CELLULAR EVALUATION OF THE ANTIOXIDANT ACTIVITY OF U.S. PECANS

[CARYA ILLINOINENSIS (WANGENH.) K. KOCH]

by

MARY ELIZABETH KELLETT

(Under the Direction of Ronald B. Pegg)

ABSTRACT

Clinical trials have shown an inverse relationship between consumption of antioxidant-rich tree nuts and the development of several chronic diseases. As the USDA considers pecans to be a top antioxidant-rich food, their biological antioxidant efficacy was evaluated using an adapted cellular antioxidant activity (CAA) assay. The CAA assay was performed in HepG2 cells and modified for use in Caco-2 cells due to their common use as a model of the intestinal barrier. Crude phenolic extracts from raw and roasted pecans, as well as fractions abundant in proanthocyanidins (PACs), were analyzed. This activity was measured by monitoring the fluorescence of 2′,7′-dichlorofluorescein; this fluorescence is highest when the cells are devoid of antioxidants. Significant reductions in fluorescence showed that raw and roasted pecans are effective antioxidants in biological systems. The primary active phenolic constituents in the extracts were determined to be (epi)catechin dimers and trimers via HPLC-ESI-MS/MS.

INDEX WORDS: Pecans, Antioxidants, Phenolics, Proanthocyanidins, (Epi)Catechin, Cell-based Assay, Caco-2 cells
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by

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BS, University of Pittsburgh, 2012

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

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Dean of the Graduate School
The University of Georgia
August 2015
DEDICATION

This work is dedicated to my three biggest cheerleaders: my parents, Matthew and Karen Kellett, and my fiancé, David Hornbeck. Collectively, they have been with me through everything these past two years, ready to encourage me to put one foot in front of the other (or one word after another, as the case may be). I never would have discerned my life path or completed this degree without their constant love and support.
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CHAPTER 1

INTRODUCTION

The pecan [Carya illinoinsis (Wangenh.) K. Koch.], known as the traditional tree nut of the United States, is very important to the South and the state of Georgia in particular. The U.S. is the world’s largest producer and Georgia single-handedly accounts for 33% of the country’s yield\(^1\); in 2013, over 133,000 tons of pecans were produced and the crop was valued at $460 million in the 2013 season.\(^2\) In Georgia, pecan revenue makes up almost half (43.3%) of the state’s fruit and nut category and is responsible for 2.32% of the state’s total agricultural farm gate value. The importance of the crop has ballooned in the past few decades, growing from a farm gate value of $45.8 million in 2002 to $315.6 million in 2013, largely due to the Chinese market and health benefits associated with this tree nut.\(^2,3\)

Pecans and other tree nuts are gaining increased public awareness of their healthfulness resulting primarily from various lipid constituents and antioxidant-rich phenolic compounds.\(^4-6\) Studies have shown that regular nut consumption can help with the prevention of cardiovascular disease, cancer and other chronic conditions.\(^7,8\) Pecans in particular have many phenolic compounds, including flavan-3-ol monomers, such as (+)-catechin/(-)-epicatechin, as well as oligomeric and polymeric proanthocyanidins (PACs).\(^9-11\) In an assessment of the antioxidant potential of tree nuts, pecans were ranked
first for overall phenolic compounds, flavonoids and PACs\textsuperscript{10,12–14}. This unique and strong phenolic profile may give pecans added benefits lacking in other types of tree nuts.

Recent data has shown that pecans have antioxidant activity both \textit{in vitro} and \textit{in vivo}.\textsuperscript{9,15,16} A clinical feeding trial was performed to examine the effects of a pecan-based meal. Postprandial concentrations of γ-tocopherol and (+)-catechin were elevated and oxidation of lipids and cholesterol was significantly reduced.\textsuperscript{16} Additionally, plasma collected after the pecan test meal showed increased antioxidant activity, as measured with both lipophilic- and hydrophilic-oxygen radical absorbance capacity (\textit{ORAC}_{\text{FL}}) assays. This study suggests that the phenolics and tocopherols (\textit{i.e.}, vitamin E) in pecans are both absorbable and bioavailable.

Though \textit{in vitro} assays such as \textit{ORAC}_{\text{FL}} are in wide use, recent evidence has shown that these measurements do not always extrapolate to the performance of a compound when tested \textit{in vivo}.\textsuperscript{17} These assays are much more affordable than feeding studies, but they miss important aspects of biological antioxidant function such as bioavailability, cellular uptake and metabolism; thus, there is a need for biologically-relevant measurement techniques. For this reason, cell-based assays are gaining traction for the measurement of dietary antioxidants in fruits and vegetables.\textsuperscript{18,19} The cellular antioxidant activity of tree nuts has not been studied and results highlighting their potential effectiveness \textit{in vivo} could strongly influence marketing efforts in the future.

The goal of the present study was to build upon limited data pertaining to the health-benefits of U.S. pecan consumption as a means to strengthen targeted marketing initiatives to distinguish U.S. pecans from other popular tree nuts, such as almonds and walnuts. Specific objectives were as follows:
1. To adapt an existing Cellular Antioxidant Activity (CAA) Assay using a human colon adenocarcinoma (Caco-2) cell line as a model for an intestinal barrier.

2. To prepare crude phenolic-rich extracts from U.S. pecans, to isolate fractions rich in pecan proanthocyanidins, and to evaluate their antioxidant efficacy using in vitro and cell models.

3. To collect biologically relevant CAA data for pecan phenolics to supplement current in vitro antioxidant data from TPC, FRAP and ORAC_{FL} assays.

4. To characterize and elucidate the chemical compounds (notably the PACs) in pecans, responsible for their antioxidant activity, by HPLC-ESI-MS/MS.

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(14) United States Department of Agriculture (USDA). *Database for the Flavonoid Content of Selected Foods*; Release 2; 2006.


2.1 Health Benefits of Nuts

It is widely known that regular consumption of tree nuts can have profound health benefits in adults. Between the favorable fatty acid profile, high fiber content, vitamin E, minerals, and many phytochemicals of interests, nuts contain an abundance of desirable nutrients.\textsuperscript{1-3} There is also substantial evidence regarding the healthfulness of regular nut consumption, to the point that the FDA granted tree nuts a qualified health claim in July 2003 stating “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.”\textsuperscript{4} The USDA echoed these views in the 2010 U.S. Dietary Guidelines for Americans, stating that varied protein sources should include nuts, as “moderate evidence indicates that eating peanuts and certain tree nuts (\textit{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.”\textsuperscript{5} The 2015 Dietary Guidelines for Americans will likely keep the recommendation, as the case for nuts and health is only getting stronger; in fact, the number of scientific studies published per year examining the health benefits of nuts has grown over 400\% from the late 1990s to 2010.\textsuperscript{6}
2.1.1 Nuts and Cardiovascular Health

Several epidemiological studies have been performed examining the link between dietary patterns and mortality, including large cohort studies with follow-up. In particular, the Nurses’ Health Study and the Health Professionals Follow-up Study showed marked inverse relationships between nut consumption and total mortality, as well as cause-specific mortality from cancer, heart disease and most major causes of death in both men and women.\(^7\)

One of the biggest reasons for encouraging nut consumption, as seen in the FDA health claim, is to improve cardiovascular health. A study of over 30,000 health-conscious Californians showed that consuming nuts four or more times per week resulted in significantly fewer fatal coronary heart disease-related events when compared to those in the cohort who consumed nuts fewer than once per week.\(^8\) Additionally, cultures adhering to the nut-friendly Mediterranean diet, characterized by its varied proteins and high healthful lipid content (25-35% of energy intake, <8% saturated fat), have seen some of the longest life expectancies and lowest rates of CHD worldwide.\(^9\) Furthermore, several studies and meta-analyses have shown the nut-heavy Mediterranean diet has reduced risk of mortality overall, as well as cardiac incident related deaths and cancer risk.\(^{10-12}\) The pronounced cardioprotective properties attributed to nuts are due to their wealth of unsaturated fatty acids (MUFA and PUFA); such fatty acid profiles are known to positively impact both blood lipid levels and cholesterol.\(^{13}\)

Consumption of modest amounts of several nut types has shown significant (up to 19%) reduction of low density lipoprotein (LDL) cholesterol.\(^{14}\) Nishi \textit{et al.} found that diabetics who consumed almonds for 12 weeks saw marked increases in oleic acid and
MUFA content in serum phospholipids fractions, which is associated with a decreased 10-year coronary heart disease risk.\textsuperscript{15} Similarly, Rajaram \textit{et al.} found that when pecans were incorporated into a diet rich in monounsaturated fats (MUFAs), consumers showed significant favorable alterations in serum lipids and lipoproteins without causing weight gain.\textsuperscript{16} Several other studies showed hazelnuts, pistachios and combinations of other tree nuts had beneficial effects on plasma cholesterol.\textsuperscript{17–20}

\subsection*{2.1.2 Tree Nuts and Weight Control}

In addition, regular consumption of nuts has helped with both satiety and weight management, despite previous fears that the high fat content and subsequent nutrient density may lead to undesired weight gain.\textsuperscript{21–23} In fact, there is an inverse relationship between regularity of nut consumption and body mass index (BMI).\textsuperscript{8,24} Numerous feeding trials have shown that feelings of fullness and satiety can be increased by adding nuts to the diet, a result of high protein and fiber content.\textsuperscript{25–27} Almond consumption has been shown to suppress hunger, as well as increase the amount of time between meals.\textsuperscript{28} With almonds and peanuts, this satiating effect has been found to be the most pronounced when consumed as a snack, rather than a meal component.\textsuperscript{29,30} Possible reasoning behind this is that nuts and nut products have been shown to reduce the desire to eat by enhancing gut satiety hormone secretion and increasing levels of gastric peptides that affect hunger.\textsuperscript{31} For example, peanut butter increased postprandial peptide YY, glucagon-like peptide-1 (GLP-1) and cholecystokinin (CCK-8); pistachios reduced GLP-1, and both free fatty acids (FFAs) and triglycerides (TGs) from pine nuts increased levels of GLP-1 and CCK-8.\textsuperscript{32–34} Tree nuts have improve markers of metabolic syndrome; in long
term studies, Mediterranean diets including tree nuts were shown to decrease both BMI and waist circumference in a serving-dependent manner.\textsuperscript{35}

2.1.3 Tree Nuts and Inflammation, Cancer and Mortality

In addition to promoting heart health and helping with weight management, regular nut consumption is known to aid with inflammation and cancer prevention. Though many studies focus on the lipid components and cardiovascular health, the phytochemicals found in tree nuts bestow many protective effects. Models for predicting cholesterol based on fatty acid consumption showed that nut-heavy diets performed better than diets with a similar fatty acid breakdown; this points to the existence of other bioactive constituents in nuts that can positively affect cholesterol levels.\textsuperscript{36,37} These bioactives, such as ellagic acid, selenium, quercetin, phytosterols, polyphenols and several vitamins are thought to have anti-inflammatory and antioxidant behavior that can reduce or delay oxidative stress, as well as slow the general loss of genetic control over cell growth that leads to malignant tumors.\textsuperscript{38} It has also been hypothesized that these phytochemicals in nuts may have protective synergies.\textsuperscript{39} The Spanish PREDIMED trial showed that cohorts consuming nuts three or more times per week died significantly less often from cancer, and in a five year follow-up such cohorts were the only group showing reduction in premature mortality.\textsuperscript{40} Several studies have looked at the chemopreventative status of various tree nuts; frequent nut consumption was associated with reduced risk of stomach cancer, colorectal cancer, prostate cancer, and pancreatic cancer in women.\textsuperscript{41–45} Despite this, many other studies suggest that much further research is needed to solidify the link between dietary intake of nuts and chemoprevention.\textsuperscript{42,46,47} As the World Cancer
Research Fund hypothesized the growth of up to 40% of cancerous tumors could have been suppressed by modified diets and exercise, one can conclude that new studies on chemopreventative behavior of various foods, such as nuts, are extremely valuable.\textsuperscript{48}

The physical make-up of pecans, particularly the phenolic compounds, lends itself to very strong purported health benefits. Epidemiological studies have shown that ingestion of pecans has marked effects on postprandial antioxidant content, oxidation of LDL cholesterol and blood lipid levels.\textsuperscript{49}

2.2 Pecans and Their Growth

The pecan \textit{(Carya illinoinensis} (Wangenh.) K. Koch.) is a tree nut belonging to the Juglandaceae family, which is related to the walnut, butternut, heartnut and hickory nut.\textsuperscript{50,51} The Juglandaceae family dates back to the Late Cretaceous period and was spread throughout North America, Europe and Asia over 50 million years ago. However, 34 million years ago the genus \textit{Carya} died out in Europe, but through its survival in North America, the pecan eventually became known as the traditional tree nut of the United States.\textsuperscript{16,51} Pecans or “nuts requiring a stone to crack” according to the Algonquian language, were an important part of the diet of Native Americans long before the land was settled by the Europeans.\textsuperscript{50,51} Pecan trees are known for their extremely long life span and large size; native specimens have been determined to be over 1000 years old and trees have been known to grow up to sixty meters in height and three and a half meters in diameter.\textsuperscript{50,52} Other characteristics include being wind-pollinated, heterodichogamous, monoecious and deciduous.\textsuperscript{53} Additionally, pecan trees are alternate-bearing, yielding maximal crops every two to three years.\textsuperscript{51}
Traditionally, pecan trees grew along the Mississippi River, but currently, they can be found stretched across a large portion of the United States, from northern Illinois to southeastern Iowa to the Gulf Coast. Isolated pockets of trees can be found in Ohio, Kentucky and Alabama, as well as Mexico.\textsuperscript{51} However, according to the USDA, the highest volume of pecans are grown in Georgia, New Mexico and Texas and account for 33, 27 and 11\% of national production, respectively.\textsuperscript{54}

Successful growth of pecans depends on a variety of factors; trees are susceptible to many environmental factors, especially water drainage, pH and topography.\textsuperscript{52} Pecans require flat land with adequate drainage as well as the relatively neutral soil pH of ~6.4.\textsuperscript{51,52} These conditions are easily met in the sandy soil near riverbeds, hence the history of pecan abundance along the banks of the Mississippi River.\textsuperscript{51} If pecan groves are overly hilly, water can pool and cause the soil to be too moist; in these conditions, oxygen has difficulty reaching the roots and growth is limited.\textsuperscript{52} From a practical perspective, flat land is also required for ease of harvest in the pecan grove.

The growth and cultivation of pecans for industrial purposes began in the late 1800s. As pecans do not grow effectively from seeds or cuttings, propagation was achieved through grafting and budding. These husbandry practices resulted in over 1000 genetically different cultivars, with various physical properties, falling into geographic categories of northern (Illinois-based), southern (Georgia) or western (Texas).\textsuperscript{55,56} Defined in terms of acres planted, the most prevalent pecan cultivar is Stuart; it is one of the oldest cultivars (documented as far back as 1874) and is recommended for planting across the southern United States from North Carolina to Arkansas.\textsuperscript{52,57} Recommendations for Georgia growers include eleven commercially-viable cultivars
(Cape Fear, Curtis, Desirable, Elliot, Gloria Grande, Kiowa, Oconee, Pawnee, Schley, Stuart and Sumner), of which the most lucrative are Stuart and Desirable. Pecans are grown worldwide, but the United States is the largest producer of pecans by a large margin, providing 80% of the world’s supply.

Pecans are known to have a pleasing aroma, crunchy texture, and a satisfying flavor that is applicable in both savory and sweet applications. They are ranked in the top three most popular nuts in America and have seen increased market demand in China in recent years, so the market potential of pecans is only expected to strengthen.

