IMPACT OF *IN VITRO* DIGESTION ON PECAN [*CARYA ILLINOINENSIS* (WANGENH.) K. KOCH.] PHENOLICS AND EVALUATING THEIR ABSORPTION USING CACO–2 MONOLAYERS

by

MICHELLE CHEUNG

(Under the Direction of Ronald B. Pegg)

ABSTRACT

Limited information on the effects of digestion and bioaccessibility of pecan phenolics exist within the literature. The effects of *in vitro* gastrointestinal digestion on pecan phenolics were evaluated using various *in vitro* antioxidant assays and changes to the phenolic profile were monitored using HPLC–ESI–MS/MS. The phenolic profile was modified during digestion and an overall decrease (30-100%) in phenolic compounds was noted. This was reflected in reduced TPC (20-50%) and antioxidant capacity (29-80%) following digestion. Interestingly, a 20-fold increase in procyanidin dimers was observed and was attributed to the dimerization of (+)–catechin and breakdown of procyanidins oligomers. Caco–2 monolayers were utilized as a model of the intestinal lining to evaluate the apical to basolateral transport of *in vitro* digested pecan phenolics. Ellagic acid, four ellagic acid derivates, epigallocatechin gallate, along with procyanidin monomers to trimers were able to undergo apical to basolateral transport across Caco–2 monolayers.

INDEX WORDS: Pecans, phenolics, antioxidants, *in vitro* digestion, Caco–2 cells, Tranwells, proanthocyanidins.

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DEDICATION

To grandpa. Thank you for sharing your love of food with me and everything you've done for me.

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CHAPTER 1

INTRODUCTION

In recent years, there has been increased interest in the pecan [*Carya illinoinensis* (Wangenh.) K. Koch.] and other tree nuts due to increased public awareness about their healthfulness. A variety of studies have shown that regular nut consumption is associated with a lowered risk of heart disease and other chronic diseases. Pecans along with almonds, hazelnuts, peanuts, some pine nuts, pistachio, and walnuts were even awarded the following qualified health claim: "Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts, such as pecans, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease (FDA, 2018)." The benefits of nut consumption have been attributed to the various lipid components and phenolic compounds with anti–oxidative and anti–inflammatory properties (Hudthagosol et al., 2001). Pecans in particular are extremely rich in antioxidant compounds, such as phenolics (Gu et al., 2004). Studies have indicated that the predominant compounds present in pecans are flavan–3–ols, more specifically monomers such as (+)–catechin, (–)–epicatechin, and oligomeric and polymeric proanthocyanins (PACs) (Robbins et al., 2014; Gong and Pegg, 2017).

While research has indicated that pecans are a rich source of dietary antioxidants and possess strong *in vitro* antioxidant properties, many of the assays used to measure antioxidant capacity do not reflect relevant biological conditions (Gong, 2016; Gong and

Pegg, 2017; Kellett, 2015; Kellett et al., 2018; Robbins, 2012; Robbins et al., 2014; Robbins et al., 2015). One major hurdle is that the antioxidant capacities of compounds *in vivo* are affected by their bioavailability and bioaccessibility. In addition, it is possible that digestion and absorption can result in changes to chemical structure. As structure is often linked to function, it is possible that the antioxidant capabilities of phenolics could be modified after undergoing digestion and absorption. Therefore, the impact of digestion and absorption on phenolics should be taken into consideration when discussing the potential health benefits of pecan consumption.

To date, limited information on the digestion and absorption of phenolics from pecans exists in the literature. However, what little information exists suggests that some antioxidants compounds from pecans could be absorbed through the intestinal lining and bestow antioxidant activity. A recent pecan feeding study conducted by Hudthagosol et al. (2011), showed that post–prandial plasma levels of α–tocopherol and (+)–catechin were elevated. In addition, the authors also observed increased post–prandial plasma antioxidant activity, as evaluated with the lipophilic– and hydrophilic–oxygen radical absorbance capacity assays.

Presently, existing research has studied the intestinal absorption of digested phenolics from various foods, such as strawberry fruits, by combining static *in vitro* digestion with the human colorectal adenocarcinoma (Caco–2) cell line as a biological model of the intestinal epithelium (Kosińska–Cagnazzo et al., 2015). However, thus far no such studies have been done for pecans. Therefore, this project seeks to fill this gap and further knowledge of the possible benefits of pecan consumption by studying the capability of pecan phenolics to remain intact throughout gastric and duodenal digestion,

as well as their absorption using the Caco–2 cell line as a model of the intestinal lining. Specific objectives are outlined as follows:

1) Investigate the impact of digestion on the antioxidant properties of phenolic extracts prepared from raw and roasted pecans using *in vitro* assays (i.e., TPC, TEAC, FRAP, and H–ORAC$_{FL}$).
2) Evaluate the transport of prepared digested LMW and HMW fractions from raw and roasted pecans across Caco–2 monolayers *via* HPLC–ESI–MS/MS.

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CHAPTER 2

LITERATURE REVIEW

2.1 Nuts and their healthfulness

As the incidence of chronic diseases, such as cardiovascular disease (CVD), has increased, interest in dietary interventions to ameliorate or reduce the risk of developing these illnesses has been growing. Diets abundant in plant foods, such as fruits, vegetables, legumes, nuts and whole grains, have been associated with a reduced risk of developing chronic diseases (Sabaté, 2003). The protective effect bestowed by the consumption of plant foods may partially be attributed to the bioactive phytochemicals, such as tocopherols and polyphenolics, present. In plants, these bioactive phytochemicals confer protection against oxidative stress (Ros, 2015). Oxidative stress plays a significant role in the development of chronic diseases in humans (Temple, 2000). Increasing evidence has suggested that these phytochemicals are bioavailable and may exert a positive effect on human health by preventing or reducing the risk of degenerative diseases by ameliorating the effects of oxidative stress (Hudthagosol et al., 2011; Ros & Hu, 2013). Nuts in particular offer an attractive package of minerals, vitamins, fiber, phytochemicals, and lipid constituents, which together impart a protective effect against chronic disease.

2.1.1 Heart disease

Perhaps one of the most studied and well–known benefits is the link between regular nut consumption and decreased risk of heart disease. Pecans, almonds, hazelnuts, peanuts, some pine nuts, pistachios, and walnuts were even awarded the following qualified health claim: "Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts, such as pecans, as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease (FDA, 2018)." The mechanism of action can be attributed to several factors. Nut consumption has been shown to ameliorate various risk factors for cardiovascular disease (CVD). Some risk factors include elevated levels of blood total cholesterol (TC) and low–density lipoprotein cholesterol (LDL–C), in addition to reduced levels of high–density lipoprotein cholesterol (HDL–C).

Within the past several decades, several epidemiological studies have investigated the relationship between nut intake and cardiovascular disease. The Adventist Health Study was one of the earliest investigations that linked frequent nut consumption with a lowered risk of CVD (Fraser et al., 1992). In 1974, the Adventist cohort was established when a census questionnaire was mailed to all Seventh–day Adventist (SDA) households in the state of California. (Beeson et al., 1989). For this study, the group members were mailed a detailed lifestyle questionnaire that also contained a food frequency section. This section included a nut consumption category, with response options ranging from "never consume" to "more than once per day" and medical records were reviewed to assess CVD. The authors noted that nut consumption among SDA was relatively high, with 24% of subjects consuming nuts at least 5 times a week. The results of this study

suggested that frequent nut intake conferred a protective effect against the incidence of non–fatal and fatal CVD.

The Nurses' Health Study was another early prospective cohort study, which linked frequent nut consumption with a lowered risk of CVD in women. This cohort was established in 1976 and the study followed the lifestyle and health of 121,700 registered female nurses between the ages of 30 to 55. Participants received follow–up questionnaires every two years to provide updated information on potential risk factors and newly diagnosed diseases. The authors noted that frequent nut intake significantly reduced the risk of CVD in women. Subjects who ate nuts more than 5 times a week reduced their risk of CVD by 35% relative to individuals who rarely ate nuts (Hu et al., 1998).

The lowered risk of CVD associated with nut consumption has been attributed to the various lipid and phenolic compounds with anti–oxidative and anti–inflammatory properties (Hudthagosol et al., 2011). Various studies have investigated the impact of nut consumption on various biomarkers of CVD, such has levels of TC and LDL–C, in addition to the ratio of LDL–C to HDL–C and the HDL–C: TC ratio.

Sabaté et al. (2010) performed a meta–analysis of 25 intervention trials conducted between 1992 and 2004 that investigated the effects of nut intake on blood lipid levels. The results of the suggested that the incorporation of nuts into the diet reduced TC, LDL–C, ratio of LDL–C to HDL–C, in addition to reducing the ratio of HDL–C to TC. The authors also noted that the cholesterol–lowering effects of nut consumption were dose dependent and more pronounced in subjects with higher baseline LDL–C or lower body mass index.

2.1.2 Studying the health benefits of pecan consumption

Within the last 20 years, several clinical trials have focused on the effect of pecan intake on ameliorating risk factors for CVD. Many of these studies have focused on changes to specific blood biomarkers that have been associated with CVD. These include total triglycerides, LDL–C, and HDL–C. However, very few studies have investigated the bioavailability of antioxidant compounds, such as tocopherols and catechins, from pecans.

Morgan and Clayshulte (2000) conducted an eight–week, randomized controlled study to investigate the impact of pecan nutmeat consumption on the serum profiles (i.e., total triglycerides, LDL–C, and HDL–C) of 19 individuals with normal lipid levels who ate self–selected diets. For eight weeks, both groups avoided the consumption of other nuts. The treatment group consumed 68 g of pecans per day along with their self–selected diets while the control groups only consumed their self–selected diets. The subject's serum levels were analyzed at 0 (baseline), 4 and 8 weeks. The outcomes of the study indicated that at 4 and 8 weeks the levels of TC and LDL–C of the pecan treatment group were lowered. It also demonstrated that after 8 weeks of pecan treatment, levels of TC and HDL–C were significantly lower than that of the control group.

Rajaram et al. (2001) compared the effects of pecan consumption on modifying serum profiles to that of consuming a Step I diet recommended by the National Cholesterol Education Program in individuals with normal to moderately high serum cholesterol. The authors conducted a 10–week single–blind, randomized, controlled crossover study with 23 individuals (9 women and 14 men). The first 2 weeks of the study consisted of a lead–in phase, where subjects were fed a typical American diet

consisting of 34% energy from total fat and 15% from saturated fat. This was followed by the Step I and pecan–enriched diets, with each lasting for 4 weeks. No washout period between diets was included in this study. Serum levels of TC, LDL–C, HDL–C and triacylglycerol levels were measured to evaluate the impact of dietary modification. The authors noted that a pecan–enriched diet decreased TC, LDL cholesterol and triglyceride levels by 6.7, 10.4, and 11.1% beyond the Step I diet, respectively. Furthermore, HDL cholesterol levels were 5.6% higher than those of the Step I group. Taken together the results indicated that a pecan–enriched diet provided a more favorable improved serum lipid profile than the Step I diet. This suggested that nuts, like pecans, which are rich in monounsaturated lipids (MUFAs) and low in saturated fat could be part of a cholesterol lowering dietary intervention for patients or healthy individuals.

Haddad et al. (2006) utilized a randomized, single–blind, crossover, controlled–feeding trial to evaluate the impact of a pecan–enriched diet on plasma tocopherol concentrations, in addition to changes in antioxidant capacity and lipid peroxidation. Twenty–four healthy volunteers (14 men and 10 women) participated in the study and were fed either a control diet or a pecan–enriched diet for four weeks each. Changes in antioxidant capacity were evaluated using the ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) assays, while concentrations of malondialdehyde, a marker of lipid peroxidation, was measured using the 2-thiobarbituric acid assay and was expressed as 2-thiobarbituric acid reactive substances (TBARS). Plasma levels of α– and γ– tocopherols were also measured *via* normal phase high performance liquid chromatography (NP–HPLC). No significant differences in antioxidant capacity, as measured by the FRAP and TEAC assays, between the pecan–

enriched and control diets were noted. The concentration of TBARS of the pecan–enriched diet group was significantly lower than that of the control group. Furthermore, a reduction in α–tocopherol serum levels of the pecan diet group was observed, while levels of γ–tocopherol increased. While antioxidant capacity was not significantly affected by a pecan–enriched diet, the significant reduction in TBARS suggested that antioxidant constituents in pecans, such as tocopherols, might play a role in the inhibiting lipid peroxidation and degradation *in vivo.*

Hudthagosol et al. (2011) investigated the impact of pecans on post–prandial antioxidant capacity (*via* hydrophilic– and lipophilic– oxygen radical capacity (H– and L–$ORAC_{FL}$) and FRAP) assays in addition to changes in serum levels of cholesterol, catechins, tocopherols, and malondialdehyde (MDA). Sixteen individuals participated in a placebo–controlled, 3–way crossover study with 1–week washout periods between treatments. Participants were randomly assigned to a test meal consisting of 90 g of whole pecans plus water, 90 g of pecans blended with water, or a control meal composed of olive oil, whey protein, white bread, and water. The control meal was equivalent in energy, nutrients, and fluid content to the pecan meals. After the consumption of the assigned meal, blood samples were drawn at 0 (baseline), 1, 2, 3, 5, 8, and 24 h after the consumption of the test meals. The post–prandial 'Area Under the Curve' from 0 to 5 h ($AUC_{0–5h}$) was analyzed to evaluate antioxidant capacity.

Both the blended and whole pecan meals exhibited higher post–prandial $AUC_{0–5h}$ of total polyphenols, H–$ORAC_{FL}$ and L–$ORAC_{FL}$ compared to the control group. The post–prandial $AUC_{0–5h}$ of FRAP did not differ after the consumption of each meal. In addition, both the blended and whole pecan meal groups exhibited higher post–prandial

plasma levels of γ–tocopherol than the control meal at 5 and 8 h. Furthermore, significantly higher levels of γ–tocopherol at 5 and 8 h were observed when compared to the baseline. Additionally, the authors reported that levels of oxidized LDL were reduced after consumption of the whole pecan meal. Epigallocatechin gallate, (+)–catechin, (–)–epicatechin, epigallocatechin, epicatechin gallate and gallocatechin gallate were all detected in plasma after the ingestion of pecans. Taken together, the study demonstrated that bioactive constituents from pecans, such as tocopherols and flavan–3–ol monomers, were bioavailable and could provide a more favorable antioxidant status.

2.2 Economic value of pecans to the United States and the state of Georgia

In 2017, the United States (U.S.) was the global leader in tree nut production accounting for 38% of production (INC, 2018a). This amounted to over 1.6 million metric tons of tree nuts, worth $9 billion (USDA Economic Research Service, 2018a). The U.S. is the top producer of nuts such as pecans. The U.S. produced 294 million pounds of pecans, valued at $684 million in 2017 (USDA Economic Research Service, 2018b). This accounted for 51% of the world's production. The next two largest producers were Mexico and South Africa, with 41% and 7% of global production, respectively (Figure 2.1) (INC, 2018a).

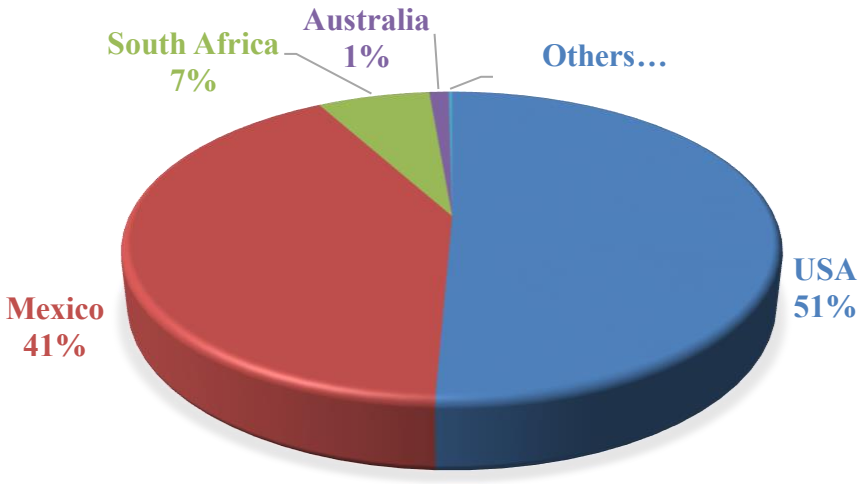


Figure 2.1 Global production of pecans in 2017.

In 2017, Georgia was the top producing state in the nation, accounting for 36% of production, followed by New Mexico and Texas, with 31 and 13% of production, respectively (Figure 2.2). Together the top three states accounted for 80% of national production (USDA National Agricultural Statistics Service, 2018). Pecans are an important agricultural commodity within the state of Georgia. According to the 2017 Georgia Farm Gate report, pecans were ranked 8th as a commodity and accounted for 2.91% of the state's agricultural revenue. When grouped by commodities, pecans accounted for over half (57%) of the fruit and nut category. Since the 2012 season, the farm gate value of pecans has significantly increased by 61% (Figure 2.3) (The University of Georgia Center for Agribusiness & Economic Development, 2018).

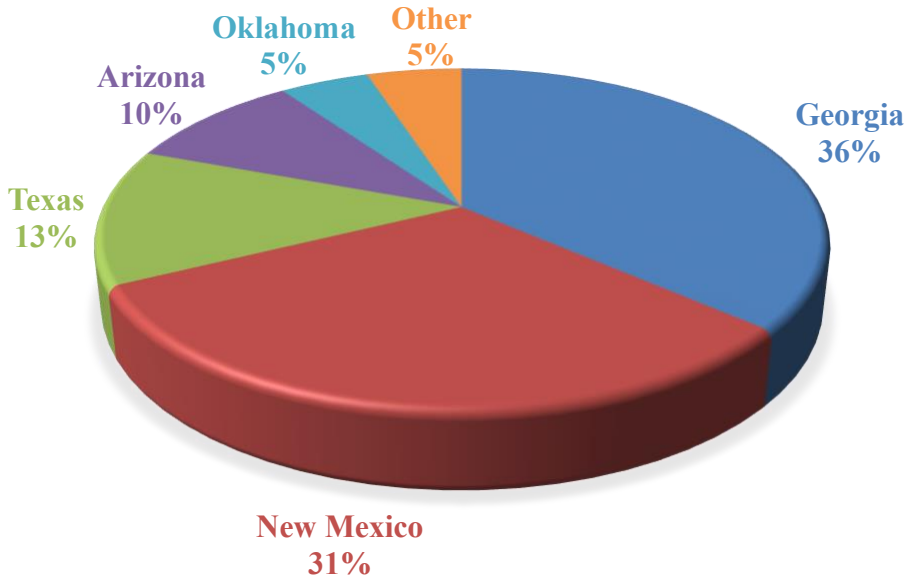


Figure 2.2 Major pecan producing states in the U.S in 2017.

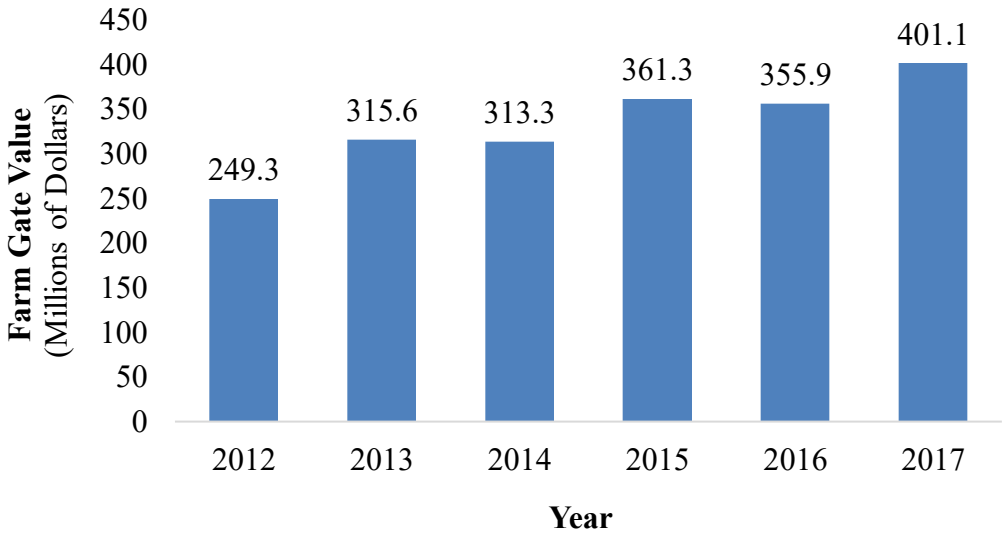


Figure 2.3 Georgia farm gate value of pecans from 2012 to 2017.

The majority of Georgia's pecans are destined for the Chinese market. Currently, China is one of the biggest export markets for American pecans. In 2017, approximately

32% of U.S. pecan production was exported to China, with 75% of the exported pecans coming from Georgia (Figure 2.4) (USDA Economic Research Service, 2019).

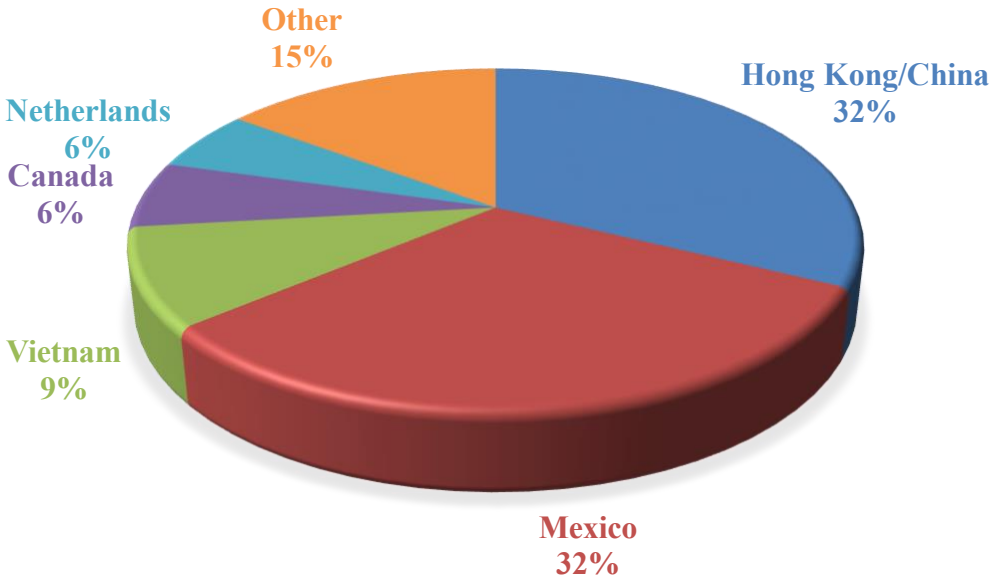


Figure 2.4 Major export markets for U.S. pecans in 2017.

The immense popularity of pecans in China began in 2007, when the Chinese were introduced to American pecans as an alternative to walnuts and the hickory nuts. During this time, walnuts were undergoing a global shortage and the demand for hickory nuts outpaced their supply (Wessel, 2011; INC, 2018b). Both the pecan (*Carya illinoinensis (Wangenh.)* K. Koch) and hickory nut (*Carya Cathayensis* Sarg) belong in the *Carya* family. Pecans are similar in taste to hickory nuts, possess a shorter growing period, and thin shell, which makes it easy to crack. These attributes have contributed to their marked popularity in China. Furthermore, an increasing trend of "chasing health" has also bolstered their attractiveness to Chinese consumers (Arn, 2018). The

healthfulness of pecans has been highlighted by various studies, especially in regards to CVD.

2.3 Composition of pecans

As aforementioned, the benefits of nut consumption have been attributed to the various lipid components and phenolic compounds present (Hudthagosol et al., 2011). Pecans are composed of a unique package of various lipid constituents such MUFAs, phytosterols, and vitamin E compounds called tocopherols. In addition, pecans are also a rich source of phenolics and antioxidants (Gu et al., 2004). Together, the various health benefits associated with pecan consumption may be attributed to these compounds.

