

**CULTIVAR AND E-BEAM IRRADIATION EFFECTS ON PHYTOCHEMICAL
CONTENT AND ANTIOXIDANT PROPERTIES OF PECAN KERNELS**

A Thesis

by

JOSE EMILIO VILLARREAL LOZOYA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Food Science and Technology

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ABSTRACT

Cultivar and E-Beam Irradiation Effects on Phytochemical Content and
Antioxidant Properties of Pecan Kernels. (May 2006)

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Pecan kernels from six cultivars were analyzed for phenolic content and antioxidant properties. In addition, kernels from two cultivars were irradiated with 0, 1.5 and 3.0 kGy using E-Beam irradiation and stored in accelerated conditions (40 °C and 55% R.H.). Changes in phytochemical profile and antioxidant properties were monitored for 134 days.

Cultivars differed greatly in their phytochemical content. Total extractable phenolic content (TP) ranged from 62 to 106 milligrams of chlorogenic acid equivalents per gram of defatted kernel. Antioxidant capacity (AC) measured by the DPPH free radical had a strong correlation with TP. Shells from each cultivar were 6, 4.5 and 18 times greater for TP, AC and condensed tannin content (CT). Gallic and ellagic acids, epicatechin and catechin were identified in hydrolyzed extracts of all cultivars. Prior to hydrolysis, no compounds were positively identified. Fatty acid profile of kernel oil had a strong inverse correlation between oleic and linoleic oil. Kernels from the same cultivar but different location differed in their fatty acid composition but had similar TP.

Irradiation of ‘Kanza’ and ‘Desirable’ kernels with 1.5 and 3.0 kGy had no detrimental effects on AC and TP by the end of experiments. Phenolic profile was similar for all treatments. Tocopherol content decreased with irradiation treatments, but no further degradation was observed throughout storage. Peroxide values increased slightly after 98 and 134 days of storage for ‘Desirable’ kernels, with slight differences between controls and irradiated samples. Color of kernels decreased in lightness and

yellowness and increased in redness with no differences between irradiated samples and controls.

For the first time the effect of pecan cultivar and E-Beam irradiation was assessed in phytochemical and antioxidant attributes of pecan kernels. Additionally, irradiation with E-Beam had no significant detrimental effects in phytochemical composition and only a slight increase in peroxide value, indicating potential as pecan kernel sanitization.

DEDICATION

Para mi familia, por su incondicional apoyo. Para los que seguimos aquí y para los que ya son parte de la eternidad. Espero algún día ser merecedor de ustedes.

A mi padre (Q.E.P.D.) y a mi madre, gracias por todo su esfuerzo. A mis hermanos, por su amistad y sabios consejos.

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CHAPTER I

INTRODUCTION

Pecans [*Carya illinoensis* (Wangenh.) K. Koch] belong to the Juglandaceae family which also includes walnuts (*Juglans* sp.), another important genus native from North America (1). Pecan is distributed over an area of geographic and climatic variation extending from northern Illinois and southeastern Iowa to the gulf coast of United States (2). It is a riparian species that grows abundantly along the Mississippi river, the rivers of central and eastern Oklahoma and the Edwards plateau of Texas. The geographic segment of the U.S. pecan-producing land area has been expanded to Georgia, California, Arizona, New Mexico and Western Texas. The species is also distributed in the form of sporadic populations and regenerating stands throughout north-central Mexico and as far south as the state of Oaxaca (2, 3).

Pecans are monoecious (both, male and female organs in the same plant) but cannot self-pollinate due to its dichogamous behavior (male and female organs mature at different times) (4). Bud break occurs approximately at the beginning of April and wind pollination can occur anytime from April to May, depending on cultivar and environmental factors. After pollination, nutlets grow very rapidly until July, when sizing decreases sensitively (5).

Pecan kernels have a high content of oil (6). Most of the oil and protein content of the kernel is formed during the last growth period, usually between September and October (6). When nuts are physiologically mature, the shucks surrounding the nuts split and trees can be shaken and harvested. After harvest, pecans can be sold in shell or processed. Process involves mechanically washing and sanitizing, cracking and separating kernels from the shells (shelling). Shelling process has been demonstrated to reduce the shelf-life of pecans. Controlling storage temperature is the single most important strategy for extending shelf-life of shelled or in-shell pecans (7). However,

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pecans in some products that can not be refrigerated or frozen require other strategies for extending their shelf life (7).

