once the *i*NOS gene is activated, there is a marked increase in the production of cytokines and other inflammatory mediators (8).

NO, a somewhat-stable free radical, is thought to be a mediator of inflammation (9) and is produced once the NF-κB complex has been activated by extracellular stimuli (2, 10). NO is also generated during everyday activities to help mediate other biological functions in the human body (9). The amino acid L-arginine is converted to NO by one of three NO synthases:constitutive synthases, endothelial (*e*NOS) and neuronal nitric oxide synthases (*n*NOS), as well as inducible nitric oxide synthase (*i*NOS). Different from the constitutive synthases, *i*NOS is not found in uninjured cells (11-12). Upon any form of injury or inflammation, the NF-κB complex will, however, initiate cytokines in the cell to begin producing *i*NOS (13).

NO serves multiple purposes in the inflammation process (14). It is a small molecule and therefore able to easily diffuse into cells. This enables the NO to easily travel from the site of formation to various sites of action (4, 15). Because it is a free radical, NOcan be extremely reactive with other free radicals found in the cells. Even though NOitself is not usually very reactive, it does form a much more damaging reactive nitrogen species (RNS), peroxynitrite anion ($ONOO^-$) (16). For this reason, the pro-oxidative NO has been shown to create oxidative damage (17-19). It is also believed that ROS may play a key role in the activation of NF-κB (20).

Studies have investigated the anti-inflammatory properties of pine bark extract (17), cocoa procyanidins (21), and a hydrophilic phenolic extract from pistachios (22) in cell culture

systems. Varying and conflicting results have been reported regarding to which extract constituents are most effective at reducing NO and ROS production. For instance, one study reportsthat HMW tannin constituents (22) are the most effective, while others have demonstrated the effectiveness of monomers and dimers (21, 23). Due to the moderately high PAC content of pecans, it was postulated that acrude phenolic extract from pecans wouldbe excellent as inhibitors of the inflammatory process. The prepared pecan crude extract was further separated via Sephadex LH-20 column chromatography to give two phenolic fractions: a LMW and a HMW one. Through the combination of results from HPLC characterization and cell culture assays, our objective is to indicate which compounds may be most effective as anti-inflammation agents.

6.2 MATERIALS AND METHODS

Chemicals and Glassware. Sea sand, glass wool, DMSO, sodium carbonate and ethanol (95%) as well as ACS-grade methanol, hexanes, and acetone plus HPLC-grade water, methanol, and acetonitrile were acquired from FisherScientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid was purchased from VWR International, LLC (Suwanee, GA, USA). *Escherichia coli* O111:B4 lipopolysaccharide (LPS), triton X-100, fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Folin&Ciocalteu's phenol reagent and (+)-catechin hydrate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The Dulbecco's Modified Eagle Medium (DMEM/high glucose + sodium pyruvate), L-glutamine, penicillin-streptomycin, and phosphate-buffered saline (PBS) were ordered from Invitrogen, Inc. (Grand Island, NY).

Collection of Samples. Pecans were harvested during the Fall 2011 season and shipped to the Department of Food Science & Technology, Athens, GA. The nuts were raw and remained in-shell through transport, packaging and storage. After arrival in Athens, GA, the pecan samples were placed in labeled pouches (Sealed Air Corporation, Elmwood Park, NJ), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to prevent any oxidative degradation, and stored at -80 °C until analyzed. On the day of analysis, pecan samples were removed from the freezer, shelled, and tempered to room temperature to ensure a proper mass reading. Approximately 15 g of nutmeat were placed into a -20 °C freezer and allowed to partially refreeze to facilitate grinding. Each nut sample was then combined with ~65 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ), to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

Lipid Extraction. A Soxhlet apparatus was employed to extract the lipids from all pecan samples. Briefly, a known quantity of the ground matter (pecan and sand) was placed into a cellulose extraction thimble (single thickness, 43 mm I.D. and 123 mm external length, Whatman International Ltd., Maidstone, England) and the mass recorded. Glass wool was placed in the top of the thimble to ensure that the contents would remain in place during extraction. Extraction was performed using ~350 mL of hexanes for 20 h. Upon completion, the thimbles were removed and allowed to dry overnight. The hexanes were removed from the lipid extract using a BüchiRotavapor R-210 (Büchi Corporation, New Castle, DE). The lipid portion was weighed for gravimetric analysis. Extracted lipids were transferred to amber-colored vials, flushed with N_2, capped, and placed in the -80°C freezer until analyzed.

Extraction of Phenolic Compounds. The defatted pecan kernel was removed from the dried thimble and placed into an Erlenmeyer flask. As employed by Wu *et al.*(18), an $(CH_3)_2CO/H_2O/CH_3COOH$ solvent mixture (70:29.5:0.5 v/v/v, 100 mL) was used to extract phenolic compounds. Extraction was carried out according to Craft *et al.* (20). Briefly, the contents in the flasks were heated at 50°C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed three times and the supernates pooled. Acetone was evaporated from the pooled supernate using the Rotavapor.The aqueous portion was poured into crystallization dishes, covered with filter paper, and placed in a -80 °C freezer until completely frozen. The samples were then lyophilized (LabconcoFreezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS). The dried extract was placed in amber-colored vials, capped and stored at 4 °C until ready to be used.The entire extraction process was conducted in triplicate.

Extract Separation.A ~2 g sample of pooled crude pecan extract was mixed in a small volume of ~75% (v/v) ethanol and sonicated to ensure that it was completely dissolved. The sample was then applied via a Pasteur pipette to the top of a chromatographic column packed with Sephadex LH-20(bead size: 25-100 μm; Chromaflex column, 30 x 400 mm [I.D. x length], Kontes, Vineland, NJ, USA). LMW phenolic compounds were eluted with ~1.5 L of 95% (v/v) ethanol. Fractions (15 mL) were collected using a Beckman Coulter SC100 fraction collector (Fullerton, CA, USA). The eluent was then switched to 50% (v/v) aqueous acetone and a HMW phenolic fractionwas eluted from the column with ~600 mL of the second mobile phase.