2.3 Pecans in the Market Place

In 2013, the United States produced over 133,000 tons of pecans and the crop was valued at $460 million. Georgia, in particular, ranked first out of U.S. producers with 89 million pounds of utilized pecans, which is 33% of the national supply. The two next largest producers were New Mexico and Texas, accounting for 27 and 11% of the supply, respectively; the breakdown of U.S. pecan suppliers can be seen in Figure 2.1. In 2013, Georgia pecans sold for an average of $1.91/pound, for an overall value of 170 million dollars, with 162 million dollars coming from improved cultivars.

According to the 2013 Georgia Farm Gate report, the fruit and nut commodity group grew by 33.2% from 2012 to 2013, which is an increase in value of $182 million. Pecans are in the top ten most important commodities in Georgia, accounting for 2.32% of the state’s agricultural revenue. This is significant growth (28.8%) since the 2012 season, where pecans were the eleventh ranked agricultural commodity and accounted for 1.80% of the state’s total farm gate value. When classified into commodity groups,
pecans account for almost half (43.3%) of the fruit and nut category farm gate value in Georgia.\textsuperscript{60} It is hard to track the growth of the pecan industry from year to year, due to pecan tree’s alternate-bearing, but it is indisputable that the importance of pecans to the Georgia economy has drastically increased in the past few decades, ballooning from a farm gate value of $45.8 million in 2002 to a value of $315.6 million in 2013.\textsuperscript{52,60,62}

A significant factor in the growth of U.S. pecan sales is the entry of China into the market; they are increasingly interested in pecans, pistachios, almonds and other nuts that are not widely grown within that country. Between 2000 and 2012, sales of U.S. tree nuts in China saw exponential growth from $77 million to over $1.6 billion.\textsuperscript{63} Since 2012, growth has slowed, but in 2015, Chinese tariffs were reduced from 24% to 10%, which

\textbf{Figure 2.1: Major Producers of U.S. Pecans and Percentage of Supply}\textsuperscript{54}
could boost sales in the coming years.\textsuperscript{63}

The primary grower of pecans outside the United States is Mexico, with minor production in other countries including Australia, Israel, Peru and South Africa.\textsuperscript{64} In 2013, the top importers of U.S. pecans were Hong Kong, China, Mexico, Vietnam, Canada and the Netherlands.\textsuperscript{65} Domestically, pecans are in the top three nuts in both production and popularity; they are third behind the almond and English walnut for total production and in terms of popularity, pecans are tied with walnuts for second, behind almonds.\textsuperscript{66,67} According to Mintel, the nut market is fairly saturated, with 85\% of households consuming tree nuts monthly; however, with marketing based on health implications, the pecan could gain ground in terms of market share and popularity.\textsuperscript{68}

\textbf{2.4 Pecan Composition and Antioxidant Potential}

The aforementioned health benefits of pecans are largely tied to their composition, which includes a healthy lipid profile, high amounts of dietary fiber, satiating vegetable protein and a wealth of phytochemicals. The proximate composition of pecans can be found in Table 2.1. For both raw and roasted pecans, the primary constituent is lipid, making up over 70\% of the nut on average. The lipid content of individual crops can range from 65-75\% depending on a host of factors, including geographic location, cultivar, harvest year, previous productivity of the tree, and growing conditions.\textsuperscript{69,70} According to the USDA National Nutrient Database for Standard Reference (Release 27), raw and roasted pecans have a lipid content of 71.97 and 74.27 g/100 g nutmeat, respectively.\textsuperscript{71} The moisture content in raw pecans is fairly low (3.52\% moisture) and as expected, drops when pecans are roasted (1.12\% moisture). Protein level
is fairly high (13.86 and 13.55% in raw and roasted, respectively) and is not changed considerably by roasting. Carbohydrates, calculated by subtraction, are similarly unaffected by roasting. Raw pecans have 13.86 g carbohydrate/100 g nutmeat, while their roasted counterparts have 13.55 g/100 g. About 69% of the carbohydrates in pecans are made up by dietary fiber.\textsuperscript{71}

<table>
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<tr>
<th>Nutrient Constituent</th>
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<th>Dry Roasted</th>
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<tr>
<td>Water</td>
<td>3.54</td>
<td>1.12</td>
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<tr>
<td>Protein</td>
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<td>9.50</td>
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<tr>
<td>Total Lipid</td>
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</tr>
<tr>
<td>Carbohydrate, by difference</td>
<td>13.86</td>
<td>13.55</td>
</tr>
<tr>
<td>Fiber, total dietary</td>
<td>9.6</td>
<td>9.4</td>
</tr>
<tr>
<td>Sugars, total</td>
<td>3.97</td>
<td>4.06</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data found in USDA National Nutrient Database for Standard Reference, Release 27\textsuperscript{71}

\textbf{2.4.1 Pecan Lipid Profile}

As mentioned above, the lipid content is very high in the pecan (~70% by weight), which makes the tree nut a very high energy density food. Despite this, when it comes to heart health, the pecan has an extremely wholesome lipid profile.\textsuperscript{16} According to the recent Dietary Guidelines for Americans, for optimum heart health, consumers should keep dietary saturated fats to a minimum because they are associated with adverse health effects.\textsuperscript{5} In line with this, reduction in saturated fats in the diet is known to improve cardiovascular risks in a variety of populations.\textsuperscript{72,73} To replace saturated fats in the diet, a variety of mono- and poly-unsaturated fats (MUFAs and PUFAs) are
recommended, as they are known to have many beneficial properties.\textsuperscript{16,20,37,73} Heart-healthy diets are rich in n-3 and n-6 PUFAs, as well as MUFAs, as these types of lipids are known for their cardioprotective characteristics. For example, PUFAs are known to help with satiety, insulin sensitivity and cardiovascular health, while MUFAs are known for their capability to regulate cholesterol levels by lowering total and low-density lipoprotein (LDL) cholesterol, while maintaining levels of high-density lipoprotein (HDL) cholesterol.\textsuperscript{74} The Mediterranean diet, which is rich in olive oil and tree nuts (namely almonds, walnuts and hazelnuts), adheres to these recommendations.\textsuperscript{9} Figure 2.2 shows the fatty acid distribution of olive oil, along with that of raw and roasted pecans. While olive oil is known for being “heart healthy”, the figure shows that pecans actually contain a smaller proportion of saturated fatty acids, and more heart-healthy PUFAs on a percentage basis. Pecans also contain a large fraction of MUFAs.\textsuperscript{71}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fatty_acid_distribution.png}
\caption{Fatty Acid Distribution of Olive Oil, Raw and Roasted Pecans\textsuperscript{71}}
\end{figure}
2.4.2 Pecan Tocopherols

The majority of the lipids (96%) in pecans are triacylglycerols (TAGs), which are comprised of glycerol backbones with three fatty acids attached (these can be saturated or unsaturated). The remaining 4% is made of complex lipids (mainly tocopherols, sterols and acylglycerols). Tocopherols are known to be beneficial lipophilic antioxidants that can exhibit protective effects in humans as well as in the nut itself. These compounds protect the germ from oxidative stress as it grows and matures, preserve reproductive capabilities of the seed, and remain post-harvest to yield a bioavailable antioxidant load after consumption. Tocopherols consist of eight different homologues: α-, β-, γ-, and δ-tocopherols and tocotrienols, which are collectively referred to as tocols. They are also known commonly as vitamin E. The difference between these classes of compounds lies in the characteristics of their side chains: tocopherols are saturated, while tocotrienols are unsaturated. The tocols increase in antioxidant activity as they increase in relative polarity from α-T to δ-T, but the most prevalent in nature are α-T and γ-T. In pecans specifically, the dominant homologue is γ-tocopherol, making up >90% of the vitamin E found in pecans. In the past, most research was focused on α-T but recent efforts have shown that the activity of γ-T has been underestimated, as it possesses important and unique benefits when compared to other homologues, including being antiatherogenic.

The total tocopherol content in multiple cultivars in two crop years ranged from 21.3-32.0 mg/100 g nutmeat, with no significant difference found among cultivars (p>0.05). These complex lipids are especially important to plants with such high levels of unsaturated fatty acids, as these fatty acids are susceptible to oxidative rancidity and subsequent reduction in quality. In fact, vitamin E was determined to be an important
predictor of kernel quality, as reduced tocopherol content during storage was correlated with rancidity development.\textsuperscript{83} Tocols have the ability to protect pecan lipids during both growth and post-harvest storage, and their effects, as well as shelf life, are maximized by storage conditions of 0 °C or cooler, in the presence of minimal oxygen and a relative humidity of 70-75%.\textsuperscript{50,84}

### 2.4.3 Pecan Phenolics

Pecans contain several other antioxidant compounds that enhance their overall healthfulness; in addition to fat-soluble vitamin E, the defatted meal of pecans contains many phenolic compounds, which are known as the most active class of dietary antioxidants.\textsuperscript{85} Similar to tocopherols, phenolic compounds have the ability to safeguard against lipid peroxidation and oxidative DNA damage in the nut itself.\textsuperscript{86}

Chemically, phenolic compounds are defined as substances containing aromatic rings with one or more hydroxyl groups attached; based on various functional groups, there are a myriad of sub-classifications, including phenolic acids, flavonoids, isoflavonoids, lignans, and stilbenes.\textsuperscript{78,85,87} Structural examples of each time of polyphenol can be found in Figure 2.3. Though there are many final phenolic products, the mechanisms of synthesis in plants are all very similar, as all phenolic compounds are secondary metabolites of L-phenylalanine or L-tyrosine.\textsuperscript{85}

The two main classes of phenolics endogenous to pecans are phenolic acids and tannins.\textsuperscript{88} In general, phenolic acids can be benzoic acids, such as gallic acid and protocatechuic acid, or \textit{trans}-cinnamic acids, such as quinic or tartaric acid. Tannins can be either hydrolyzable or condensed (such as the flavan-3-ol monomers (+)-catechin and
(-)-epicatechin and their polymers known as proanthocyanidins of PACs). PACs are phenolic polymers and are defined by their size and subsequent degree of polymerization (DP), which can range from 2 to over 10.\textsuperscript{88,89}

![Diagram of major phenolic classes present in food](image)

\textbf{Figure 2.3: Major Phenolic Classes Present in Food.}\textsuperscript{87}

In pecans, the most prevalent phenolic acids are ellagic and gallic acids, though several others have been identified. Using gas chromatography-mass spectrometry (GC-MS), early research by Senter \textit{et al.} determined that pecans contain eight phenolic acids:
gallic, gentisic, vanillic, protocatechuic, \( p \)-hydroxybenzoic, and \( p \)-hydroxyphenylacetic acids, with trace amounts of coumaric and syringic acids.\(^9\) Of these hydroxybenzoic acid derivatives, the most prominent was gallic acid, accounting for 78\% of the measured phenolics at 138 \( \mu \)g/g defatted meal.\(^9\) Using a base then acid hydrolysis preparation procedure for high performance liquid chromatography (HPLC), Villarreal-Lozoya et al. determined that pecan kernels contain gallic acid, ellagic acid, catechin and epicatechin.\(^9\)

The pecan samples from different cultivars contained 651-1300 \( \mu \)g gallic acid/g and 2505-4732 \( \mu \)g ellagic acid/g nutmeat and the phenolic acids were found to be present in much higher quantities than the hydrolyzable tannins.\(^9\) Additionally, the research of Senter et al. found that decreases in levels of hydroxybenzoic acids correlated with reduced sensory quality of pecan kernels. This strongly suggests that not only are these compounds present, but they exhibit antioxidant activity, which preserves flavors during storage by slowing lipid peroxidation.\(^9\)

Besides these two phenolic acids, the most prevalent endogenous phenolic compounds in pecans are condensed tannins or proanthocyanidins (PACs). As mentioned above, they can vary drastically in size and degree of polymerization.\(^8\) These structural differences also affect their antioxidant and anti-mutagenic potential.\(^8\) Condensed tannins are reported to comprise anywhere from 0.5-1.71\% of the pecan kernel weight, depending on cultivar.\(^9\)–\(^9\) Gu et al. characterized the degree of polymerization for condensed tannins using GC-MS and found that of the 494±86 \( \text{mg/100 g nutmeat} \) was as follows: monomers – 17.2 \( \text{mg} \), dimers – 42.1 \( \text{mg} \), trimers – 26 \( \text{mg} \), tetraters through hexamers – 101 \( \text{mg} \), heptamers through decamers – 84 \( \text{mg} \), and polymers with over ten subunits – 223 \( \text{mg} \).\(^9\) The level of PACs present in a cultivar highlights the potential of a
crop to resist insect and disease problems. Tannins are also a key colorant in pecans, so the hue of the shuck and middle divider between the two nut halves can help quickly determine quality. Furthermore, it is important to note that several other tree nuts, such as pine nuts, Brazil nuts and macadamia nuts, were tested for PACs and no detectable amounts were found, suggesting that pecans have unique advantages compared to other similar foodstuffs.

Tree nuts have been examined extensively for antioxidant benefits using a variety of in vitro methods, whose methodology will be discussed in the next section. A summary of these results can be found in Table 2.2. Pecans are ranked as the top nut for antioxidant content, and the reasoning can be seen by its high amount of phenolics (20.16 mg GAE/g), flavonoids (34.01 mg/100g), and proanthocyanidins (494.1 mg/100g), as well as their capability to quench free radicals, as seen in the ORACFL assay.

<table>
<thead>
<tr>
<th>Tree Nut</th>
<th>Total ORACFL (μmol Trolox equivalents/g)</th>
<th>Total Phenolics (mg gallic acid equivalents/g)</th>
<th>Total Flavonoids (mg/100g)</th>
<th>Total PACs (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almond</td>
<td>44.54</td>
<td>4.18</td>
<td>15.24</td>
<td>184.0</td>
</tr>
<tr>
<td>Brazil Nut</td>
<td>14.19</td>
<td>3.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cashew</td>
<td>19.97</td>
<td>2.74</td>
<td>1.98</td>
<td>8.7</td>
</tr>
<tr>
<td>Hazelnut</td>
<td>96.45</td>
<td>8.35</td>
<td>11.96</td>
<td>500.7</td>
</tr>
<tr>
<td>Pecan</td>
<td>179.40</td>
<td>20.16</td>
<td>34.01</td>
<td>494.1</td>
</tr>
<tr>
<td>Pine Nut</td>
<td>7.19</td>
<td>0.68</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td>Pistachio</td>
<td>79.83</td>
<td>16.57</td>
<td>14.37</td>
<td>237.3</td>
</tr>
<tr>
<td>Walnut</td>
<td>135.41</td>
<td>15.56</td>
<td>2.71</td>
<td>67.3</td>
</tr>
</tbody>
</table>
2.5 Antioxidants and Their Measurement

Antioxidants are necessary for protection from damage caused by free radicals. Free radicals are molecules with one or more isolated, unpaired electrons, which make them extremely reactive and unstable. The two main forms of free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some common ROS are the superoxide anion radical ($\text{O}_2^-$), alkoxy radical (RO’), peroxy radical (ROO’), hydrogen peroxide ($\text{H}_2\text{O}_2$), hydroxyl radical (HO’) and singlet oxygen ($^1\text{O}_2$). The most common RNS are the nitric oxide (NO’), nitric dioxide (NOO’), and peroxynitrite (OONO$^-$). In the body, ROS are formed naturally in the mitochondria of cells as a byproduct of metabolizing oxygen; though mitochondria have built-in ROS scavenging capabilities, about 1-3% of consumed oxygen’s metabolism to water is incomplete. Additionally, ROS concentrations are increased through environmental stresses, including chemicals, pollution, cigarette smoke and other environmental toxins. ROS are especially problematic because they are capable of attacking many macromolecules in the body, oxidizing lipids and proteins, breaking DNA strands and modulating gene expression. These pathways can damage cellular organelles and even induce apoptosis. Because ROS and other free radicals are naturally occurring in vivo, and amplified through environmental stressors, the body has several defense systems in place. These defenses include antioxidant enzymes, such as superoxide dismutase, glutathione S-transferase, and glutathione peroxidase, metal ion-binding proteins like ferritin, the packaging of DNA in chromatin, and various DNA repair systems. However, despite the effectiveness of endogenous antioxidant systems, they fail to provide complete protection; over time, detrimental changes in cells and tissues result in aging and
increased risk of various diseases, such as cancer, asthma, Alzheimer’s, Parkinson’s, retinal damage, and arthritis, just to name a few. For these reasons, dietary antioxidants, such as vitamins C and E, polyphenols and carotenoids, are required to maintain antioxidant balance in the body.