2.3.1 Pecan lipid profile

Pecans are a rich source of lipids. Robbins et al. (2014) reported that on a gravimetric basis the lipid content of pecans ranged from 68 to 78 g oil/100 g nutmeat depending on the cultivar. The lipid constituents can be divided into saponifiable and unsaponifiable lipids. Saponifiable lipids include triacylglycerols, while unsaponifiable lipids include tocopherols and phytosterols.

2.3.2 Fatty–acid composition

Robbins et al. (2011) investigated the healthful lipid constituents of commercially important tree nuts, such as pecans. The results of this study indicated that pecans were a rich source of MUFAs with the predominant MUFA being oleic acid (18:1 ω–9), which consisted of 62.36 ± 7.75% of total lipids.

Gong et al. (2017a) studied the chemical and nutritive characteristics of various tree nut oils available in the U.S. market. In this study, 4 pecan oils of various origins were analyzed. This included 3 oils extracted from raw pecans and 1 from roasted pecans. The results indicated that the pecan oil was also a rich source of MUFAs with 54–56% of the oil consisting of oleic acid.

The MUFA rich fatty acid profile of pecans may contribute to some of its heart healthy properties. Consumption of MUFAs has been suggested to reduce the risk of CVD by 20% (Gillingham et al., 2011). The favorable cardioprotective effects of MUFA consumption has been attributed to their capability to modulate several parameters. Studies have indicated MUFA consumption can reduce levels of plasma TC and LDL–C, while improving the HDL–C:TC ratio (Hammad et al., 2015). Furthermore, several studies have demonstrated that diets rich in MUFAs enrich HDL–C better than diets rich in polyunsaturated fatty acids (PUFAs) or carbohydrates (Keys et al., 1986; Kris–Etherton, 1999). Regular consumption of dietary MUFAs was also shown to significantly reduce the risk of mortality in patients with myocardial infarction by 24% (Venturini et al., 2015).

2.3.3 Tocopherols

Tocopherols are the major group of lipophilic antioxidants present in pecans. Tocopherols, along with tocotrienols, are lipid–soluble molecules that belong to a group of vitamin E compounds which play an essential role in human nutrition and health (Munne–Bosch & Alegre, 2002). In the human body, vitamin E plays a wide variety of roles. It is an essential nutrient for reproduction (Evans & Bishop, 1922). It also functions

as an antioxidant to protect lipids against oxidative damage (Bunyan et al, 1961; Tappel, 1962). Most importantly, it protects PUFAs in both biological membranes and plasma lipoproteins from oxidative damage (Burton & Ingold, 1983).

Tocopherols are compounds that are synthesized exclusively by photosynthetic organisms (Sen et al, 2006). Tocopherol levels in plants have been linked to the level of unsaturated fatty acids, as the increased concentrations of unsaturated fats results in the formation of higher levels of antioxidants to protect the oil from oxidation (Eskin et al, 1996).

Tocopherols exist in four homologous isomers, which are α–, β–, γ– and δ–tocopherol and the structures differ due to the number or position of methyl groups in the molecules (Oomah et al, 1997). The isomers also differ in their biological activities and abilities to protect fats and oils from oxidation. During digestion, vitamin E is absorbed in the intestine and enters the circulation *via* the lymphatic system. Here it is absorbed together with lipids, packed into chylomicrons, and transported to the liver where it is up–taken by the hepatic α–tocopherol transfer protein (α –TPP). This protein controls the distribution of α–tocopherol to cells and tissues throughout the body. α–TPP is has preferential affinity for α–tocopherol and as a result this isomer possesses the highest nutritional importance. While α–tocopherol has been designated the isomer with the highest biological importance, the unique properties of γ–tocopherol have drawn much interest and pecans have been shown to a good source of γ–tocopherol (Robbins et al., 2011; Robbins et al., 2015).

Compared to α–tocopherol, γ–tocopherol has been shown to possess superior abilities to scavenge reactive nitrogen species (RNS), such as peroxynitrites ($ONOO^-$)

and nitrogen dioxide (NO_2) (Cooney et al., 1993; Hoglen et al., 1993; Christen et al., 1997). γ–Tocopherol and its metabolite, γ–carboxyethyl hydroxychroman (γ–CEHC), have also been shown to possess anti–inflammatory properties. Jiang et al. (2000) observed that γ–tocopherol and γ–CEHC inhibited the activity of cyclooxygenase–2 (COX–2), a protein involved in inflammation, in macrophage and epithelial cells. Jiang and Ames (2003) reported that γ–tocopherol and γ–CEHC reduced the production of pro–inflammatory compounds in male Wistar rats by down regulating the expression of COX–2 and 5–lipoxygenase (5–LOX). As various studies have indicated that the γ–tocopherol is major isomer present in pecans, the unique biological properties of γ–tocopherol and its metabolite, γ–CEHC, may contribute to some of the health benefits of pecan consumption (Robbins et al., 2011; Robbins et al., 2015).

2.3.4 Phytosterols

Phytosterols are another class of lipophilic compounds found in plants that play a role in maintaining the fluidity of the cell wall. They are similar to cholesterol in structure but possess a different side chain at C_{24} and the number and location of double bonds differs in these molecules. Phytosterols are comprised of plant sterols 3and stanols: both which are similar in structure; however, stanols are fully saturated molecules.

It has been suggested that the dietary consumption of phytosterols can play a role in reducing the risk of CVD. It has been hypothesized that phytosterols disrupt cholesterol absorption by displacing cholesterol from intestinal micelles, thus reducing the amount of absorbable cholesterol (Ostlund, 2004). Studies have shown that

phytosterols reduce TC and LDL–C without affecting HDL–C and triglycerides (Ostlund, 2002).

Blair et al. (2000) demonstrated that phytosterol consumption in conjunction with statin drugs were effective in reducing TC and LDL–C levels. The authors suggested the phytosterols combined with statin drugs could be an attractive treatment for treating patients with elevated levels of LDL–C by targeting two different mechanisms of cholesterol reduction; where phytosterols help eliminate cholesterol and statins targets the biosynthesis of cholesterol.

A cross–sectional study performed by Andersson et al. (2004) investigated the relationship between intake of plant sterols and serum lipid concentrations. The authors noted that phytosterol intake reduced TC and LDL–C without interfering with cholesterol–lowering medications.

Analysis of commonly consumed nuts showed that β–sitosterol is the major sterol present (Phillips et al., 2005; Robbins et al., 2011). Robbins et al. (2011) reported that pecans possessed 130.1 mg of β–sitosterol per 100 g nutmeat. Together, the favorable lipid profile of pecans consisting of MUFAs, tocopherols, and phytosterols may contribute to their healthfulness.

2.4 Phenolics

Phenolics are compounds that contain an aromatic ring substituted with one or more hydroxyl groups. These compounds are essential for the plant's growth, well–being, and play a role in reproduction. In addition, phenolics offer the plants protection against ultraviolet (UV) light, insects, bacteria, and animals (Shahidi & Naczk, 2003). The

properties of plants, such as color and astringency, can be attributed the presence of certain phenolics, such anthocyanins and tannins, respectively (Craft et al., 2010; Santos–Buelga & Scalbert, 2000). A variety of phenolic compounds exist and the classification of phenolics is shown in Figure 2.5.

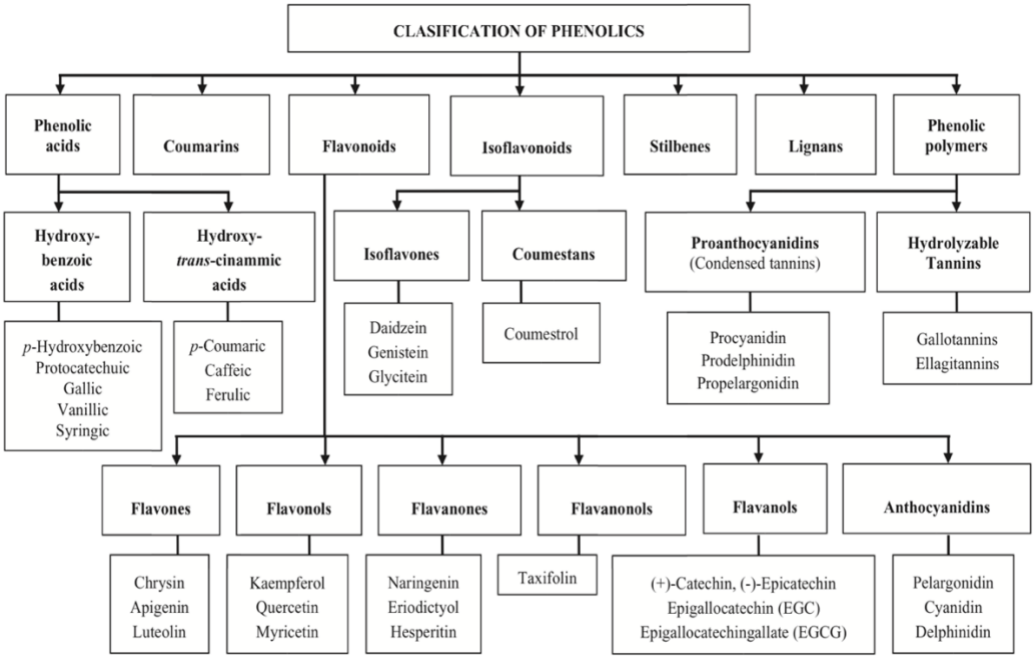


Figure 2.5 Classification of phenolics (Craft et al., 2010).

While a myriad of phenolics exist, they are all synthesized from L–phenylalanine (Phe), and to a lesser extent L–tyrosine (Tyr) *via* several closely related pathways. These include the shikimate, phenylpropanoid, and flavonoid pathways (Vogt, 2010; Jaganath & Crozier, 2010). Synthesis of phenolics begins with the shikimate pathway and the production of Phe and Tyr. These compounds are key substrates for the phenylpropanoid pathway (Figure 2.6) (Iandolino & Cook, 2010).

The first step of the phenylpropanoid pathway is the deamination of Phe *via* phenylalanine ammonia lyase, which produces *trans*–cinnamic acid. *Trans*–cinnamic acid is then converted to *p*–coumaric acid by P450 mono–oxygenase cinnamic acid 4–hydroxylase, which can subsequently be converted into a variety of free (i.e., caffeic, ferulic, 5–hydroxyferulic, and sinapic acid) and esterified (i.e., with organic acids) hydroxycinnamic acids by additional enzymes (Boerjan et al., 2003). The enzyme 4–coumaroyl–CoA ligase converts *p*–coumaric acid into *p*–coumaroyl–CoA, which can enter the flavonoid and stilbene biosynthesis pathways (Iandolino & Cook, 2010).

Flavonoids are the most common and widespread group of phenolics present in plants (Craft et al., 2012). The first step of flavonoid biosynthesis is an irreversible condensation reaction between *p*–coumaroyl–CoA and malonyl–CoA. This is followed by reactions catalyzed by polyketide synthases and chalcone synthase that produce a linear tetraketide intermediate. This intermediate then undergoes subsequent condensation, decarboxylation, and aromatization steps to yield the C15 (6:3:6) skeleton that is the basic structure common to all flavonoids as shown in Figure 2.7 (Iandolino & Cook, 2010). Examples of other sub–categories of flavonoids are depicted in Figure 2.8.

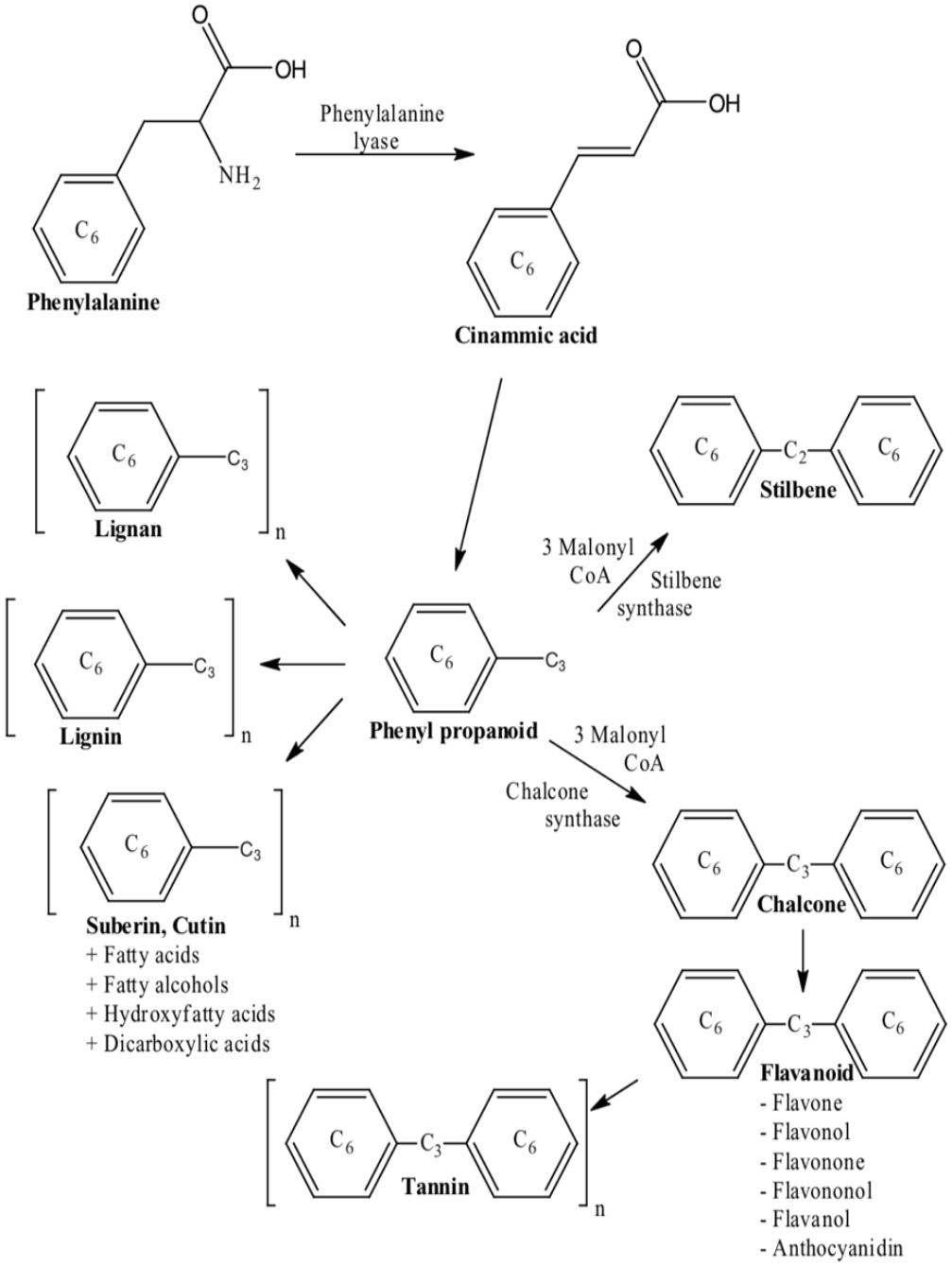


Figure 2.6 Phenolic pathway (Craft et al., 2010)

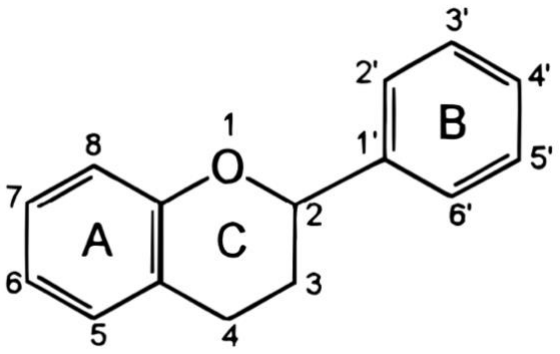


Figure 2.7 Basic flavonoid backbone **(**Pietta, 2000).

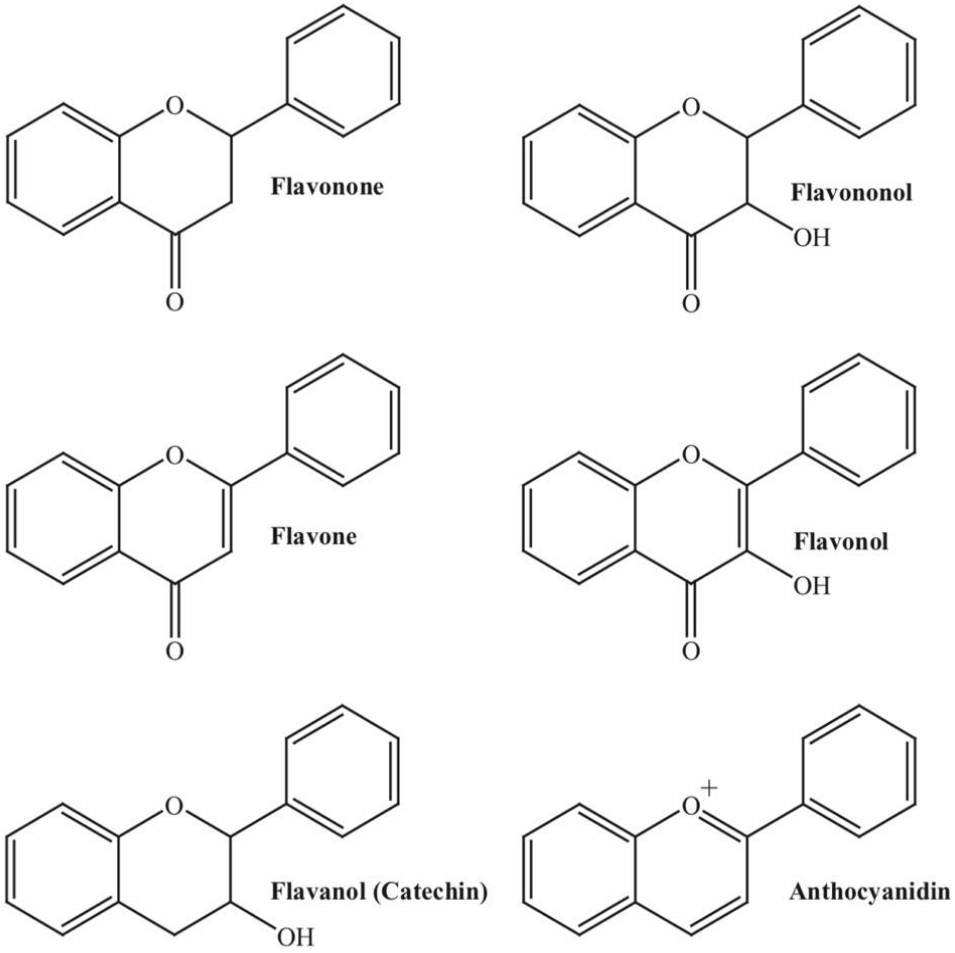
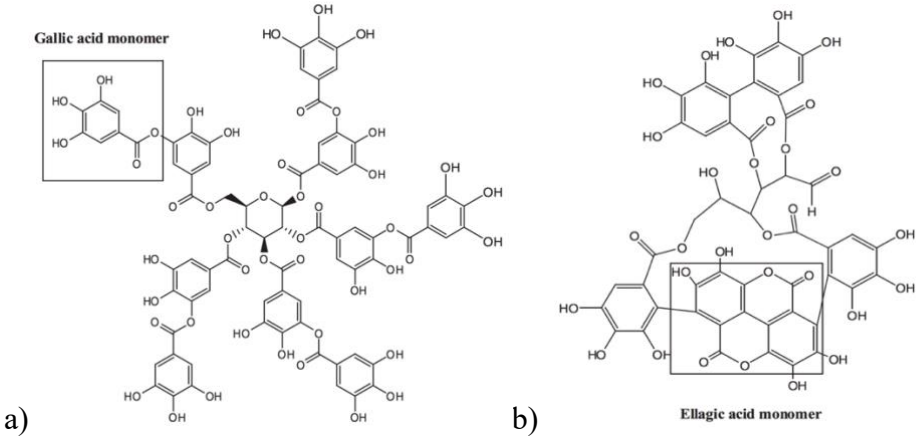


Figure 2.8 Structures of various flavonoids (Craft et al., 2010).

The term tannin refers to a group of oligomeric and polymeric phenolic compounds with molecular weights ranging from 500 to 3000 Da (Shahidi & Naczk, 2006). Tannins can be classified as hydrolysable or condensed tannins depending on their structural configuration. Hydrolysable tannins can be further sub–divided into gallotannins or ellagitannins depending on their hydrolysis products. Gallotannins or ellagitannins consist of a central sugar molecule that is partially or completely esterified to gallic acid or hexahydroxydiphenic acid (Craft et al., 2010). Examples of a gallotannin and ellagitannin are shown in Figures 2.9 a and b. Base, acid, or enzymatic hydrolysis of gallotannins and ellagitannins yield gallic and ellagic acids, respectively.



a) b)

Figure 2.9 Examples of hydrolysable tannins a) tannic acid, a gallotannin, and b) punicalagin, an ellagitannin (Craft et al., 2010).

Condensed tannins or proanthocyanidins (PACs) are oligomers or polymers consisting of flavan–3–ol subunits. When discussing PACs, there are several commonly utilized terms. The first refers to the type of interflavanol linkage between the flavan–3–ol subunits. There are two types of linkages, B–type and A–type. The B–type linkage is

more common and occurs when the flavan–3–ol units are linked *via* C4 → C8 or sometimes C4→ C6 bonds. A–type linkages are less common and occur when an additional ether linkage is formed (mainly) between C2 and C7 (Figure 2.10).

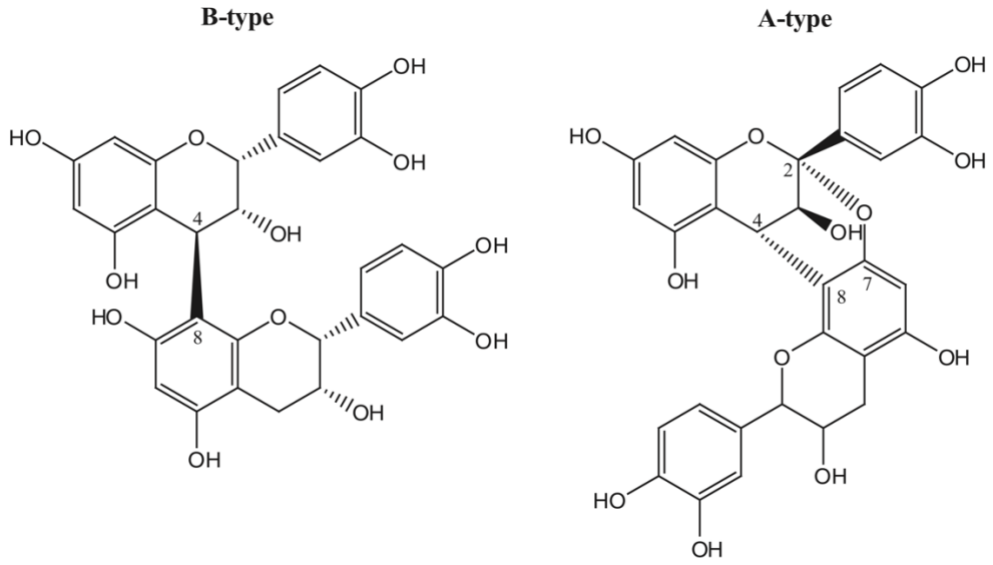


Figure 2.10 Examples of B–type and A–type condensed tannins (Craft et al., 2010).