Fraction Determination. LMW phenolic fractions were analyzed using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) scanning across a wavelength range of 200 to 400 nm. Spectral differences were used as a guide to group the

collected fractions into five pooled lots. After combining, the ethanol was evaporated using the Rotavapor with the water bath set at 45°C. To ensure complete removal of solvent and moisture, the pooled fractions were lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS) and then stored in amber-glass bottles at 4°C until analysis. Apropos the HMW fraction: acetone was also removed *in vacuo* and the aqueous residue lyophilized and stored as described above.

TPC assay. The total phenolics contents (TPC) of the ethanolic fractions were determined based on a method adapted from Swain and Hillis (13) employing Folin-Ciocalteu's phenol reagent. Each fraction was diluted to 0.20 mg/mL in anhydrous methanol. The assay was performed using 1 mL of methanolic extract, 7.5 mL deionized water, 0.5 mL Folin-Ciocalteu's phenol reagent, and 1 mL of saturated sodium carbonate.The resultant solution was vortexed for 30 s. The tubes sat for 1 h to allow for optimal color development. The absorbance of the resulting chromophore from this colorimetric reaction was measured at $\lambda = 750$ nm using the Agilent spectrophotometer. A standard curve was prepared using known concentrations of working solutions of (+)-catechin (1.6 to 8.0 μg/mL).

Based on the low TPCs of fractions I and II, fractions III-V were combined to give the LMW fraction used in the cell culture studies.Ethanolic solutions were prepared with phenolic levels of the following specifications: LMW – 2 mg CE/mL in 70% (v/v) ethanol, HMW– 5 mg CE/mL in 50% (v/v) ethanol, and crude extract – 5 mg/mL CE in 75%(v/v) ethanol. Differences in ethanol concentration were necessary in order to ensure proper solubilization.

Cell Culture. Murine RAW 264.7 macrophage cell lines were purchased from the American Cell Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % endotoxin-free, heat-inactivated

fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), and L-glutamine (2 mM) and maintained at 37°C in a humidified 5% (v/v) CO_2 incubator. After scraping cells from a Corning 25-cm^2 culture flask, cells were plated (3.0×10^5) in MULTIWELL™ 24-well flat bottom tissue culture-treated dishes (Falcon, Franklin Lakes, NJ) and incubated until confluent. Three different treatment application methods were employed:

(1) The co-incubation treatment involved adding 0.5 mL of 100 ng/mL LPS in DMEM media supplemented with 5 % FBS.The cells were incubated at 37°C for 1 h. Then, 0.5 mL of the 3 ethanolic phenolic solutions with phenolic concentrations of either 25, 50, or 100 µg CE/mL (prepared in 5% FBS DMEM) were added to the appropriate well in quadruplicate, and the cells were incubated for 18 h.

(2) The extract pre-treatment involved adding 0.5 mL of each of the 3 ethanolic phenolic solutions prepared in 5% FBS DMEM and incubating at 37 °C for 1 h. The cells were then washed 2 times with PBS and 1 mL of 100 ng/mL LPS in DMEM + 5% FBS was added, and the cells were incubated for an additional 18 h.

(3) The final treatment, a pre-treatment with LPS, involved adding the 100 ng/mL LPS in DMEM + 5% FBS and incubating for 1 h. At this point, the cells were washed twice and then the 3 ethanolic phenolic solutions in DMEM were added for the final 18 h.

Ethanol concentrations were held constant across all treatments and concentrations. Final concentrations of the extracts in the wells were 12.5, 25, and 50 µg CE/mL.

MTT Cell Viability Assay. Cell viability was determined using the method of Gentile *et al.* (22) with slight modifications. Briefly, the cells were washed with PBS (pH 7.4) following the 18 h incubations at 37 °C. Then, 300 µL of FBS-free DMEM medium containing 5

mg/mLMTT was added to each well, and the plate was incubated for 4 h at 37 °C. The medium was discarded after incubation and the cells were washed again with PBS. DMSO (1 mL) was added to each well and the plate was allowed to sit until all of the formazan blue formed in the cells was dissolved. The absorbance at $\lambda = 540$ nm of each well was measured in a spectrophotometer (Beckman DU 650 Spectrophotometer, Beckman Coulter, Inc., Indianapolis, IN).

Measurement of ROS. Production of ROS was measured using a method described by Zhang *et al*. (27) with slight modifications. Briefly, 300 μL of 10 μM CM-H_2DCFDA in 1% ethanol (final concentrations) was added to each well 30 min prior to harvesting. The plate was incubated for an additional hour, after which the medium was removed and cells were detached using 300 μL of 0.5% Triton X-100. An aliquot (100 μL) was removed from each well and placed in a 96-well microtitre plate. The fluorescence was measured using a BMG FLUOstar Omega (Ω) microplate reader (BMG Laboratories, Cary, NC) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Cellular Release of NO. After 18 h of incubation at 37 °C, the culture media was pipetted off, centrifuged, and used to measure the NO generated by the cells from LPS activation of the NF-κB pathway. NO production was measured as nitrite using a Griess assay kit.The Molecular Probes Griess Reagent Kit for nitrite determination wasemployed for the analysis (Life Technologies, Eugene,OR). A Griess reagent was prepared by combining equal parts (v/v) of*N*-(1-napthyl)ethylenediamine•dihydrochloride and sulfanilic acid. To a microplate, 20 μL of the Griess reagent, 150 μL of sample or standard, and 130 μL of deionized water were added to each well. Sodium nitrite was used as the standard solutions with 10working concentrations ranging from 1 to 100 μM. The plate was allowed to rest at room temperature for 30 min to

allow for optimum color development of the azo dye. The absorbance of each well was read at λ = 548 nm using the spectrophotometric setting of themicroplate reader, and the readings were converted to nitrite concentrations based on the standard curve.