As foodstuffs, pecan kernels are commonly used as an ingredient for desserts, candies or ice cream and, until recently, they were not considered of value for their nutritional properties. Lately, pecan kernels have been observed to be beneficial for human health in numerous ways. They improve serum lipid profile, due to their high monounsaturated fatty acid content (8). Most recently, they have been identified to have phenolic compounds (9), which, according to several studies (10, 11), act as antioxidants and have the ability to lower the incidence of chronic diseases, such as Alzheimer's, Parkinson's, some types of cancer, and other degenerative diseases.

Information of the types of phenolic compounds present in kernels of different cultivars pecan is not available. Studies mention the presence of different phenolic acids (12), condensed tannins (13) and other antioxidants such as tocopherols (14), but no reference has been done to their antioxidant capacity and their dependence on cultivar or variety. One of the objectives of this study was to analyze and characterize phenolic compounds in cultivars of economic relevance to increase our knowledge on the composition and potential health benefits of pecan kernels. Better knowledge of cultivar differences can also serve as reference for future breeding programs.

Another issue of concern in pecan industry is food safety. Current horticultural practices involve harvesting pecans after they have been shaken from the trees onto orchards ground (this process is also used in walnuts, almonds and similar horticultural products) (15). This practice causes pecans to be in contact with dirt and soil-dwelling microorganisms. In many occasions, managed native pecan groves are not restricted from domesticated animals and grazing cattle or other, providing additional risks for pathogenic contamination. Due to pecan kernels low water activity ($a_w \approx 0.68$) (7), pathogenic growth is usually not of major concern. Nevertheless, there could be occasions when nut surface increases its moisture allowing pathogenic growth. In 2004 the Food and Drug Administration (FDA) issued a press release regarding an outbreak of *Salmonella enteritidis* due to the consumption of raw almonds. Seven people from five

different states in the U.S. showed symptoms of infection apparently due to almond consumption. This led to investigate different procedures, such as irradiation, to decontaminate almonds from pathogenic microorganisms.

Food safety issues are an even bigger concern if we consider that larger amounts of food are being transported across nation boundaries every year and food safety potential problems arise with them. Approximately 80% of U.S. pecan production (~250 million pounds per year) is consumed internally showing the great demand for this product (16). The U.S. also imports pecans, with 95% of them coming from Mexico, averaging 54 million pounds per year in the last 10 years. Mexico's pecan crop for 2004 is estimated at 150 million pounds and only 55% is necessary to satisfy domestic consumption while the remaining will be exported mainly to the U.S (16). This implies a food safety concern when importing pecans, in-shell or shelled. Consequently there is the need to assure safety when trading pecans across borders (16).

The process of food irradiation involves exposing the food to ionizing radiation (gamma rays) so that a prescribed quantity of ionizing energy is absorbed (17). There is information on the effects of irradiation on almonds (18), cashews (19, 20), walnuts (18), pecans (21), and others, with different responses depending on the nut and irradiation dose.

Foodstuffs with high lipid content have greater probabilities of becoming rancid after irradiation due to an increased oxidation rate. In pecans, Silva et al. (17) studied the effects of gamma irradiation and the use of packaging materials sprayed with synthetic antioxidants throughout storage. Irradiation levels of 0.1 kGy to 1 kGy effectively decreased the amount of pecan kernels infested with *Aspergillus* spp. without increasing peroxide values or decreasing sensory panel scores. However, applied irradiation doses were not sufficient to inhibit total growth of the fungus. Further research was suggested using higher irradiation doses.

In another study, Sajilata et al. (20) found that ethanol extracts of irradiated cashew nuts decreased their antioxidant activity by assessing their ability to inhibit lipid peroxidation using the 1,3-diethyl-2-thiobarbituric acid (DETBA) assay. No

phytochemical profile or antioxidant content was evaluated. There is the need to evaluate how irradiation affects the antioxidant capacity and phytochemical content of pecans.

This thesis is divided in three main chapters. Chapter II describes the characterization of kernels from different pecan cultivars for their antioxidant and nutraceutical attributes. Phenolic compounds, antioxidant capacity, vitamin E content and fatty acid profile are some of the attributes analyzed. Chapter III describes the storage stability of phenolic compounds after different irradiation treatments and their relationship to quality attributes values such as color and oxidative status. Finally, Chapter IV is dedicated to general conclusions and gives recommendations for future research.