Degree of polymerization (DP) is another term often used to describe the size of PACs and refers the number of monomeric subunits in a molecule (Kelm et al., 2006). PACs can further be classified by the composition of their subunits. PACs consisting exclusively of (+)– catechin or (–)–epicatechin subunits are called procyanidins, while PACs containing afzelechin or gallocatechins are respectively called propelargonidins or prodelphinidins (Ou & Gu, 2014). The majority of PACs present in nature exist as procyanidins (Cos et al., 2003).

Research has demonstrated that PACs from various sources possess a wide variety of bioactive properties and strong antioxidant activity (Bagchi et al., 2002). It has

been hypothesized that these unique characteristics of PACs play a role in preventing and ameliorating chronic diseases. While studies have suggested that PACs can exert a wide range of physiological activities, this is confounded by their poor bioavailability (Beecher, 2004). This may suggest that the beneficial health benefits of consuming PACs could possibly be attributed to their metabolites.

2.4.1 Pecan phenolic profile

Knowledge of the health benefits of pecan consumption has improved, as various studies have focused on identifying and characterizing the antioxidant properties of phenolic constituents found in various pecan cultivars. Robbins et al. (2014) profiled the phenolic compounds from U.S. pecans *via* liquid chromatography–tandem mass spectrometry. In this study, the crude extracts of eight commercially significant cultivars with relatively high antioxidant capacities were pooled and then separated *via* Sephadex LH–20 column chromatography into five ethanolic low–molecular–weight (LMW) fractions and one acetonic high–molecular–weight (HMW) fraction. This study found that ellagic acid and (+)–catechin were the major phenolics present in the low LMW fraction, while the HMW fraction was comprised of mostly procyanidins (PACs) as dimers. The presence of PACs with various degrees of polymerization, monomers to hexamers, were also detected in the HMW fraction. Gong and Pegg (2017b) optimized a method for the separation and characterization of ellagitannin–rich phenolics from U.S. pecans and Chinese hickory nuts extracts using fused core columns. These studies have helped further the knowledge and characterize the phenolic compounds responsible for the high antioxidant potential of pecans.

2.4.2 Impact of roasting on the phenolic profile

Raw or roasted tree nuts are often eaten as healthful snacks or used as an ingredient in a variety of processed foods, especially baked and confectionary products (Chang et al, 2016). The roasting process can improve desirable characteristics such as flavor, color, crispiness, and texture. Roasting also results in microstructural and chemical changes. These include decreased moisture content, modification of lipids, changes in color, and the formation of compounds responsible for the typical roasted nut flavor, which are due to Maillard reaction products (Alamprese et al., 2009; Amaral et al., 2006, Saklar et al., 2001). Although roasting can improve several desirable traits, the chemical and microstructural changes may lead to increased susceptibility of the roasted nuts to lipid oxidation when compared to raw nuts (Alamprese et al., 2009). Roasting may also alter the content of phenolics and therefore impact the antioxidant capacities of the phenolics.

Robbins (2012) studied the impact of roasting on pecan phenolics and observed that roasting resulted in a significant decrease in PAC content. Robbins also noted that H–ORAC$_{FL}$ and TPC values of roasted pecans were significantly lower than that of their unroasted counterparts but FRAP values did not vary significantly. These results contrast with those published by Kellett et al. (2019) who employed the roasting profile utilized by Robbins (2012). Kellett et al. (2019) reported that roasting did not degrade antioxidant constituents present in pecans.

The type of roasting and conditions used can also influence the quality of phenolics. Craft et al. (2010) studied the antioxidant properties of extracts from raw, dry–roasted, and oil–roasted skinless U.S. peanuts of commercial importance. In this study,

characterization *via* RP–HPLC indicated that the raw peanut methanolic extracts were mainly comprised of free *p*–coumaric acid and their potential *p*–coumaric acid derivatives. When subjected to thermal processing, either dry or oil roasting, the concentration of free *p*–coumaric acid increased at the expense of its derivatives with oil–roasting exhibiting the highest increase in *p*–coumaric acid. The overall findings indicated that while thermal processing altered the phenolics composition of peanut kernels, TPC and radical–scavenging activities were retained. Yang et al. (2015) noted that stir–frying Zhejiang pecans at low temperatures over short periods of time resulted in an apparent increase in extractable chlorogenic acid, gallic acid, and antioxidant activity. However, with increased heating time and temperature these values decreased. In addition, this study demonstrated that roasting negatively affected individual phenolics but not the total phenolics content (TPC) and the antioxidant activity (Yang et al., 2015). As nuts, such as pecans, are commonly consumed in their roasted forms, it is important to document the effect of roasting on their phenolic profile.

2.5 Antioxidants

Antioxidants possess the capability to slow or prevent the oxidation of other molecules through their interactions with free radical species (Zhang et al., 2017). Antioxidants can function *via* one of two mechanisms, Hydrogen Atom Transfer (HAT) or Single Electron Transfer (SET). During the HAT mechanism, free radical species are quenched when an antioxidant donates a hydrogen atom thus forming a more stable species *via* resonance stabilization. In the case of the SET mechanism, a single electron is transferred to quench the reactive species (Craft et al., 2012). As antioxidant activity can

function *via* one of two different mechanisms, it is important to utilize various *in vitro* antioxidant assays to gain a complete representation of a sample's true antioxidant activity (Prior et al., 2005).

Oxidative stress has been recognized to potentiate the production of reactive oxygen species (ROS) and has been implicated in the development of a wide spectrum of chronic diseases. ROS can damage cellular components such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), lipids, proteins and carbohydrates and membranes. Damage to these components can produce more free radicals, which if left unchecked can result in inflammation and chronic conditions, such as CVD, diabetes, and cancer (Temple, 2000). In particular, damage to DNA can result in mutagenesis, which may lead to carcinogenesis.

2.5.1 Dietary antioxidants

The human body has developed various endogenous antioxidant defense mechanisms to keep ROS in check. These mechanisms include antioxidant enzymes (i.e., catalase, glutathione reductase, glutathione peroxidase, superoxide dismutase), endogenous factors (i.e., glutathione, coenzyme Q), and metal–ion sequestration systems (Craft et al., 2012). While the body has developed a bevy of endogenous antioxidant defense mechanisms, there are times when these mechanisms are overwhelmed; resulting in an increased risk of various chronic and degenerative diseases. Therefore, dietary antioxidants (i.e., vitamins E and C, polyphenols, and carotenoids) help maintain the antioxidant balance within the body (Craft et al., 2012).

2.5.2 *In vitro* antioxidant assays

Over the years a number of *in vitro* antioxidant assays have been developed and are used to measure the concentration and antioxidant capabilities of phenolic compounds. Some examples include the Total Phenolics Content (TPC), Hydrophilic Oxygen Radical Absorbance Capacity (H–ORAC$_{FL}$), and Ferric Reducing Antioxidant Power (FRAP).

2.5.3 Total phenolics content (TPC)

The TPC assay is a classic *in vitro* assay used to measure the concentration of phenolics in a sample. It does not measure the antioxidant potential; however, it is often used in tandem with other assays to help determine antioxidant potential relative to the concentration of phenolics. The TPC assay is a colorimetric assay. Samples and standards are mixed with the Folin & Ciocalteu (F–C) phenol reagent and are then treated with a saturated carbonate solution to obtain a final pH of 10–11. F–C's phenol reagent is light yellow/green in appearance, but in the presence of a phenolic compound in a basic environment, it reacts with phenolic residues and turns blue in color (Singleton & Rossi, 1965). The absorbance of the resulting mixtures can be measured at 750 nm after a 30–minute incubation using a spectrophotometer.

2.5.4 Hydrophilic–oxygen radical absorbance capacity (H–ORAC$_{FL}$)

The H–ORAC$_{FL}$ assay is an *in vitro* antioxidant assay commonly used to assess the antioxidant potential of phenolics and predominantly follows the HAT mechanism. It was developed to assess the antioxidant capability against ROS, more specifically

peroxyl radicals. The assay uses a fluorescent probe, fluorescein (3′6′–dihydroxy–spiro[isobenzofuran– 1[3*H*],9′[9*H*]–xanthen]–3–one), and a free radical generator, 2,2′–azobis[2–amidinopropane] dihydrochloride) (AAPH). During the assay, peroxyl radicals are generated when AAPH undergoes thermal decomposition. If fluorescein reacts with a ROS, there will be a loss of fluorescence signal. However, if antioxidants are present, they can scavenge ROS and as fluorescein is not able to react with ROS, there is no loss of fluorescent signal. Thus, if antioxidants are present in sample, the reduced loss of fluorescence will be observed as the antioxidant reacting with the radical before it can react with the florescent probe (Ou et al., 2001)

The H–ORAC$_{FL}$ is an antioxidant assay that has some biological relevance. The assay is performed 37 °C and at a relatively neutral pH of 7.4, which is similar to the conditions within the body. The assay also evaluates the ability of antioxidants to scavenge peroxyl radicals, which are ROS found throughout the body (Ou et al., 2001). While this assay is performed using biologically relevant conditions, it does not take into consideration the uptake and metabolism of antioxidants, which is an important aspect of understanding the mechanisms of antioxidants within biological systems.

2.5.5 Ferric reducing antioxidant power (FRAP).

The FRAP assay is a colorimetric assay that measures the reducing power of antioxidants and is based on the SET mechanism. The assay is performed under acidic conditions (pH 3.6) in an acetate buffer. In this assay, a single electron transfer leads to the reduction of the colorless ferric 2,4,6–tripyridyl–*S*–triazine (Fe(III)–TPTZ) to ferrous 2,4,6–tripyridyl–*S*–triazine (Fe(II)–$(TPTZ)_2$), yielding an intense blue color which can be

measured at a wavelength of 550 nm (Pulido et al. 2000). As the assay is performed at a low pH of 3.6 and biological systems are at a neutral pH, there are concerns that *in vitro* results do not reflect the antioxidant properties *in vivo*.

2.5.6 *In vitro* antioxidant assays and their biological relevance

Various *in vitro* test tube methods have been developed and are used to extensively study the antioxidant properties of phenolic compounds extracted from various commodities such as tea, nuts, fruits and vegetables. These include assays such as FRAP and H–ORAC$_{FL}$ which are relatively cheap, simple and easy to operate (Craft et al., 2012). While these assays have shown that phenolic compounds possess strong *in vitro* antioxidant activities, there is controversy over whether *in vitro* results can be translated into *in vivo* results as the assays are performed under non–physiological conditions and do not take into consideration a variety of factors, such as bioavailability and cellular uptake and metabolism, that may influence the function of dietary antioxidants. Nevertheless, these *in vitro* assays can be valuable tools when screening phytochemicals for possible *in vivo* antioxidant activity.

The antioxidant capacities of a substance *in vivo* are affected by several factors, with one of the major factors being bioavailability. Upon consumption, antioxidants may degrade or undergo changes to its chemical structure. Changes in structure may prevent it from being absorbed and thus prevent it from being distributed throughout the body. As a result, exogenous antioxidants cannot prevent oxidation within the body. Currently, there is a lack of knowledge on how digestion and absorption can modify the antioxidant power of phenolic compounds. It is highly likely that the structure of phenolic

compounds could be altered while undergoing digestion and absorption. As a result, the activities and effectiveness of phenolic compounds observed using classic *in vitro* antioxidant assays may not be biologically relevant.

2.5.7 Bioaccessibility and bioavailability

Bioaccessibility and bioavailability are two important factors that should be taken into consideration when evaluating the potential health benefits of dietary antioxidants. Bioaccessibility is defined as the quantity or fraction of nutrients that are released from the food matrix in the GI tract and is available for absorption. Bioaccessibility takes into consideration the digestion of compounds (i.e., proteins into peptides), absorption by intestinal epithelium cells, and pre–systemic metabolism which includes metabolism by intestinal epithelium cells and the liver (Carbonell–Capella et al., 2014).

Bioavailability is a broader term that bioaccessibility falls under. It refers to the amount of an ingested nutrient or compound that is able to reach systemic circulation and be utilized for physiological function. Bioavailability takes into consideration GI digestion, intestinal absorption, metabolism, tissue distribution, and physiological function (Carbonell–Capella et al., 2014; Galanakis, 2017)

2.6 Digestion and Absorption of Phenolics

2.6.1 Digestion of phenolics

Research has focused on elucidating the impact of digestion on the structure of phenolics and the absorption of phenolics. Before phenolics can be distributed throughout the body and utilized, they are digested and absorbed through the intestinal lining. During

their passage through the gastrointestinal (GI) tract, phenolics may interact with micro–and macro–molecules present in the GI tract. These interactions may chemically modify phenolics, thus altering their function and antioxidant capabilities (Dominguez–Avila et al. 2017). Therefore, to better understand the health benefits of consuming a phenolic rich diet the impact of digestion on phenolics should be investigated.

Rios et al. (2002) investigated the stability of cocoa procyanidins from a cocoa beverage during gastric transit in humans. The authors found that cocoa procyanidins were relatively stable in the acidic gastric stomach environment. This suggested that ingested cocoa procyanidins could reach the small intestine relatively unchanged. These results contradict those of Spencer et al. (2000), who demonstrated that procyanidin oligomers, trimers to hexamers, from cocoa under *in vitro* gastric conditions can depolymerize and break down to form epicatechin monomers and dimers.

Bermudez–Soto et al. (2007) studied the stability of polyphenols present in chokeberry (*Aronia melanocarpa*) juice subjected to *in vitro* gastric and pancreatic digestion. The study noted that most of the polyphenols appeared to be quite stable during gastric digestion. However, once subjected to the neutral or slightly alkaline conditions of pancreatic digestion the concentrations of polyphenols decreased. The authors saw a ~43% decrease in cyanidin–3–O–glucoside after *in vitro* pancreatic digestion of the chokeberry juice. In addition, the recovery of flavonols in chokeberry juice ranged from 70 to 85% depending on the compound. The results of this study suggested that polyphenols are highly sensitive to the slightly alkaline intestinal conditions and that the structure of some polyphenols may be modified, which may result in different chemical properties (Bermudez–Soto et al., 2007).

Neilson et al. (2007) studied the degradation of catechins during *in vitro* digestion and the formation of possible dimers under simulated gastrointestinal conditions. The authors found that digestion significantly degraded (–)–epigallocatechin gallate (EGCG), (–)–epigallocatechin (EGC), and (–)–epicatechin gallate (ECG), resulting in losses of 71–91, 72–100 and 60–61%, respectively. The authors also noted that catechin and epicatechin were more resistant to digestive degradation, with losses of 8–11% and 7–8%, respectively. The majority of the loss of catechins was attributed to intestinal degradation. Overall, the results indicated that catechins are much more stable in the gastric environment when compared to that of the intestinal environment. The study also found catechins subjected to *in vitro* digestion conditions resulted in the formation of dimers. EGCG formed theasinensins (THSNs) A and D and P–2, its autoxidation homodimers, while EGC produced the homodimers THSN C and E and homodimers similar to P–2. The presence of ECG homodimers was not observed. Furthermore, EGCG and EGC formed heterodimers similar to the THSNs and P–2. In addition, EGCG and ECG formed homodimers similar to the THSNs (Neilson et al., 2007). The results of this study indicate, that while digestion can result in the degradation of phenolics it can also provide conditions that result in the formation of new polyphenols. This helps to highlight the importance of understanding how digestion can affect phenolics.

2.6.2 Gastric absorption of phenolics

Studies have indicated that some phenolics, such as phenolic acids, can be absorbed in the stomach. Lafay et al. (2006) studied the bioavailability of chlorogenic acid using rat feeding studies and found that chlorogenic acid can be absorbed in the

stomach. An animal study done by Zhao et al. (2004) demonstrated that ferulic acid (FA) is absorbed in the stomach and is metabolized by the liver. Konishi et al. (2006) reported that various phenolic acids are absorbed at different rates in the stomach in increasing order as follows: gallic acid = chlorogenic acid < caffeic acid < *p*–coumaric acid = FA. Anthocyanins, which belong to the phenolic class of flavonoids, have also been shown to be absorbed in the stomach (Talavéra et al., 2003; Manach et al., 2004). While the majority of absorption takes place in the intestinal tract, the gastric absorption of phenolics should not be dismissed. Future studies may need to investigate the gastric absorption of phenolics to gain a complete understanding of their passage through the gastrointestinal tract.

2.6.3 Absorption of phenolics using *in vitro* and *in vivo* models

While the gold standard for studying the digestion and absorption of phenolics are animal and human studies, these are time consuming and expensive. Therefore, *in vitro* gastrointestinal digestion and intestinal absorption models have been developed to assess bioavailability and bioaccessibility (Xie et al., 2013). Cell culture models are a good alternative to animal and human trials, as they are less expensive and provide some biologically relevant information.

The bioavailability of phenolics has been evaluated using *in vitro* and *in vivo* systems. *In vitro* systems usually consist of *in vitro* digestion combined with either dialysis membranes or Caco–2 monolayers to study the capability of phenolics to pass through a model of the intestinal barrier. *In vivo* studies often consist of animal or human trials, where the bioavailability of phenolics is assessed by evaluating plasma or urine

samples. One limitation of this method is the low concentration of phenolics and metabolites in these biological samples. Furthermore, the bioavailability of phenolics and their metabolites can be underestimated as accumulation within tissues is not taken into consideration. It is highly possible that the accumulated polyphenols can confer some protection against free radical damage. For animal studies, the distribution and uptake of phenolics to various tissues can be evaluated; however, this is not feasible for human trials.

2.6.4 Caco–2 transport studies

The Caco–2 cell line, a human colon epithelial cancer cell line, has been used extensively by the pharmaceutical industry to study the mechanisms of drug transport and absorption of drug molecules using special transwell plates (Zhang et al., 2017). Caco–2 cells possess the capability to differentiate spontaneously, which leads to the formation of a monolayer of cells that possess morphological and functional characteristics of mature enterocytes. Once differentiated, they exhibit epithelial characteristics such as brush–border microvilli and tight junctions when cultured on impermeable supports. Over time the production of brush border enzymes, such as sucrase, alkaline phosphatase, and aminopeptidase, that are unique to the adult human small intestine, is observed (Sambuy et al, 2005). These properties have made the Caco–2 cell line a good alternative to animal studies and an invaluable tool for predicting the intestinal absorption of various substances.

Transport studies are performed using Transwell® inserts. Caco–2 cells are seeded on the apical side of the Tranwell inserts placed in 12 well plates. Over a course of 21

days, the cells are allowed to grow and differentiate to form a confluent monolayer. The culture medium is replaced every 2–3 days, with the apical and basolateral compartments containing 0.5 mL and 1.5 mL of culture medium, respectively (Kosińska & Andlauer, 2012; Kosińska–Cagnazzo et al., 2015). The integrity of the monolayer is monitored using Transepithelial Electrical Resistance (TEER) readings. An epithelial voltmeter and an electrode are used to take TEER readings. An alternating current is applied across the electrode and the voltage and current is measured. The electrical resistance of the cell layer is calculated using Ohms Law, V= I*R; where V is voltage (V), I is current (amps), and R stands for resistance (Ω).

Reports in the literature have indicated that TEER readings >250 $\Omega*cm^2$ indicate that the monolayer is tight. This means that the tight junctions, which play a role in sealing the spaces between cells, are correctly sealing and a tight barrier is formed. Under these conditions, the majority of the transport through the monolayer should primarily be transcellular as opposed to paracellular.

On the day of the transport experiment, media is removed, and the cell monolayers are washed with pre–warmed Hank's Balanced Salt Solution (HBSS) (pH 7.4, 37 °C) to remove residual medium. The digested or undigested phenolics samples are added to the apical side and the basolateral well is sampled immediately to assess the concentration at time 0 min. The plates are incubated in for 120 min and the basolateral side is sampled at various time points. The samples are then analyzed using RP–HPLC and HPLC–MS to evaluate the transport of undigested and digested phenolics (Kosińska & Andlauer, 2012; Kosińska–Cagnazzo et al., 2015; Xie et al., 2013).

Caco–2 cells have been used to examine the intestinal absorption of polyphenols such as phenolic acids, flavonoids, and mixtures of polyphenols extracted from plant sources. Kosińska and Andlauer (2012) investigated the intestinal absorption of cocoa polyphenols and found that the dominant compounds present in the permeates were (+)–catechin, procyanidin B2, and (–)–epicatechin. In addition, the transport of cocoa procyanidins across the Caco–2 monolayers was found to be limited to monomers and dimers. These are similar to the results obtained by Déprez et al. (2001). Déprez et al. (2001) also used Caco–2 cells to examine the movement of B–type proanthocyanidin (PACs) dimers, trimers, and polymers. The results of this study suggested that PAC dimers and trimers could be absorbed *in vitro* and that PACs with higher degrees of polymerization were not absorbed. Ou et al. (2012) studied the transport of A–type procyanidins from cranberries across a Caco–2 monolayer. The study found that A–type procyanidins dimers, trimers, and tetramers could potentially be transported across a Caco–2 monolayer with a low transport ratio.

Caco–2 cells have also been used to study the absorption of polyphenols from digested foodstuffs. Kosińska–Cagnazzo et al. (2015) identified bioaccessible phenolic compounds from strawberry fruits using *in vitro* digestion and a Caco–2 absorption model. After the strawberry phenolic extract was subjected to *in vitro* gastrointestinal digestion, changes in anthocyanins and ellagic acid derivatives were noted. A 20% decrease in anthocyanins was noted, while the contents of ellagic acid pentose and ellagic acid increased. The increase in ellagic acid was attributed to the decomposition of ellagitannins. The results of the transport studies showed that pelargonidin–3–O–glucoside and coumaric acid hexose were transported across the monolayer. The study

also found that dihydrocoumaric acid sulfate and *p*–coumaric acid were metabolites produced by the Caco–2 cells.

2.6.5 *In vivo* studies

Serra et al. (2010) studied the bioavailability of procyanidin dimers and trimers isolated from grape seed using a combination of *in vitro* and *in vivo* methods. For this study, grape seed procyanidin extract (GSPE) was subjected to *in vitro* gastrointestinal digestion and was used in conjunction with dialysis as an *in vitro* model of absorption. *In vivo* bioavailability was done by evaluating the pharmacokinetics of rats fed GSPE. For the *in vitro* digestion, the authors noted that dimers and trimers were stable under gastric and duodenal digestions and that there was an increase in their concentrations after digestion. This increase was attributed to the depolymerization of larger oligomers into procyanidin dimers and trimers. For the dialysis study, procyanidin monomers, dimers, and trimers were detected in the dialyzable fraction which suggested that smaller procyanidins would be available for absorption *via* passive diffusion. The results of the *in vivo* study detected the glucuronidated forms of (+)–catechin and (–)– epicatechin in rat plasma. Procyanidin dimers and trimers were also detected in rat plasma after the consumption of GPSE. Together, the results of this study suggested that larger procyanidins were broken down into more absorbable phenolics which were shown to be bioavailable.

Sano et al. (2003) who studied the bioavailability of procyanidins from a grape seed extract (GSE) in humans were able to demonstrate that procyanidin B1 from the GSE extract was bioavailable. Holt et al. (2002) investigated the bioavailability of

procyanidins from cocoa in humans. Participants were fed cocoa, which contained procyanidins with degrees of polymerization from monomers to decamers. Blood samples were taken at baseline (0 h) and 0.5, 2, and 6 h after consumption and the plasma was then analyzed using LC–MS. After the consumption of cocoa, (–)–epicatechin, (+)–catechin, and procyanidins B2 and B5 were detected in the plasma which indicated that these compounds were bioavailable. Together these studies have demonstrated that procyanidin monomers, dimers, and trimers are small enough to pass through the intestinal epithelium.

2.6.6 Factors influencing the intestinal absorption of phenolics

Structural characteristics

The intestinal absorption and cellular absorption of phenolics is influenced by a variety of factors. One such factor is molecular structure. Some structural factors that have been studied include molecular weight, glycosylation and esterification. For proanthocyanidins, the DP plays a large role in its capability to pass through the gastrointestinal lining. The absorption of PACs has been shown to be limited to monomers to tetramers (Déprez et al., 2001; Kosińska & Andlauer, 2012; Ou et al., 2012; Zumdick et al., 2012).