HPLC Characterization. *Reversed-Phase HPLC Characterization (RP-HPLC).* The RP-HPLC method reported by Srivastava *et al.* (15) was followed to characterize the crude extracts of the pecan cultivars. An Agilent 1200 series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV-vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA) was employed for the chromatography. A reversed-phase Luna $C_{18}(2)$ column (4.6 x 250 mm, 5 μm; Phenomenex, Torrance, CA) was utilized. A gradient elution consisting of mobile phase A ($H_2O/CH_3CN/CH_3COOH$, 93:5:2, v/v/v) and mobile phase B ($H_2O/CH_3CN/CH_3COOH$, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 mL/min was employed. A volume of 100 μL was injected for each of the acetonic extracts (4.0 mg/mL methanol). Detection wavelengths were λ = 255 nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, catechin, epicatechin), 320 nm (phenolic acids, notably of the *trans*-cinnamic acid family), and 360 nm (flavonols). Tentative identification of separated components was made by matching UV-VIS spectra and retention time mapping with standard compounds.

Normal-Phase HPLC Characterization (NP-HPLC). PACs were separated based on their degree of polymerization according to Kelm *et al.* (16). The same Agilent 1200 Series HPLC for the reversed-phase analyses was used, but with an AstecDiol HPLC column (250 mm × 4.6 mm, 5 μm particle size; Supelco, Bellefonte, PA) and a fluorescence detector. A gradient elution consisting of mobile phase A [(A) CH_3CN:CH_3COOH (98:2, v/v) and (B) CH_3OH:H_2O:

CH_3COOH (95:3:2, v/v/v) was utilized. The linear gradient was completed at 1.0 mL/min as follows: 0-35 min, 0-40% B; held for 5 min; 40-45 min, 40-0% B and then an additional 5 min hold in order to equilibrate the system. A volume of 20 µL was injected for each sample; they were first dissolved in anhydrous CH_3OH (20 mg/mL) and then further diluted 1:9 (v/v) with mobile phase A to a final concentration of 2 mg/mL. Samples were passed through a 0.45-µm PTFE syringe filter prior to injection. Fluorescence detection was utilized with excitation/ emission at 276/316 nm, respectively. Commercial purified standards were analyzed to determine the retention times and area values for two monomers [(+)-catechin and (-)-epicatechin], three dimers (B2, 2a, 2b), and a set of polymers of varying degrees ranging from three to ten. Quantification of samples was based on standard retention times and area values.

Statistical Analyses.Differences in the means of each ethanolic extractwere determined for each of the measured quantities with the PROC GLM procedure. Differences in means from control were determined with the means procedure and a Tukey's mean separation test with $P < 0.05$ using the Statistical Analysis System software, Version 9.2 (SAS Institute, Cary, NC).

6.3 RESULTS

Three extracts (LMW fraction, HMW fraction, and crude extract) were tested for their capacity to reduce ROS production within the cell and the production of a cellular inflammatory biomarker (NO). The production of NO was analyzed under three different experimental conditions: (1) co-incubation of LPS and the pecan extracts for 18 h; (2) pre-treatment of the cells with LPS followed by a washing step and application of the extracts; and (3) pre-treatment with the extracts followed by a washing step and then incubation with LPS. To ensure that proper activation of the cells with the LPS occurred, a control sample was included in every

experiment. As expected, the addition of LPS caused a dramatic increase in NO and ROS production in the cells. The level of ethanol in the cells was controlled at a final concentration of 0.9% in order to remove ethanol concentration as a variable in the experiment. No cell death was observed at this low ethanol concentration.

The level of NO production was measured as nitrite production using the Griess assay. For the co-incubation experiments, the LMW extracts showed a classical dose-dependent decrease in NO production with declines of 22, 29, and 78% with the 12.5, 25, and 50 µg CE/mL extracts, respectively (Figure 1a).The 25 and 50 µg CE/mL tests were shown to be significantly different from the control ($P < 0.05$). The HMW and crude extracts did not impart significant reductions from that of the control ($P > 0.05$) for any of the concentrations investigated. For the experiments with the pre-treatment of the extracts, there were no significant differences from control for any of the concentrations or extracts investigated (Figure 1b). Moreover, there was no dose-dependent effect observed for any of the extracts. In the pre-treatment with LPS experiments, the results were similar to the co-incubation experiments. A dose-dependent decrease of NO production in the presence of the LMW extracts was once again observed (Figure 1c). All three concentrations of the LMW extract, 12.5, 25, and 50 µg CE/mL, displayed significant decreases from the LPS control ($P < 0.05$). The decreases were 28, 37, and 77 % for the 12.5, 25, and 50 µg CE/mL extracts, respectively. The lowest concentration for both the HMW and crude extracts also reduced NO production, though these effects were small (11 and 14%, respectively). It should be noted that there was no dose dependent effect observed with either of these extracts. To test the cytotoxicity of the extracts, all treatments weresubjected to the MTT viability assay. The 12.5, 25, and 50 µg CE/mL exhibited decreases from LPS control of 9.5, 12.0, and 14.7 %, respectively.

The production of ROS inside the cell was evaluated using H_2-DCFDA, a non-fluorescent compound that fluoresces upon reaction with a ROS. A dramatic difference in fluorescence was observed between control cells and cells treated with LPS. The middle concentration, 25 µg CE/mL of each of the three extracts was tested in cells pre-treated with LPS (Figure 6.3). While no significant differences ($P > 0.05$) were observed for the HMW and crude extracts, a significant decrease was observed for the LMW extracts ($P<0.05$). These results are in agreement with those results observed in the NO production experiments (Figure 6.1). The intracellular ROS production was decreased by almost 50% with incubation of the LMW extract. The ROS decreased from 21.2 x 10^4 FU for the LPS control samples to 11.4 x 10^4 FU for the LMW samples.

To determine the phenolic compounds which may be present in these LMW fractions, the fractions present in the extract were analyzed using RP-HPLC and the major constituents were identified and quantified (see Chapter 5). Two main groups of phenolic acids were identified across the 3 fractions utilized for the cell culture: catechin and ellagic acid (including its derivatives). These acids were quantified in each sample and reported as mg phenolic/g fraction. It was discovered that fraction III contained 22.18 mg ellagic acid/g fraction while fraction IV had 47.69 mg catechin/g fraction and 4.97 mg ellagic acid/g fraction.Fraction V did not appear to contain any phenolic acids or monomers identifiable by RP-HPLC. NP-HPLC was employed to further investigate fraction V (Figure 6.4). Monomers and dimers were identified, but no compounds with higher degrees of polymerization were detected.