Antioxidants are defined as “any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.” They are considered to be reductants and can operate with primary or secondary antioxidant mechanisms; primary antioxidants directly quench free radicals, while secondary antioxidants work indirectly to inhibit initiation through binding pro-oxidants or scavenging oxygen. According to Shahidi, the antioxidant activity of a particular compound is a fairly general term that depends on its capability to scavenge free radicals and/or singlet oxygen, decompose free radicals, act as metal-ion chelators, or produce synergies with other similar compounds. In food samples, antioxidants can be split into hydrophilic and lipophilic antioxidants. The most prevalent hydrophilic antioxidants in nature are phenolic compounds, while the most common lipophilic antioxidants are tocopherols (section 2.4.2).

2.5.1 Mechanisms of Antioxidant Activity

There are two main mechanisms for antioxidants to quench free radicals: hydrogen atom transfer (HAT) and single electron transfer (SET). Individual antioxidants may use both mechanisms in a single system, or they may have one response that varies with oxidant and radical sources. For example, carotenoids are extremely effective at quenching singlet oxygen, but not peroxyl radicals, while phenolic compounds do not
quench singlet oxygen nearly as effectively as they do peroxyl radicals. Both HAT and SET mechanisms usually occur concurrently, but the dominant mechanism depends on a variety of factors including system characteristics (polarity, solvent, etc.) and the chemical properties of the antioxidant.

The HAT reaction is considered to be the more biologically-relevant mechanism and involves the transfer of a hydrogen atom to a radical, effectively stopping the radical reaction, as the resultant antioxidant radical is inherently stable. The basic reaction equation is as follows:

$$X' + ArOH \rightarrow XH + ArO'$$

As mentioned above, the resultant radical (ArO') is more stable, frequently due to resonance stabilization made possible by the antioxidant’s structure. As the radical is more stable and much less reactive, subsequent reactions are much less likely to continue. Understandably, the capability of an antioxidant molecule to perform a HAT reaction depends on its likelihood of losing a hydrogen atom and its subsequent stability as a radical. The capability of a compound to give up a hydrogen atom is highly dependent on the hydrogen’s bond dissociation energy (BDE); the lower the BDE for a particular hydrogen attached to a compound, the easier it is for a hydrogen to be transferred, and thus the higher the antioxidant activity will be. In the case of plant phenolic compounds, the BDE of the phenolic O—H bond is relatively low (~87-95 kcal/mol depending on the phase and polarity), making them good antioxidants. The BDE can be even further lowered by the regiochemistry of other substituents: in the case of α–tocopherol, the BDE of the phenolic O—H bond is lowered to ~77-79 kcal/mol due to the alkyl groups at the ortho position. Phenolic antioxidants are typically stable radicals after donating a
hydrogen atom; however, stability depends on structure and substituents. A ring structure can be deactivated by the presence of electron withdrawing groups, especially at the meta-position, whereas the presence of electron donating groups can activate the ring. Resonance stabilization of aromatic compounds plays a large part in the delocalization of the unpaired electron and subsequent stability of the radical. For example, as seen in Figure 2.4, after donating a hydrogen atom, a phenolic compound will shift from an unstable peroxyl radical to a stable phenoxy radical.

![Resonance Stabilization of a Phenolic Antioxidant](image)

The other major antioxidant mechanism is the SET reaction, which involves the transfer of a single electron to a free-radical species. This mechanism requires multiple steps in order to stabilize the aromatic compound; this is achieved through deprotonation reaction with water. The reaction scheme is as follows:

\[ X^- + ArOH \rightarrow X^- + ArOH^+ \]

\[ ArOH^+ \overset{H_2O}{\rightarrow} ArO^- + H_3O^+ \]

\[ X^- + H_3O^+ \rightarrow XH + H_2O \]
As this reaction scheme involves the creation of an ionic compound, the ionization potential (IP) of the antioxidant comes into play.\textsuperscript{113,115} If high ionization energy is required for the donation of an electron, it is much less likely that a compound will scavenge free radicals via SET pathways.\textsuperscript{113,118} In general, highly alkaline environments are preferred for SET reactions because IP decreases as pH increases.\textsuperscript{113}

2.5.2 Antioxidant Measurement Methods

There are a myriad of ways to measure antioxidant capacity, according to the literature today. Antioxidant capacity refers specifically to the “total radical-scavenging capability of a test solution, independent of individual antioxidant activity constants”.\textsuperscript{119} As discussed in the previous section, there are two methods in which antioxidants quench radicals: the Hydrogen Atom Transfer (HAT) mechanism, where an antioxidant donates a atom hydrogen to a radical or other unstable, highly reactive molecule, and the Single Electron Transfer (SET) mechanism, where an electron is transferred to the radical to halt its progress through a redox-type reaction. Both of these mechanisms can be employed in a single system (food or otherwise), or even by a single compound, as is the case for vitamin E.\textsuperscript{115,120,121} For this reason, when analyzing a compound or the antioxidant capacity of a food, it is important to choose multiple assays so each mechanism is examined and considered; in many cases, the results of a HAT and SET assay will not correlate, as different factors are in play.\textsuperscript{122}

It is important to note that many of the methods used to measure the effectiveness of antioxidants are purely in vitro assays. It is unclear whether antioxidant effectively in vitro truly correlates with effectiveness in vivo. One reason for this is that the human
digestive tract breaks dietary phenolic acids and polyphenols into various phenolic metabolites that have different bioavailability; metabolism and breakdown may alter the chemistry of an antioxidant so much that by the time it reaches the lower intestine, it is not absorbable and therefore incapable of bestowing antioxidant activity. In fact, consumption of 10-100 mg of a single compound rarely results in a postprandial plasma concentration of more than 1 μM. That being said, \textit{in vitro} assays are widely used in antioxidant research, particularly in screening, because it is widely accepted that compounds that fail to protect against oxidation and proliferation of free radicals \textit{in vitro} will rarely have good efficacy \textit{in vivo} for preventing oxidative reactions. Additionally, it is fairly common to administer various dietary antioxidant treatments and perform \textit{in vitro} tests on postprandial biological fluids, such as plasma, as a middle ground between \textit{in vitro} and \textit{in vivo} tests. In recent years, cell culture-based assays have been gaining ground and showing promise for \textit{in vivo} efficacy measurements.

\textbf{2.5.3 Total Phenolics Content (TPC) Assay}

The total phenolics content in a given sample does not measure the antioxidant activity of the compound in question. A number of researchers frequently report TPC values as antioxidant activity, but this is wrong. Nonetheless, there is frequently a strong correlation between the total phenolics content and antioxidant response. Direct and specific measures of antioxidant activity must be employed as well, but as TPC results frequently correlate, both methods are typically employed and reported together in antioxidant literature. In the cases where they are strongly correlated, it is typically concluded that the phenolics are the prime source of antioxidant activity within
the sample; similarly, if the correlation is not strong, it can be concluded that other non-phenolic compounds are present in large quantities, and are responsible for the quenching.\textsuperscript{113}

The TPC assay was originally developed in 1912 as a way to use colorimetric reagents comprised of a phosphotungstic-phosphomolybdic compounds to quantitatively measure tyrosine residues in proteins.\textsuperscript{132,133} In 1927, the method and reagent was improved by incorporating additional molybdenum to improve redox sensitivity.\textsuperscript{113,134} The method was further improved by Singleton and Rossi in 1965, who were the first to employ the assay for a food product (wine), and has not been changed much since then.\textsuperscript{135} They recommended various changes to improve repeatability and reliability in the assay, including a specific volume ratio, proper temperature and time constraints for the development of color, absorbance readings at a specific wavelength (765 nm) and gallic acid as a typical standard.\textsuperscript{115,135} The mechanism showing the development of a blue color, likely the result of a Keggin structure, as the molybdenum ion from the F-C reagent interacts with the present phenolics can be seen below:\textsuperscript{113}

\[ Mo^{6+} (yellow) + ArOH \rightarrow Mo^{5+} (blue) + [ArOH]^+ \]

Though the TPC assay is widespread in its use, there is still some dissent over how to standardize conditions and reporting styles.\textsuperscript{115} Though Singleton and Rossi gave very specific recommendations, different standards and wavelengths are used: for example, the literature shows gallic acid, \textit{p}‐coumaric acid, (+)‐catechin and other standards, which all have different \(\lambda_{\text{max}}\) values ranging from 745 to 765 nm.\textsuperscript{113,136,137} Opinions differ on whether or not gallic acid should be used explicitly, as many believe using representative standards for phenolics found in one’s sample is more important.
Other concerns about the TPC assay involve its lack of specificity, slow reaction time at acidic pH and interference from a variety of compounds found in wine and most fruits.\textsuperscript{113,115} Additionally, if one tries to increase the speed of reaction by increasing the temperature (T > 60°C), using an excess of F-C reagent (> 5 mL/100 mL) or making the solution extremely alkaline (pH > 10), the phenol reagent can precipitate out.\textsuperscript{113,115,138} Despite the varying opinions on the use of the assay and its effectiveness, its popularity in literature is clearly evident; when used in tandem with specific antioxidant activity measures that employ HAT or SET mechanisms, the TPC assay is a useful tool in predicting a sample’s antioxidant behavior.

2.5.4 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay follows the hydrogen atom transfer (HAT) mechanism and was originally developed in the 1990s for determining the effect of ROS species and subsequent radical damage through the monitoring of fluorescence of B-phycoerythrin (B-PE) in biological systems.\textsuperscript{139} It is unique in that it measures the total antioxidant activity of a substance by allowing the oxidation reaction to run to completion, showing results for lag time and initial rate.\textsuperscript{115,140} Additionally, experimental investigations have been conducted to explore the mechanism of action and Ou et al. showed that the ORAC assay is, in fact, following hydrogen atom transfer patterns and that it is specific to antioxidants.\textsuperscript{141} Many other antioxidant measurements only account for lag phase and early stages of oxidation; however, ORAC can avoid underestimation of antioxidant activity because its extended run time (30 min or more) can follow the effects of secondary oxidation products as well. Trolox\textsuperscript{®} is the standard traditionally used and the
area under the fluorescent decay curves (AUC) is all blank corrected compared to the sample and various Trolox standards and reported as Trolox equivalents. The excitation wavelength used is 493 nm, while the emission wavelength is 523 nm.

The principle behind this competitive assay is that a controlled azo initiator creates peroxyl radicals in the system by degrading AAPH. The radicals then interact with a fluorescent probe, causing the level of fluorescence to reduce over time. If an antioxidant is present in the system, there is a competitive HAT reaction, which causes fluorescence to be diminished to a lesser degree, as the antioxidant can intercept the radical before it can reach the probe. A possible reaction scheme can be found below:

\[ \text{ROO}^\cdot + \text{probe (fluorescent)} \rightarrow \text{ROOH} + \text{oxidized probe (loss of fluorescence)} \]

\[ \text{ROO}^\cdot + \text{AH} \rightarrow \text{ROOH} + A^\cdot \]

\[ \text{ROO}^\cdot + A^\cdot \xrightarrow{\text{fast}} \text{ROOA} \]

The assay has seen continual improvement over the past few decades, and is widely used in antioxidant research. Cao et al. adapted the assay to be able to assess antioxidants, such as α-tocopherol, vitamin C, and β-carotene, in human serum. The assay was automated in 1995 for a Cobas Fara II centrifugal analyzer and can now be performed in a microplate fluorescence reader, which drastically increases efficiency.

A decade after its development, the ORAC assay was drastically improved by Ou et al., who substituted fluorescein (3,6-dihydroxyxyspiro[isobenzofuran-1[3H],9[9H]-xanthen]-3-one) (FL) for B-PE as the fluorescent probe of choice; assays using this probe use the ORAC_{FL} method. B-PE was replaced because of poor reproducibility due to natural variability in its reactions with peroxyl radicals, tendencies toward
photobleaching effects, and binding with PACs that skewed results.\textsuperscript{115,145} It was also cited that commercially-available B-PE was only \textasciitilde30\% pure.\textsuperscript{141} Ou \textit{et al.} found that FL was a very suitable fluorescent probe, as it was much more affordable than P-BE, did not interact with phenolics and could be used in a 96-well plate without photobleaching. The only complaint with ORAC\textsubscript{FL} is that pH must be closely monitored: the pK\textsubscript{a} of FL is 6.5 and if the pH drops below 7, the fluorescent intensity is greatly reduced.\textsuperscript{141} The pH of the system for the ORAC\textsubscript{FL} assay is typically \textasciitilde7.4; as long as the pH is controlled with buffers, the assay and probe are very reliable. In addition to pH sensitivity, the ORAC\textsubscript{FL} assay is extremely temperature sensitive and must be performed at 37 °C. As the assay has a longer run time, it is essential to make sure all reagents and buffers are preheated to 37 °C before being added and the microplate reader used must have good temperature control. Previous studies show that variation in results is greatly reduced if the buffer used is preheated before AAPH is added and dissolved.\textsuperscript{142} Additionally, as this assay is performed in a plate, the outside wells are avoided to further reduce variation.

Originally, the ORAC\textsubscript{FL} assay was only suitable for hydrophilic antioxidants, such as phenolics, but it was a poor choice for lipophilic antioxidants, such as tocopherols, due to solubility issues. As ORAC\textsubscript{FL} was meant to be relevant to biological applications, the ability to measure the activity of lipophilic antioxidants was important, since they have \textit{in vivo} importance because of their capability to cross the lipid bilayers of cellular membranes for storage in the body.\textsuperscript{146} Through the work of Huang \textit{et al.}, the solubility of lipophilic antioxidants was improved by the use of randomly methylated β-cyclodextrin (RMCD) to the point where a 10-40\% addition of RMCD improves solubility of lipophilic antioxidants in an aqueous environment by 1000-fold.\textsuperscript{147} The lipophilic
ORAC_{FL} (L-ORAC_{FL}) method has been determined to be robust and reliable and has been used for a variety of foodstuffs including pecans, dates, broccoli and twenty-five other foods.\textsuperscript{147,148}

Despite requiring rigorous pH and temperature control, as well as changes for lipophilic solubility, the ORAC_{FL} assay is considered one of the best candidates for the standardization of antioxidant measurement.\textsuperscript{115}

2.5.5 Ferric Reducing Antioxidant Power (FRAP) Assay

In contrast with ORAC_{FL}, the FRAP assay is a single electron transfer (SET) mechanism assay. It was originally developed in the mid 1990s to examine the reducing power of plasma, and as such was named the Ferric Reducing Ability of Plasma.\textsuperscript{149} Since its development, the FRAP assay has undergone various changes to be able to examine and quantify the antioxidant power in other samples, including tea, fruits, vegetables other botanicals.\textsuperscript{115,150–152} A few years later, Benzie and Strain renamed the assay with its current name (Ferric Reducing Antioxidant Power assay).\textsuperscript{153} The method commonly used today is that of Pulido \textit{et al.}, which was developed in 2000.\textsuperscript{154}

The FRAP assay is a colorimetric assay that examines the reduction of ferric 2,4,6-tripyridyl-S-triazine (TPTZ). When a single electron is transferred to Fe\textsuperscript{3+}-TPTZ to form Fe\textsuperscript{2+}-TPTZ, a deep blue color develops in a very short amount of time. The reaction scheme can be found below:

\[
Fe^{3+} - TPTZ(\text{colorless}) + ArOH \rightarrow Fe^{2+} - TPTZ(\text{blue}) + [ArOH]^+ 
\]

The color development is fairly rapid, as the assay is traditionally touted as a quick assay that can be completed in just a few minutes. Despite this, Pulido \textit{et al.} found that
extending the reaction time beyond the traditional four minutes, showed increased absorbance values of several phenolics, including quercetin, caffeic acid, tannic acid and ferulic acid.\textsuperscript{154} They found that polyphenols require reaction times closer to 30 min.\textsuperscript{154} Because of this discrepancy, one must remember that some cases may underestimate the FRAP values depending on reaction time employed.