Glycosylation is another factor that can influence uptake through the intestinal epithelium. Absorption of quercetin glucosides from onions was shown to be higher than that of the quercetin aglycone. The type of attached sugar moiety can also affect uptake. For example, quercetin 3–O–β–glucoside had improved absorption when compared to its

aglycone counterpart; however, under similar conditions the rhamnosides of quercetin were poorly absorbed (Morand et al., 2000).

Esterification can also influence intestinal absorption. Walden et al. (2001) studied the absorption and excretion profiles of EC, ECG, EGC, and EGCG in subjects consuming black tea. The authors observed that recovery of galloylated catechins in human urine was 10 times lower than that of non–galloylated catechins. Similar results were obtained by Chen et al. (1997) who studied the pharmacokinetic behavior of catechins in rats fed a decaffeinated green tea extract. Olthof et al. (2001) studied the absorption of caffeic acid and chlorogenic acid, an ester of caffeic acid and quinic acid, in humans. The study found that the absorption caffeic acid was higher than that of chlorogenic acid. In subjects, the intestinal absorption was caffeic acid reached 95%, while uptake of chlorogenic acid was only 33%. Furthermore, the recovery of intact chlorogenic acid in urine did not exceed 3% of the ingested dose.

Synergistic effects between polyphenols

Differences in the absorption of pure compounds and mixtures of polyphenols has also been observed. Chen et al. (1997) investigated the pharmacokinetic behavior and bioavailability of tea polyphenols in rats. The rats were fed either a decaffeinated green tea extract, which contained (–)–epicatechin (EC), (–)–epigallocatechin (EGC), (–)–epicatechin–3–gallate (ECG), and (–)–epigallocatechin–3–gallate (EGCG), or pure EGCG. Differences in the pharmacokinetic behavior of EGCG was observed between the extract in comparison to pure EGCG. EGCG levels were higher in the plasma of rats administered the extract than the plasma of the rats given the pure compound. In addition,

it was noted that pure EGCG seemed to be eliminated from the body more readily than EGCG from the extract. The authors suggested these phenomena could be explained by the synergy between the compounds present in the extract. One theory was that other compounds present in the extract may compete for metabolic pathways that convert EGCG or competitively bind to plasma and tissue proteins that may decrease the bioavailability of EGCG. A study by Shoji et al. (2006) investigated the absorption of PACs from apples in rats and noted that the presence of PACs with DP $\geq$ 8 increased the uptake of oligomers with DP ranging from 2 to 5 in rats. The authors postulated that PACs with DP $\geq$ 8 interacted with mucosal proteins present in the digestive tract and as oligomers were not bound to proteins, they were able to be absorbed.

Polyphenols can also play a role in modulating their uptake and metabolism. Cellular transport of polyphenols can occur either transcellularly or paracellularly. Transcellular transport refers to the passage of compounds through the cell by crossing the apical and basolateral membranes. Paracellular transport refers to the transit of compounds through the spaces between cells that are sealed by tight junctions, which are responsible for producing a proper tight monolayer. The mechanism by which paracellular transport occurs is passive diffusion. Paracellular transport is limited to the passive diffusion of water–soluble and low molecular weight molecules (<600 Da) (Kosińska & Andlauer, 2013; Bohn, 2015). Transcellular uptake can occur *via* facilitated, active transport, or passive diffusion. Transcellular passive diffusion is reserved for the transport of small, non–polar molecules, which possess the capability to readily pass through the cell membrane. On the other hand, facilitated and active transport both

require the presence of transmembrane transport proteins. Over 400 transport membrane proteins have been identified.

Research has indicated that polyphenols can affect the function of the tight junctions involved in paracellular transport and the transcellular activity of transmembrane proteins. The effects of which are dependent on the type of polyphenol, concentration, and length of exposure (Bohn et al., 2015). Kosińska and Andlauer (2012), who studied the transport of polyphenols from a cocoa extract suggested that the paracellular transport of flavan–3–ols, such as (+)–catechin and (–)–epicatechin, could be influenced by the presence PACs with higher DP. Redan et al. (2017), who investigated the effects of acute versus extended chronic exposure to blackberries on polyphenol absorption and metabolism in Caco–2 monolayers, proposed that chronic exposure to blackberry phenolics influenced metabolism and transport by down–regulating genes related to metabolic enzymes and transport proteins. The authors suggested that studies assessing bioavailability with acute exposure may not be representative of daily consumption habits and more research on the chronic exposure of phenolic compounds should be conducted to better understand polyphenol absorption.

Effect of matrix composition

The composition of the food matrix can also play a role in influencing the absorption of polyphenolics and the effects can vary depending on the phenolic and the matrix composition. Serra et al. (2010) observed that the absorption of procyanidin dimers and trimers in rats was negatively affected by a carbohydrate rich meal. Serafini et al. (2003) who studied the oral bioavailability of (–)–epicatechin from chocolate in

humans noted that the addition of milk, through ingestion or processing, decreased the absorption of (–)–epicatechin. These results contrast with those obtained by Xie et al. (2012) who studied the impact of milk on the absorption of catechins from green tea using *in vitro* digestion and Caco–2 monolayers. Xie et al. (2012) reported that the addition of milk resulted in improved permeability of all catechins present in the green tea extract. It is possible that the differences in the matrix composition between green tea and chocolate can account for the contrasting results obtained by Serafini et al. (2003) and Xie et al. (2012). The differences between the results obtained by the two authors may also be attributed to other post–absorption events. Serafini et al. (2012) employed an *in vivo* system, which provides a complete picture of phenolic absorption and metabolism, while Xie et al. (2012) utilized an *in vitro* system that is limited to the intestinal absorption and metabolism that does not take into account post–absorption events, such as systematic distribution and elimination.

Dupas et al. (2006) assessed the effect of milk on chlorogenic bioavailability present in coffee using *in vitro* digestion in conjunction with Caco–2 monolayers, in addition to animal models. The authors noted that chlorogenic acid interacted with milk proteins, however *in vitro* digestion was able to disrupt these interactions. These results are similar to those of Xie et al. (2012) who also observed a similar trend with catechins from green tea. However, the results reported by Dupas et al. (2006) indicated that chlorogenic acid was poorly absorbed by Caco–2 monolayers regardless of the presence of milk, while Xie et al. (2012) noted that the presence of milk improved the permeability of green tea catechins. As the structures of chlorogenic acid, a phenolic acid, and catechins, which are flavan–3–ols, differ vastly, it is possible that the differences can be

attributed to their structural characteristics. Dupas et al. (2006) also reported that the presence of milk had no significant effect on the bioavailability of chlorogenic acid in rats and that Maillard reaction products slightly reduced the absorption of chlorogenic acid.

Martínez–Huélamo et al. (2015) investigated the influence of a lipid matrix on the absorption and metabolism of phenolics from tomatoes in humans. In this randomized cross–over study, five participants were fed tomato sauce without oil, with virgin oil or with refined oil. Plasma and urine samples were collected and analyzed to evaluate the effect of the lipid matrix on the absorption and metabolism of tomato phenolics. The authors reported that naringenin, ferulic acid, and caffeic acid along with their corresponding glucuronided counterparts were detected in urine after consumption of all three tomato sauces. In addition, naringenin– and ferulic–glucuronide were detected in plasma after ingestion of each of the tomato sauces. No significant differences in the pharmacokinetic behaviors of naringenin– and ferulic–glucuronide detected between the three sauces were detected. However, the authors noted that for the oil–enriched tomato sauces the plasma concentration of naringenin glucuronide exhibited a biphasic absorption profile over time. Martínez–Huélamo et al. (2015) suggested that this may be attributed to the re–absorption events during enterohepatic circulation due to enterohepatic and enteric recycling. Enterocytes may possess β–glucuronidases or sulfatases that can deconjugate glucuronidated or sulfated phenolics, respectively, thus reverting the conjugates back to their parent compounds, which are then absorbed in the GI tract. These parent compounds may then be re–conjugated and de–conjugated. Because of this recycling, the apparent plasma half–life is extended.

Taken together, these studies have shown that the type of phenolic and matrix composition can play a role in influencing the bioaccessibility and bioavailability of phenolics. As a result, the impact of matrix composition should be investigated in order to fully understand the metabolism of polyphenolics.

2.7 Polyphenol metabolism

Metabolism of polyphenols within an *in vivo* system is a complex topic. Prior to appearing in the circulatory system, phenolics may undergo a variety of transformations and the structures of the resulting metabolites may not resemble that of their precursors and are functionally distinct from their dietary form (Jaganath & Crozier, 2010). After absorption at the intestinal epithelium, phenolics may be metabolized by enterocytes and the liver. Furthermore, phenolics not absorbed in the small intestine may be metabolized by the gut microflora and the resulting products may possibly be taken up in the colon. These colonic metabolites may also be further modified by the liver after absorption. The low levels of dietary phenolics present in the bloodstream (<10 μM) may suggest that much of the positive health benefits associated with the consumption of phenolic rich foods are attributed to their metabolites (Williamson & Manach, 2005).

2.7.1 Metabolism *via* the intestinal epithelium and the liver

Research has also shown that the small intestinal epithelium can play a role in metabolizing phenolic compounds. This includes phase II metabolism consisting of glucuronidation, sulfation, and methylation (Aragones et al., 2017). Kern et al. (2003) characterized the metabolites of hydroxycinnamic acids that were absorbed and

metabolized by Caco–2 cells. The study indicated that Caco–2 cells possessed intracellular sulfotransferases and glucuronosyltransferases that could produce sulfate and glucuronide conjugates of various hydroxycinnamic acids. Soler et al. (2010) studied the metabolism and transport of olive oil polyphenols and found that there was limited metabolism of olive oil phenolics. However, methylated conjugates were detected.

After the initial metabolism *via* the small intestinal epithelium, phenolics can be further metabolized by the liver. Piskula and Terao (1998) investigated possible sites of (–)–epicatechin metabolism in rats by screening tissues for enzymatic activities involved in (–)–epicatechin metabolism. Results suggested that the glucuronidation of (–)–epicatechin takes place in the intestinal mucosa of the small and large intestine, while sulfation occurs in the liver. It was proposed that methylation takes place in the liver and kidney. Donovan et al. (2001) who investigated the metabolism of (+)–catechin in mice, also postulated that glucuronidation takes place in the small intestine. The results suggested that methylation occurs in the small intestine and the liver, while sulfation takes place in the liver.

These conjugation reactions play an important role in detoxification, which prevents, in most cases, toxicity, and improves the hydrophilicity of phenolics, which enhances elimination *via* biliary or urinary routes. As these reactions are extremely efficient, limited concentrations of the aglycone counterparts are present in the circulatory system after the consumption of phenolics.

2.7.2 Metabolism *via* colonic microflora

Numerous studies have demonstrated that colonic microflora possess the capability to metabolize phenolics. Déprez et al. (2000) studied the metabolism of polymeric procyanidins by human colonic microflora. This study showed that procyanidins were metabolized by colonic microflora into phenolic acids. These were identified as [2– (*p*–hydroxyphenyl)acetic acid, 2–(*p*–hydroxyphenyl)– propionic acid, 2–(*m*–hydroxyphenyl)acetic acid, 2–(*m*–hydroxyphenyl)propionic acid, 5–(*m*–hydroxyphenyl)valeric acid, and phenylpropionic acid. In addition, studies have suggested the ability of the gut microbiota to metabolize PACs is limited by their DP (Gonthier et al., 2003). Metabolism of larger PACs may be hindered due to reduced accessibility of the substrate due to increased DP. Furthermore, larger PACs may be bound to other macromolecules present, such as proteins and enzymes, and would not be available for metabolism by the gut microbiota.

Consumption of ellagitannin rich foods, such as pomegranates, has also been associated with reducing the risk of chronic diseases; however, studies have shown that these compounds possess poor bioavailability. While ellagic acid and their derivatives possess poor bioavailability, interest in their gut microbiota metabolites, has grown and the health promoting benefits of ellagitannins may be attributed to these compounds. Ellagic acid has been shown to be metabolized by the gut microbiota resulting in the production of urolithins, which possess improved bioavailability (García–Villalba et al., 2013; Tulipani et al., 2012; Tomás–Barberán, 2014). Urolithins have been detected in plasma as glucuronide and sulfate conjugates at concentrations ranging from 0.2 to 20 μM (Espín et al., 2013). *In vitro* studies have also shown that urolithins possess anti–

inflammatory properties and anti–antioxidant activity. Piwowarski et al. (2015) demonstrated that urolithins were able to inhibit lipopolysaccharide–induced inflammation in RAW 264.7 murine macrophages and reduced the expression of genes involved in the production of proinflammatory molecules, such as IL–1β, TNF–α, and IL–6.

A variety of models have been developed to study the colonic microflora metabolism of phenolics and their absorption. *In vitro* fecal incubation is one frequently method used to study the metabolism of phenolics. Fecal samples are collected from healthy individuals and incubated with phenolics. The resulting metabolites are extracted and analyzed. *In vitro* fecal incubation can also be combined with transport experiments using Caco–2 cells as an intestinal epithelium model. Wang et al. (2013) observed that the colonic metabolites of a grape seed extract were able to be absorbed through a Caco–2 monolayer.

In vivo studies using rodents are also commonly used. One popular method is to inoculate germ–free mice with fecal micro–biota derived from healthy humans. Bioavailability of compounds in the germ–free and inoculated mice is then investigated. These studies that have shown that the presence of certain polyphenol derivatives is attributed to the presence of intestinal microbiota.

2.7.3 Methods used to detect metabolites

Currently, one of the major limitations to studying phenolic metabolites is the lack of standards (Barron et al., 2012). However, chemical procedures and biochemical labelling methods have been developed to aid with studying phenolic metabolites. This

includes the chemical synthesis and biosynthesis of isotopic compounds. The use of isotopically labelled molecules provides researchers the ability to trace the parent compound through its metabolic journey, in addition to elucidating their subsequent metabolites. These isotopically labelled molecules also provide investigators the ability to study the excretion routes and tissue distribution of the parent compounds and metabolites (Déprez & Scalbert, 1999).

Isotopically labelled polyphenols can be produced *via* chemical or biochemical synthesis. Chemical synthesis of radiolabelled compounds provides high yields and the reaction can easily be scaled up. Furthermore, there is high isotopic purity and the location of the labelled compound is known. However, one of the main disadvantages is the production of more complex molecules is difficult (Déprez & Scalbert, 1999; Barron et al., 2012).

Biolabeling or biosynthesis uses a biological system, such as cell culture or shoot explants, to produce isotopically labeled molecules that are more structurally complex, which cannot be synthesized easily. As it is easier to trace metabolites by the carbon skeleton rather than by hydrogen, which can be exchanged or lost, carbon isotopes (^{13}C and ^{14}C) are often used. However, biosynthesis does have some downsides. One of the main disadvantages is the lack of specific activity, meaning the location of the isotope present in the compound can vary. Furthermore, isotopic precursors may also be incorporated into other compounds that are not of interest. Additionally, rigorous purification steps are required to isolate the compounds of interest (Déprez & Scalbert, 1999; Barron et al., 2012).

Enzymatic treatment is another frequently used technique to aid with identifying polyphenolic metabolites, more specifically conjugated metabolites. Samples are treated with sulfatase and β–glucuronidase, which will release sulfate and glucuronic acid resulting in unconjugated phenolics. The enzymatically treated and untreated samples can be analyzed using HPLC–MS. If sulfated and glucuronidated phenolics are present, the loss of peaks and an increase in the area of the corresponding unconjugated phenolics during analysis will be observed (Bohn et al., 2015). For methylated compounds, there are no enzymes that can remove the added methyl group. However, identification can be done using MS and nuclear magnetic resonance (NMR) to confirm the structure (Boulton et al., 1999).

2.7.4 Cellular accumulation of phenolics

As previously mentioned, the bioaccessibility and bioavailability of polyphenols is a complex subject. The presence of limited amounts of phenolics in the circulatory system can be attributed to a variety of factors. Polyphenols can be poorly absorbed from the intestine. Furthermore, phenolics can be highly metabolized, such that metabolites do not resemble their precursors. It is also highly possible that up–taken phenolics can accumulate within the cells and as a result, may not be present in the circulatory system.

To fully understand the metabolism and distribution of phenolics within an *in vivo* system, the uptake and accumulation of phenolics within cells, tissues, and organs should also be investigated. Vaidyanathan et al. (2003) investigated the cellular uptake of tea flavonoids and noted that there was no cellular accumulation of (–)–epicatechin, while (+)–catechin exhibited limited cellular accumulation. In contrast, epicatechin gallate and

epigallocatechin gallate, which are gallated flavonoids, both exhibited significantly higher amounts of accumulation compared to their non–gallated counterparts.

D'Antuono et al. (2015) demonstrated that polyphenols from digested artichoke heads could be transported across a Caco–2 monolayer and can accumulate within the cells. The authors noted that chlorogenic acid, 4,5– *O*–Dicaffeoylquinic acid, 3,4– *O*–Dicaffeoylquinic acid, 1,4–*O*–Dicaffeoylquinic acid, 3,5–*O*–Dicaffeoylquinic acid, 1,5–*O*–Dicaffeoylquinic acid, 1–*O*–caffeoylquinic acid, 3–*O*–caffeoylquinic acid, and cynarin had accumulated in Caco–2 cells. The authors were also able to detect small amounts of coumaric and caffeic acids, which were not present in artichoke hearts. Previous literature has indicated that the gut microbiota can metabolize chlorogenic acid resulting in the formation of coumaric acid. Based on this information, the authors suggested that the presence of coumaric acid could possibly be attributed to the intracellular metabolism of chlorogenic acid.

Furthermore, it is highly likely that accumulated phenolics can bestow a protective antioxidative effect within the cell, however the mechanisms are not well known. It is possible that phenolics may preemptively react with ROS, thus preventing the oxidation of cellular components such as DNA and proteins. There is also some evidence that phenolics may bind to DNA and proteins, inhibiting the binding of carcinogenic substances or free radical interactions that can produce oxidative damage (Whitley et al., 2003). Whitley et al. (2003) studied the uptake and transport of ellagic acid (EA) using Caco–2 monolayers. The authors noted that there was low apical to basolateral absorption of EA, which was in agreement with previous mice feeding studies that indicated that EA possessed poor bioavailability (Teel & Martin, 1988).

Interestingly, Whitley et al. (2003) noted that there was extensive cellular accumulation of EA which suggested EA possessed the ability to cross the apical membrane. The authors also observed that the majority of cellularly accumulated EA was bound to DNA and proteins, with DNA binding being 5 times higher than that of protein binding.

2.8 Impact of digestion and absorption on antioxidant capacity

As previously mentioned, phenolics may be chemically modified or degraded prior to arriving at the site of activity (Dominguez–Avila et al. 2017). As a result, antioxidant potential observed *in vitro* for undigested samples may not be representative of what occurs *in vivo*. Thus, to advance understanding of the advantages of consuming antioxidant rich foods, it is pertinent to investigate the impact of metabolism on antioxidant capacity.

2.8.1 Digestion

The impact of digestion on the antioxidant capacity has been evaluated for a variety of foods. However, the impact of digestion on the antioxidant potential of foods is not conclusive. Huang et al. (2014) investigated the impact of digestion on the antioxidant capacity of Chinese bayberry and reported that digestion reduced TPC and antioxidant capacity. These results contrast to those of Chiang et al. (2013) who reported that *in vitro* digestion of gooseberry increased TPC and antioxidant capacity. Furthermore, Chen et al. (2014) investigated the impact of digestion on 33 different fruits and the results of this study were mixed. The antioxidant capacity and TPC of some fruits increased after GI digestion, while others decreased. Ryan and Prescott (2010) who

studied the effect of *in vitro* digestion on the antioxidant capacity of 25 commercially available fruit juices reported that the antioxidant capacity for the majority of juices was enhanced after digestion. Wootton–Beard et al. (2011), who studied the effects of digestion on the 23 vegetable juices, also reported a similar trend. Celep et al. (2015) reported that *in vitro* GI digestion reduced the antioxidant potential of Turkish fruit wines, while Chen et al. (2013) reported that digestion reduced the antioxidant capacity and TPC content of commercially prepared teas.

The inconclusive effects of digestion may be attributed to a variety of factors. The first is differences in the *in vitro* GI digestion model, specifically the concentration of enzymes used. Differences in the models used make it difficult to directly compare results. Another factor is the matrix of the sample. The matrix may offer phenolics protection from the harsh GI conditions. In addition, the types of phenolics present can vary from sample to sample. Differences in the structure of phenolics may play a role in determining their stability during GI digestion.

2.8.2 Absorption

Various studies have shown that polyphenol conjugates and metabolites possess antioxidant and biological activity. However, the scope of understanding is limited, and results have been mixed. The impact of glucuronidation, sulfation, and methylation has not been extensively investigated. Manach et al. (1998) was able to detect quercetin conjugates in human plasma after the consumption of a quercetin–rich meal. The authors were also able to demonstrate that some conjugated derivatives of quercetin possessed the

capability to delay Cu^{2+} induced oxidation of LDL. However, the antioxidant effect seen was about half of what was displayed by its aglycone counterpart.

Yamamoto et al. (1999) also investigated the antioxidant activity of quercetin conjugates (quercetin 3–O–*β–D–*glucopyranoside (Q3G), quercetin 4′*–O–β–D–* glucopyranoside (Q4′G, quercetin 7–O– *β–D–*glucopyranoside (Q7G)), but in rat plasma. The authors demonstrated that conjugated quercetin metabolites suppressed Cu^{2+} induced oxidation of LDL, which is in agreement with the results obtained by Manach et al. (1998). The results also indicated the conjugates possessed differing antioxidant activities and lipid oxidation inhibiting abilities. Antioxidant activity measured by the DPPH• assay was as follows from lowest to highest: Q4′G < Q3G < Q7G ~ quercetin. Inhibition of AAPH induced oxidation of LDL followed the same trend. The results of this study suggest that position of the conjugate group can alter antioxidant activity.

Da Silva et al. (1998) investigated the effect of the oral administration of (–)–epicatechin on the antioxidant ability of rat plasma. Metabolites present in the plasma were identified as sulfate, glucuronide, and sulfogluronide conjugates of epicatechin and methylated epicatechin. Glucuronide conjugates were the major conjugates present. Like the aforementioned quercetin conjugates, the (–)–epicatechin conjugates present in plasma were also able to inhibit Cu^{2+} induced oxidation.

Spencer et al. (2001) evaluated the effects of glucuronidation and methylation on epicatechin's capability to mitigate hydrogen peroxide (H_2O_2) induced cell death in neurons and fibroblasts. The study demonstrated the (epi)catechin and its glucuronidated and methylated conjugates were able to mitigate H_2O_2 induced cell death in both neurons

and fibroblasts. In fact, the glucuronidated conjugate was able to bestow a similar level of activity as its unconjugated counterpart.

Piazzon et al. (2012) synthesized and purified sulfate conjugates of ferulic (ferulic acid 4′–O–sulfate) and caffeic acid (caffeic acid 3′–*O*–sulfate and caffeic acid 4′–O–sulfate) and glucuronides of ferulic acid (ferulic acid acyl glucuronide and ferulic acid 4′–O–glucuronide) and antioxidant activity was evaluated using FRAP and ABTS assays. The authors noted that ferulic acid 4′–O–sulfate and ferulic acid 4′–O–glucuronide possessed lower antioxidant activity compared to their aglycone counterpart. The mono–sulfated derivatives of caffeic acid, caffeic acid 3′–O–sulfate and caffeic acid 4′–O–sulfate, exhibited less antioxidant activity than caffeic acid. FAG exhibited similar antioxidant activity to that of its parent compound, ferulic acid, while ferulic acid 4′–O–glucuronide possessed very low antioxidant activity. Together these studies highlight the importance of studying the properties of phenolic conjugates, as they may also contribute to favorable *in vivo* activity.