6.4 DISCUSSION

The moderately high levels of beneficial lipids (*i.e*. MUFAs, PUFAs, and tocopherols) have prompted investigation into their effect on inflammation (30). Recent investigations concerning the phenolic constituents of commodities such as pine extract (17), pistachios (22), and tea extracts (16) have shown that their PACs and phenolic compounds can act as anti-inflammatory mediators. The present study is the first study examining the use of pecan phenolic extracts as mediators of inflammation in RAW 264.7 macrophage cells. Through our investigation, we determined that the LMW fraction of crude pecan extract displays a dose-dependent decrease in the NO production in LPS-stimulated RAW 264.7 macrophage cells. This dose-dependent response was observed for both the co-incubation of the extract with LPS and pre-treatment of LPS prior to the addition of the extract (Figure 6.1). In fact, similar results were notedin both cases. No significant differences ($P > 0.05$) from LPS control were observed for both the HMW fraction and the crude extract in any of the NO trials except for the most dilute concentration when LPS was pre-incubated with the cells. The LMW fraction also exhibited a significant reduction ($P < 0.05$) in ROS production from that of the LPS control (Figure 3). No significant differences were observed from the LPS control for either the HMW or the crude extract samples.

Other studies investigating the anti-inflammatory properties of natural products have produced contradictory results in terms of the compounds most likely responsible for the observed anti-inflammatory effect. Our results support those articles that show this effect is most likely due to phenolic acids and small condensed tannin compounds (21, 23), whereas other studies point to the high-molecular-weight tannin fractions as being responsible for decreasing inflammation (22). The only similar study with tree nuts investigated the effect of a pistachio

phenolic extract on the LPS-induced inflammatory response of RAW 264.7 macrophage cells (22). Gentile *et al*. reported that the polymeric PACs displayed a much stronger effect than that of the smaller oligomeric PACs.

Park *et al*. (23) conducted a study using commercial standards of compounds with varying degrees of polymerization (i.e., catechin and epicatechin monomers, dimers, and trimers). They found that the monomers were the most effective at reducing the level of NO production in cells stimulated by IFN-γ, a similar inflammatory stimulator to LPS. It was also reported that when a commercial trimer, PCA C2, was used in the cell culture experiment there was actually an increase in the production of NO. These results are similar to those observed in our study.It should be noted that the LMW fraction of pecans contained a high proportion of catechin monomers (Fraction IV and V). An additional study conducted by Zhang *et al*. (21), showed that the COX-2, an additional enzyme linked to the inflammatory process, expression levels were greatly reduced by procyanidin B2 in human monocyte cells. Based upon the results of the NP-HPLC characterization, fraction V did contain some monomeric and dimeric compounds but no trimeric or larger compounds (Figure 6.4). It is suspected that the ethanol may have eluted some of the lower-molecular-weight tannin compounds. The effect of the LMW fraction of pecans can be attributed to not only the presence of ellagic acid and catechin compounds, but also to the inclusion of some of these LMW tannin constituents.

As previously mentioned, one argument for the effect of phenolics on NO and ROS production is cytotoxicity. Using the MTT cytotoxicity study, the LMW extract showed no significant differences ($P > 0.05$) from the LPS control in causing cytotoxicity. Another possible explanation is an interaction between the phenolic compounds and LPS. It has been suggested that the phenolic compounds might bind the LPS making it difficult for the LPS to successfully

initiate inflammation in the cells (2, 23, 31).For this reason the pre-treatment LPS experiments were performed. There were no large differences observed between the pre-treatment with LPS and the co-incubation of LPS and extracts experiments.

There is also evidence for the importance of extract concentration (22) and time of extract incubation (17) on the effect of NO and ROS production. In the present study, both effects were observed. The dose-dependent observations from the LMW extracts prove that concentration did indeed play a key role in the efficacy of the extract. In addition with the extract pre-treatment experiment, no significant differences ($P > 0.05$) were observed in any of the assays. The short length of extract incubation time is probably responsible for the lack of effect. Pecan phenolic compounds may require longer incubation times in order to be effective as inflammatory mediators. This delay was not observed with hydrophilic phenolic extracts from pistachios (22), but was observed in the use of an extract isolated from pine bark (17).

The present study focused on the effect of different phenolic compounds isolated from pecans as inflammatory mediators. Future studies are necessary to determine the mechanisms for the observed effects. Possible mechanisms proposed in the literature include changes in the expression of both COX-2 and *i*NOS enzymes, changes in the activation of NF-κB, and changes in the level of mRNA expression (17, 21, 23, 32-33). The antioxidant activity of the phenolic compounds may act in quenching key ROS involved in the activation of the NF-κB complex.

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(a)