The FRAP assay is only capable of showing the reducing effects of compounds with a redox potential less than 0.7 V, which is that of the Fe$^{3+}$-TPTZ indicator; this could be seen as a drawback, but this is a reasonable redox potential for cells and tissues.\textsuperscript{115,149,153} However, it is important to state that any antioxidants operating with the HAT mechanism will not be detected, so FRAP is likely used in tandem with other antioxidant measures. This combining of methods is particularly useful when measuring serum, where high thiol and protein content will show serious underestimation if FRAP alone is used.\textsuperscript{115,152} Additionally, carotenoids are not well detected.\textsuperscript{154}

Performing the FRAP assay requires maintaining a very acidic pH, which can be seen as a downside; to preserve iron solubility, the assay must be performed at a pH of 3.6, which is much lower than physiological pH. Additionally, as pH is known to affect ionization potential, it must be monitored to maintain proper redox conditions in the system so electrons will be transferred as expected.\textsuperscript{155} The FRAP assay has also been called into question because there is no clear connection between a compound’s capability to reduce iron and it’s capability to quench radicals.\textsuperscript{115}

Despite the concerns about its relevancy, the FRAP assay continues to be a popular choice for a variety of reasons. It is rapid, inexpensive, requires no specialized equipment and is easily automated. Furthermore, its mechanism of action (purely SET) is
unique, as many antioxidant measurement techniques look only at HAT mechanisms or combine the two; this allows the FRAP assay to reveal antioxidant power that other antioxidant measures would miss, as well as highlight the dominant mechanism in compounds that use both. Therefore, though FRAP is rarely used alone, when combined with other methods it remains a powerful tool.

2.5.6 Cellular Antioxidant Activity (CAA) Assay

Though there are several *in vitro* antioxidant assays that are in wide use, such as the aforementioned ORAC<sub>FL</sub> and FRAP assays, there remains the fact that chemical assays cannot extrapolate the performance of the tested sample *in vivo*. The chemical tests are inexpensive, can be performed quickly and yield results that are good for comparing or ranking compounds based on their relationship to standard compounds such as Trolox. However, growing evidence shows that little can be deduced from these results when it comes to human health: in fact, the USDA Nutrient Data Laboratory has removed all their ORAC<sub>FL</sub> Database for Selected Foods from their website as of 2010, citing “there is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the antioxidant capacity of these foods” determined via *in vitro* studies. Antioxidant assays typically look at the scavenging and quenching of free radicals, but antioxidant action is not limited to this; it also encompasses up-regulation of antioxidant enzymes, modulation of gene expression and changes in cell signaling. Oxidative stress may be better understood biologically and mechanistically as a “disruption of redox signaling and control” rather than an imbalance between pro-oxidants and antioxidants.
Additionally, one must consider the reaction kinetics of a biological system, the liposolubility of antioxidants, which determines if they can reach radicals in compartmentalized systems, such as cells, and their ability to deal with secondary reactions.\textsuperscript{156,159,160} Since biological systems are so complex, cell culture assays are gaining traction as a viable intermediate testing method between traditional \textit{in vitro} tests and full-fledged clinical trials or feeding studies for gaining relevant information about dietary antioxidants.

One increasingly popular measurement technique for examining the effectiveness of antioxidants in a biological system, especially after employing \textit{in vitro} chemical methods, is the Cellular Antioxidant Activity (CAA) assay.\textsuperscript{126,156,161} The CAA assay is useful for examining the bioavailability of antioxidants because it can begin to take uptake, metabolism and membrane partitioning into consideration in ways that \textit{in vitro} assays cannot.\textsuperscript{156} They also require a lesser investment in time or resources than animal models and clinical studies involving humans. The proposed mechanism of the CAA assay can be seen in Figure 2.5.
Figure 2.5: Mechanism of the Cellular Antioxidant Activity (CAA) Assay\textsuperscript{126}

Briefly, the CAA assay measures the inhibition of a peroxyl radical-induced oxidation of dichlorofluorescin (DCFH) in cells. The ester form of the fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA) is first added to the cell because it can permeate the cellular membrane and cellular uptake is considered rapid and relatively stable, as the compound is nonionic and non-polar.\textsuperscript{161–163} Once inside, the DCFH-DA is hydrolyzed by cellular enzymes and left in the more polar and vulnerable DCFH form.\textsuperscript{126,164} The efficacy of antioxidants in the cellular system is assessed when the free radical generator 2,2-azobis (2-amidinopropane) dihydrochloride (ABAP) is added to the system. Peroxyl radicals are formed in a variety of ways, including at the cell membrane
or intracellularly as the ABAP decomposes. Cells also create ROS/RNS naturally through regular cellular function. The radicals formed create secondary ROS/RNS in the cell, which can cause oxidative damage, or oxidize DCFH directly to form the highly fluorescent DCF; however, if antioxidants are present, a competitive reaction occurs. Fluorescence diminishes as less DCFH is oxidized, due to the antioxidant activity of the sample applied.

The CAA assay is quantified by plotting the fluorescent response of the oxidized DCFH using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence is recorded every 5 min for an hour and the area under the resultant curve is compared for samples and a control. The CAA unit is determined by the following equation, which is the same as % reduction in fluorescence that is resultant from any given treatment.

\[
CAA \text{ unit} = 100 - \left( \frac{\int \text{sample curve}}{\int \text{control curve}} \times 100 \right)
\]

The first recorded instance of this type of cellular assay using a fluorescent microplate reader was by Wang and Joseph in 1999. DCFH had previously been shown as an effective chemical for analyzing ultramicro quantities of hydrogen peroxide, so Wang and Joseph examined the oxidation of DCFH over time and quantified oxidative stress by studying the changes in fluorescence. They showed a dose-dependent change in fluorescence based on the amount of ABAP applied and determined that DCFH was a good choice of indicator, as it was indiscriminate in its reaction with free radicals, and could thus could show the effect of multiple oxidation pathways.

Originally, the assay was developed using PC12 cells (cancer cells derived from the adrenal glands of rats), but in the past few decades has been modified for use in
Wolfe and Liu developed the assay for the HepG2 hepatocellular carcinoma cell line and also gave the assay its current name (CAA) in 2007. Sessa et al. examined the antioxidant activity of nano-encapsulated resveratrol in Caco-2 (human colorectal cells), showing that the antioxidant was readily absorbed in the cells of the GI tract. A modified CAA assay was also performed by Xu and Chang to look at the antioxidant effects of lentils and several other common legumes in gastric adenocarcinoma (AGS) cells. Erythrocytes have also been commonly used, as red blood cell (RBC)-centered antioxidant measurements are considered biologically relevant due to the role of RBCs in reducing oxidative stress in the circulatory system; this technique is frequently referred to as CAA-RBC assay. Recently, the conditions of the CAA assay have been optimized for Caco-2 cells, which are considered a more appropriate cell line than HepG2 cells based on their positioning in the digestive system and general similarity to epithelial cells in the small intestine. Additionally, as dietary antioxidants are taken into the body via oral routes, intestinal absorption is required before such compounds can take effect; thus, showing a compound’s capability to pass through the intestinal barrier is crucial for the true assessment of possible biological antioxidant power.

The CAA assay has been used to assess many different foods, including dietary supplements and pure antioxidant compounds, along with many common vegetables and fruits. Processing conditions have also been evaluated, such as the work of Roy et al., who examined steamed broccoli and deduced the antioxidant power was higher for the steamed samples when compared to raw samples. In many cases, cellular antioxidant capacities are heavily dependent on total phenolic content, where other
compounds, such as saponin and phytic acid have much lower effects as cellular antioxidants.\textsuperscript{126,156,160} Though the CAA assay has been used for a variety of food groups, tree nuts and pecans in particular, have not been assessed. Considering the wealth of phenolic compounds in pecans, one can expect promising results using this biologically relevant assay, especially in the Caco-2 cell line.

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CHAPTER 3

MODIFICATION OF CELLULAR ANTIOXIDANT ACTIVITY (CAA) ASSAY
TO STUDY PHENOLIC ANTIOXIDANTS IN CACO-2 CELLS

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ABSTRACT

Multiple *in vitro* assays are widely used to analyze the antioxidant potential of compounds; however, they cannot accurately predict antioxidant behavior in the body. Cell-based assays are gaining traction as they give a more comprehensive biological picture. One such assay is the cellular antioxidant activity (CAA) assay, which uses changes in fluorescence from a probe to monitor oxidative stress. It has recently been used to analyze dietary antioxidants using hepatocellular carcinoma (HepG2) cells, which are not directly representative of the human intestinal barrier. When HepG2 cells were employed in this experiment, concentrations of quercetin 25x greater than those in the literature were needed before similar results were found in this study; 25 μM quercetin reduced fluorescence by 17.1±0.9% and 250 μM quercetin reduced fluorescence by 58.6±2.4%. (+)-Catechin, a flavan-3-ol abundant in pecans, bestowed virtually no antioxidant activity within HepG2 cells. When modified for the Caco-2 cells, which better represent the human intestine, the CAA assay not only yielded higher antioxidant activity, but also showed catechin and quercetin yielding similar results, reducing fluorescence by 54.1±1.4% and 63.6±0.9%, respectively, when applied at 50 μM concentrations. Based on these results, which are likely due to differences in active membrane transport, the Caco-2-based CAA assay was deemed more appropriate for analyzing dietary phenolics, such as those found in U.S. pecans.

3.1 INTRODUCTION

*In vitro* methods of analyzing dietary antioxidants are widely used today; however, their overall usefulness has recently been called into question. This is because
the results from chemical tests simply cannot be extrapolated to predict the performance of the tested sample in the body. In fact, the importance of the most popular in vitro assays (the oxygen radical absorbance capacity or “ORACFL” test) has been minimized by the U.S. Department of Agriculture (USDA); they no longer maintain their ORACFL Database for Selected Foods, stating “there is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the antioxidant capacity of these foods” as measured via ORACFL.

Most in vitro tests analyze a compound’s capability to quench radicals via one of two mechanisms: the hydrogen atom transfer (HAT) method or the single electron transfer (SET) method. However, quenching radicals are not the whole role of antioxidant defenses; to protect from oxidative stress, antioxidant compounds may up-regulate antioxidant enzymes, modulate gene expression, or change cell signaling. “Test tube” assays, such as ORACFL and FRAP, are incapable of examining these additional factors, and for that reason, new methods of antioxidant activity analysis are needed.

Cell-based assays are gaining traction as a more biologically-relevant middle ground between in vitro assays and full-fledged feeding studies or clinical trials. One such assay is the Cellular Antioxidant Activity (CAA) assay, popularized in the last decade by Wolfe and Liu. The CAA assay is useful for examining bioavailability of antioxidants because it can take several additional factors into consideration including cellular uptake, metabolism and membrane partitioning. Originally, the assay used PC12 cells from the adrenal gland of rats; today HepG2 cells, derived from human hepatocellular carcinoma, are used frequently in the literature. Though the HepG2
cell line is an improvement over PC12 cells, as they are of human origin, the liver is not the most biologically-relevant cell for measuring the effectiveness of dietary antioxidants. For this reason, there is a desire to move towards the use of human colorectal adenocarcinoma (Caco-2) cells, as they are frequently used for modeling the intestinal barrier.\textsuperscript{9,10}

Briefly, the CAA assay uses a fluorescent probe (2,7-dichlorofluorescin diacetate or DCFH-DA) to monitor the inhibition of peroxyl radical-induced oxidation inside the cell. The ester form of the dye (DCFH-DA) is used because it is nonionic and non-polar and thus can rapidly transport across the cell membrane.\textsuperscript{6,11} Once inside the cell, the DCFH-DA is hydrolyzed and left in the more vulnerable DCFH form. A free radical generator (2,2’-azobis (2-amidinopropane) dihydrochloride or ABAP) is added to the system and begins to form peroxyl radicals. Other reactive oxygen species (ROS) are formed naturally through cellular function.\textsuperscript{12} If an antioxidant, such as quercetin or a food sample rich in phenolics is added which can enter the cell, it can compete with the radicals and quench them in a variety of ways, keeping the DCFH from being oxidized to form the fluorescent DCF. The principle of the CAA assay, along with possible pathways of radical quenching can be found in Figure 3.1.\textsuperscript{2} Antioxidant samples may competitively quench radicals before they can reach the DCFH dye (1), react with the ABAP directly to inhibit the formation of radicals (2), inhibit lipid peroxidation in the cell membrane (3), react with peroxyl radicals to stop propagation of other radicals (4) or inhibit intracellular redox pathways that can also oxidize the DCFH (5)\textsuperscript{1,2,13} The effectiveness of the sum total of these pathways is evaluated by monitoring the fluorescence resulting from DCFH over
time: reduced fluorescence compared to a control (wells with no antioxidant treatments) means the sample has substantial antioxidant activity within the cell.\textsuperscript{2,1,6}

Figure 3.1: Mechanisms and Antioxidant Pathways of CAA Assay

The CAA assay is quantified by looking at the CAA unit (the relative reduction in fluorescence, expressed in percent), which looks at relative areas under the curves (AUCs) of sample wells and control wells. The higher the CAA unit, the more effective the antioxidant is in a cellular system.

\[
CAA \text{ unit} = 100 - \left( \frac{\int \text{sample curve}}{\int \text{control curve}} \right) \times 100
\]
The assay has been used in HepG2 cells for a variety of pure phytochemicals, such as quercetin, gallic acid and caffeic acid, as well as several common fruits and vegetables.\textsuperscript{1,7,8} Commercial products, such as the OxiSelect\textsuperscript{TM} Cellular Antioxidant Assay Kit, are available for easy performance of the test.\textsuperscript{14} Recently, the assay has been modified for optimal analysis of pure phytochemicals in the Caco-2 cell line; Wan \textit{et al.} slightly changed cell plating density and probe concentration as well as incubation and measurement time for better results.\textsuperscript{10} As the assay is still relatively new and is thus continually being optimized and modified for a variety of applications, standardization has not yet occurred. In fact, there is no universally accepted method for the measurement of antioxidants, in cells or otherwise.\textsuperscript{15} For these reasons, discrepancies between labs are common.

In this study, the CAA was performed with quercetin, (+)-catechin and phenolic compounds derived from pecans following the directions of Wolfe and Liu, as well as the OxiSelect\textsuperscript{TM} kit.\textsuperscript{1,14} The assay was then modified to yield more biologically-relevant results in Caco-2 cells. The results demonstrated that the CAA assay was thus improved through the use of Caco-2 cells, for the cellular antioxidant activity of pure antioxidant standards and phenolics derived from U.S. pecans were measured and compared in both HepG2 and Caco-2 cells.