CHAPTER II

ANTIOXIDANT PROPERTIES AND PHYTOCHEMICAL COMPOSITION OF KERNELS FROM SIX PECAN CULTIVARS

Summary

Recently, nuts have been included into the high phenolic content food group, being pecans one with the highest content. Nevertheless, information of pecan genotypes has not been assessed for this matter. Pecan kernels from ‘Desirable’, ‘Kanza’, ‘Kiowa’, ‘Nacono’, ‘Pawnee’ and ‘Shawnee’ cultivars were collected from two different locations and analyzed for their antioxidant capacity (AC), total phenolic content (TP), condensed tannin content (CT), HPLC phenolic profile, tocopherol content and fatty acid composition. The strongest correlation was found between AC measured by DPPH free radical and TP ($R^2 = 0.89$). Values obtained for AC assessed with the ORAC assay (AC_{ORAC}) followed the trend ‘Kanza’ > ‘Nacono’ \geq ‘Shawnee’ \geq ‘Kiowa’ \geq ‘Pawnee’ > ‘Desirable’. Similar trends were observed for TP, CT, and AC measured with the DPPH assay (AC_{DPPH}). ‘Kanza’ had the greatest AC_{ORAC} value and ‘Desirable’ the least, 817 and 372 μ moles of TE/g defatted kernel, respectively. Cultivars differed greatly in their phenolic content; CT ranged from 23 to 47 mg CE/g defatted kernel and TP from 62 to 106 mg CAE/g defatted kernel. Before hydrolysis, phenolic compounds were minimally detected on HPLC phenolic profile. After basic-acid hydrolysis gallic and ellagic acids were identified suggesting the presence of hydrolyzable tannins. ‘Desirable’ grown in two distinct locations showed similar phenolic profile but different fatty acid composition. Environmental conditions, horticultural practices, and post-harvest procedures need to be assessed for their effect on phytochemical content and profile of pecan kernels. Presence of phenolic compounds with antioxidant activity in pecan shells suggests a new potential source of natural antioxidants. The high flavonoid content together with a high monounsaturated fatty acid profile, suggests diverse health benefits of pecan kernels.

Introduction

In the last decade, extensive analysis of phenolic compounds suggests a preventive effect against degenerative diseases such as Alzheimer's, Parkinson's, different types of cancer, and others (22-25). Pecans have been classified as a high phenolic content food (26). Wu et al. (9) screened different common foods and vegetables across the U.S. and reported pecan kernels to have the greatest antioxidant capacity (AC) and total extractable phenolic content (TP) within the nut group and ranked among the foods with the greatest TP (9). Senter et al. (12) screened cultivars 'Stuart' and 'Schley' for phenolic acid content using a gas chromatographic technique revealing the presence of gallic, gentisic, vanillic, protocatechuic, coumaric, syringic, p-hydroxybenzoic and p-hydroxyphenyl-acetic acids (12). Gallic acid constituted 138 µg/g of defatted kernel and accounted for 78% of the phenolic acid constituents. The authors also found that the content of phenolic acids decreases rapidly during storage and that a linear correlation can be found between phenolic acid degradation and sensory scores. Gallic, gentisic, and vanillic acid decreased 39, 36, and 28%, respectively, by wk 3 of accelerated storage (21 °C, 65% RH) and all major phenolic acids progressively decreased during the 12 wk period. Sensory evaluations of pecan kernels revealed a significant score decrease at each storage interval up to wk 9.

Condensed tannins (CT) is another group of phenolic compounds which are present in pecan kernels (13). Tannin antioxidant and antimutagenic properties are affected by degree of polymerization, monomer structure, and bond type between monomers (27). Differences between cultivars were found using the acid-butanol assay, with CT content as high as 1.71% of total kernel weight for 'Shoshoni' and as low as 0.70% for 'Jackson' (13). Gu et al. (26) analyzed different foodstuffs and vegetables, including pecan kernels, for their proanthocyanidin (PA) structural composition and revealed the presence of B type PA's (C4-C8 and C4-C6 bonds). Prodelphinidins (3-*O*-gallates) were found including epigallocatechin, epicatechin-3-*O*-gallate, and the more common flavan-3-ols, catechin and epicatechin. Degree of polymerization of CT has been tested in pecans (28) and CT differing in polymer sizes have been identified. From

a total PA content of 494.1 ± 86.2 mg/100 g FW ($\approx 0.5\%$ of total weight), 17.2, 42.1, 26, 101, 84, and 223 mg/100 g corresponded respectively to monomers, dimmers, trimmers, tetramers through hexamers, heptamers through decamers, and polymers above 10 subunits.