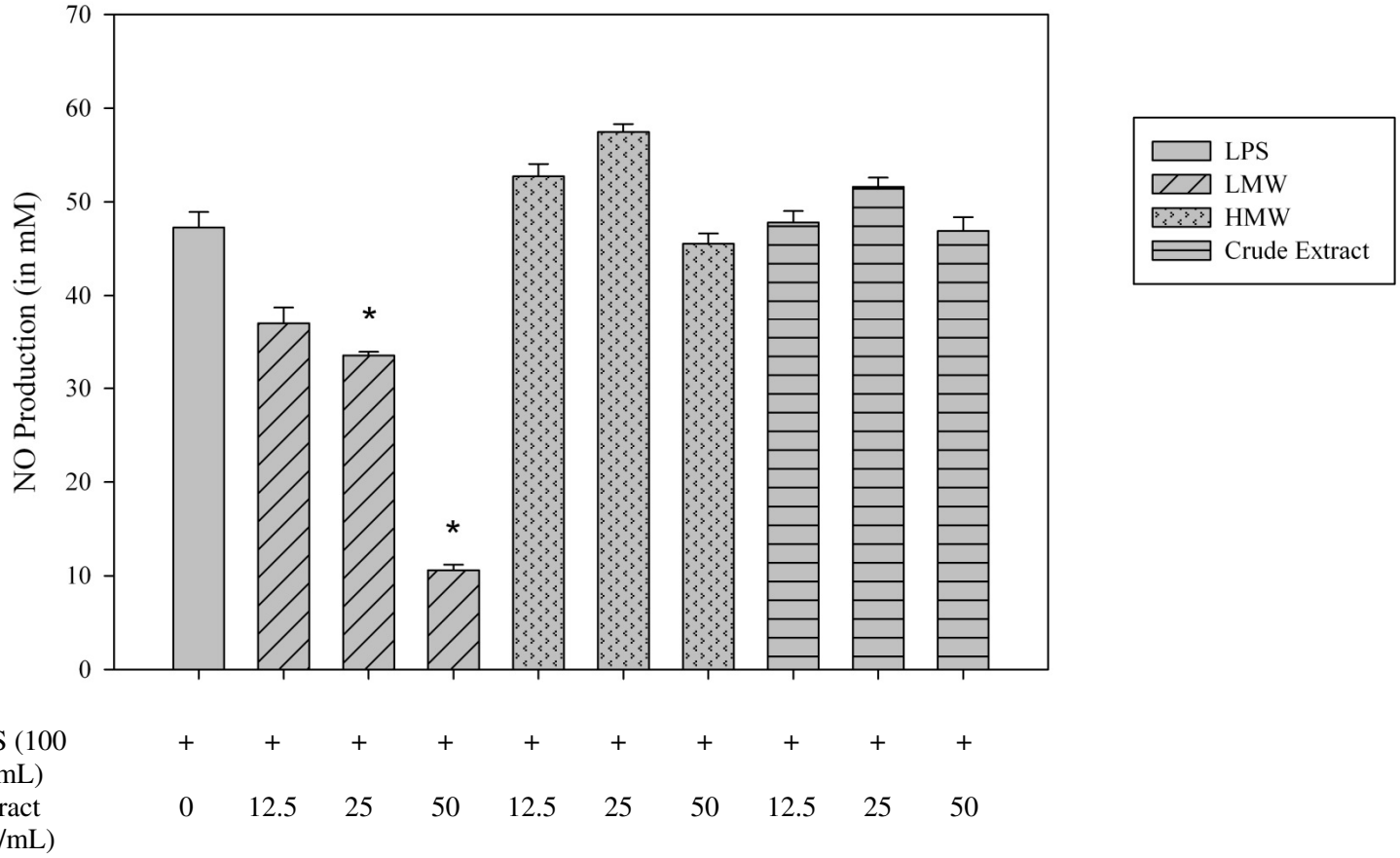
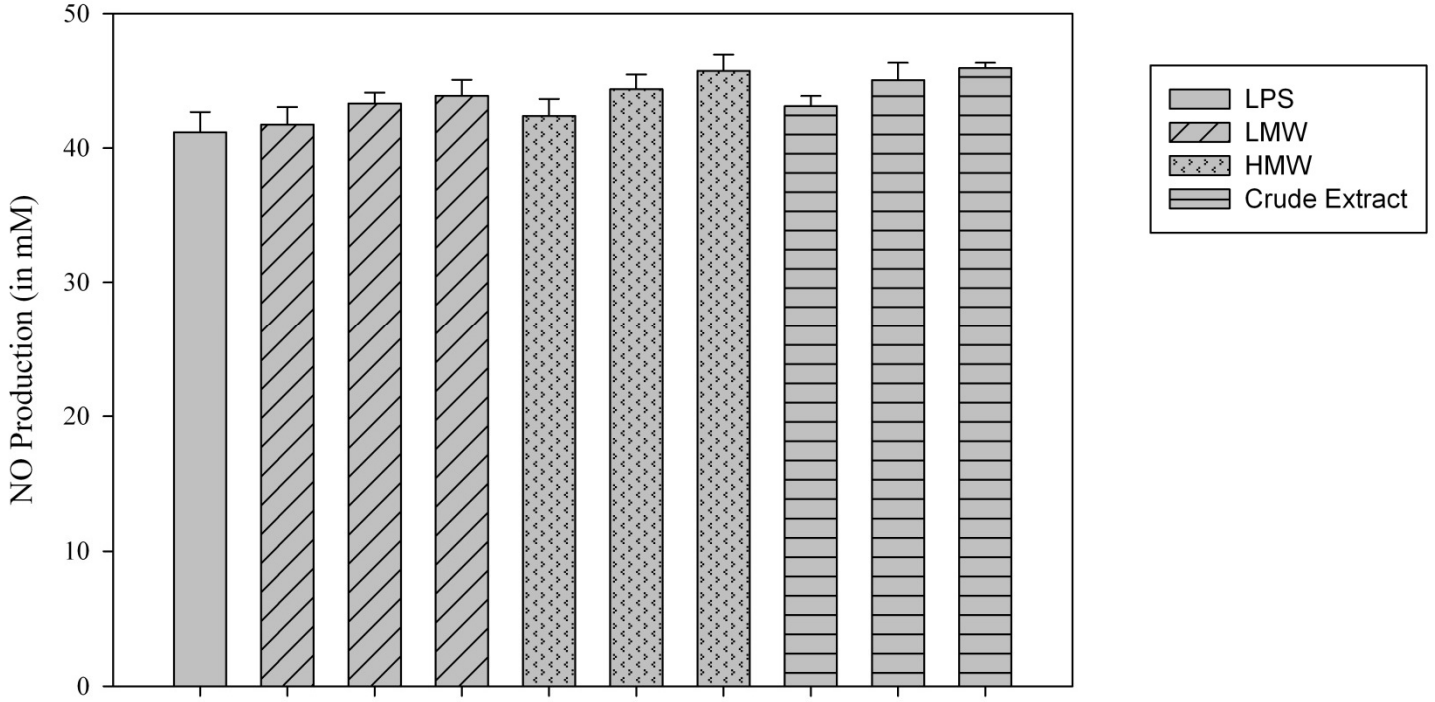