3.2 METHODS

\textbf{Materials and Reagents.} 2',7'-Dichlorofluorescin diacetate (DCFH-DA), 2,2-azobis[2-amidinopropane] dihydrochloride (ABAP), (+)-catechin hydrate, and quercetin dihydrate were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). Both
advanced and regular DMEM (Dulbecco's Modified Eagle Medium), Williams’ Medium E, phosphate-buffered saline (PBS), Hanks’ Balanced Salt Solution (HBSS), fetal bovine serum (FBS), L-glutamine, HEPES buffer, insulin, gentamicin, and penicillin-streptomycin were purchased from Life Technologies (Grand Island, NY).

**Extraction of Phenolic Compounds.** Raw in-shell pecans from the 2013 season were shipped to the Department of Food Science and Technology in Athens, GA from pecan orchards in Ocilla, GA. They were promptly shelled and frozen an -80 °C. Once frozen, pecan kernels were ground to a powder and defatted over 18 h using a Soxhlet apparatus and hexanes. The defatted nutmeat was allowed to dry overnight, then removed from the dried cellulose thimble and placed in a 500 mL Erlenmeyer flask. Following the method of Wu *et al.*, a (CH$_3$)$_2$CO/H$_2$O/CH$_3$COOH mixture (70.0/29.5/0.5 v/v) was used as the extraction solvent for the phenolic compounds. According to the methods of Craft *et al.*, 100 mL of solvent was added to the flask and covered with foil. Flasks were placed in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ) and heated at 60 °C for 30 min. The extraction liquid was then poured through Whatman #1 filter paper (185mm diameter, Whatman International Ltd., Maidstone, England) into a round-bottomed flask. The process was repeated three times and supernates pooled. The acetone and acetic acid were removed from the phenolic extract using a BüchiRotovapor R-210 (Büchi Corporation, New Castle, DE). The remaining aqueous liquid was placed in a crystallization dish and frozen at -80 °C overnight. Frozen samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MO). The dried phenolic extract powder was weighed, placed in amber colored vials, capped and stored at -80 °C.
**Cell culture.** Human hepatocellular carcinoma (HepG2) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured via the method employed by Wolfe and Liu.¹ Briefly, cells were cultured in Williams’ Medium E (WME) along with 5% FBS, 10 mM Hepes, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin and 100 μg/mL gentamicin. Cells were incubated at 37 °C with 5% (v/v) CO₂. Human colon adenocarcinoma (Caco-2) cells were also obtained from the American Type Culture Collection (ATCC, Manassas, VA). Following the method of Xie et al.,¹⁶ cells were cultured in Advanced Dulbecco’s Modified Eagle’s Medium (Advanced DMEM) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin (10,000 U/mL) and 1% streptomycin (10,000 μg/mL) and incubated in a humidified 5% (v/v) CO₂ environment at a temperature of 37 °C. Both cell lines were maintained in Corning 25-cm² canted-neck cell culture flasks and split using 1:2 ratios when confluent.

**Preparation of Chemical and Phenolic Sample Solutions.** Stock solutions of the phenolic extract from pecans were prepared by dissolving lyophilized powders in DMSO at a concentration of 25 mg/mL. On the day of analysis, samples were then diluted to final concentrations ranging from 0.05 mg/mL to 0.20 mg/mL in culture media; final treatment solutions contained < 2% DMSO. For chemical compounds, quercetin dihydrate and (+)-catechin hydrate were similarly dissolved in DMSO to form 50 mM stock solutions. On the day of analysis, standards were diluted to concentrations ranging from 5-1000 μM in cell culture media with final treatment solutions containing < 2% DMSO. A 12.5 mM stock solution of the fluorescent probe DCFH-DA (2,7-
dichlorofluorescin-diacetate) in methanol was prepared monthly; before each experiment, working solutions of 25 μM DCFH-DA in media were used. A 60 mM stock solution of ABAP in HBSS was prepared weekly and further diluted to 600 μM concentrations before experimental use. All DCFH-DA and ABAP solutions were kept frozen at -20 °C between uses while samples in DMSO were kept refrigerated at 4 °C.

Cellular Antioxidant Activity (CAA) Assay. Cellular antioxidant measurements were made following the method of Wolfe and Liu with modification.1 After HepG2 or Caco-2 cells reached confluence in Corning 25-cm² culture flasks, cells were washed with PBS twice, then dissociated from the surface using 0.05% trypsin-EDTA. Cells were plated (6.0 x 10⁴) in 100 μL cell culture media/well in Corning Costar® 96-well, black, flat bottom tissue culture-treated dishes and incubated until confluent (24-48 hours). Wells on the perimeter were left empty to reduce any variation due to plate location. Growth medium was removed after confluence was reached and cells were washed with PBS to remove any non-adherent cells.

Next, 50 μL of 25 μM DCFH-DA working solution was added to each well, followed by 50 μL of antioxidant treatments (in triplicate wells). Later, crude pecan extracts were added at 0.20 mg pecan phenolics/mL cell culture media. Final concentrations ranged from 2.5 to 500 μM for quercetin and catechin and were 0.10 mg/mL for pecan phenolics. For a control, 50 μL of dye and 50 μL of cell culture media (no antioxidant included) were applied to triplicate wells. Once the dye and antioxidant treatments were added, cells were incubated for 1 h at 37°C.

After 1 h, cells were washed with PBS three times to ensure any antioxidant effect was due to compounds internalized by the cells. One hundred μL of the free radical
generator, ABAP (600 μM), were then added. Cells were immediately placed in a BMG FLUOstar Omega microplate reader (BMG LABTECH Inc., Cary, NC), where the fluorescence was read initially and every five minutes after for 1 h (13 readings total). Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

**Quantification of CAA Assay.** Effectiveness of antioxidant treatments was quantified by examining the percent reduction in fluorescence. Briefly, a curve was generated by the 13 fluorescent response readings of each treatment over the course of the 1 h assay. The area under each curve was calculated through integration with MARS Data Analysis Software (BMG LABTECH Inc., Cary, NC). Control wells yielded the maximum fluorescence, as there was no inhibition of the ABAP and DCFH-DA reaction. Percent reduction (or CAA unit) was calculated by subtracting the ratio of the sample well (with antioxidant) to the control well (without antioxidant) from 1 and multiplied by 100. The equation can be found below.

\[
CAA \text{ unit} = \%\text{reduction} = \left(1 - \frac{AUC_{sample}}{AUC_{control}}\right) \times 100
\]

CAA units were found for quercetin, catechin, and pecan phenolics.

### 3.3 RESULTS AND DISCUSSION

**CAA results from HepG2 cells.** The CAA assay was performed using HepG2 cells and concentrations of quercetin ranging from 25 to 250 μM. The CAA unit increased in a dose-dependent manner based on how much quercetin was applied; the trend strongly followed a semi-log pattern as the increased AOx effect of greater antioxidant concentrations tapered off. Fluorescence was reduced by up to 58.6±1.0%
when 250 μM was applied. These results were in line with commercial products, such as the OxiSelect™ Cellular Antioxidant Activity Kit (Cell Biolabs, Inc., San Diego, CA); the enclosed methodology and literature recommended using concentrations of quercetin ranging from 31.3 to 2000 μM to develop a standard curve. These results showed very little in common with the work of Wolfe and Liu, who popularized the HepG2 version of the assay in 2007. In fact, concentrations of quercetin had to be multiplied by twenty-five to put CAA values in a similar range; Wolfe and Liu cited CAA values of up to 70.0 units with quercetin concentrations as low as 10 μM. For easy comparison, these results have been placed concurrently in Figure 3.2. The results were similar for three different experiments. Each time, this study yielded similar results to those expected from the commercial OxiSelect™ kit.

As quercetin is not a prevalent antioxidant found in tree nuts, (+)-catechin (a more representative standard for tree nut phenolics) was also analyzed using the CAA assay. Results can be found in Figure 3.3. The CAA of catechin increased only modestly with higher concentrations of catechin in HepG2 cells. The overall antioxidant effectiveness was diminished greatly; while 250 μM quercetin reduced fluorescence considerably for a CAA value of 58.6±1.0, the same amount of catechin resulted in a CAA value of 16.5±0.9.

Wolfe and Liu examined the structure-activity relationships of pure compounds and their effectiveness in HepG2-based CAA measurement and found that catechin had virtually no antioxidant activity in cells, while quercetin was extremely active. However, structural characteristics in catechin such as the ortho-dihydroxyl group on the B-ring and the C-3 hydroxyl group both increase its radical-scavenging capability.
monomeric and polymeric catechin is very prevalent in tree nuts, it was determined that
the HepG2-based CAA assay would not be useful for gaining biologically-relevant
information on the antioxidant potential of U.S. pecans. For this reason, in addition to its
increased biological relevancy, the Caco-2 cell line was employed.

Results for both catechin and quercetin were quite different when the Caco-2 cell
line was compared to the HepG2 cell line. As seen in Figure 3.4, with the use of Caco-2
cells, catechin CAA values were greater; lesser catechin concentrations (2.5-200 μM)
yielded greater fluorescent reduction than previously seen with HepG2 trials. In Caco-2
cells, fluorescence was reduced by 25.5-61.0%, whereas in HepG2 cells, the maximum
reduction in fluorescence was much less, at 16.5%. The results for quercetin followed a
similar pattern; Caco-2 results ranged from 19.8 to 71.1% reduction in fluorescence,
while similar amounts in HepG2 cells showed a maximum of 54.2% fluorescent
reduction.

Due to the large amounts of fluorescent reduction, Caco-2 cells were deemed
superior not only for the analysis of catechin and subsequently, pecan phenolics, but also
for quercetin. As quercetin, the standard used in common HepG2-based CAA analyses,
showed better cellular antioxidant activity in the GI-based Caco-2 model, subsequent
trials solely used the Caco-2 cell line.

One possible explanation for the difference in CAA in the two cells is their
membrane transport systems. As diffusion through membranes is similar for all cells,
differences in active membrane transport likely created the observed differences in
antioxidant activity. If HepG2 cells have an efflux transporter for the immediate removal
of catechin, it would be very difficult for the compound to effectively quench radicals or
help antioxidant defenses. Additionally, if HepG2 cells have an uptake transporter for quercetin but are lacking a similar transporter for catechin, a lesser CAA could be seen for catechin trials. Similarly, if Caco-2 cells had the uptake transporter for both compounds, increased CAA would result for both quercetin and catechin, as was seen in this study. As studying transporters was outside the scope of this project, further pharmacological research is necessary to pinpoint these transporters and their effects.

Phenolic extracts from pecans were then studied with the Caco-2 cells. When phenolics were applied to Caco-2 cells at a concentration (0.10 mg/mL), fluorescence was reduced by 48.2%. Roasted phenolics were also examined to see if thermal processing significantly degraded the antioxidant potential of the sample; no degradation was observed as the same level of roasted phenolics reduced fluorescence by slightly more (48.6%) in the Caco-2 cell line. For reference, catechin concentrations ~30 μM exhibited similar results, reducing fluorescence by ~50%.

There have been no previous CAA studies using phenolics derived from tree nuts so direct comparison is not possible. Previous research performed by Wan et al. aimed to optimize the CAA assay for Caco-2 cells. However, they used pure phytochemicals only and worked to streamline the area under the curve for best results; they did not examine the same compounds in multiple cell lines. Wan et al. concluded that Caco-2 cells could be used very effectively for such measurements of biological antioxidant activity, which was further demonstrated here.
Figure 3.2: CAA of Quercetin in HepG2 Cells

R² = 0.9971

Wolfe and Liu, 2007
Kellett, 2015
Figure 3.3: CAA of Catechin and Quercetin in HepG2 cells
Figure 3.4: CAA of Catechin and Quercetin in Caco-2 and HepG2 cells
3.4 SUMMARY AND CONCLUSION

As more questions have been raised about the relevancy of chemical tests in determining the biological effectiveness of antioxidants, such as the ORAC<sub>FL</sub> and FRAP assay, the need for new measurement techniques has grown. Cell-based assays are gaining interest in the scientific community, because they account for additional factors including metabolism of antioxidants, cellular uptake and secondary reactions. Recently, a HepG2-based Cellular Antioxidant Activity assay has been popularized; however, Hep-G2 cells are not suitable for every type of dietary antioxidant (possibly due to uptake or efflux transporters) and are not derived from the digestive system. Instead, Caco-2 cells (known to be good models of the intestinal barrier), were used and when results using the two cell lines were compared, Caco-2 cells were deemed superior. They provide more antioxidant activity (shown as substantial reductions in fluorescence) for multiple pure phytochemicals, as well as tree nut extracts. As HepG2 cells were not suitable for flavan-3-ols, such as catechin, the Caco-2 cell model would be more appropriate for assessing foods rich in these phenolics.

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CHAPTER 4

CELLULAR EVALUATION OF THE ANTIOXIDANT ACTIVITY OF U.S. PECANS [CARYA ILLINOINENSIS (WANGENHL.) K. KOCH]²

² Kellett, M.; Gong, Y.; Greenspan, P.; Pegg R.B. To be submitted to Journal of Agricultural and Food Chemistry
ABSTRACT

Clinical trials have shown an inverse relationship between consumption of antioxidant-rich tree nuts and the development of several chronic diseases. As the U.S. Department of Agriculture considers pecans to be a top antioxidant-rich food, their antioxidant efficacy was evaluated using a variety of *in vitro* assays (H-ORAC<sub>FL</sub>, TPC and FRAP) and a modified cellular antioxidant activity (CAA) assay. Crude phenolic extracts from raw and roasted pecans in both low- and high-molecular-weight fractions rich in proanthocyanidins (PACs) were analyzed. In the CAA assay, pecan phenolics entered human colorectal adenocarcinoma (Caco-2) cells (known to model the intestinal barrier) and bestowed cellular antioxidant activity. This activity was measured by monitoring the fluorescence of 2,7-dichlorofluorescein, which is highest with no antioxidants present. Concentrations of phenolics ranging from 0.025 to 0.10 mg/mL demonstrated reduced fluorescence by 37-69% for raw and 26-67% for roasted pecans. The primary active phenolic constituents in the extracts were determined to be epi(catechin) dimers and trimers *via* HPLC-ESI-MS<sup>n</sup>. These oligomeric PACs, ranging in size from ~560-840 g/mol appear to be small enough to be absorbed. In summation, these results show that pecans are an effective antioxidant in biological systems, regardless of roasting profile or method of measurement.
4.1 INTRODUCTION

In recent years, tree nuts have gathered a lot of positive attention due to their myriad of health benefits, which include fewer instances of cancer, longer lifespans, and better weight management.\(^1\)\(^-\)\(^3\) The healthfulness of tree nuts was asserted officially in July 2003 when the FDA granted tree nuts a qualified health claim due to strong evidence that regular consumption reduced the risk of heart disease; this point was reinforced with the 2010 Dietary Guidelines for Americans which state, “moderate evidence indicates that eating peanuts and certain tree nuts (\textit{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.”\(^4\)\(^,\)\(^5\) Many of these benefits are attributed to the favorable fatty acid profile of nuts as well as other lipid constituents, such as tocopherols and phytosterols; however, several phytochemicals, particularly dietary phenolics and proanthocyanidins (PACs), bestow several favorable effects.\(^6\)\(^,\)\(^7\)

Phenolic compounds are known for their antioxidant activity and capability to protect the body from oxidative stress. This is especially important as the damage caused by reactive oxygen species (ROS) and other free radicals are known to play a role in aging, as well as the development of several diseases, including cancer and heart disease.\(^8\)\(^,\)\(^9\) ROS such as the superoxide anion (O\(_2^•\)), alkoxyl radical (RO•), peroxyl radical (ROO•), hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radical (HO•) and singlet oxygen (\(^1\)O\(_2\)) are produced naturally in the body and enhanced by environmental stresses. For this reason, there are endogenous antioxidant systems in place including antioxidant enzymes, metal-ion binding proteins, and DNA repair systems.\(^10\)\(^,\)\(^11\) However, when the ROS loads increase beyond levels that antioxidant systems can handle, extensive damage to cells,
organelles and DNA can occur, leading to the development of many chronic diseases.\textsuperscript{12,13} With rising awareness of chronic diseases and their causes, the importance of dietary antioxidants is gaining ground in the public sphere. Through consuming a diet rich in a variety of tocopherols, carotenoids, and phenolics, antioxidant balance can be maintained in the body.\textsuperscript{14,15} This was shown for pecans specifically through a 2011 clinical trial performed by Hudthagonsol \textit{et al.}; they reported tocopherol levels in the blood were doubled, oxidized LDL cholesterol levels were reduced by over 25\% and ORAC\textsubscript{FL} values for lipophilic- and hydrophilic-antioxidants were increased significantly in the hours following test meals with pecans.\textsuperscript{16}

In general, antioxidants function \textit{via} two main mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET).\textsuperscript{17} The HAT mechanism is considered more biologically relevant and involves the donation of a hydrogen atom to a radical, effectively stopping its propagation. The antioxidant then undergoes resonance stabilization to create a stable, nonreactive radical.\textsuperscript{17} The SET mechanism is more similar to a classical redox reaction and in it an electron is transferred to quench a radical.\textsuperscript{17} As these are both very different reaction mechanisms and some compounds use a combination of both to quench radicals, it is important to use multiple assays to fully analyze a sample’s antioxidant activity.\textsuperscript{18} A common HAT assay is the Oxygen Radical Absorbance Capacity (ORAC\textsubscript{FL}) assay and a common SET assay is the Ferric Reducing Antioxidant Power (FRAP) assay.