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CHAPTER 3

Evaluating the potential bioaccessibility of pecan phenolics: Changes to the phenolic profile and antioxidant properties following *in vitro* digestion

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ABSTRACT

Acetonic crude extracts from raw and roasted Georgia pecans were prepared and subjected to *in vitro* gastrointestinal digestion. Digested and undigested extracts were separated into low– and high–molecular–weight (LMW and HMW) fractions via Sephadex LH–20 column chromatography, afterwards they were characterized by RP–HPLC–ESI–MS. The LMW fraction consisted primarily of flavan–3–ols and ellagic acid derivatives and following digestion there was an overall loss in phenolics from 16 to 100%. In the HMW fraction, procyanidins with degrees of polymerization ranging from dimers to hexamers were present. Following digestion, a loss in trimers to hexamers was observed, and a significant increase in dimers. This increase was attributed to the dimerization of (+)–catechin/(–)–epicatechin and the scission of larger procyanidins, mainly tetramers to hexamers. The loss of phenolics following digestion, as seen by HPLC characterization, was reflected in reduced total phenolics content and antioxidant capacity, as determined by selected *in vitro* antioxidant assays.

3.1 Introduction

In recent years, there has been increased interest in the pecan [*Carya illinoinensis* (Wangenh.) K. Koch] and other tree nuts due to improved public awareness about their healthfulness. A variety of studies has shown that regular nut consumption is associated with a lowered risk of heart disease and other chronic diseases. Pecans, along with almonds, hazelnuts, peanuts, some pine nuts, pistachios, and walnuts, were even awarded the following qualified health claim: Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts (*such as pecans*), as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease. The benefits of nut consumption have been attributed to the various lipid components and phenolic compounds with anti–oxidative and anti–inflammatory properties (Hudthagosol, Haddad, McCarthy, Wang, Oda, & Sabaté, 2011). Pecans in particular are extremely rich in antioxidants, such as phenolics (Gu et al., 2004).

Previous studies have focused on identifying and characterizing the antioxidant properties of phenolic constituents found in various pecan cultivars (Gong & Pegg, 2017; Robbins, Gong, Wells, Greenspan, & Pegg, 2015; Robbins, Ma, Wells, Greenspan, & Pegg, 2014). These studies have shown that the major phenolics present were (+)–catechin, ellagic acid and its derivatives, along with procyanidins consisting of various degrees of polymerization ranging from monomers to hexamers. Studies have also looked at the biological efficacy of pecan phenolics. For instance, Robbins, Greenspan and Pegg (2016) demonstrated that pecan phenolics were capable to inhibit the production of mediators of inflammation in lipopolysaccharide–stimulated RAW 264.7 murine macrophages. Kellett, Greenspan, Gong and Pegg (2019), who investigated the cellular

antioxidant activity of U.S. pecans using a Caco–2 cell line, suggested that procyanidins from pecans were taken up by Caco–2 cells and could scavenge reactive oxygen species. Clinical data by Hudthagosol et al. (2011) also demonstrated that bioactive constituents from pecans, like tocopherols and flavan–3–ol monomers, improved postprandial antioxidant capacity and reduced levels of oxidized low–density lipoprotein. Together these studies have demonstrated that pecan phenolics possess strong antioxidant activity and biological properties. While previous research has helped to characterize the antioxidant constituents, identify their health promoting properties, and evaluate their contributions to post–prandial antioxidant defenses, little is known about the impact of digestion on pecan phenolics and other nut phenolics. There has been concern that in vitro antioxidant assays do not represent in vivo conditions, because factors such as digestion have not been taken into consideration. During their passage through the gastrointestinal tract, phenolics may interact with micro– and macro–molecules. These interactions may chemically modify phenolics, thus altering their function and antioxidant capabilities (Dominguez–Avila, Wall– Medrano, Velderrain–Rodriguez, Chen, Salazar–Lopez, Robles–Sanchez, & Gonzalez–Aguilar, 2017). To better understand the health benefits of consuming a phenolic–rich diet, the impact of digestion and absorption on phenolics ought to be investigated.

Within the past decade, several studies have investigated the impact of digestion on antioxidant rich extracts from a variety of foodstuffs such as tea and various fruits; however, few studies have examined the effects of gastrointestinal digestion on nut phenolics and to our knowledge, none have looked at pecans. More specifically, no study has characterized changes to the pecan phenolic profile and their antioxidant capacity

following *in vitro* digestion conditions. In the present work, acetonic crude phenolic extracts were prepared from raw and roasted Georgia pecans and were then subjected to *in vitro* digestion. Traditional chemical assays were used to assay the changes to TPC and antioxidant activity (i.e., H–ORAC$_{FL}$, FRAP, and TEAC) following digestion treatments. Sephadex–LH 20 column chromatography was used to prepare low– and high– molecular weight (LMW and HMW) fractions to aid with identifying changes to the antioxidant constituents following digestion, and characterization was performed using HPLC–ESI–MS/MS.

3.2 Materials and methods

3.2.1. Chemicals

Glass wool, cellulose thimbles, sodium carbonate, ACS–grade acetone, methanol, ethanol (95%), hexanes, acetone, and Whatman No. 1 filter paper, as well as HPLC–grade water, methanol, and acetonitrile were acquired from Fisher Scientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid was obtained from VWR International, LLC (Suwanee, GA, USA). Sephadex LH–20, Folin & Ciocalteu's phenol reagent, (+)–catechin hydrate, gallic acid, ellagic acid, protocatechuic acid, pepsin, pancreatin, fluorescein 3′,6′– dihydroxyspiro[isobenzofuran–1[3H]9′[9[H]xanthen]–3–one), Trolox (6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid), AAPH (2,2′–azobis[2–amidinopropane] dihydrochloride), ABTS (2,2′–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid) diammonium salt), iron(II) sulfate heptahydrate, iron(III) chloride hexahydrate, and potassium persulfate were purchased from the Sigma–Aldrich Chemical, Co. (St. Louis, MO, USA).

3.2.2. Sample preparation

Different lots of raw in–shell 'Desirable' pecans were shipped from three Georgia pecan orchards in the fall of 2019 to the Department of Food Science & Technology in Athens, GA. The pecans were frozen in–shell at –80 °C until analyzed.

3.2.3. Roasting

Pecans were removed from the –80 °C freezer, shelled, and the nutmeat roasted prior to lipid and phenolic extractions. A roasting method designed by Erickson, Santerre, and Malingre (1994), later optimized by Robbins (2012) to match the color of commercially roasted pecans using the Commission internationale de l'éclariage (CIE) L*C*h system was employed. In summary, an impingement oven (Model 1450, Lincoln Foodservice Products, Fort Wayne, IN, USA) was employed to roast pecan halves at 175 ± 10 °C for 8 min. The roasted pecans were then cooled, vacuum packaged, and stored at –80 °C until subsequent lipid and phenolic extractions.

3.2.4. Lipid extraction

Lipids were extracted from raw and roasted pecans using a Soxhlet apparatus. First, pecan samples were taken from the –80 °C freezer, shelled, and the halves immersed in liquid nitrogen. The cryogenically treated pecans were then ground to a very fine powder in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ, USA), after which ~20 g were transferred into a cellulose extraction thimble (single thickness, 43 mm i.d. × 123 mm external length, Whatman International Ltd., Maidstone, England) and a thin plug of glass wool placed in the top of the thimble

to prevent movement of the sample during extraction. Lipids were extracted for ~18 h with ~350 mL of hexanes. The defatted nutmeat samples in the thimbles were allowed to dry overnight in a fumehood before proceeding with phenolic extractions.

3.2.5. Phenolic extraction

Acetonic crude phenolic extracts were prepared from defatted nutmeat using an extraction solvent of $(CH_3)_2CO$:H_2O:CH_3COOH in a ratio of 70:29.5:0.5 (v/v/v) at a solid–to–solvent ratio of 1:6 (w/v) in Erlenmeyer flasks. Prepared samples were placed in an orbital–shaking water bath (New Brunswick Scientific, New Brunswick, NJ, USA) at 60 °C for 30 min. After this period, the slurries were vacuum filtered through Whatman No. 1 filter paper. The residue was then re–extracted with fresh solvent three more times for a total of four extractions, and the supernatants were pooled. Acetone was removed from the collected supernatant using a Büchi Rotavapor R–210 and a V–700 vacuum pump connected to a V–850 vacuum controller (Büchi Corporation, New Castle, DE, USA) and a water bath at 45 °C. The aqueous residue was transferred to a crystallization dish (100 × 50 mm, dia. × H), covered with Whatman No. 1 filter paper, and placed in a –80 °C freezer until frozen. These samples were then lyophilized using a Labconco Freezone 2.5 L freeze dryer (Labconco Corp., Kansas City, MO, USA). The lyophilized extracts were weighed (for mass balance purposes), transferred to an amber vial, capped, and stored at –4 °C until analysis.

3.2.6. Fractionation via Sephadex LH–20 chromatography

To further elucidate the impact of digestion on proanthocyanidins (PACs), crude extracts from raw and roasted pecans were separated into LMW and HMW fractions. Following the method of Robbins et al. (2014), ~2 g of crude phenolic extract were dissolved in ~50 mL of 75% (v/v) ethanol using a Ultrasonik 104x sonicator (Ney Dental Inc., Bloomfield, CT) set to maximum power, minimum degassing, without heating. The sample was then loaded onto a chromatographic column packed with Sephadex–LH 20 (bead size: 25–100 μm; Chromaflex column, 400 mm × 30 mm [length × i.d.], Kontes, Vineland, NJ, USA). The LMW fraction was eluted using 95% (v/v) ethanol. After the LMW fraction had eluted, the HMW fraction was then eluted with 50% (v/v) aqueous acetone. Ethanol and acetone were removed from the LMW and HMW fractions, respectively, using the Rotavapor. The residual aqueous solutions were then frozen and lyophilized as described above.

3.2.7. Digestion procedure

Crude phenolic extracts were subjected to simulated *in vitro* gastric and intestinal digestion according to Kosińska–Cagnazzo, Diering, Prim, and Andlauer (2015), but with some modifications. Samples were digested both with and without enzymes: this was performed to help differentiate between the effects of pH and the combination of pH with digestive enzymes. In addition, control digestions without phenolic samples were run in parallel. Briefly, ~100 mg of pecan crude phenolic extract were weighed into a 125–mL Erlenmeyer flask and dispersed in 10 mL of 0.9% (w/v) saline. To initiate the gastric phase, the pH was lowered to 2.0 using 1 M HCl. Two mL of a pepsin solution (17.5 g/L) prepared in 0.1 M HCl were added. The headspace of samples in the Erlenmeyer flasks

were flushed with nitrogen to remove oxygen, capped with a rubber stopper, and incubated for 1 h in a shaking water bath set at 37 °C. This was followed by a simulated intestinal digestion process, which was initiated by adjusting the pH to 8.0 using 1.0 M NaOH and 0.1 M $NaHCO_3$. Two mL of a pancreatin solution (7 g/L) prepared in 0.1 M $NaHCO_3$ were added. Samples were again flushed with nitrogen and intestinal digestion was carried out for 2 h at 37 °C in the aforementioned shaking water bath. Digestion was terminated placing the Erlenmeyer flasks in ice water for 10 min. Digests were transferred to 50–mL Falcon centrifuge tubes (VWR International) and brought to 50 mL with 0.9% (w/v) saline. The contents were centrifuged at 1,000 rpm for 20 min to remove precipitates, which would not be bioaccessible. A 30–mL aliquot of the supernatant, which will be referred to as the bioaccessible fraction, was lyophilized and then fractionated as described above. The remaining supernatant was divided into 5–mL aliquots and stored at –80 °C until analysis.

3.2.8. Total phenolics content (TPC) determination

The TPC of samples was determined using Folin & Ciocalteu's phenol reagent as described by Robbins et al. (2015). Raw and roasted pecan crude phenolic extracts were first dissolved in methanol then diluted to 0.2 mg/mL with deionized water. A standard curve using concentrations of (+)–catechin ranging from 1.6 to 8.0 μg/mL was prepared in a similar fashion. For digested samples, aliquots were removed from the –80 °C freezer, thawed, and then diluted to 0.2 mg/mL with deionized water. One mL of each diluted extract, 7.5 mL of deionized water, 0.5 mL of Folin & Ciocalteu's phenol reagent, and 1 mL of a saturated Na_2CO_3 solution were combined to give a final volume of 10

mL. Each sample was vortexed for 30 s after the addition of each component. A quiescent period of 1 h followed to allow for maximum color development of the chromophore. Afterwards, 200 µL of each sample were pipetted into a black, clear–bottomed 96– well microplate, and the absorbance was measured at 750 nm using a FLUOstar Omega microplate reader (BMG LABTECH Inc., Cary, NC, USA). Results were reported as mg (+)–catechin equivalent (CE)/100 g of either raw or roasted pecan nutmeat. Samples were prepared in triplicate and analyzed in triplicate (n=9) and then averaged.

3.2.9. Hydrophilic–oxygen radical absorbance capacity (H–ORAC$_{FL}$) determinations

The H–ORAC$_{FL}$ assay was performed to assess the *in vitro* antioxidant power of the undigested and digested pecan phenolics, based on the hydrogen–atom transfer mechanism of primary antioxidants. Robbins et al. (2015) described the method that was employed. Phosphate buffer (0.075 M, pH 7.4) was used as the blank and diluent. The control digest was used as the blank. ABAP (80 µM in buffer) and fluorescein (0.1 µM in buffer) were employed as the radical initiator and fluorescent probe, respectively. For the duration of the experiment, the temperature of both solutions was maintained at 37 °C. The crude phenolic extracts were first dissolved in 95% (v/v) ethanol at a concentration of 0.5 mg/mL and were then further diluted with phosphate buffer to a concentration of 0.025 mg/mL. Likewise, digested samples were diluted with phosphate buffer to the appropriate concentrations.

The BMG LABTECH microplate reader, equipped with two internal 500 µL lead pumps, was used. During the experiment, the temperature of the microplate reader was

maintained at 37 °C. Fluorescence was measured at excitation/emission wavelengths of 485/520 nm, respectively, over a run time of 3 h (i.e., 60 cycles). Twenty microliters of the blank, sample, or Trolox standard were pipetted into the COSTAR 96–well clear, non–sterile, non–treated microtiter plate. Two hundred μL of fluorescein and 20 μL of AAPH were introduced automatically into each well with the addition of each reagent was separated by one cycle. Upon completion of the run, areas under the standard and sample curves were calculated and blank corrected. Quantitation was carried out from a standard curve constructed based on five different concentrations of Trolox (12.5, 25, 50, 80, and 100 μM in the phosphate buffer) and results were reported as mmol Trolox equivalent/100 g of either raw or roasted pecan nutmeat. Samples were prepared in triplicate and analyzed in triplicated (n=9).

3.2.10. Ferric reducing antioxidant power (FRAP) determinations

The FRAP assay was carried out to assess the *in vitro* antioxidant power of the undigested and digested pecan phenolics, based on the single–electron transfer mechanism of primary antioxidants, according to Pulido, Bravo, and Saura–Calixto (2000). Lyophilized extracts were first dissolved in methanol at a concentration of 0.2 mg/mL. The FRAP reagent, prepared on the day of use, consisted of 2.5 mL of 10 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$, and 25 mL of 0.3 M acetate buffer at pH 3.6. The reagent was incubated at 37 °C until ready for use. A standard curve using aqueous iron sulfate ($FeSO_4 \bullet 7H_2O$) solutions was prepared at five concentrations ranging from 250 to 1600 μM. For the duration of the experiment, the temperature was maintained at 37 °C. Two hundred microliters of FRAP reagent, 20 μL of deionized

water, and 6.6 µL of either the blank, standard, or sample were pipetted in to a 96–well microplate and immediately analyzed in the plate reader. The absorbance at λ = 595 nm was recorded and results were reported as mmol Fe^{2+} equivalent/100 g of either raw or roasted pecan nutmeat. Samples were prepared in triplicate and analyzed in triplicate (n=9).

3.2.11. Trolox equivalent antioxidant capacity (TEAC) determinations

TEAC values were determined according to Re, Pellegrini, Proteggente, Pannala, Yang, and Rice–Evans (1999), which involved assessing the capability of undigested and digested crude extracts to quench the free–radical cation ABTS potassium persulfate solutions were prepared in deionized water. Equal volumes of the two solutions were combined and left to stand in the dark at room temperature for 12–16 hours to generate a stock solution of ABTS stock solution was diluted with 95% ethanol (v/v) until an absorbance of 0.7 ± 0.02 at λ = 734 nm was reached, when measuring the absorbance with an 8453 UV/Vis diode–array spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). For the development of a standard curve, Trolox solutions were prepared in 95% (v/v) ethanol at concentrations ranging from 500 to 1500 µM. Ten microliters of test sample or Trolox standard were mixed with 1 mL of the $ABTS^{\bullet+}$ working solution, pre–warmed for 5 min at 30 °C, and absorbance readings at λ = 734 nm recorded. Digested and undigested samples were diluted with 95% (v/v) ethanol such that they gave a 20 to 80% inhibition of the $ABTS^{\bullet+}$ working solution. Samples were prepared in triplicate and assayed in triplicate (n=9). Results were expressed as mmol Trolox equivalent (TE)/100 g of either raw or roasted pecan nutmeat.

3.2.12. High performance liquid chromatography

LMW and HMW fractions of digested and undigested pecan crude phenolic extracts recovered from the Sephadex LH–20 column were chromatographed on an equi–mass basis using an Agilent 1200 series HPLC system comprising a quaternary pump, degasser, autosampler, thermostatic column compartment, UV–Vis diode array detector, and fluorescence detector with standard flow cell, and 3D ChemStation software (Agilent Technologies).

Compounds in the LMW fraction were analyzed according to Gong and Pegg (2017). Briefly, the LMW fraction was chromatographed with a Kinetex pentafluorophenyl (PFP) fused– core column (150 mm × 2.6 mm i.d., 2.6–µm particle size, 100 Å; Phenomenex, Torrance, CA, USA) and a SecurityGuard cartridge of the same material. LMW fraction samples were dissolved in mobile phase A at a concentration of 10 mg/mL. Samples were then passed through a 0.2–µm regenerated cellulose syringe filter. Detection wavelengths for the diode–array detector were set at λ = 255 nm (ellagic acid and their derivatives), 280 nm (benzoic acids and flavan–3–ols, more specifically catechins), 320 nm (hydroxycinnamic acids), and 360 nm (flavonols). Mobile phases A and B comprised $H_2O/CH_3CN/CH_3COOH$ (94:5:1, v/v/v) and $H_2O/CH_3CN/CH_3COOH$ (59:40:1, v/v/v), respectively. A linear gradient elution was employed at a flow rate of 0.8 mL/min as follows: 0–30 min, 0–60% B; 30–32 min, 60% B; 32–35 min, 60–0% B, followed by a 5–min hold to re–equilibrate the system. Tentative identification was achieved by matching UV–Vis spectra and retention time (t_R) mapping with commercial standards.

Procyanidins present in the HMW fraction were separated by their degree of polymerization (DP) with the aforementioned Agilent 1200 system, but with a hydrophilic interaction liquid chromatographic (HILIC) analytical column (Luna 150 mm × 4.6 mm i.d., 3–µm particle size, 200 Å; Phenomenex), a SecurityGuard cartridge of the same material, and fluorescence detection. The method of Kelm, Johnson, Robbins, Hammerstone, and Schmitz (2006), later modified by Gong and Pegg (2017), was used. Mobile phases A and B consisted of CH_3CN/CH_3COOH (98:2, v/v) and $CH_3OH/H_2O/CH_3COOH$ (95:3:2, v/v/v), respectively. A flow rate of 1 mL/min was employed with the following gradient elution: 0–25 min, 0–45% B; 25–30 min, 45–0% B; with an additional 2–min hold to re–equilibrate the system. The lyophilized HMW fraction was dissolved in methanol (10 mg/mL) followed by a 1:1 (v/v) dilution with mobile phase A to give a final concentration of 5 mg/mL. Samples were passed through a 0.2–µm regenerated cellulose syringe filter before 20 µL were injected by the autosampler onto the HILIC column. Fluorescence detection at excitation/emission wavelengths of λ = 276/316 nm was employed.

3.2.13. Statistical analysis

Results from the TPC, H–$ORAC_{FL}$, FRAP, and TEAC assays were analyzed by analysis of variance, and data were reported as mean ± standard deviation. Significant differences were evaluated using the Tukey–Kramer honest significant difference test using JMP Pro Software, Version 13 (Cary, NC, USA).

3.3. Results and discussion

3.3.1. Impact of digestion on antioxidant capacity of raw and roasted pecan phenolic extracts

In this study, lipids, which comprise 70-80% of the pecan's mass, were removed to help concentrate pecan phenolics in the form of 'crude extract' prior to *in vitro* digestion (Robbins, Gong, Wells, Greenspan, & Pegg, 2015). Employing this approach, we hoped to better characterize the resultant phenolics, antioxidant capacity, and TPC after simulated *in vitro* digestion in the upper gastrointestinal tract. Differences in antioxidant capacity and TPC of the undigested, digested with pH conditions, and digested with pH conditions and digestive enzymes were assessed. The results of which are given in Table 3.1. Undigested raw extracts had TPC, H–$ORAC_{FL}$, and FRAP values of 988 ± 15 mg CE/100 g nutmeat, 15.4 ± 6.0 mmol TE/100 g nutmeat, and 7.0 ± 1.2 Fe^{2+} eq./100g nutmeat, respectively.

Undigested roasted extracts possessed TPC, H–ORACFL, and FRAP values of 932 ± 6 mg CE/100 g nutmeat, 16.2 ± 1.6 mmol TE/100 g nutmeat, 5.7 ± 0.6 Fe^{2+} eq./100g nutmeat, respectively. TEAC values of the raw and roasted pecans were 13.4 and 14.4 mmol TE/100 g pecans, respectively. No significant differences between the TPC and various measures of antioxidant capacity of the undigested crude extracts prepared from raw and roasted pecans were noted. These results suggest that the antioxidant capacity of pecan phenolics are not significantly affected by thermal degradation during roasting, which was also observed by Kellett et al. (2019).

The TPC, H–$ORAC_{FL}$, FRAP and TEAC values of raw and roasted extracts all followed similar trends following digestion treatment with pH conditions or pH

conditions with enzymes. A significant reduction in TPC and various measurements of antioxidant capacity was observed following pH treatment of raw and roasted pecans phenolics. In addition, the TPC and antioxidant capacities of raw and roasted phenolics were also significantly decreased following pH treatment with the addition of digestive enzymes ($p < 0.05$). Overall, TPC and antioxidant capacity decreased following pH treatment and was further reduced with the addition of digestive enzymes. The reduction in TPC and various antioxidant capacity measurements seen following gastrointestinal pH treatment may be attributed to the instability of phenolics under alkaline conditions. Bermúdez–Soto, Tomás–Barberán, and García–Conesa (2007) noted that polyphenols are highly sensitive to the slightly alkaline intestinal conditions and that the structure of some polyphenols may be modified or degrade during digestion. It is possible that these altered compounds may not be detected by the TPC assay (Li et al., 2015). These metabolized compounds could also possess lower antioxidant activity relative to their parent compounds. The additional reduction in TPC and antioxidant capacity seen following pH and enzymatic digestion may be attributed to the interactions of phenolics with digestive enzymes. Li et al. (2015) observed a similar trend with TPC and attributed the loss to interactions between pancreatin enzymes and phenolics.