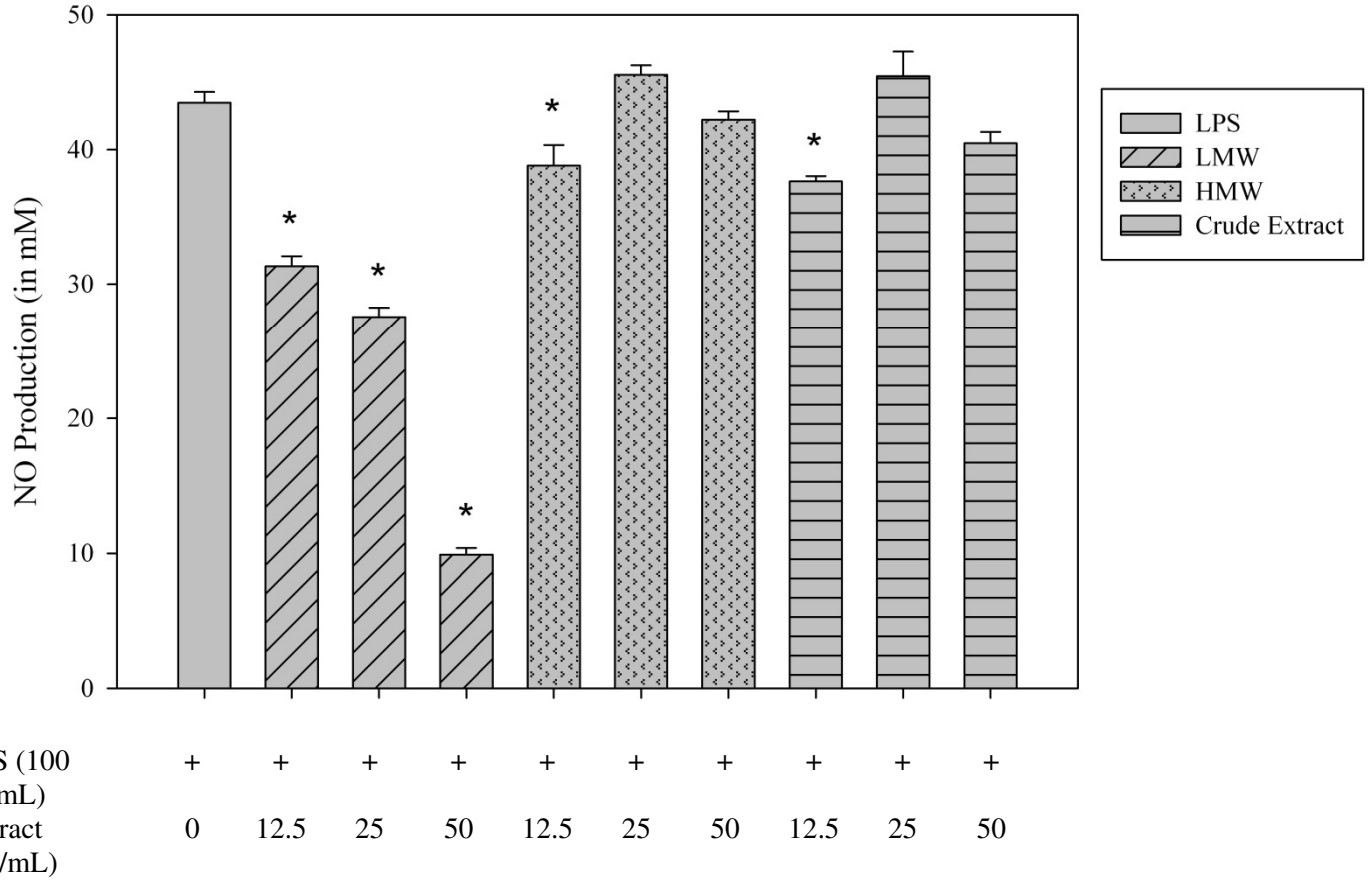
Figure 6.1.The effect of pecan extracts on mean NO production in cells (a) co-incubated with LPS, (b) pretreated with the extracts, and (c) pretreated with LPS. Bars indicate standard error. Stars indicate significant differences from LPS control ($P<0.05$) as determined by Tukey's.

(b)



LPS (100 ng/mL)	+	+	+	+	+	+	+	+	+	+
Extract (µg/mL)	0	12.5	25	50	12.5	25	50	12.5	25	50

(c)


NO Production (in mM)
50
40
30
20
10
0
*
LPS
LMW
HMW
Crude Extract
LPS (100 ng/mL) + + + + + + + + + +
Extract (µg/mL) 0 12.5 25 50 12.5 25 50 12.5 25 50