Though \textit{in vitro} assays such as ORAC\textsubscript{FL} and FRAP are in wide use, recent evidence has shown that these measurements do not correlate to the performance \textit{in vivo}.\textsuperscript{19} Their biological relevance has been held in question and the USDA currently no
longer report ORAC\textsubscript{FL} values, citing that “there is no evidence that the beneficial effects of polyphenol-rich foods can be attributed to the antioxidant capacity of these foods” as seen in this chemical test.\textsuperscript{20} On the whole, these assays miss important aspects of biological antioxidant function such as bioavailability, uptake and metabolism of antioxidant compounds.\textsuperscript{13,21} Effective \textit{in vivo} antioxidants may even modulate gene expression and up-regulate endogenous antioxidant enzymes.\textsuperscript{19,22} For these reasons, the popularity of cell-based assays is on the rise; these methods are gaining traction as they are a good middle ground between chemical tests with questionable applicability in the body and expensive, time-consuming clinical trials and feeding studies.\textsuperscript{13} Though many studies use the HepG2 cell line (hepatocellular carcinoma), it has been said that the Caco-2 (colorectal adenocarcinoma) cell line is more representative, as it is in the digestive system and bears marked similarity to epithelial cells in the small intestine.\textsuperscript{13,23–25} As the capability to pass through the intestinal barrier is crucial for the effectiveness of dietary antioxidants, trials that simulate this environment are needed for accurate assessment of antioxidant activity.\textsuperscript{25}

While there are several studies using the CAA assay for a variety of foodstuffs (mainly fruits and vegetables) as well as pure antioxidant compounds, there has been no research examining the antioxidant activity of pecans or other tree nuts using a cell-based assay.\textsuperscript{13,24–26} In this present study, phenolic compounds from raw and roasted Georgia pecans were extracted and subsequently fractionated in order to assess their antioxidant properties using a variety of methods. Through the preparation of low-molecular-weight and high-molecular-weight fractions, it was possible to isolate the effects of condensed tannins (PACs) and compare the results with those from crude phenolic extracts. The
biological efficacy of phenolic extracts was assessed with a CAA assay using human adenocarcinoma (Caco-2) cells. Three traditional “test tube” assays (TPC, H-ORACFL and FRAP) were performed in order to relate CAA results with the in vitro antioxidant power of the same extracts, using both HAT and SET mechanisms. Additionally, characterization of the compounds responsible for strong antioxidant effects was performed using diol-phase HPLC-ESI-MS/MS.

4.2 METHODS

Chemicals and Glassware. Glass wool, sodium carbonate, ACS-grade methanol, ethanol (95%), acetone and hexanes, as well as HPLC-grade water, methanol, hexanes, 2-propanol and acetonitrile were purchased from Fisher Scientific Co., LLC (Suwanee, GA). Glacial acetic acid, hydrochloric acid and dimethyl sulfoxide (DMSO) were acquired from VWR International, LLC (Suwanee, GA). Sephadex LH-20, Amberlite, XAD-16, Folin & Ciocalteu’s phenol reagent, 2,7-dichlorofluorescin diacetate (DCFH-DA), 2,2-azobis[2-amidinopropane] dihydrochloride (ABAP), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), (+)-catechin hydrate, quercetin dehydrate, TPTZ (2,4,6-tripyridyl-S-triazine) and iron chloride were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). Both advanced and regular DMEM (Dulbecco's Modified Eagle Medium), Williams’ Medium E, phosphate-buffered saline (PBS), Hanks’ Balanced Salt Solution (HBSS), fetal bovine serum (FBS), L-glutamine, HEPES buffer, insulin, gentamicin, penicillin-streptomycin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Life Technologies (Grand Island, NY).
Sample Preparation. Raw in-shell pecans from the 2013 season were shipped from pecan orchards in Ocilla, GA to the Department of Food Science and Technology in Athens, GA where they were kept frozen until analyzed. On the day of analysis, pecans were shelled, stored in polyethylene plastic bags and stored at -80 °C between runs. This temperature maintained freshness in the pecans, as well as prepared samples for grinding.

Roasting. A fraction of the shelled pecans were removed from the -80 °C freezer and roasted before subsequent lipid and phenolic extraction. A proper roasting profile was developed by Erickson et al. and modified by Robbins.\textsuperscript{27,28} Briefly, a Lincoln Impinger impingement oven (Model 1450, Lincoln Foodservice Products, Fort Wayne, IN) was used to roast pecan halves at 175±10°C for 8 min. These parameters were optimized by Robbins to match commercially the color of roasted pecans using the Commission Internationale de l’Éclairage (CIE) L*C*h system.\textsuperscript{28} The roasted pecans were cooled and subsequently stored in the -80 °C until further analyzed.

Lipid Extraction. Lipid constituents were removed from all samples, raw and roasted, through the use of a Soxhlet apparatus. Shelled pecans (raw and roasted) were taken from the -80 °C freezer and promptly ground. Approximately 60g of nutmeat at a time was ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ) using an intermittent pulsing technique until a fine powder was achieved. The frozen temperature and pulsing kept any oil from being expelled during the grinding. Approximately 20 g of ground pecan nutmeat were placed in a cellulose extraction thimble (single thickness, 43 mm I.D. and 123 mm external length, Whatman International Ltd., Maidstone, England). A thin plug of glass wool was placed at the top of the thimble to ensure the entirety of the sample would remain in place throughout the
extraction. The extraction of lipids occurred over 18 h with the use of ~300 mL of hexanes. When the extraction was complete, the thimbles were removed and allowed to dry overnight. Hexanes were removed from the lipid portion using a Büchi Rotovapor R-210 (Büchi Corporation, New Castle, DE) and dried in a 103 °C oven for 1 h. After drying, the lipid portion was weighed for gravimetric analysis.

**Extraction of Phenolic Compounds.** The defatted nutmeat was removed from the dried cellulose thimble and placed in a 500 mL Erlenmeyer flask. Following the method of Wu et al.,\textsuperscript{29} a (CH\textsubscript{3})\textsubscript{2}CO/H\textsubscript{2}O/CH\textsubscript{3}COOH mixture (70.0/29.5/0.5 v/v) was used as the extraction solvent for the phenolic compounds. According to the methods of Craft et al.,\textsuperscript{30} 100 mL of solvent were added to the flask and covered with foil. Flasks were placed in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ) and heated at 60°C for 30 min. The extraction liquid was then poured through Whatman #1 filter paper (185 mm diameter, Whatman International Ltd., Maidstone, England) into a round-bottomed flask. The process was repeated three times and supernates pooled. The acetone and acetic acid were removed from the phenolic extract using a Büchi Rotovapor R-210 (Büchi Corporation, New Castle, DE). The remaining aqueous liquid was placed in a crystallization dish and frozen at -80°C overnight. Frozen samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MO). The dried phenolic extract powder was weighed, placed in amber colored vials, capped and stored at -80 °C.

**Desugaring and Fraction Separation.** In order to further analyze the phenolic compounds in pecan samples and isolate the effects of proanthocyanidins (PACs), the crude phenolic extracts were desugared and separated into low-molecular-weight (LMW)
and high-molecular-weight (HMW) fractions. Following the method of Srivastava *et al.*³¹ with slight modification, to desugar samples, ~2 g of phenolic extract were mixed in a small amount of 10% (v/v) methanol and sonicated until dissolved. The sample was applied to the top of a chromatographic column packed with Amberlite XAD-16 (bead size: 20-60 mesh) using a Pasteur pipette. The column was then washed with deionized water until 0.0% Brix registered on an Atago Digital Hand-held Refractometer PAL-1 (Atago USA, Inc., Bellevue, WA). This occurred after ~500 mL were run through the column. At this point, the eluent was changed to methanol to remove the desugared phenolics from the resin. The methanol was then removed using a Büchi Rotovapor R-210 (Büchi Corporation, New Castle, DE) with water bath set to 30 °C, and the aqueous remainder was placed in a crystallization dish and frozen at -80 °C overnight. Once frozen, samples were lyophilized in a FreeZone 2.5 L freeze dryer (Labconco Corp., Kansas City, MO). Next, samples were mixed in a small volume of ~75% (v/v) ethanol and sonicated until dissolved. Using a Pasteur pipette, the desugared phenolic sample was then applied to 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). Compounds making up the LMW fraction were eluted using ~1.5L of 95% (v/v) ethanol. Once the LMW fraction was removed, the eluent was changed to 50% (v/v) aqueous acetone and with ~600mL of mobile phase, the HMW fraction was eluted. Ethanol was removed from the LMW fraction with a Rotovapor with water bath set to 35 °C and acetone was removed from the HMW fraction with a Rotovapor with water bath set to 32 °C. Aqueous remainders were frozen and lyophilized as described above.
**Cell culture.** Human colon adenocarcinoma (Caco-2) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Following the method of Xie et al., cells were cultured in Advanced Dulbecco’s Modified Eagle’s Medium (Advanced DMEM) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin (10,000 U/mL) and 1% streptomycin (10,000 μg/mL) and incubated in a humidified 5% (v/v) CO₂ environment at a temperature of 37°C. Both cell lines were maintained in Corning 25-cm² canted-neck cell culture flasks and split using 1:2 ratios when confluent.

**Preparation of Chemical and Phenolic Sample Solutions.** Stock solutions of each of the phenolic extracts from pecans were prepared by dissolving lyophilized powders in DMSO a concentration of 25 mg/mL. On the day of analysis, samples were then diluted to final concentrations ranging from 0.05 mg/mL to 0.20 mg/mL in culture media; final treatment solutions contained <2% DMSO. For standard curves, quercetin dihydrate and (+)-catechin hydrate were similarly dissolved in DMSO for 50 mM stock solutions. On the day of analysis, standards were diluted to concentrations ranging from 5 to 400 μM in cell culture media with final treatment solutions containing <2% DMSO. A 12.5 mM stock solution of the fluorescent probe DCFH-DA (2′,7′-dichlorofluorescin-diacetate) in methanol was prepared monthly; before each experiment, working solutions of 25 μM DCFH-DA in media were used. A 60 mM stock solution of ABAP in HBSS was prepared weekly and further diluted to 600 μM before experimental use. All DCFH-DA and ABAP solutions were kept frozen at -20 °C between uses while samples in DMSO were kept refrigerated at 4 °C.
Cellular Antioxidant Activity (CAA) Assay. Cellular antioxidant measurements were made following the method of Wolfe and Liu with modification.\textsuperscript{13} After Caco-2 cells reached confluence in Corning 25-cm\textsuperscript{2} culture flasks, cells were washed with PBS twice, then dissociated from the surface using 0.05% trypsin-EDTA. Cells were plated (6.0 x 10\textsuperscript{4}) in 100 μL cell culture media/well in Corning Costar\textsuperscript{®} 96-well, black, flat bottom tissue culture-treated dishes and incubated until confluent (24-48 hours). Wells on the perimeter were left empty to reduce any variation due to plate location. Growth medium was removed after confluence was reached and cells were washed with PBS to remove any non-adherent cells. Next, 50 μL of 25 μM (DCFH-DA) were applied to each well, followed by 50 μL of antioxidant treatments (in triplicate wells). Final treatments ranged from 2.5 to 200 μM quercetin and catechin, as well as 25-100 μg pecan phenolics/mL. For a control, 50 μL of dye and 50 μL of cell culture media (no antioxidant included) were applied to triplicate wells. Once the dye and antioxidant treatments were applied, cells were incubated for 1 h at 37°C. After 1 h, cells were washed with PBS three times to ensure any antioxidant effect was due to compounds internalized by the cells. One hundred μL of the free radical generator, ABAP (600 μM), were added. Cells were immediately placed in a BMG FLUOstar Omega microplate reader (BMG LABTECH, Inc., Cary, NC), where the fluorescence was read initially and every five minutes after for 1 h (13 readings total). Fluorescent readings were taken at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Quantification of CAA Assay. Effectiveness of antioxidant treatments was quantified by examining the percent reduction in fluorescence. Briefly, a curve was generated by the 13 fluorescent response readings of each treatment over the course of
the 1 h assay. The area under each curve was calculated through integration with MARS Data Analysis Software (BMG LABTECH, Inc., Cary, NC). Control wells yielded the maximum fluorescence, as there was no inhibition between ABAP and DCFH-DA reactions. Percent reduction was calculated by subtracting the ratio of the sample well (with antioxidant) to the control well (without antioxidant) from 1 and multiplied by 100. In other words,

\[ \%\text{reduction} = \left(1 - \frac{\text{area under sample curve}}{\text{area under control curve}} \right) \times 100 \]

**Cell Cytotoxicity Assay.** To test the viability of cells after pecan treatments were applied, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used. DCFH-DA and pecan phenolics were applied in the same fashion as described for the CAA assay and allowed to incubate for 1 h. Then, the cells were thoroughly washed with PBS to remove all phenolic residue and traces of phenol red indicator, which interferes with MTT absorbance readings. Control wells contained MTT and media, but no phenolics. One hundred microliters of culture media without phenol red were then applied to the cells along with 10 μL of 12 mM MTT. This was allowed to incubate at 37 °C with 5% CO\(_2\) for 4 h. After the MTT incubation, 85 μL of media were removed and 50 μL of DMSO were added to each well to dissolve the formazan blue chromagen formed in the cells and mixed thoroughly with a multi-channel pipette. The samples were incubated at 37 °C once more for 10 minutes and mixed again to allow for thorough dissolving. The absorbance was measured at 540 nm using a BMG FLUOstar Omega microplate reader (BMG LABTECH, Inc., Cary, NC). The assay was quantified by looking at the absorbance of samples versus that of the control; treatments resulting in significantly lower absorbance readings are considered cytotoxic.
Antioxidant and Phenolic Assays. All tests were performed on the following six powdered extracts of pecan phenolics: Raw Crude, Raw LMW, Raw HMW, Roasted Crude, Roasted LMW and Roasted HMW. Antioxidant tests were all performed using a BMG FLUOstar Omega (Ω) microplate reader (BMG LABTECH, Inc., Cary, NC), equipped with temperature control, in clear-bottomed, black 96-well microplates.