3.3.2. HPLC characterization of the impact of digestion

3.3.2.1 LMW fraction

Fractionation via Sephadex LH–20 column chromatography facilitated the separation of extracts into LMW and HMW fractions to aid with HPLC–ESI–MS/MS identification. Differences in the chromatograms of the LMW fractions before and after

digestion are depicted in Figure 3.1. Analysis of the undigested LMW fraction revealed that this fraction consisted of mostly ellagic acid derivatives and flavan–3–ols, which are in agreement with the results previously published by Robbins et al. (2014) and Gong and Pegg (2017). Following digestion, there were losses ranging from 30 to 100% in the quantity of many of the compounds present in the LMW fraction (Table 3.2).

HPLC analysis revealed losses of flavan–3–ols ranging from 16 to 100% following *in vitro* digestion. The loss of flavan–3–ols, such as (+)–catechin and (–)–epicatechin, may be attributed to their instability under intestinal digestive conditions which has been previously established in the literature. Zhu et al. (2002) reported that catechin monomers were relatively stable under acidic conditions but unstable in an alkaline pH environment (pH > 7.4). *In vitro* digestion of pure (+)–catechin standard was performed to elucidate potential by–products. Following *in vitro* digestion, a 39% reduction in (+)–catechin was observed (Figure 3.2). Previous studies that looked at the stability of catechin standards under *in vitro* digestion conditions reported varying amounts of losses. For instance, Tagliazucchi, Verzelloni, Bertolini, and Conte (2010), Laurent, Besançon, and Caporiccio (2007), and Bermúdez–Soto et al. (2007) reported 7.2. 41.6, and 58% losses, respectively.

Various phenolic acids, such as caffeic acid, protocatechuic, and gallic acid along with their derivatives were also present in the LMW fraction and in this study, their losses ranged from 71 to 100%. Previous work has reported significant losses of caffeic and gallic acid pure standards, as well as phenolics in foodstuffs, following *in vitro* digestion (Goulas & Hadjisolomou, 2019; Tagliazucchi et al. 2010). Friedman and Jürgens (2000) concluded that gallic and caffeic acids were susceptible to degradation

under highly alkaline pH and suggested that the ionized and resonance forms of these mono–ring phenols were highly unstable under high pH conditions.

As aforementioned, ellagic acid along with its derivatives are one of the predominant phenolics present in pecans. While previous studies have indicated that the digestion of ellagitannins can result in an increase of ellagic acid and ellagic acid pentose (Kosińska– Cagnazzo et al., 2015; Mosele, Macià, Romero, Motilva, & Rubió, 2015), the results of this study demonstrated a 30 to 100% loss of ellagic acid and its derivatives. This might be attributed to differences in handling samples post–digestion. As this study was focused on the bioaccessibility of pecan phenolics, the digesta was separated via centrifugation into a bioaccessible fraction; that is, the soluble aqueous fraction, which was analyzed for this study and the non–bioaccessible fraction that consisted of insoluble substances. The limited amount of ellagic acid present in the bioaccessible fraction has been reported in previous studies that used dialysis to determine the bioaccessibility of various commodities. These studies have reported an overall increase in total ellagic acid following digestion due to the breakdown of ellagitannins; however, the dialyzable fraction contained little ellagic acid when compared to the non–dialyzable fraction (Gil–Izquierdo, Zafrilla, & Tomás–Barberán, 2002; Mosele et al., 2015). The lack of ellagic acid present in the dialyzable fraction may be attributed to the notoriously poor solubility of ellagic acid in aqueous solutions (Rommel & Wrolstad, 1993). Ayrton, Lewis, Walker, and Ioannides (1992) noted that precipitated ellagic acid was present within the abdominal cavity upon autopsy when rats were administered ellagic acid intraperitoneally. The precipitation of ellagic acid out of aqueous solution may explain the limited amount of ellagic acid present in the bioaccessible fraction in previous

studies, as well as our present study. It is possible that there was an increase in the total amount of ellagic acid following digestion, but the majority of the total ellagic acid would be present in the non–bioaccessible fraction that was not analyzed in this study. This should be addressed in future studies to provide a better picture of the effects of digestion on pecan phenolics.

3.3.2. HMW fraction

Procyanidins with DPs of 2 to 6 were present in undigested samples (Table 3.3). Analysis of the HMW fraction of the digested crude raw extract revealed that there was a loss of larger procyanidins with DPs of 4 to 6 (Figure 3.3). An 84% reduction in procyanidins with DP 4 was also found. Interestingly, there was only an 8% reduction in trimers and a 20–fold increase in dimers was noted. The slight loss of trimers may have been mitigated by the breakdown of procyanidins with larger DPs into smaller compounds. Furthermore, the significant increase in dimers could possibly be attributed to the depolymerization of larger procyanidins with DP > 4.

Spencer, Chaudry, Pannala, Srai, Debnam, and Rice–Evans (2000) demonstrated that procyanidin oligomers, trimers to hexamers, from cocoa under *in vitro* gastric pH conditions can depolymerize and breakdown to form (–)–epicatechin monomers and dimers. The results suggested that pH plays a role in the degradation of PACs during gastric digestion (Ortega, Reguant, Romero, Macià, & Motilva, 2009; Zhang, Wang, Li, Ho, Li, & Wan, 2016). Ortega et al. (2009) later employed an *in vitro* gastrointestinal model to investigate the effects of digestion on cocoa procyanidin (namely dimers to nonamers). The authors noted that there was an increase in monomers and dimers, which

was attributed to the hydrolysis of larger procyanidins (i.e., pentamers to nonamers). Furthermore, Serra, Macia, Romera, Valls, Blade, Arola, and Motilva (2010) reported that there was an increase in dimers and trimers following the *in vitro* digestion of a grapeseed procyanidin extract and suggested this was due to the depolymerization of larger oligomers.

In addition to the depolymerization of larger procyanidins, the increase in dimers could also be attributed to the dimerization of monomers. To illustrate, a commercial (+)–catechin standard (purity ≥ 98%) was subjected to *in vitro* digestion, and the digesta was analyzed by HPLC–ESI– MS/MS. It eluted from the Kinetex PFP column at 10.75 min. The appearance of four by– products with differing retention times (Figure 3.2; t_Rs at 7.8, 9.3, 9.8 and 10.2 min) and an $[M - H]^-$ for each with a *m/z* of 577 were also noted. These metabolites were tentatively identified as procyanidin dimers. Previous studies have suggested that the alkaline intestinal environment may offer a favorable environment which drives epimerization and auto–oxidation reactions that generate homo– and hetero–dimerization products (Green, Murphy, Schulz, Watkins, & Ferruzzi, 2007; Neilson, Hopf, Cooper, Pereira, Bomser, & Ferruzzi, 2007; Sun & Miller, 2003). The dimers evident in Figure 3.2 after (+)–catechin was digested suggest that (+)–catechin was capable of undergoing epimerization under the gastrointestinal conditions prior to dimerization (Zhu et al., 2002). The capability of (+)–catechin to epimerize and form (–)–epicatechin may explain the formation of the various dimers observed in this study. Higher–molecular–weight PACs have long been considered anti–nutritive, because they can bind to and inhibit the activity of digestive enzymes; thus, reducing the digestibility and absorption of nutrients and proteins. The loss of larger PACs due to their capability

to bind to digestive enzymes cannot be ignored and should be investigated in future works to better understand the effects of digestion on pecan PACs.

PACs have been suggested to be the predominantly consumed form of flavonoids in the western diet. Their ubiquitous presence and potent antioxidant capacity have drawn interest as their consumption has been thought to play a role in ameliorating the effects of chronic oxidative stress (Santos- Buelga & Scalbert, 2000). Increased DP has been associated with increased radical–scavenging capability per molecule (Shahat & Marzouk, 2013). Craft (2009) demonstrated that the tannin fraction of dry blanched and dry roasted peanut skins possessed strong antioxidant capacity and attributed this to the presence of PACs. Ma, Kosińska–Cagnazzo, Kerr, Amarowicz, Swanson, & Pegg (2014), who later characterized phenolics in dry–blanched peanut skins via LC–ESI–MS, showed that PACs with DPs ranging from dimers to nonamers were present. Liao, Greenspan and Pegg (2019) reported that the majority of the antioxidant capacity of peaches was attributed to PACs ranging from dimers to heptamers. Kellett et al. (2019) also suggested that procyanidins are responsible for the antioxidant properties of pecans.

While larger procyanidins possess stronger antioxidant activity *in vitro*, their capability to antioxidant activity in vivo is limited as they are too large to pass through the intestinal What is unique about pecans is not only are they are a rich source of PACs, but ~80% of their PAC profile is predominantly composed of procyanidin dimers and trimers (Gong & Pegg, 2017; Kellett et al., 2019). A few agricultural by–products, such as peanut skins, are rich in PACs with DPs > 6 (Ma et al., 2014). Studies that have investigated the bioavailability and biological efficacy of procyanidins with varying DPs using *in vitro* or *in vivo* studies have shown that larger procyanidins with DP > 4 possess

limited permeability through Caco–2 cells and have not been detected in plasma following ingestion (Deprez, Mila, Huneau, Tome, & Scalbert, 2001; Holt et al., 2002; Zumdick, Deters, & Hensel, 2012). Kellett et al. (2019) demonstrated that pecan procyanidins possessed strong cellular antioxidant activity and postulated that this phenomenon could be attributed to procyanidin dimers and trimers with molecular masses ranging from 560 to 840 g/mol, which were small enough to be taken up by Caco–2 cells. Kosińska and Andlauer (2012) investigated the intestinal absorption of cocoa polyphenols and found that transport of cocoa procyanidins across the Caco–2 monolayers was limited to monomers and dimers. This is in agreement with the results obtained by Deprez et al. (2001), who also used Caco–2 cells to examine the absorption of B–type PAC dimers, trimers, and polymers. Zumdick et al. (2012) also reported that significant amounts of procyanidin dimers to tetramers were present in Caco–2 cell lysates, which suggests that these compounds are small enough to enter cells. Together, these studies demonstrate that the uptake of PACs by Caco–2 monolayers is limited to smaller compounds such as monomers to tetramers.

In vivo studies have also demonstrated that the bioavailability of procyanidins is limited by their molecular weight. Holt et al. (2002) reported that (–)–epicatechin, (+)–catechin, and procyanidin dimers B2 and B5 from cocoa were detected in post–prandial plasma. Serra et al. (2010) showed that procyanidin dimers and trimers were present in rat plasma following the consumption of a grape seed procyanidin extract. Clinical data by Hudthagosol et al. (2011) showed that flavan–3–ol monomers from pecans were bioavailable at levels higher than those reported for brewed green tea. Brewed green tea is a commodity rich in monomers (126.6 mg/100g), while pecans contain less monomers

(13.2 mg/100g) and are rich in procyanidins. The authors suggested that the digestive process might play a role by breaking down larger flavan–3–ols like PACs into smaller more bioavailable compounds. Thus, the modification of the procyanidin profile following *in vitro* digestion as seen in our study could play a beneficial role in the bioavailability of pecan phenolics.

3.4 Results and conclusions

The present study demonstrated that pecan phenolics still possessed *in vitro* antioxidant capacity, albeit at a lower level, following *in vitro* gastrointestinal digestion. The reduction in antioxidant capacity and TPC was mirrored by the reduction in overall phenolics, as evidenced via HPLC analysis. HPLC–ESI–MS/MS results revealed that digestion altered the phenolic profile of the LMW and HMW fractions. There was an overall loss of pecan phenolics due to digestion; however interestingly, an increase in procyanidin dimers was noted. The increase in dimers was postulated as being attributed to the depolymerization of procyanidins with higher DP (4–6), and the dimerization of catechin/epicatechin. This increase is noteworthy as these procyanidin dimers have been shown to be small enough to be absorbed in the intestine and are present in the systemic circulation following the ingestion of foods rich in PACs. The alteration of the procyanidin profile during the digestive process may be an important factor for their bioavailability.

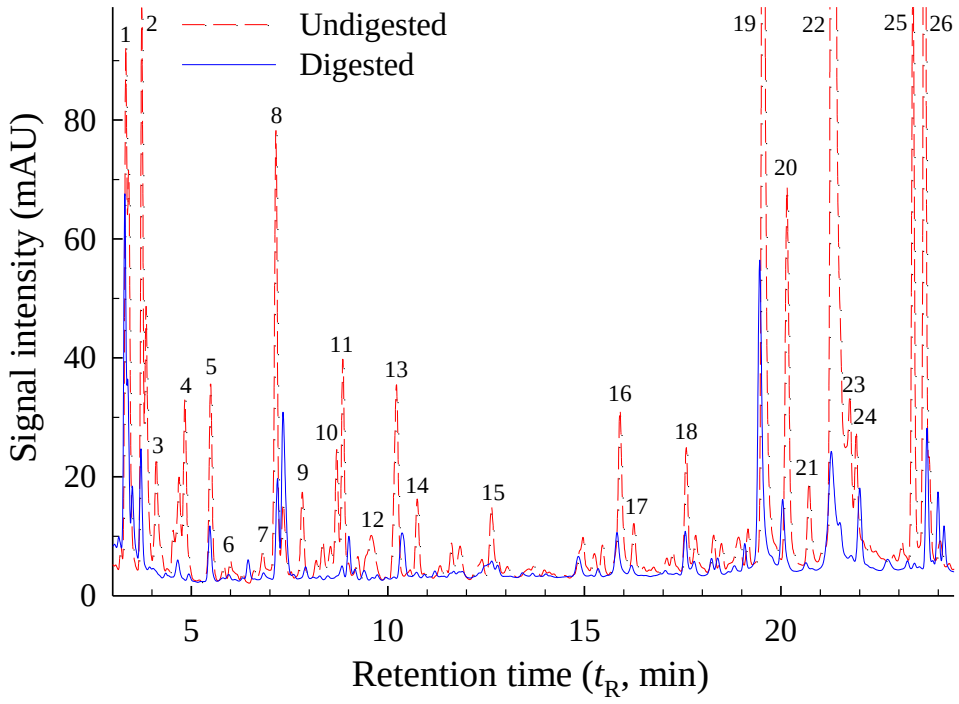

Figure 3.1

Chromatogram of low–molecular–weight (LMW) compounds, previously isolated from a Sephadex LH–20 column, of undigested and digested pecan phenolics from raw pecans analyzed on a Kinetex PFP analytical column with UV detection at 255 nm.

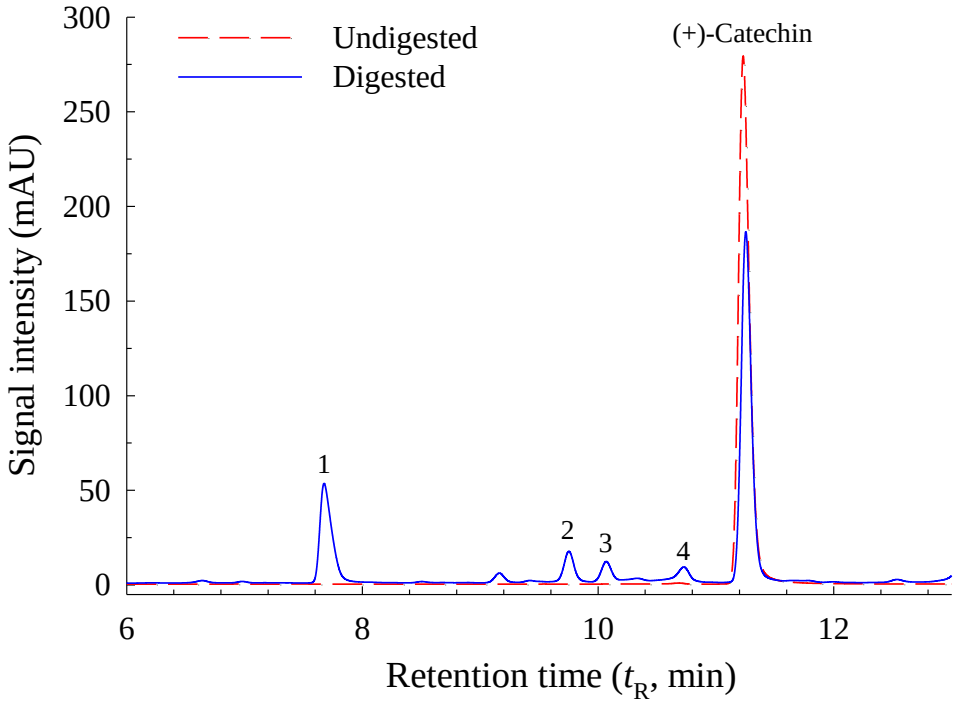

Figure 3.2

Reversed–phase HPLC chromatogram of (+)–catechin, before and after *in vitro* digestion, analyzed on a Kinetex PFP analytical column with UV detection at 280nm.

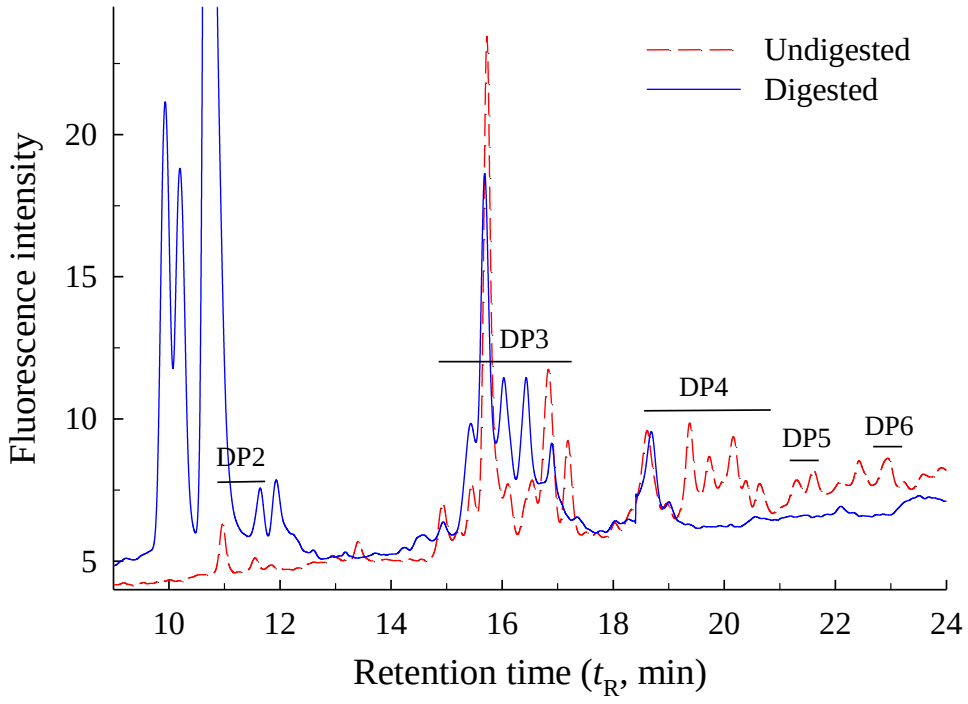

Figure 3.3
Chromatogram of high–molecular–weight (HMW) compounds (namely procyanidins), previously isolated from a Sephadex LH–20 column, of undigested and digested pecan phenolics from raw pecans analyzed on a HILIC analytical column with fluorescence detection at an excitation/emission wavelength of 276/316 nm, respectively.

Table 3.1
TPC, H–ORAC$_{FL}$ and FRAP values of undigested and digested (gastrointestinal pH conditions or pH conditions with digestive enzymes) crude pecan phenolic extracts (n=9).

Sample	Treatment	TPC mg (+)–catechin eq./100g pecans	H–ORAC$_{FL}$ mmol Trolox eq./100g pecans	FRAP mmol Fe^{2+} eq./100g pecans	TEAC mM Trolox eq./100 g pecans
Raw crude extract	Undigested	988 ± 15^{A}	15.4 ± 6.0^{A}	7.0 ± 1.2^{A}	13.4 ± 2.6^{A}
	pH	767 ± 12^{B}	8.5 ± 0.9^{B}	5.7 ± 0.6^{B}	8.5 ± 0.5^{B}
	pH + enzymatic digestion	624 ± 7^{B}	7.5 ± 1.3^{C}	2.8 ± 0.4^{C}	2.7 ± 0.2^{C}
Roasted crude extract	Undigested	932 ± 6^{A}	16.2 ± 1.6^{A}	5.7 ± 0.6^{A}	14.4 ± 1.5^{A}
	pH	691 ± 9^{B}	10.1 ± 0.9^{B}	4.2 ± 0.1^{B}	4.7 ± 0.6^{B}
	pH + enzymatic digestion	489 ± 4^{C}	8.5 ± 0.7^{B}	2.6 ± 0.4^{C}	3.6 ± 0.2^{B}

[a] Means ± standard deviation (n=9) followed by the same letter in a column for raw or roasted are not significantly ($p > 0.05$) different according to Tukey–Kramer honest significant difference test.
Abbreviations are as follows: TPC =Total phenolics content, H–ORAC$_{FL}$ = Hydrophilic–oxygen radical absorbance capacity, FRAP = Ferric reducing antioxidant power, and TEAC= Trolox equivalent antioxidant capacity.

Table 3.2
Tentative identification of compounds isolated from the low–molecular weight fractions of undigested and digested crude raw pecan phenolic extracts.

Peak No.	t_R[a] (min)	$[M–H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification[b]	% Reduction
1	3.34	481	421–301–275	HHDP–glucose isomer[c]	30
2	3.74	481	421–301–275	HHDP–glucose isomer	77
3	4.11	169	125	gallic acid	100
4	4.84	315	153	protocatechuic acid hexoside	97
5	5.49	331	313–169	monogalloyl glucose	75
6	6.0	341	179	caffeic acid hexoside	74
7	6.8	331	169	gallic acid hexoside	85
8	7.16	483	331–313–169	digalloyl glucose	77
9	7.83	613	289	unknown (flavan–3–ol)	87
10	8.7	443	301	unknown (ellagic acid derivative)	81
11	8.86	785	633–483–301	digalloyl HHDP–glucose	93
12	9.58	451	289	catechin hexoside	100
13	10.22	387	179–161	caffeic acid derivative	71
14	10.75	289	245–205–179	(+)–catechin	100
15	12.64	575	423–407–289	procyanidin A–type dimer	45
16	15.91	729	577–559–289	procyanidin dimer monogallate	71
17	16.26	463	301	ellagic acid hexoside	76
18	17.59	619	457–323	unknown (flavan–3–ol)	53
19	19.55	615	463–301	digalloyl ellagic acid	65
20	20.16	433	301	ellagic acid pentoside	79
21	20.72	487	469–425–301	valoneic acid dilactone hydrate	88
22	21.31	477	315–301	methyl ellagic acid hexoside	91
23	21.75	301	217	ellagic acid	53
24	21.92	457	323–293	epigallocatechin gallate	16
25	23.36	489	301	ellagic acid acetyl hexoside	100
26	23.65	447	315–300	methyl ellagic acid pentoside	89

[a] Retention time (t_R) from RP–HPLC analysis performed on the Kinetex PFP column.
[b] Tentative identification was achieved through t_R mapping and comparisons of fragmentation patterns to those of available commercial standards, as well as relevant literature.
[c] Abbreviation is as follows: HDDP–glucose = HHDP–glucose, bis(hexahydroxydiphenoyl) glucose

Table 3.3
Characterization of procyanidin compounds present in the high–molecular–weight fractions of undigested and digested crude raw pecan phenolic extracts.

t_R[a] (min)	DP[b]	Unit type	Linkage[c]	[M–H]– (*m/z*)
11.37	2	2 (epi)catechin	B	577
11.96	2	(epi)catechin + (epi)gallocatechin	B	593
15.53	3	3(epi) catechin	B	865
16.32	3	2 (epi)catechin + (epi)gallocatechin	B	881
17.431	3	1 (epi)catechin + (epi)gallocatechin	B	897
19.21	4	4 (epi)catechin	B	1153
19.97	4	3 (epi)catechin + (epi) gallocatechin	B	1169
20.76	4	2 (epi)catechin + 2 (epi)gallocatechin	B	1185
21.9	5	5 (epi)catechin	B	1441
22,2	5	4 (epi)catechin + (epi)gallocatechin	B	1457
23.5	6	6 (epi)catechin	B	1729

[a] Retention time (t_R) performed on a HILIC column.
[b] DP= Degrees of polymerization.
[c] The letter B denotes a B–type linkagebetween the flavan–3–ol units either (C4→C8) or (C4→C6).