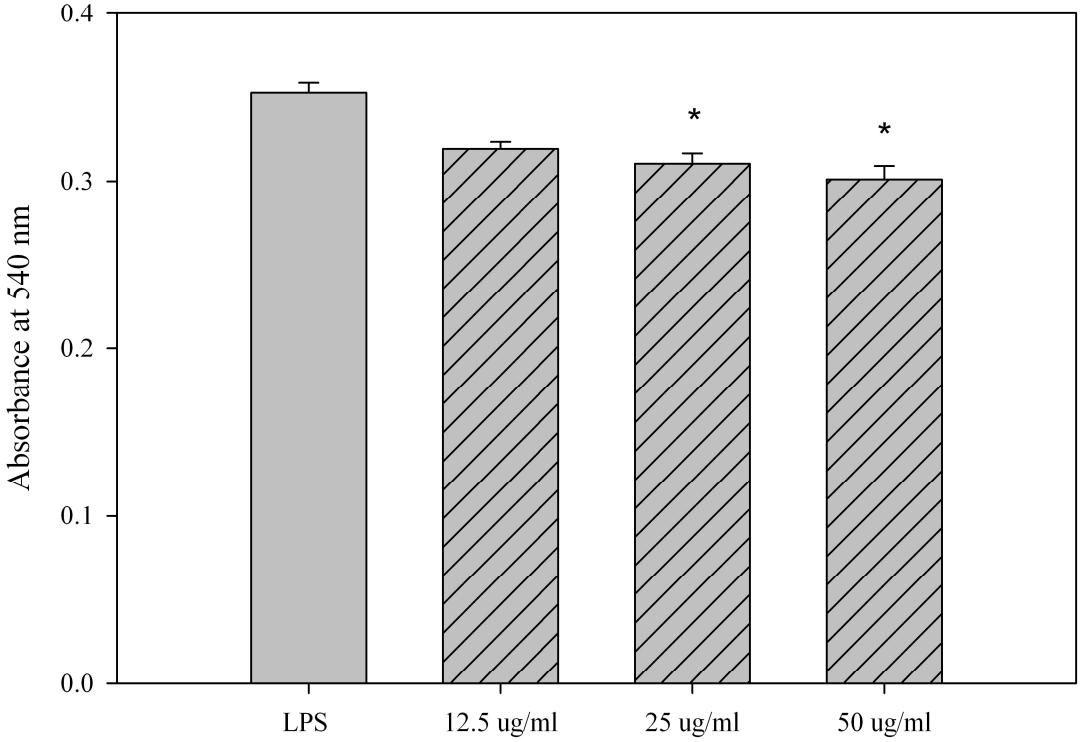


Figure 6.2. The effect of LMW pecan extracts on cellular viability. Mean absorbance values reported with bars indicating standard error. Stars indicate significant differences from LPS control ($P<0.05$) according to Tukey's mean separation.

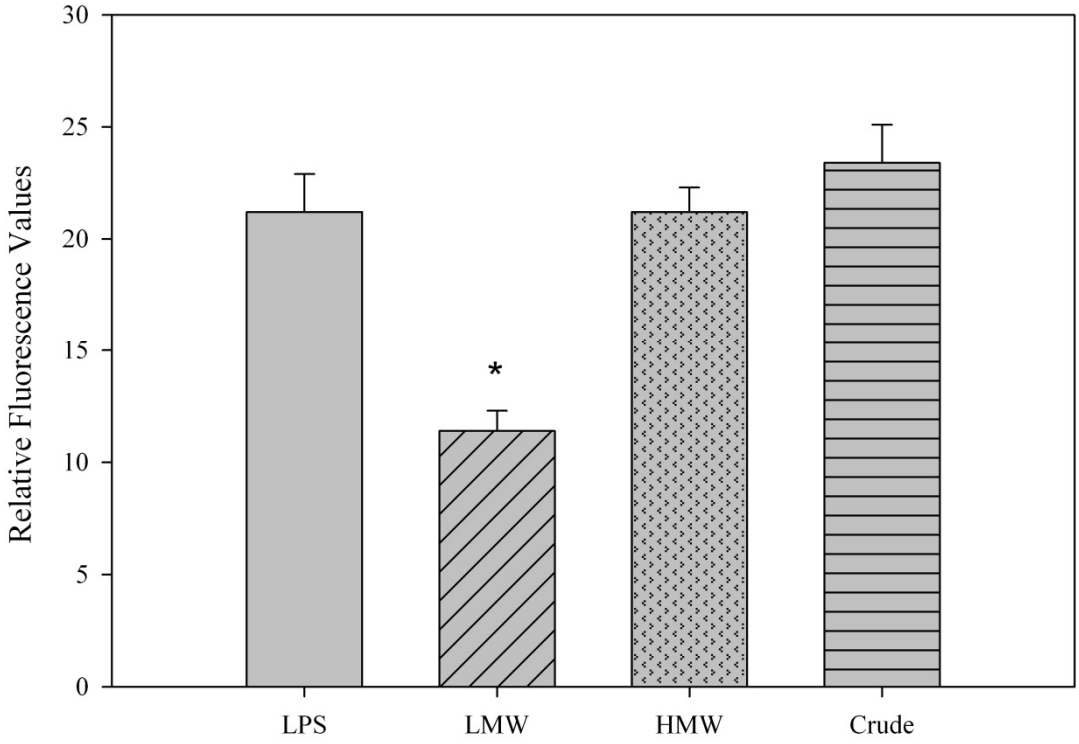


Figure 6.3.The effect of pecan extracts (25 µg CE/mL) on mean ROS production. Bars indicate standard error and stars indicate significant differences from LPS control ($P < 0.05$) as indicated by Tukey's mean separation.