Hydrophilic-ORAC<sub>FL</sub> Assay. To assess the antioxidant power of pecan phenolics in vitro using the HAT mechanism, the hydrophilic-ORAC<sub>FL</sub> assay was used; the method employed was that of Prior et al. The blank and diluent for the assay were phosphate buffer (0.075 M, pH 7.4). In each experiment, ABAP (80 μM in buffer) was used as the radical initiator and fluorescein (0.1 μM in buffer) was used as the fluorescent probe. Both the initiator and probe were maintained at 37 °C for the duration of the experiment. Phenolic extract powders from pecans were diluted to 0.5 mg/mL in ethanol and then further diluted to 0.025 mg/mL in the phosphate buffer. Twenty microliters of each sample, Trolox standard or blank were pipetted into a 96-well microplate.

The microplate reader was set to maintain 37 °C for the duration of the experiment; additionally, an external lead system and two 500-μL reagent pumps were used to add FL (fluorescein) and ABAP during the experimental run. A 3-h run time was employed for the analysis, and the fluorescence was assessed using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. During analysis, 200 μL of FL and 20 μL of ABAP were added to each well using automated addition. There was one cycle between additions of each reagent. Upon completion of the run, the area under the standard and sample curves was calculated and blank corrected. Samples and standards were compared and results were quantified using a Trolox standard curve.
comprised of concentrations ranging from 12.5 to 100 μM; values were reported as mmol Trolox eq./100 g nutmeat. Samples were prepared in duplicate and analyzed in triplicate for a total of six replicates that were averaged.

**TPC Assay.** The total phenolics content (TPC) of pecan samples was assessed using the method of Swain and Hills, through the use of the Folin & Ciocalteu’s (F-C) phenol reagent. Phenolic extract powders were dissolved in methanol and further diluted to 0.2 mg/mL. Using 1 mL of the methanolic extract, a final volume of 10 mL was reached through adding 7.5 mL deionized water, 0.5 mL F-C reagent, and 1 mL of saturated sodium carbonate (Na₂CO₃). The sample was vortexed for 30 s between additions of each component. The sample was allowed to develop color over the course of 1 hour. After the quiescent period, 200 μL of sample were added to a black, clear-bottomed 96-well microplate and absorbance was measured at λ = 750 nm. A standard curve was developed in a similar fashion using concentrations of (+)-catechin ranging from 1.6 to 8.0 μg/mL. TPC values were reported as mg (+)-catechin eq./100 g pecan nutmeat. Samples were analyzed in quadruplicate and averaged.

**FRAP Assay.** The Ferric Reducing Antioxidant Power (FRAP) assay was employed to determine the antioxidant activity of pecan extracts using the SET mechanism. The method of Pulido et al. was used. The freeze-dried phenolic extracts (crude, LMW and HMW) were dissolved in anhydrous methanol at a concentration of 0.2 mg/mL. The FRAP reagent was prepared by combining 2.5 mL of 10 mM TPTZ in 40 mM HCl and 2.5 mL of 20 mM FeCl₃•6H₂O and 25 mL of 0.3 M acetate buffer at pH 3.6. The FRAP reagent was incubated at 37°C until ready for use. A standard curve of aqueous iron sulfate (FeSO₄•7H₂O) was developed using five concentrations ranging
from 250 to 1600 μM. The temperature was maintained at 37 °C for the duration of the experiment. To perform the assay, 200 μL of FRAP reagent, 20 μL of deionized water and 6.6 μL of the blank, sample or standard were pipetted into the microplate and immediately analyzed in the plate reader, as the assay is time sensitive. The absorbance at λ = 595 nm was recorded and reported as mmol Fe$^{2+}$ eq./100 g nutmeat. Samples were prepared in duplicate and analyzed in triplicate for a total of six replicates that were averaged.

**Characterization and Quantification of Phenolic Components.** PACs in the HMW fractions were separated based on their degree of polymerization (DP) using diol-phase HPLC with fluorescence detection. An Agilent 1200 Series HPLC was utilized for the normal-phase analyses coupled with a Princeton SPHER DIOL column (4.6 x 250 mm, 5 μm particle size, 60 Å; Princeton Chromatography, Inc., Cranbury, NJ), equipped with a guard cartridge/holder system, a thermostatic column compartment set at 30 °C, and a fluorescence detector. A gradient elution consisting of mobile phase (A) CH$_3$CN/CH$_3$COOH (98:2, v/v) and (B) CH$_3$OH/ H$_2$O/CH$_3$COOH (95:3:2, v/v/v) was employed. 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; extracts were first dissolved in anhydrous CH$_3$OH (20 mg/mL) and then further diluted 1:1 (v/v) with mobile phase A to a final concentration of 10 mg/mL. Samples were passed through a 0.45 μm PTFE syringe filter prior to injection. Fluorescence detection was employed with excitation/emission at λ = 276/316 nm, respectively. The photomultiplier tube gain was held constant at 10 for the duration of the run time. Tentative identification based on
the DP was made by $t_R$ mapping of these authentic standards prior to ESI-MS/MS analysis. Quantification of samples was based on proanthocyanidin B2 standard $t_R$’s and area values.

Further characterization of the PAC sample was accomplished by diol-phase HPLC-ESI-MS/MS. Briefly, an Agilent 1100 HPLC system was coupled to a Waters® Q ToF (time-of-flight) micro™ Mass Spectrometer equipped with an ESI interface (Waters Corporation, Milford, MA) operating in both positive- and negative-ion modes using capillary voltages of +3.5 kV and −2.5 kV, respectively. The HPLC conditions of separation were those described above. The microchannel plate detector voltage was set at +2.35 kV. Nitrogen was employed as the desolvation gas at a temperature of 100 °C and flow rate of 150 L/h. Argon was used as the collision gas. For normal MS, the collision voltage was set at 5 V, but for MS/MS, the collision voltage was increased to 30 V. A full scan was performed over the mass range of 200 to 3000 Da at a rate of $m/\text{z}$ 13,000 per second. The MS/MS analyses were acquired by automatic fragmentation where the three most intense mass peaks where fragmented. Each mass spectrum generated was based on an average of five scans. PAC identity was determined based on $t_R$ mapping, $m/\text{z}$ comparison with those reported in the literature, and MS/MS fragmentations.

**Statistical Analysis.** To analyze the data from the CAA assay and the MTT assay, an analysis of variance was used for all treatments. CAA data was gathered from a minimum of 12 wells and reported as means ± standard error. Differences were analyzed among all raw and roasted samples, sample preparations (crude, LMW and HMW) and concentrations (25-100 μg phenolics/mL media). Differences in means were determined
using LS means procedure and a Student–Newman–Keuls (SNK) means separation test with $p<0.05$ using IBM SPSS Statistics 23 (IBM, Armonk, NY).

4.3 RESULTS AND DISCUSSION

*In vitro Antioxidant Assays.* Three extracts were prepared from both raw and roasted pecans (crude phenolic, LMW and HMW) for a total of six treatments, which were assessed using three popular *in vitro* measurements of antioxidant potential and determination of phenolics: TPC, H-ORAC\textsubscript{FL} and FRAP. These three tests gave a fairly comprehensive picture, as they accounted for both HAT and SET reaction mechanisms. The results from these tests can be found in Figure 4.1.

The TPC values were reported as mg (+)-catechin equivalents (CE)/100 g nutmeat. Values ranged from 344±16 mg CE/100 g nutmeat to 4080±167 mg CE/100 g nutmeat for the raw low-molecular-weight (LMW) fraction and roasted high-molecular-weight (HMW) fraction, respectively. In general, the LMW fractions had the lowest amount of total phenolics and the HMW fractions had the greatest. The crude phenolic extracts had a TPC value of 1240±26 and 2300±24 for raw and roasted, respectively; this marked difference is likely due to the heat during roasting liberating phenolics within the pecan. Significant differences ($p<0.05$) were seen between nearly all extracts, showing that the total phenolics content is highly dependent on sample preparation. These differences were expected, as the LMW and HMW fractions required additional preparation steps and were considered more purified samples.

Comparison to previous research proved difficult, as most TPC values in literature are reported as mg gallic acid eq./100 g fresh weight; in many cases there isn’t any
consideration relating the chemistry of the dominant phenolic compound in the samples analyzed. This study used a (+)-catechin standard, as it is a more accurate and representative standard of phenolics found in pecan kernels. Average values from this study were slightly different from pecan samples reported by Wu et al. (2016±103 mg GAE/100 g nutmeat), which were higher than the raw extracts in our study, but slightly lower than the roasted extracts. Bolling et al. performed a review of all available antioxidant literature and found pecans had an average TPC value of 1588 mg GAE/100 g nutmeat, which also falls between the values for raw and roasted pecans in this study. Both raw and roasted phenolics showed TPC values that were higher than studies such as those performed by De La Rosa et al. and Yang et al. (1170-1250 and 1227.3 mg/100 g nutmeat, respectively); this was likely due to differences in sampling and extraction solvent choice.

Overall, the H-ORAC$_{FL}$ assay showed less variability than other antioxidant measures, especially if LMW fractions are regarded separately. The values shown in Table 4.1 ranged from 8.14±0.05 mmol TE/100 g nutmeat for the raw LMW fraction to 24.2±0.9 mmol TE/100 g nutmeat for the roasted HMW fractions. Unlike the TPC assay, both raw and roasted crude extracts showed no significant difference ($p$>0.05), and both were statistically the same as the raw HMW fraction. LMW fractions showed no significant differences from each other, while the roasted HMW fraction was significantly different from all other samples.

The data in this study is comparable to the work of de la Rosa et al., where three Mexican pecan cultivars were reported to have H-ORAC$_{FL}$ values of 23.1±1.5, 26.2±3.8 and 22.7±5.0 mmol TE/100 g nutmeat. The average value for crude extracts in this
study was 22.2±1.0 mmol TE/100 g nutmeat, which was slightly lower but consistent with their work. Wu et al. reported data for the H-ORAC_{FL} that was also similar; they reported an average antioxidant capacity of 17.5±1.0 mmol TE/100 g nutmeat, which is slightly lower than our study. They reported an average antioxidant capacity of 17.5±1.0 mmol TE/100 g nutmeat, which is slightly lower than our study. This test shows good precision but values clearly vary due to cultivar, growing conditions and locations, and extraction schema, making larger pooled samples a good idea for consistent pecan data.

The FRAP results shown in Table 4.1 were the most varied of all the in vitro data, covering a range from 4.1±0.2 to 43.0±3.8 mmol Fe^{2+} eq./100 g nutmeat. Once again, the LMW fractions showed the lowest antioxidant activity, with 4.1±0.2 and 5.1±0.6 mmol Fe^{2+} eq./100 g nutmeat for raw and roasted pecans, respectively. The raw crude extract had a FRAP value of 14.7±1.5 mmol Fe^{2+} eq./100 g, while the roasted crude extract was almost double at 26.7±1.8 mmol Fe^{2+} eq./100 g. Interestingly, the raw HMW fraction was the most effective SET antioxidant with a FRAP value of 43.0±3.8 mmol Fe^{2+} eq./100 g, while the roasted HMW fraction was much lower at 23.6±1.7 mmol Fe^{2+} eq./100 g. The means of all samples were significantly different (p<0.05), with the exception of the LMW fractions.

Previous data from this laboratory showed a range of 14.2±1.2 to 20.7±0.7 mmol Fe^{2+} eq./100 g for a variety of cultivars from Georgia, New Mexico and Texas. Research by Blomhoff et al. showed pecans from the U.S., Mexico and Norway had FRAP values ranging from 6.3 to 11.1 mmol Fe^{2+} eq./100 g. In general, these were much lower than the results seen here. This is likely due to different methods of sample preparation, as well as the concentration effect that occurred through the fractionation process. Additionally, as the FRAP assay is time dependent, slight differences in reaction
time before readings were taken could have a large effect.\textsuperscript{35}

As mentioned above, all of these antioxidant measures can be affected by crop year, growing location, cultivar and a variety of other factors.\textsuperscript{28} Additionally, it is normal for the trends seen in different antioxidant measures to vary, as they measure antioxidant activity based on completely different mechanisms. For example, H-ORAC\textsubscript{FL} is a hydrogen atom transfer (HAT) assay while FRAP is a single electron transfer (SET) assay. As antioxidants in food frequently operate using a combination of both mechanisms, multiple measures, as seen here, are required for thorough analysis of any given sample.

**Cellular Antioxidant Activity (CAA) Assay.** The six extracts prepared for the \textit{in vitro} tests were also examined using the Cellular Antioxidant Activity (CAA) assay. These included crude phenolic extracts as well as LMW and HMW fractions from both raw and roasted pecans. CAA results were measured against (+)-catechin standards. Phenolic treatment of cells ranged from 0.025 to 0.10 mg phenolic/mL while standards ranged in concentration from 2.5 to 50.0 μM. Effectiveness of the antioxidant in Caco-2 cells was measured by monitoring fluorescence as 2′,7′-dichlorofluorescin (DCFH) was oxidized to 2′,7′-dichlorofluorescein (DCF) over the course of 1 h. Results were obtained by looking at the area under the curve (AUC) for a control well compared to the AUC for various sample treatments. The control wells contained the maximum possible fluorescence, as no antioxidants were present to stop azo-initiated free radicals from attacking the DCFH probe. Results were recorded as average % reduction in fluorescence (when compared to the control) with standard error and can be seen in Figures 4.1 and 4.2 for (+)-catechin standards and pecan phenolics, respectively.
For the (+)-catechin standards, fluorescence was reduced in a dose-dependent manner; fluorescence was reduced by 25.5% in the lowest concentration (2.5 μM) and 61.0% in the highest concentration (200 μM). These results differ from the previous research by Wolfe and Liu as the development of the CAA assay for HepG2 cells showed virtually no antioxidant activity (less than 3% reduction in fluorescence when compared to quercetin), citing the lack of a 2,3-double bond and 4-keto group in the B-ring of catechin as the reason. However, in Caco-2 cells this was not a problem as even modest concentrations significantly reduced fluorescence. As active membrane transport systems play a large role in cell-based assay, these discrepancies are likely due to differences in uptake or efflux transporters between the two cells. The substantial CAA of catechin standards in Caco-2 cells supports our approach, as catechin and other flavan-3-ols are the basis for most phenolic compounds in pecans.

Raw and roasted pecan phenolics entered the cell or interacted with the cell membrane in such a way that the DCFH probe was protected and fluorescence was significantly reduced. As seen in Figure 4.2, crude acetonic phenolic extracts reduced fluorescence by 37.1±3.0% to 49.9±1.7% from raw pecans and 25.9±3.8% to 48.6±1.3% from roasted pecans. High molecular weight (HMW) fractions outperformed all other samples and reduced fluorescence by a range of 50.1±1.2 to 69.1±1.2% in raw samples and 52.3±1.1 to 67.3±1.5% in roasted samples. In general, LMW fractions were less effective cellular antioxidants than HMW fractions. Interestingly, there was substantial overlap between LMW and crude treatments. Only the HMW fractions were significantly different ($p<0.05$) than the crude and LMW treatments when applied at concentrations of 0.05-0.10 mg extract/mL media. No significant differences existed ($p > 0.05$) between
raw and roasted samples, with the exception of the lowest concentration of roasted phenolics. Interestingly, this large amount of overlap between the crude and HMW phenolic treatments matches that of the H-ORAC FL assay and differs from the trends found in the FRAP results.