Table 3.4
Tentative identification of (+)–catechin digestion by–products.

Peak No.	t_R (min)	Tentative identification	$[M–H]^-$ (*m/z*)
1	7.8	Procyanidin dimer	577
2	9.3	Procyanidin dimer	577
3	9.8	Procyanidin dimer	577
4	10.2	Procyanidin dimer	577

[a] Retention time (t_R) from RP–HPLC analysis performed on the Kinetex PFP column.

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CHAPTER 4

Transepithelial transport of digested phenolic fractions prepared from raw and roasted pecans in Caco–2 cell monolayers

[1]Cheung, M.; Robinson, J.A.; Greenspan, P.; & Pegg, R.B. To be submitted to *Food Chemistry*

Abstract

Studies have suggested that phenolics, such as those from pecans, play a role in ameliorating the effects of oxidative stress and inflammation. However, limited information on the bioavailability and bioaccessibility of pecan phenolics exist. In this study, crude acetonic extracts were prepared from raw and roasted pecans and were separated into low– and high–molecular weight (LMW and HMW, respectively) fractions *via* Sephadex LH–20 column chromatography. The bioaccessibility and bioavailability of each fraction was evaluated using a combined *in vitro* digestion/Caco–2 absorption model. Ellagic acid, four ellagic acid derivatives, and epigallocatechin gallate from the digested LMW fraction were detected in the basolateral well following 1 and 2 h of incubation. The profile of the HMW fraction was significantly modified by both the pH changes and digestive enzymes, leading to an increase in smaller molecular weight procyanidins (DP 1–2). Monomers, dimers, and trimers of these procyanidins in the digested HMW fraction were also able to traverse across the Caco–2 monolayer. These experiments further our understanding on the importance of certain phenolics in bestowing antioxidant activity after human consumption of pecans.

4.1 Introduction

The burden and presence of chronic illnesses has increased within the past century. According to the United States' Center for Disease Control (CDC), as approximately one–half of American adults suffer from or have two or more chronic illnesses, respectively. Dietary intervention strategies to ameliorate or reduce the risk of developing chronic illness are gaining popularity due to the sometime undesirable adverse side effects of pharmaceutical drugs (Srivastava et al., 2010). Diets abundant in plant foods, such as fruits, vegetables, legumes, nuts, and whole grains, have been associated with a reduced risk of developing chronic diseases (Sabaté, 2003). In particular, the consumption of tree nuts, such as pecans, has been linked to numerous health benefits. These include weight management and a decreased risk for chronic illnesses, such as cardiovascular disease and diabetes (Haddad et al., 2006; McKay et al., 2018).

As excessive oxidative stress has been linked to the development of numerous chronic degenerative diseases, the favorable benefits of nut consumption have been attributed to antioxidant compounds, such as polyphenols. Pecans have been shown to be a rich source of antioxidants and possess strong *in vitro* antioxidant activity (Robbins et al., 2015). Previous studies have shown that EA derivatives and proanthocyanidins (PACs), comprised of epicatechin and epigallocatechin subunits, are the major phenolics found in pecans (Gong & Pegg, 2017; Robbins et al., 2014). Furthermore, cell–based assays have demonstrated that pecans possess bioactivity. Robbins et al. (2016) demonstrated that pecan phenolics exhibited anti–inflammatory properties in LPS–

stimulated RAW 264.7 murine macrophage cells and Kellett et al. (2019) showed that pecan phenolics could bestow antioxidant activity in Caco–2 cells.

While much work has been done to characterize the phenolic constituents of pecans and their *in vitro* biological properties, limited information exists on the bioavailability of antioxidant compounds from pecans. However, existing studies have indicated that pecan consumption can inhibit lipid oxidation *in vivo* and contribute to post–prandial antioxidant defenses. Haddad et al. (2006) suggested that antioxidant constituents in pecans, such as tocopherols, might play a role in the inhibiting lipid peroxidation and degradation *in vivo*. Marquardt et al. (2019) and Guarneiri et al. (2021) also reported that the consumption of pecan–enriched diets contributed to increased plasma antioxidant capacity and decreased lipid peroxidation. Furthermore, a clinical trial conducted by Hudthagosol et al. (2011) demonstrated that bioactive constituents from pecans, such as flavan–3–ol monomers, were bioavailable and could improve antioxidant status.

Post–prandial plasma levels following the consumption of a 10–100 mg dose of a single phenolic compound rarely exceeds 1 μM. It is important to note that post–prandial plasma concentrations may be higher as these studies do not consider phenolic metabolites or cellularly accumulated phenolics. As phenolics pass through the gastrointestinal (GI) tract and are absorbed, they may chemically degrade or be highly metabolized, such that their metabolites do not resemble their precursors. Thus, the bioavailability and antioxidant activity of metabolites may differ from that of their parent compounds.

While human and animal trials are the gold standard for studying the digestion and absorption of phenolics, as they provide a complete picture of metabolism and absorption, these studies are time consuming and expensive. Thus, *in vitro* gastrointestinal digestion and intestinal absorption models have been developed to assess the bioavailability and bioaccessibility of phenolics under physiological conditions (Xie et al., 2013). Cell culture models are an alternative to animal and human trials, as they offer some biologically relevant insight. The Caco–2 cell line, a human colon epithelial cancer cell line, has been used extensively by the pharmaceutical industry to study the mechanisms of drug transport and absorption of drug molecules using special Transwell® plates (Zhang et al., 2017). Caco–2 cells possess the capability to differentiate spontaneously, which leads to the formation of a monolayer of cells that possess morphological and functional characteristics of mature enterocytes. Once differentiated, they exhibit epithelial characteristics such as brush–border microvilli and tight junctions when cultured on impermeable supports. Over time the production of brush border enzymes, such as sucrase, alkaline phosphatase, and aminopeptidase, that are unique to the adult human small intestine, increases (Sambuy et al, 2005). These properties have made the Caco–2 cell line a good alternative to animal studies and an invaluable tool for predicting the intestinal absorption of various phytochemicals, such as phenolics. Previous studies have investigated the bioavailability of single phenolic compounds and phenolic extracts from various fruits and vegetables, however, to the best of our knowledge none have utilized Sephadex LH–20 column chromatography for sample preparation. Furthermore, limited information on the absorption of nut phenolics, in particular pecan phenolics, exist in the literature.

In the present study, crude acetonic pecan phenolics extracts from raw and roasted pecans were prepared and separated into LMW and HMW fractions using Sephadex LH–20. An *in vitro* gastrointestinal model combined with Caco–2 monolayers was used to evaluate the absorption of each fraction and characterization of transported compounds was performed using HPLC–ESI–MS/MS.

4.2 Materials and methods

4.2.1 Chemicals

The following were obtained from Fisher Scientific Co., LLC (Suwanee, GA, USA): glass wool, cellulose thimbles, ACS–grade acetone, ethanol (95%), hexanes, and Whatman No. 1 filter paper, as well as HPLC–grade water, methanol, acetonitrile. Glacial acetic acid was acquired from VWR International, LLC (Suwanee, GA). Sephadex LH–20, (+)–catechin hydrate, ellagic acid (EA), and epigallocatechin gallate (EGCG) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Procyanidin B2 (PDB2) was acquired from the Indofine Chemical Company, Inc. (Hillsborough, NJ, USA). Advanced DMEM (Dulbecco's Modified Eagle Medium), phosphate–buffered saline (PBS, 7.4), Hank's Buffered Salt Solution (HBSS), fetal bovine serum (FBS), L–glutamine, and penicillin–streptomycin were purchased from Life Technologies (Grand Island, NY, USA).

4.2.2 Sample preparation

Raw in–shell 'Desirable' pecans from the 2018 season were shipped from pecan orchards to the Department of Food Science and Technology in Athens, GA. The pecans were shelled, vacuum packaged and stored at –80 °C until analysis.

4.2.3 Lipid extraction

Lipids from raw pecans were removed using a Soxhlet apparatus. Pecans were removed from the –80 °C freezer and then immersed in liquid nitrogen. Roughly sixty g of the cryogenically treated pecans was ground to a fine powder in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ, USA). Twenty g of ground pecans was transferred to a cellulose extraction thimble (single thickness, 43 mm *i.d.* x 123 mm external length) (Whatman International Ltd., Maidstone, England). Lipids were extracted for 18 h with ~350 mL of hexanes. Afterwards, hexanes were removed from the lipid portion using a Büchi Rotavapor R–210 and a V–700 vacuum pump connected to a V–850 vacuum controller (Büchi Corporation, New Castle, DE) and a water bath at 45 °C. The lipid residue was then dried in a 103 °C oven for 1 h and transferred to a desiccator to cool. The lipid portion was then weighed for gravimetric analysis. The thimbles containing the defatted meal were air–dried in a fume hood overnight prior to subsequent phenolic extractions.

4.2.4 Extraction of phenolic compounds

Extraction of phenolic compounds was as follows. As described by Wu et al. (2004), phenolics were extracted from defatted pecan meal using an extraction solvent

consisting of $(CH_3)_2CO$: H_2O:CH_3COOH in a ratio of 70.0:29.5:0.5 *v/v/v* at a solid–to–liquid ratio of 1:6 (w/v). The flasks were covered with foil, placed in an orbital–shaking water bath (New Brunswick Scientific, New Brunswick, NJ) and heated at 60 °C for 30 min. The sample was then filtered through a Whatman #1 filter paper. Phenolics were then re–extracted from the residual residue with fresh solvent three more times and the supernatants were pooled. Acetone was removed from the pooled supernatant using a Büchi Rotavapor R–210 and a V–700 vacuum pump connected to a V–850 vacuum controller (Büchi Corporation, New Castle, DE) and a water bath at 45 °C. The aqueous residue was transferred to a crystallization dish (100 x 50 mm, dia. x H), covered with filter paper, and placed in a –80 °C freezer until completely frozen. Samples were then lyophilized using a Labconco Freezone 2.5 L freezer dryer (Labconco Corp., Kansas City, MO, USA). The lyophilized extract was then weighed, transferred to an amber vial, capped, and stored at –4 °C until analysis.

4.2.5 Fractionation of Pecan Phenolics

Fractionation was performed according to the method of Robbins et al. (2014). In summary, ~2 g of the crude phenolic extract was dissolved in ~50 ml of 75% ethanol. The solution was then applied with a transfer pipette 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, USA). LMW compounds were eluted using 95% ethanol followed by the elution of the HMW fraction using 50% aqueous acetone. Organic solvent from both fractions were removed with the Rotavapor system. The

remaining aqueous residues were transferred to crystallization dishes and lyophilized as described above.

4.2.6 In vitro gastrointestinal digestion

In vitro gastrointestinal digestion was performed according to Kosińska–Cagnazzo et al. (2015) with modifications. Exactly 100 mg of the lyophilized LMW or HMW fraction was dissolved in 10 mL of 0.9% saline. Samples were prewarmed in an orbital–shaking water bath (New Brunswick Scientific, New Brunswick, NJ) set to 37 °C for 5 min. Gastric digestion was initiated by adjusting the pH to 2.0 with 1 M HCl. 2 mL of a 17.5g/L pepsin solution prepared in 0.1 M HCl was then added. The samples were purged with nitrogen to remove oxygen and were then incubated for 1 h in a shaking water bath set to 37 °C. Intestinal digestion was then initiated by adjusting the pH to 8.0 using 1.0 M NaOH and 0.1 M $NaHCO_3$. 2 mL of a pancreatin solution (7g/L) prepared in 0.9% NaCl was then added. Samples were flushed again with nitrogen and the intestinal digestion was carried out for 2 h at 37 °C in the shaking water bath. Digestion was terminated by placing the samples on ice. Samples were centrifuged for 20 minutes at 1,000 rpm to remove precipitates. The supernatant was removed and brought up to 25 mL (final concentration of 4 mg of LMW or HMW extract/mL). The supernatant, which was referred to the bioaccessible fraction, was then aliquoted and stored at –80 °C until use.

4.2.7 Cell culture

The Caco–2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured following the protocol of Xie et al.

(2013). Cells were cultured in Advanced Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% endotoxin free fetal bovine serum (FBS), 1% L–glutamine, 1% penicillin and 1% streptomycin at in 25 cm^2 canted–neck culture flasks. Cells were incubated at 37 °C in a humidified 5% (v/v) CO_2 environment (NAPCO Series 8000 WJ, Thermo–Scientific, Waltham, MA, USA). Confluence was confirmed using a Zeiss Primo Vert inverted microscope (Carl Zeiss, Inc., Oberkochen, Germany) and cells were split using a 1:2 ratio.

4.2.8 Transepithelial transport studies

Cells were seeded at a density of 2.6 x 10^5 in 12–well Transwell® permeable inserts (12 mm *i.d.,* 1.12 cm^2 growth area, 0.4 μm pore polycarbonate membrane insert; Corning Inc., NY, USA) and were cultured with 0.5 and 1.5 mL of medium on the apical and basolateral sides, respectively. The cells were allowed to grow for 21 days post–seeding to form a differentiated monolayer and media was replaced every 2–3 days. Transepithelial electrical resistance (TEER) values were used to monitor monolayer integrity and were measured using an EVOM2 resistance meter equipped with an STX2 chopstick electrode (World Precision Instruments, Sarasota, FL, USA). On the day of the experiment, medium was removed, and the cell monolayers were washed 3 times with prewarmed Hank's balanced salt solution (HBSS). For the final wash, the monolayers were incubated for half an hour and TEER measurements were taken. The TEER values of the monolayers were obtained by subtracting the resistance of a blank well without cells and multiplying the resistance by the area of the polycarbonate membrane insert (1.1 cm^2). The final values were expressed as Ω • cm^2 and wells with TEER values between

280 and 350 Ω •cm^2 were used for all experiments. A half mL of the predigested or undigested sample was added to the apical compartment and the 1.2 mL of HBSS was added to the basolateral compartment. The Transwell® plates were then incubated at 37 °C for 60 and 120 min. The solutions in basolateral wells were collected. The wells were rinsed with HBSS and the rinsing solution was also collected. Samples were stored at –80 °C until analysis.

4.2.9 Solid phase extraction

Solid phase extraction (SPE) was done to concentrate samples and to remove substances, such as buffer salts that would interference with ionization during MS analysis. The basolateral samples were purified using a polymeric reversed phase Phenomenex Strata–X SPE column. The column was first activated with methanol, followed by deionized water. The basolateral sample was loaded onto the column, followed by washing with 3 mL of deionized water, and samples were then eluted with 3 mL of methanol. The eluent was then reduced to dryness in a RT400 Speed Vac System (Savant, Holbrook, NY, USA). The LMW and HMW samples were both reconstituted in 50 µL of methanol, followed by a 1:1 dilution with mobile phase A (MP A) consisting of either H_2O: C_2H_3N:CH_3COOH (94:5:1, *v/v*) or C_2H_3N:CH_3COOH (98:2, *v/v*), respectively. The total volume of the reconstituted sample was 100 µL.

4.2.10 HPLC –ESI–MS

All digested and undigested LMW and HMW fractions were analyzed following the methods of Gong and Pegg (2017) with modifications. An Agilent 1200 series HPLC

system (Agilent Technologies, Inc., Wilmington, DE) equipped with a quaternary pump, degasser, autosampler, thermostatic column compartment, UV–Vis diode array detection and fluorescence detector with standard flow cell was used to chromatograph all samples.

LMW samples were analyzed using a Kinetex PFP column with a pore size of 100 Å (Phenomenex, Torrance, CA) and a SecurityGuard cartridge of the same material was used to chromatograph the LMW fractions. Mobile phases consisted of A: H_2O: CH_3CN/CH_3COOH (93:5:2, *v/v*) and B: H_2O:CH_3CN/CH_3COOH (58:40:2, *v/v*). A flow rate of 0.8 mL/min was utilized and the gradient elution was as follows: 0–30 min, 0–60% B; hold for two min (30–32 min); 32–35 min, 60–0%; followed by an additional 5 minute hold to re–equilibrate the system. 50 µL of the reconstituted sample was injected. Detection wavelengths were set to 255 nm (i.e., EA and its derivatives) and 280 nm (phenolic acids and catechins).

Procyanidins in the HMW fractions and samples were separated by their degree of polymerization (DP) using the same Agilent 1200 system, but with a Luna hydrophilic interaction liquid chromatography (HILIC) column (4.6 × 150 mm, 3–µm particle size, 200 Å; Phenomenex) equipped with a guard cartridge of the same material. Mobile phase A consisted of CH_3CN/CH_3COOH (98:2, *v/v*) and B of CH_3OH/H_2O/CH_3COOH (95:3:2, *v/v/v*). A flow rate of 1 mL/min was utilized and the gradient elution was as follows: 0–25 min, 0–45% B; 25–30 min, 45–0% B; followed by an additional 2 min hold to re–equilibrate the system. 50 µL of the reconstituted sample was injected and fluorescence detection at excitation/emission λ= 276/316 nm was employed.

4.2.10.1 HPLC–ESI–MS

Identification of compounds was done following the method of Robbins et al. (2015) using an 1100 HPLC system (Agilent) coupled to a QToF micro mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters Corporation, Milford, MA). Tentative identification was done by comparing the molecular ions and fragmentation patterns to that of known standards as well as relevant reported literature values.

4.2.11 Statistical Analysis

One–way analysis of variance (ANOVA) was performed using JMP Pro software, Version 14 (Cary, NC, USA) to test for significant differences between raw and roasted, in addition to digested and undigested samples. Data from triplicate wells are reported as mean ± standard deviation. Tukey's honestly significant difference (HSD) test was performed to identify the significance of the difference between raw and roasted.

4.3 Results and discussion

4.3.1 Transport of phenolics from the in vitro digested LMW fraction

In this study, Sephadex LH–20 was used to prepare a LMW fraction that then underwent *in vitro* gastrointestinal digestion. The transport of phenolics present in the resulting digesta across Caco–2 monolayers was then evaluated. Figure 4.1a shows a representative chromatogram of the digested LMW fraction which was loaded into the apical wells for the transport experiment. Following *in vitro* digestion, the dominant group of phenolics present were ellagic acid and its derivatives.

Six phenolics from the digested LMW fraction from raw and roasted pecans were transported across the Caco–2 monolayer and were detected in the basolateral well following 60 and 120 min of incubation (Figure 4.1b). No significant differences between the transport of phenolics from the digested raw and roasted LMW fractions, as evaluated by the % transport and concentrations present in the basolateral wells at 60 min and 120 min, were detected by Tukey HSD test ($p > 0.05$). The results of this study, along with that of Kellett et al. (2019) who previously reported that roasting didn't significantly affect the cellular antioxidant activity of LMW fraction, suggests that roasting does not influence the transport of LMW phenolics across the Caco–2 monolayer.

In Table 4.1, limited amounts (1–3% transport of apically loaded samples) of EA, several EA derivatives, and EGCG were detected in the basolateral well and increased over time in a non–linear fashion. The non–linear increase may be attributed to concurrently occurring cellular mechanisms. The first being basolateral to apical efflux. Studies have shown that EA and EGCG have significantly higher rates of basolateral to apical efflux (Zhang et al., 2004; Mao et al., 2016). While digested phenolic compounds from pecans were found in the basolateral compartment, these compounds are also simultaneously transported in the opposite direction to the apical compartment. A second mechanism is cellular accumulation of phenolics; compounds are internalized by the cells but are not excreted into the basolateral compartment. Elendran et al. (2019) reported that 46% of EA present in the apical compartment accumulates in Caco–2 cells with 3% released into the basolateral compartment. This phenomenon was also seen by Teel et al. (1987) and Whitley et al. (2003) who suggested that limited amounts of EA were transported because of its capability to bind to intracellular DNA and proteins.

EA and its derivatives were the main compounds transported, ranging from 1–3% of that present in the apical compartment. Previous studies have reported limited amounts of EA being transported across Caco–2 monolayers. Mao et al. (2016) reported ~0.9% transport of EA from a prepared *Fructus phyllanthi* tannin fraction, while Elendran et al. (2016) reported 3% transport of an EA standard. Furthermore, to the best of our knowledge no other studies have reported the transport of EA derivatives from other commodities (Kosińska–Cagnazzo et al., 2015; Mao et al, 2016).

The transport of EGCG across a Caco–2 monolayer has been previously reported by Xie et al. (2013) who studied green tea. More importantly, a clinical study by Hudthagosol et al. (2011) reported the presence of EGCG following the consumption of pecans. In this study, the % transport of EGCG from the raw and roasted digested LMW samples were 1.8 and 2.6%, respectively. Faralli et al. (2019) previously reported ~2% transport of EGCG across Caco–2 monolayers. Human clinical trials have shown that limited concentrations of EGCG (<1% of the ingested dose) is present in post–prandial plasma (Hudthagosol et al., 2011). Although we reported slightly higher levels of transport of EGCG, EA and its derivatives in our study, it is important to note that this study only provides a snapshot of the overall story of bioavailability. The process of digestion and absorption *in vivo* is a highly dynamic process and is not static like the Caco–2 cell model. During absorption *in vivo*, the intestine is bathed by interstitial fluid and transported molecules such as glucose are continuously removed by the solute equilibrium between the interstitial fluid and splanchnic circulation. As a result, the importance of the Caco–2 cell model is it can only demonstrate which phenolics can cross at the intestinal barrier. The deposition of phenolics in tissues represents the

ultimate fate of these compounds. Yan et al. (2014) reported a high distribution of EA in various tissues, especially the liver and kidney. Twenty–eight percent of administered radioactive labelled EA was absorbed at 2h, but 22% was eliminated in urine with <1% present in the bloodstream (Teel & Martin,1988). Chen et al. (1997) reported that high levels of EGCG were found in intestinal tissues following intravenous administration.

4.3.2 In vitro digestion of the HMW fraction

In this study, an isolated HMW fraction containing PACs was prepared using Sephadex LH–20 and was subjected to *in vitro* gastrointestinal digestion. The concentrations of monomers, dimers, pentamers, and hexamers present in the prepared HMW fractions from raw and roasted pecans were not significantly different ($p > 0.05$) (Table 4.2). However, the concentration of trimers and tetramers in the raw samples were significantly higher than that of the roasted sample ($p < 0.01$). Following digestion, a reduction in larger PACs (DPs ranging from trimers to hexamers) was noted (Figure 4.2). The concentration of trimers and tetramers present in the HMW fraction of raw and roasted significantly decreased by 71.1 and 84.8%, respectively ($p < 0.01$). Furthermore, following digestion pentamers and hexamers were not detected. Interestingly, there was a 500% increase in dimers following *in vitro* digestion ($p < 0.01$). The reduction in larger PACs with DP 3–6 may have contributed to the increase of dimers. Ortega et al. (2009) later reported an increase in procyanidins dimers and trimers following *in vitro* digestion of cocoa. The authors attributed the increase to the breakdown of larger procyanidins, pentamers to nonamers, and these results are in agreement with those previously reported by Spencer et al. (2000).