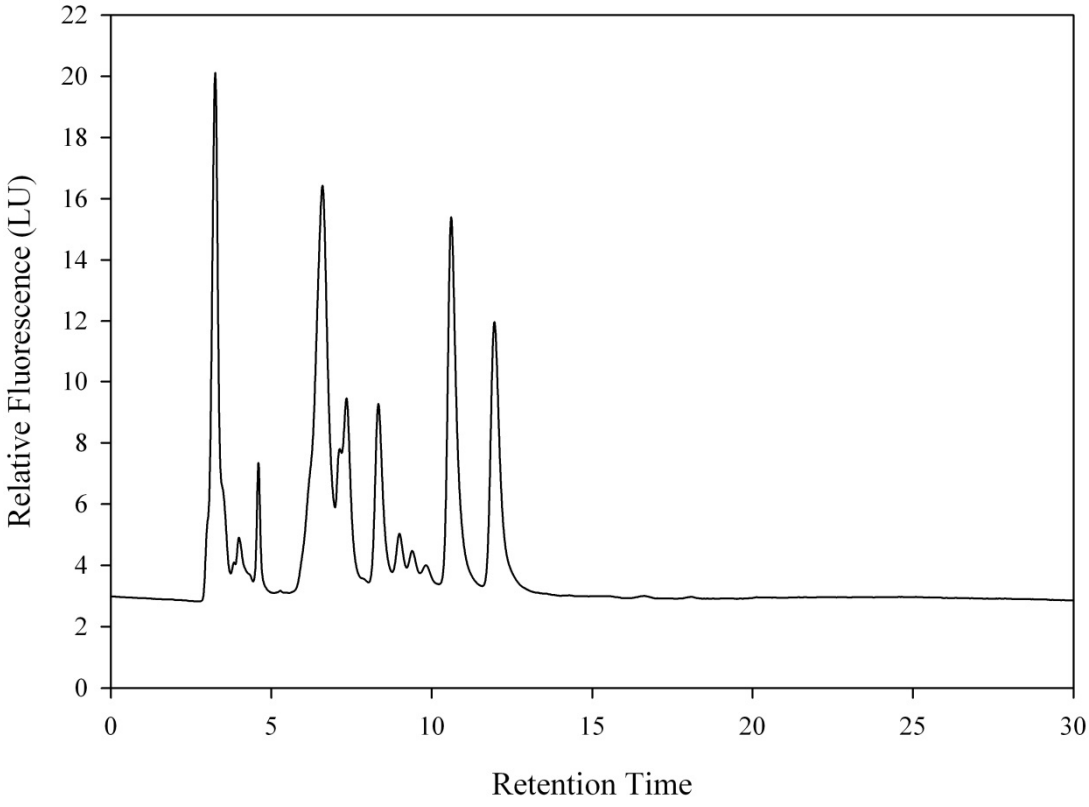


Figure 6.4. NP-HPLC chromatogram of fraction V isolated using Sephadex LH-20 column chromatography.

CHAPTER 7

CONCLUSION

The pecan, an important crop for Georgia farmers, has potential to gain increased commercial acceptance due to its beneficial phenolic profile. A proper evaluation of antioxidant activity and phenolic acid constituents is necessary in order to help prepare marketing materials and publications which could raise awareness, in the public mind, of these healthy components.

In current databases and literature, pecans have been shown to have prominent levels of antioxidants in their nutmeat compared to other nut types, fruits, and vegetables. This present study chose to investigate eighteen different cultivar samples from three different states to gain more knowledge on the antioxidant properties of pecan. To help develop a broader picture of the mechanisms involved, four different antioxidant assays were utilized (*i.e.,* H-ORAC$_{FL}$, FRAP, TPC and total proanthocyanidins). The levels of H-ORAC$_{FL}$ ranged from 13.5 ± 3.5 to 25.5 ± 3.0 mmol TE/100 g nutmeat and the levels of FRAP ranged from 14.0 ± 1.2 to 20.7 ± 0.7 mmol Fe^{2+} equivalents/100 g nutmeat. Furthermore, the TPC ranged from 1.11 ± 0.12 to 1.60 ± 0.04 g CE/100g nutmeat and the total proanthocyanidins ranged from 428 ± 51 to 655 ± 43 mg procyanidin B2 eq/100 g nutmeat. Significant differences were found between the eighteen cultivars and between the pooled cultivars for all four assays utilized. High levels of PACs are believed to be responsible for the higher antioxidant activity of the pecan compared to the other nut types as indicated by the high correlation between TPC and DMAC values as well as H-ORAC$_{FL}$and DMAC values. No significant differences were observed when growing locationwas considered. It is believed that pecans work well at quenching antioxidants by both

HAT and SET reaction mechanisms due to the high antioxidant levels in both the H-ORAC$_{FL}$ and FRAP assays. This flexibility may give pecans added benefit as an antioxidant, in biological systems. Initial feeding studies looking at the effect of pecans on the postprandial plasma antioxidant levels has recently been published, however, further *invivo* studies are necessary to gain a better understanding of the full benefit of pecans and human health.

HPLC methodology was employed to gain a better understanding of the constituents and the potential for biological activity in pecan crude phenolic extracts. Through RP-HPLC and LC-ESI-MS analyses, key phenolics were determined to be gallic acid, ellagic acid, protocatachuic acid, *p*-hydroxybenzoic acid, and catechin. Additionally, an ellagic acid derivative, valoneic acid dilactone, was identified in hydrolyzed crude samples. The lack of the dilactone in unhydrolyzed samples may indicate that the compound is produced through the breakdown of ellagitannins in the hydrolysis steps. Supporting this hypothesis, no dilactone was found in LMW fraction samples, separated via classical chromatography and Sepahadex LH-20, even after hydrolysis steps were performed confirming the presence of dilactone in the HMW fraction. NP-HPLC analysis was employed in order to characterize the tannin compounds of HMW pecan fractions; it was determined that this fraction was made up primarily of dimeric compounds, but constituents as large as pentamers were identified. Additional studies in the literature present data showing the presence of decamers and polymers in crude phenolic extract, however, further investigation into these compounds is necessary to properly identify the degrees of polymerization present in the samples. Further development of NP-HPLC and LC-MS/MS methods may be useful in further elucidating structure and degrees of polymerization for these PAC compounds.

Roasting was shown to significantly decrease H-ORAC$_{FL}$, TPC, and DMAC content of the pecan samples. No significant changes were shown in the FRAP values. Initially, the high heat treatment causes a breakdown of the PAC and phenolic compounds resulting in a reduction in antioxidant activity. Through the Maillard browning reaction, new antioxidant compounds are created, but not enough production occurs to counteract the thermal reduction in antioxidant activity. It is suspected that the newly formed antioxidants are most likely reductones that have been shown to have redox activity. This observation could account for the lack of a significant change in the FRAP values. Additional studies need to be conducted investigating different roasting temperature and time profiles to determine the full effect of roasting on antioxidant activity. It is suspected, that there may be an optimal set of conditions in which a higher level of new antioxidants are created to compensate for the thermal degradation.

The pecan's ability to act as an inflammatory mediator was tested by investigating the nitric oxide production of RAW 264.7 macrophage cells. It was found that the LMW fractions limited the nitric oxide production of RAW 264.7 macrophage cells in a dose-dependent manner. This effect is attributed to gallic acid, ellagic acid, as well as the PAC monomers, catechin and epicatechin. Further investigation into the mechanisms behind this effect is necessary. A Western blot or PCR analysis may be able to determine whether the activity of these compounds is due to a redox event or by directly affecting gene and mRNA transcription.

Pecans have been shown to have antioxidant and anti-inflammatory capabilities. This information can be used to raise public awareness on the health benefits of pecans. Accurately identifying phenolic composition is key to a better understanding of the compounds responsible for the antioxidant activity of pecans. This information may lead to identification of other plant-based products which could have similar properties as those observed in the pecan samples.

Currently, pecans are thought of as a holiday, high-fat treat. This research has the opportunity to raise awareness on the unique health benefits of pecans, increasing demand for a commodity that is so important to our state's economy.