As the CAA assay was developed less than 10 years ago, standardization has not yet occurred. This makes results difficult to compare to others in literature based on the difference in choice of cell line, food being tested and optional wash steps with PBS. Wolfe and Liu used the assay in HepG2 cells for a variety of extracts from common fruits; their 2007 study standardized all results in relation to the fluorescent reduction of quercetin and found that for fruits, blueberry extracts were the most effective cellular antioxidant. This study considered washing with PBS between applying treatments and running the assay to be optional; different results were found with and without this wash step. With no PBS wash, blueberry extracts had the equivalent CAA of 171±12 μmol QE/100 g fruit, but with the PBS wash, CAA was drastically reduced to the equivalent of 47±1.9 μmol QE/100 g fruit. The CAA assay has also been performed on a variety of vegetables using gallic acid standards; beets and red pepper were found to be the most effective with CAAs equivalent to 41.9±6.2 and 41.4±1.8 μM quercetin, respectively. Much like Wolfe and Liu’s work, the vegetable study showed that the addition of a PBS wash dropped CAA values for beets and red pepper to 4.78±0.38 μM quercetin and 4.64±0.19 μM quercetin, respectively. Sessa et al. used Caco-2 cells to look at the CAA of nano-encapsulated resveratrol and found that antioxidant activity was very high (>80% reduction in fluorescence), which validated the use of the human colorectal cell line for these analyses.
As this is the first reporting of CAA results for tree nuts, little direct comparison is possible. However, it is important to note that the presence of a PBS wash in this study helps validates the method, as all reductions in fluorescence can be attributed to strong interactions in the cell, rather than surface phenomena. As this study performed a PBS wash with all runs and continued to see high CAA values for pecan phenolic extracts, these results are on par with and sometimes higher than others in literature, confirming that pecans can be an effective antioxidant in cells. This complements clinical data that showed that the phytochemicals in pecans are not only absorbable but also contribute to postprandial antioxidant defenses. More research is needed to determine the specific pathways of antioxidant defense and the transporters involved, but these results assert that a cell-based mechanism likely plays a role.

**Cytotoxicity Results.** To test the cytotoxicity of the pecan extracts and confirm that the reduced fluorescence was resultant from antioxidant defenses and not cell death, the MTT viability assay was performed. MTT absorbance readings can be seen for pecan treatments and the control wells in Figure 4.3. Crude pecan extracts of varying concentrations were applied to the cells for 4 h and no significant differences ($p>0.05$) were seen between sample cells and control cells. From this, we concluded that the pecan phenolics were not toxic to the cells at any of the concentrations used in the study.

**HPLC Characterization.** The HMW tannin fractions from both raw and roasted pecans were analyzed via NP-HPLC. Quantification was achieved through $t_R$ mapping and area values from proanthocyanidins standards. Chromatograms can be seen in Figure 4.4 and characterization and quantification for each peak can be found in Table 4.2. As seen in the chromatogram, the HMW fractions from raw and roasted had very similar
characteristics, with roasted fractions having higher amounts of PACs overall. Varying degrees of polymerization (DP) were seen, but all were comprised of (epi)catechin and (epi)gallocatechin units with B-type linkages.

As seen in Table 4.2, there were some monomers found in these HMW fractions, but the majority of these smaller constituents (and some of the smaller polymers) were likely removed from the Sephadex LH-20 column with the first mobile phase (95% EtOH), as seen in similar research by Robbins et al.\textsuperscript{42} Interestingly, the vast majority (~80\%) of the PACs in HMW pecan extracts (using 50% (v/v) aqueous acetone) were comprised of dimers and trimers. Studies by Robbins et al. reported similar results with dimers and trimers making up 81.9\% of the HMW phenolic fraction (56.6\%, 25.3\% trimers).\textsuperscript{42} In this study, dimers accounted for 44.1\% and 46.4\% of the PACs in raw and roasted pecans, respectively, while trimers accounted for 34.3\% of the PACs in raw pecans and 33.8\% of the PACs in their roasted counterparts.

Pecan HMW extracts also included tetramers and pentamers, but these were at lesser concentrations, making up 13.8\% of raw PACs and 17.2\% of roasted PACs. Because of the large proportion of dimers and trimers (molecular weights ranging from 560 to 840 g/mol), rather than larger polymers, coupled with clinical data and cellular data, one can conclude that many of the PACs may be absorbable for use in the body’s antioxidant defenses. This is supported by work by Déprez et al. that proved colonic microflora can break down polymeric PACs into smaller pieces for absorption and utilization in the body.\textsuperscript{43} In line with this, clinical data by Hudthagosol et al. showed that despite the fact that pecan phenolics are complex, phenolic bioactives were found in plasma after pecans were consumed.\textsuperscript{16} If the compounds were too large, they would not
be as readily absorbed in the epithelial cells and therefore could not bestow antioxidant activity within biological systems. However, these studies suggest that pecan phenolics are in fact small enough for effective antioxidant activity in the body. This large proportion of oligomeric PACs is somewhat unique to pecans; in contrast, other food sources of PACs, such as peanut skins, have much bigger polymers (DP>6), which are less biologically available due to size. When these results are combined with the strong fluorescent reduction seen in the CAA experiments, it is clear that pecan phenolics are very promising biological antioxidants.

4.4 SUMMARY AND CONCLUSION

Pecans have garnered increased interest recently, as their health benefits and the compounds behind them are being examined, explained and validated. Many of these benefits are due to their phenolic content, particularly the PACs. Through both in vitro and cellular assays, the antioxidant efficacy of pecan phenolics was examined and HMW fractions outperformed all others. No significant differences existed between the cellular antioxidant activity of raw and roasted pecans. As HPLC-ESI-MS/MS revealed that the majority of constituents in HMW fractions were (epi)catechin dimers and trimers, it is believed that these are the compounds most responsible for the antioxidant activity of both crude and HMW extracts. As pecan extracts displayed significant antioxidant potential in human cell lines, it is believed that their phenolic compounds are small enough to be absorbed through the intestinal barrier and can therefore bestow antioxidant activity on the cellular level, positively affecting human health.
Table 4.1: TPC (n=4) and Two Antioxidant Capacity Assays (n=6) for Assorted Pecan Phenolics*

<table>
<thead>
<tr>
<th>Pecan Phenolic Sample</th>
<th>TPC (mg (+)-catechin eq./100 g nutmeat)</th>
<th>H-ORAC&lt;sub&gt;FL&lt;/sub&gt; (mmol TE/100 g nutmeat)</th>
<th>FRAP (mmol Fe&lt;sup&gt;2+&lt;/sup&gt; eq./100 g nutmeat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Crude</td>
<td>1240 ± 26a</td>
<td>22.0 ± 1.7a</td>
<td>14.7 ± 1.5a</td>
</tr>
<tr>
<td>Raw LMW</td>
<td>344 ± 16b</td>
<td>8.9 ± 0.1b</td>
<td>4.1 ± 0.2b</td>
</tr>
<tr>
<td>Raw HMW</td>
<td>3560 ± 13c</td>
<td>22.0 ± 0.2a</td>
<td>43.0 ± 3.8c</td>
</tr>
<tr>
<td>Roasted Crude</td>
<td>2300 ± 24d</td>
<td>22.5 ± 1.6a</td>
<td>26.7 ± 1.8d</td>
</tr>
<tr>
<td>Roasted LMW</td>
<td>399 ± 4b</td>
<td>8.1 ± 0.1b</td>
<td>5.1 ± 0.6b</td>
</tr>
<tr>
<td>Roasted HMW</td>
<td>4070 ± 167e</td>
<td>24.2 ± 0.9c</td>
<td>23.6 ± 1.7e</td>
</tr>
</tbody>
</table>

*Means±SD followed by the same letter in a column are not significantly different according to ANOVA and SNK (p>0.05) done with IBM SPSS Statistics (version 18, IBM Corporation, Armouk, NY.)
Figure 4.1: CAA Results for (+)-Catechin Standards
Figure 4.2(a): CAA of Phenolic Extracts from Raw Pecans
4.2(b): CAA of Phenolic Extracts from Roasted Pecans
Figure 4.3: Cytotoxicity Results from MTT Assay
Figure 4.4: NP-HPLC Chromatograms for HMW Extracts from Raw and Roasted Pecans
<table>
<thead>
<tr>
<th>Peak No.</th>
<th>$t_R$</th>
<th>DP</th>
<th>Unit Type</th>
<th>Linkage</th>
<th>$[\text{M-H}^-]$ $(m/z)$</th>
<th>Raw (mg/g fr.)</th>
<th>Roasted (mg/g fr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.1</td>
<td>1</td>
<td>Catechin</td>
<td>N/A</td>
<td>289</td>
<td>2.81 ± 0.33</td>
<td>1.02 ± 0.10</td>
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<tr>
<td>2</td>
<td>5.6</td>
<td>2</td>
<td>(epi)catechin</td>
<td>B</td>
<td>577</td>
<td>12.83 ± 1.35</td>
<td>14.81 ± 2.35</td>
</tr>
<tr>
<td>3</td>
<td>7.1</td>
<td>2</td>
<td>(epi)catechin+(epi)gallocatechin</td>
<td>B</td>
<td>593</td>
<td>3.17 ± 0.21</td>
<td>3.63 ± 0.71</td>
</tr>
<tr>
<td>4</td>
<td>8.6</td>
<td>3</td>
<td>(epi)catechin</td>
<td>B</td>
<td>865</td>
<td>7.02 ± 1.33</td>
<td>7.31 ± 1.04</td>
</tr>
<tr>
<td>5</td>
<td>9.4</td>
<td>3</td>
<td>2 (epi)catechin+1(epi)gallocatechin</td>
<td>B</td>
<td>881</td>
<td>5.43 ± 0.35</td>
<td>6.12 ± 0.72</td>
</tr>
<tr>
<td>6</td>
<td>10.5</td>
<td>4</td>
<td>(epi)catechin</td>
<td>B</td>
<td>1153</td>
<td>2.52 ± 0.18</td>
<td>2.25 ± 0.20</td>
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<tr>
<td>7</td>
<td>12.2</td>
<td>5</td>
<td>(epi)catechin</td>
<td>B</td>
<td>1441</td>
<td>0.96 ± 0.08</td>
<td>2.28 ± 0.13</td>
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<tr>
<td>8</td>
<td>14.5</td>
<td>5</td>
<td>4 (epi)catechin+1(epi)gallocatechin</td>
<td>B</td>
<td>1457</td>
<td>1.52 ± 0.21</td>
<td>2.32 ± 0.58</td>
</tr>
</tbody>
</table>
REFERENCES


(20) U.S. Department of Agriculture Nutrient Data Laboratory. *Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, Release 2*; 2; 2012.


CHAPTER 5

CONCLUSIONS

Pecans are a very important crop to the Georgia economy. Current literature shows that pecan nutmeat contains some of the highest amounts of antioxidant compounds of any fruit, vegetable or tree nut. Strategic marketing initiatives that play on the pecan’s healthfulness could play a large role in distinguishing them from other popular tree nuts (almonds, walnuts, etc.). In order to best equip the pecan industry for new publications, accurate and complete evaluation of the antioxidant profile using both chemical and biological assessments is needed.

In recent years, the applicability of in vitro antioxidant measurements to biological systems has been called into question. For this reason, cell-based assays are gaining popularity to supplement widely used chemical tests. In the present study, the Cellular Antioxidant Activity (CAA) assay, recently used in HepG2 cells, was modified to use Caco-2 cells. Caco-2 cells are part of the GI system and thus, are more appropriate for analyzing dietary antioxidant compounds. This study showed that a variety of pure phytochemicals (quercetin and catechin) performed better in the Caco-2 cell line, showing more overall fluorescent reduction, which directly correlates with antioxidant activity. (+)-Catechin was nearly 4x as effective in Caco-2 cells as in HepG2 cells (16.5% and 61.0% reduction at 200 μM concentrations, respectively). Similarly, quercetin was also more effective, as it increased from a 54.2% reduction in HepG2 to a 71.1% reduction in Caco-2 at 200 μM. As the phenolic compounds in pecans are largely
comprised of (+)-catechin (in both monomeric and polymeric forms), subsequent research on the antioxidant activity of pecans and their phenolics was performed using the Caco-2 cell line.

The literature shows that pecans are one of the richest natural sources of antioxidants, in large part due to their favorable phenolic profile. In order to have a comprehensive picture of pecan-based antioxidants, phenolic extracts from both raw and roasted pecans were prepared and analyzed in a variety of in vitro methods (TPC, H-ORACFL, and FRAP) and in the modified CAA assay. Values for roasted pecan phenolics were significantly higher ($p<0.05$) than raw for both the TPC and the FRAP assays: raw and roasted phenolics had a TPC value of $1240\pm26$ and $2300\pm24$ mg CE/100 g, respectively. FRAP values were $14.7\pm1.5$ mmol Fe$^{2+}$ eq./100 g nutmeat for raw phenolics and $26.7\pm1.8$ for roasted. H-ORACFL values did not differ between the two samples: raw phenolics had an ORAC value of $22.0\pm1.7$ TE/100g nutmeat, while roasted phenolics had an ORAC value of $22.5\pm1.6$ TE/100g nutmeat. When applied at concentrations of 0.05-0.10 mg phenolics/mL, raw and roasted phenolics were not significantly different in the CAA assay; phenolic treatments reduced fluorescence anywhere from $39.5\pm2.1$ to $48.6\pm1.8\%$, exhibiting strong antioxidant character. As the phenolics showed high antioxidant levels in both the ORAC and FRAP assays, it is believed that pecan phenolics operate under both the HAT and SET reaction mechanisms.

A substantial amount of the antioxidant activity of pecans is suspected to derive from the proanthocyanidins (PACs) found in these tree nuts. For this reason, a LMW and a HMW fraction were prepared from the raw and roasted phenolic extracts in order to isolate and evaluate the effect of PACs. As hypothesized, the HMW fraction, known to
be rich in PACs, performed better in each assay employed. HMW fractions ranged from 3560±13 to 4070±167 CE/100 g nutmeat for TPC, 22.0±0.2 to 24.2±0.9 TE/100 g nutmeat for ORAC_{FL} and 23.6±1.7 to 43.0±3.8 mmol Fe^{2+}_eq./100g nutmeat for FRAP. Similarly, the HMW fractions also showed more antioxidant activity in the CAA assay: for concentrations of 0.05-0.1 mg/mL, these fractions reduced fluorescence between 50.1±1.2 and 69.1±1.2% for raw phenolics and 52.3±1.1 and 67.3±1.5 % for roasted.

To validate the results found in the antioxidant assays and confidently state that the majority of the effects were from PACs, HPLC separation techniques were employed to identify the primary compounds in the HMW phenolic extracts. We found that the majority of these fractions were comprised of (epi)catechin dimers and trimers, which are likely small enough for use in the body; monomers, tetramers and pentamers of both (epi)catechin and (epi)gallocatechin were all present in smaller amounts.

Pecans are known to have strong antioxidant character in *in vitro* tests, such as ORAC, FRAP and TPC. Recent clinical data has shown that postprandial plasma antioxidant data is elevated after consumption of pecans, suggesting that phenolics are absorbable and functional *in vivo*. Further studies are needed to strengthen this point; this study validated the biological effectiveness of pecan phenolics by showing that a variety of modest treatments can exhibit substantial antioxidant activity within cells that model the human GI tract. This is likely due to the (epi)catechin dimers and trimers in pecans, which are small enough to be absorbed in the body. This research may illuminate the unique healthful properties of pecans and advance pecan consumption, changing it from a rare holiday treat to an everyday indulgence that consumers (and producers alike) can feel good about.