4.3.3 Transport of PACs present in the HMW fraction

For procyanidins, prior studies using Caco–2 monolayers, animals, or humans have all demonstrated that DP plays a major role in determining *in vivo* bioavailability. Specifically, uptake has been limited to smaller PACs ranging from monomers to trimers, and possibly tetramers (Déprez et al, 2001; Kosińska & Andlauer, 2012; Zumdick et al., 2012). In this study, the transport of digested procyanidins from both raw and roasted pecans was evaluated. For both raw and roasted samples, monomers, in addition to procyanidin dimers, and trimers were detected in the basolateral well following 60 and 120 min of incubation (Figure 4.3). Interestingly, significant differences between the apical to basolateral transport of monomers, dimers, and trimers from raw and roasted pecans were not detected ($p > 0.05$). Previously, Kellett et al (2019) reported that roasting did not affect the cellular antioxidant activity of pecan procyanidins. Together with the results of this study, thermal treatment, in the form of roasting, does not influence the transport of digested pecan procyanidins.

Procyanidins with DP greater than 3 were not detected in the basolateral well following 120 min of incubation. The transport of smaller procyanidins noted in this study agree with previous reports, which have shown that the transport of PACs across the Caco–2 monolayer is limited to smaller oligomers. The percent transport of monomers, dimers, and trimers reported in this study ranged from 1.0–1.3, 0.1, and 0.1–0.2%, respectively. These values are lower than those reported by Ou et al. (2011), who reported 1.6% transport of epicatechin, 0.6–4.8% of A–type procyanidin dimers, 3% of procyanidin B2, and 0.4% of A–type trimers from cranberries. Kosińska and Andlauer (2012) showed that the transport of cocoa procyanidins across Caco–2 monolayers was

limited to monomers and dimers. Déprez et al (2001) who investigated the absorption of B–type PAC dimers, trimers, and polymers also reported similar results. A study by Zumdick et al. (2012) also reported that significant amounts of procyanidin ranging from dimers to tetramers were present in Caco–2 cell lysates, which suggests that these compounds can enter cells.

In vivo studies have also shown that their DP limits the bioavailability of PACs. Appledorn et al. (2009) demonstrated that various procyanidins dimers were absorbed in the small intestine of rats. Serra et al. (2010) showed that 1.69% of procyanidin dimers and 0.04% of trimers were present in rat plasma following the consumption of grape seed procyanidin extract. Serra et al. (2011a) also reported micromolar (μM) concentrations of procyanidin dimers and trimers were present in rat plasma following the ingestion of a hazelnut skin extract. Holt et al. (2002) demonstrated that nanomolar (nM) concentrations of catechin, epicatechin and procyanidin B2 could be detected in the plasma of humans following the ingestion of a cocoa extract. A clinical study performed by Hudthagosol et al. (2011) also demonstrated that flavan–3–ol monomers from pecans were bioavailable.

The capability of smaller PACs from the HMW fractions to traverse Caco–2 monolayers may also play a role in alleviating oxidative stress. Kellett et al. (2019) demonstrated that HMW fraction from raw and roasted pecans could bestow cellular antioxidant activity in Caco–2 cells. Additional *in vitro* studies have also shown that procyanidin dimers, trimers and tetramers from cocoa were effective in inhibiting AAPH–induced hemolysis of rat and human erythrocytes (Zhu et al., 2002).

Although the concentration of smaller PACs increased following *in vitro* digestion (Table 4.2), limited concentrations (30–200 ng/mL) of these compounds were

able to pass through Caco–2 monolayers (Table 4.3). Zumdick et al. (2012) reported that depending on the DP, 10–30% of total transported PACs, which included compounds present in the basolateral well and cell lysates, were found in the cell lysates. Other studies have shown that procyanidins can accumulate in the tissues of the lower GI tract. Serra et al (2011a) reported that dimers and trimers had accumulated in intestinal tissue (ng/g tissue range) of rats following the ingestion of a hazelnut skin extract. The accumulation of procyanidin monomers, dimers, and oligomers from grape seed extract in the tissues of various parts of the large intestine (the cecum, proximal and distal colon) was also reported by Goodrich and Neilson (2014). The authors reported that high levels of procyanidin dimers (8.0 –91.6 ng/mg dry weight) were present in the tissues of the cecum and colon, with the highest levels found in the cecum. Studies have also shown that limited amounts of procyanidin dimers can accumulate in various tissues after entering systematic distribution. Serra et al. (2011b) reported the presence of procyanidin B2 in brain, aortic, and adipose tissues at concentrations of 1.16, 1.05. and 0.17 nmol/g of tissue. Ardévol et al. (2013) reported similar findings and showed that nanomolar concentrations of procyanidin B2 could accumulate in subcutaneous adipose tissues of obese mice. Together, these studies document that procyanidins can be absorbed at the intestinal epithelium and can be distributed to various tissues around the body.

4.4 Conclusion

The bioavailability and bioaccessibility of pecan phenolics using a combined *in vitro* digestion/Caco–2 absorption model was investigated. To the best of our knowledge, this is also one of the first studies to utilize Sephadex LH–20 column chromatography to

aid with an investigation to the effects of digestion on the phenolic constituents present in LMW and HMW fractions and their transport through Caco–2 monolayers. After digestion, EA and four of its derivatives, along with flavan–3–ols monomers (epicatechin and EGCG) and oligomeric procyanidins (dimers and trimers) from pecans were found in the basolateral compartment in the Caco–2 monolayer model system. Our results are in fundamental agreement with the limited knowledge we possess of the absorption of nut phenolics in humans.

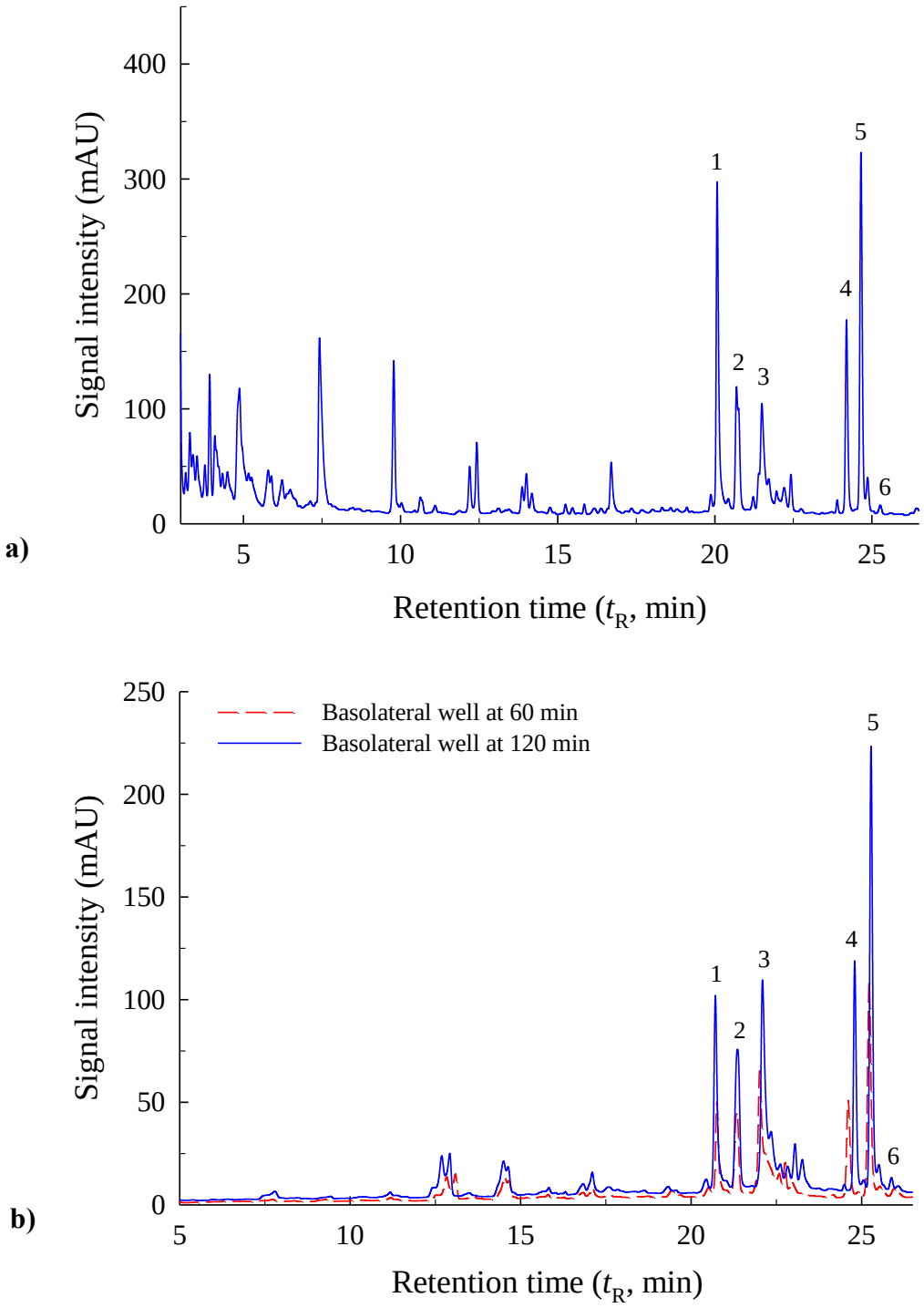

Figure 4.1
Representative reversed–phase HPLC chromatogram at λ = 255 nm of the a) digested LMW fraction added to the apical well and b) the 10x concentrated phenolics present in the basolateral compartment following at t= 120 min.

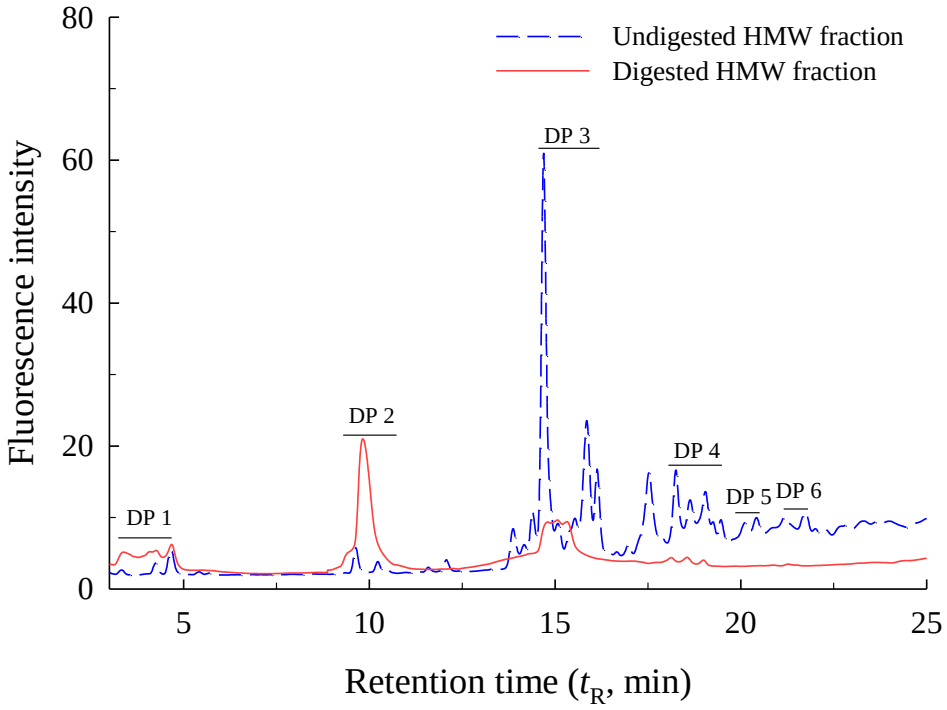

Figure 4.2

Chromatographic separation of a) undigested and b) digested HMW fractions from raw pecans at excitation and emission wavelengths of 276/31

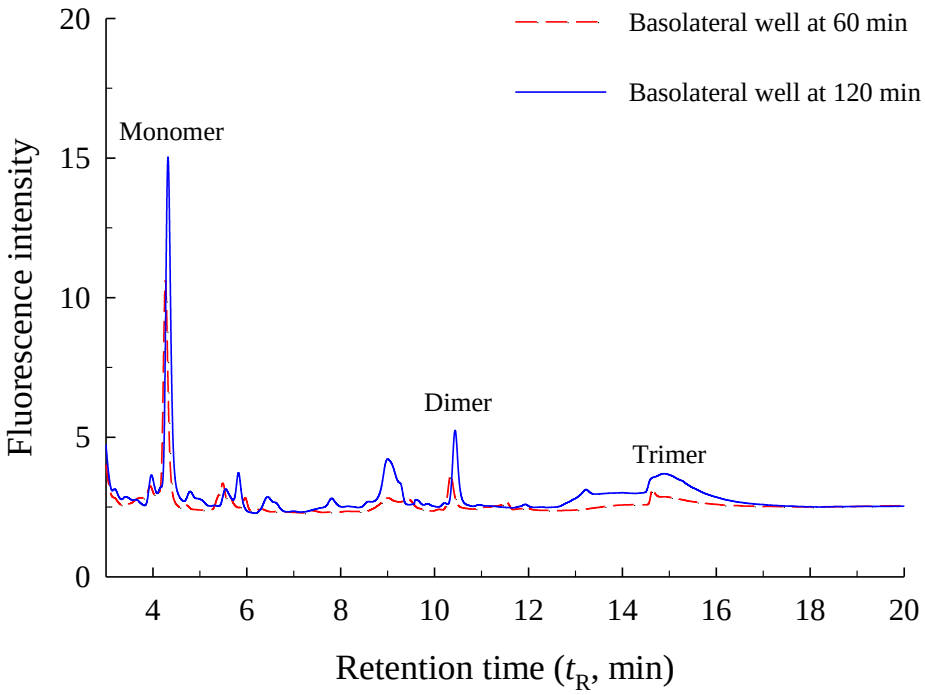

Figure 4.3

HPLC chromatogram of PACs present in the a) apical compartment at t= 60 min and b) the basolateral well at t= 120 at excitation and emission wavelengths of 276/316 nm

Table 4.1
Transport of phenolic compounds from digested LMW fractions from raw and roasted pecans across Caco–2 cell monolayers.

Peak No.	t_R[b] (min)	[M–H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative Identification[c]	Sample	Concentration (µg/mL)[a]			Transport (%)[g]
						A_0[d]	B_{60}[e]	B_{120}[f]	
1	20.51	433	301	Ellagic acid pentose	Raw	23.5 ± 1.9^x	0.42 ± 0.0^x	0.49 ± 0.0^x	1.4 ± 0.3^x
					Roast	26.2 ± 3.6^x	0.40 ± 0.0^x	0.54 ± 0.0^x	2.0 ± 0.1^x
2	21.16	477	315–300	Methyl ellagic acid hexoside	Raw	22.4 ± 0.8^x	0.37 ± 0.0^x	0.46 ± 0.0^x	1.4 ± 0.1^x
					Roast	22.8 ± 1.5^x	0.35 ± 0.0^x	0.44 ± 0.0^x	1.9 ± 0.1^x
3	22.04	301	217	Ellagic acid	Raw	24.4 ± 5.5^x	0.41 ± 0.1^x	0.34 ± 0.0^x	1.1 ± 0.4^x
					Roast	22.6 ± 1.6^x	0.32 ± 0.0^x	0.38 ± 0.0^x	1.7 ± 0.3^x
4	24.59	491	328	Dimethyl ellagic acid hexoside	Raw	27.7 ± 2.2$_x$	0.53 ± 0.0^x	0.65 ± 0.0^x	1.6 ± 0.3^x
					Roast	24.2 ± 2.4^x	0.42 ± 0.0^x	0.55 ± 0.0^x	2.2 ± 0.1^x
5	25.03	447	315	Methyl ellagic acid pentoside	Raw	37.4 ± 3.2^x	0.65 ± 0.2^x	0.83 ± 0.^{x}	1.5 ± 0.0^x
					Roast	29.9 ± 3.2^x	0.56 ± 0.0^x	0.78 ± 0.1^x	2.5 ± 0.3^x
6	25.62	457	325–293–203–163	Epigallocatechin gallate	Raw	20.5 ± 0.3^x	0.39 ± 0.1^x	0.56 ± 0.0^x	1.8 ± 0.1^x
					Roast	19.6 ± 1.0^x	0.33 ± 0.0^x	0.51 ± 0.1^x	2.6 ± 0.3^x

[a] Quantitation based on RP–HPLC analysis and data are reported as µg equivalents (eq.)/mL of available commercial standards (i.e., ellagic acid or epigallocatechin gallate) or the most comparable standard. Data are reported as means ± standard deviation (n=3). Means followed by the same letter for each phenolic from raw and roasted are not significantly different as analyzed using one–way ANOVA followed by Tukey's HSD test ($p > 0.05$).
[b] t_R= Retention time from RP–HPLC analysis performed on a Kinetex PFP column with a pore size of 100 Å (Phenomenex, Torrance, CA) equipped with a guard cartridge of the same material.
[c] Tentative identification was done by comparison to relevant literature.
[d] A_0 = Apical well at 0 min
[e] B_{60}= Basolateral well at 60 min
[f] B_{120} = Basolateral well at 120 min
[g] Transport ratio (%) = ($[B_{120}]/[A_0]$) × 100

Table 4.2
Changes to the HMW fraction of raw and roasted pecans following *in vitro* digestion.

t_R (min)[b]	DP[c]	[M–H]$^-$ (*m/z*)	Concentration (mg/g fr.) [a]			
			Undigested		Digested	
			Raw	Roasted	Raw	Roasted
4.3	Monomer	289	0.2 ± 0.0^x	0.2 ± 0.0^x	0.4 ± 0.1^x	0.4 ± 0.1^x
4.7		289				
9.6	Dimer	577	0.9 ± 0.0^x	0.6 ± 0.0^x	4.8 ± 0.5^y	4.2 ± 0.2^y
10.2		593				
14.7	Trimer	865	13.5 ± 0.4^x	11.3 ± 0.5^y	3.9 ± 0.4^z	3.8 ± 0.6^z
15.9		881				
16.1		897				
18.3	Tetramer	1153	3.3 ± 0.4^x	2.2 ± 0.1^y	0.5 ± 0.1^z	0.3 ± 0.1^z
18.6		1169				
19.1		1185				
20.1	Pentamer	1441	0.8 ± 0.1^x	0.6 ± 0.1^x	N.D.	N.D.
21.5		1457				
21.2	Hexamer	1729	0.9 ± 0.1^x	0.7 ± 0.1^x	N.D.	N.D.
21.7		2033				

[a] Contents are expressed as mg /g fr and are reported as means ± standard deviations (n=3). Dimers to hexamers are expressed as procyanidin B2 eq. Means followed by the same letter in the same row are not significantly different as analyzed using one–way ANOVA followed by Tukey's HSD test ($p > 0.05$).
[b] t_R= Retention time from NP–HPLC analysis performed on a HILIC column (4.6 × 150 mm, 3–µm particle size, 200 Å; Phenomenex) equipped with a guard cartridge of the same material
[c] DP = degree of polymerization

Table 4.3
Transport of procyanidins from digested HMW fraction from raw and roasted pecans across Caco–2 cell monolayers.

t_R (min)[b]	DP[c]	$[M–H]^-$ (*m/z*)	Sample	A_0[d] (µg/mL)	B_{60}[f] (ng/mL)	B_{120}[f] (ng/mL)	Transport ratio (%)[g]
4.3	Monomer	289	Raw	14.6 ± 4.8^x	70.5 ± 11.7^x	132.7 ± 24.3^x	1.0 ± 0.3^x
			Roast	12.6 ± 1.8^x	110.0 ± 26.8^x	159.5 ± 32.4^x	1.3 ± 0.2^x
10.2	Dimer	593	Raw	37.9 ± 4.8^x	28.2 ± 1.7^x	45.2 ± 3.9^x	0.1 ± 0.0^x
			Roast	32.6 ± 2.3^x	26.2 ± 2.4^x	30.8 ± 3.4^x	0.1 ± 0.0^x
14.7	Trimer	865	Raw	31.4 ± 3.3^x	$27.5\ 6.0^x$	47.6 ± 2.5^x	0.2 ± 0.0^x
			Roast	31.6 ± 1.1^x	25.1 ± 3.6^x	29.4 ± 1.6^x	0.1 ± 0.0^x

[a] Data are expressed as ng/mL and are reported as mean ± standard deviation (n=3). Dimer and trimer contents are expressed as procyanidin B2 equivalents. Mean ± standard deviation followed by the same letter for each DP of raw and roasted are not significantly different as analyzed using one–way ANOVA followed by Tukey's HSD test ($p > 0.05$).
[b] t_R = Retention time (t_R) from NP–HPLC analysis performed on a HILIC column (4..6 × 150 mm, 3–µm particle size, 200 Å; Phenomenex) equipped with a guard cartridge of the same material
[c] DP = degree of polymerization
[d] A_0– Apical chamber at 0 min
[e] B_{60}– Basolateral well at 60 min
[f] B_{120}– Basolateral well at 120 min
[f] Transport ratio = $([B_{120}]/[A_0]) \times 100\%$

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CHAPTER 5

CONCLUSIONS

Clinical trials have demonstrated that the consumption of tree nuts, such as pecans, has been associated with a reduced risk for chronic disease and better health outcomes. Additionally, the literature has demonstrated that the consumption of pecan phenolics can improve the antioxidant status of post–prandial plasma. This been attributed to the presence of limited quantities of pecan phenolics present in post–prandial plasma. Furthermore, previous findings have shown that pecans can inhibit oxidation in cellular systems. While these studies have shown that pecan phenolics can are able to bestow antioxidant activity in biological systems, there is a lack of knowledge regarding events prior to systemic distribution. This includes how these compounds are modified during their journey through gastrointestinal tract and their absorption at the intestinal lining.

Thus, the present work sought to understand the potential effects of digestion phenolic profile of pecan phenolics and their antioxidant capacity. Crude phenolic extracts prepared from raw and roasted pecans were subjected to *in vitro* digestion. HPLC–ESI–MS/MS results demonstrated that the pecan phenolic profile was modified during digestion. The LMW fraction isolated from the digested raw crude extract showed a 30–100% reduction in the concentrations of compounds present in the LMW fraction. For the HMW fraction there was a loss of larger procyanidins with DP 4 to 6 and only an 8% reduction in trimers. Interestingly there was a 20–fold increase in dimers. The large

increase in dimers and limited reduction of trimers was attributed to the breakdown of larger procyanidins into smaller compounds. The increase in dimers was also attributed to the dimerization of monomers during *in vitro* digestion. The reduction in the quantity of phenolics was mirrored by a marked loss of antioxidant capacity across various *in vitro* antioxidant assays (i.e., H–ORAC$_{FL}$, TEAC, FRAP) and TPC.

Caco–2 monolayers were used as a cell–based model of the intestinal lining to evaluate the apical to basolateral absorption of digested pecan phenolics. Sephadex LH–20 column chromatography was used to prepare LMW and HMW fractions from raw and roasted pecans. The prepared fractions were then then subjected to *in vitro* digestion. The ability of phenolics present in the digested fractions to be transported across Caco–2 monolayers were evaluated.

Six phenolics from the digested LMW fractions prepared from raw and roasted pecans were able to undergo apical to basolateral transport across Caco–2 monolayer following 1 and 2h of incubation. The transported phenolics were identified as EA, four EA acid derivatives along with EGCG. The concentration of phenolics present in the basolateral well increased over time with limited concentrations, 1–3% of the apically loaded samples, being transported.

From the HMW fraction procyanidin monomers, dimers and trimers were able to undergo apical to basolateral transport across Caco–2 monolayers. The ability of monomers, dimers, and trimers from pecans to be transported across Caco–2 monolayers agrees with previous reports that demonstrated transport of procyanidins is limited by their DP. The percent transported of monomers, dimers, and trimers ranged from 1.0–1.3, 0.1, and 0.1–0.2%, respectively.

The results from these studies contribute to better understanding of the potential benefits of pecan consumption. Although digestion may negatively affect pecan phenolics by reducing their antioxidant capacity, the modification of the phenolic profile by breakdown of larger procyanidins into more bioavailable compounds, such as monomers, dimers, and trimers, may be beneficial. The absorption of procyanidin monomers, dimers, and trimers through a model of the intestinal lining was also demonstrated.