Despite the studies mentioned above, little is known of how cultivars affect phytochemical content and profile of kernels. There is the need to elucidate which types of phytochemical compounds are present in different pecan cultivars and how these compounds affect the antioxidant properties of the food system.

The objective of the present investigation was to characterize kernels from different pecan cultivars for their antioxidant and nutraceutical attributes, such as phenolic compounds, antioxidant capacity, vitamin E content and fatty acid profile.

Materials and Methods

Chemicals and Apparatus. Solvents were HPLC grade and purchased from Fisher Scientific (Houston, TX). Folin-Ciocalteu reagent, vanillin reagent, trolox (6-hydroxy -2,5,7,8- tetramethylchroman-2-carboxylic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein sodium salt (FL), and phenolic standards were purchased from Sigma Chemical Co. (St. Louis, MO). AAPH (2,2'-Azobis (2-amidino-propane)-dihydrochloride) was obtained from Wako Chemicals (Richmond, VA). Nanopure water was used in all solutions. A Synergy HT plate reader equipped with fluorescence and absorbance and KC-4 v. 3.4 analytical software (Bio-Tek Instruments, Inc., Winooski, VT) were used with different 96-well plates depending on fluorescence or absorbance measurements.

Sample Preparation and Extraction. Pecan nuts were mechanically harvested during Fall 2004 from trees of six different cultivars, 'Desirable', 'Kanza', 'Kiowa', 'Nacono', 'Pawnee', and 'Shawnee' (Figure 1). Cultivars were chosen due to their commercial relevance or, in case of the recently-released 'Nacono' (29), for its prospective increase diffusion and production. All cultivars but 'Kiowa' were grown at the USDA Experiment Station located in Brownwood, Texas (BW). 'Kiowa' and an

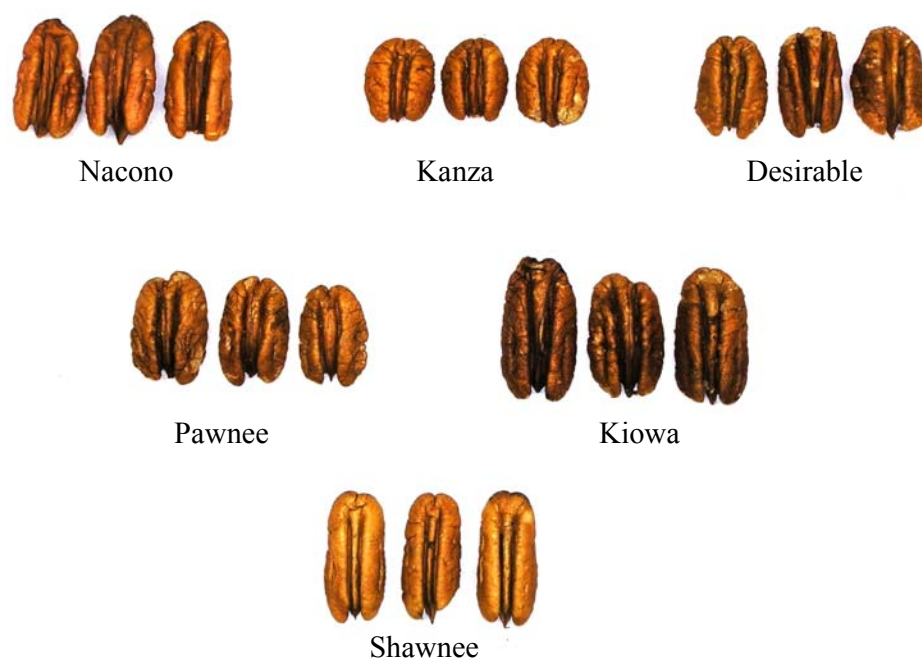


Figure 1. Pecan kernels from six different cultivars harvested in Texas.

additional batch of 'Desirable' nuts were harvested from a commercial orchard located near Caldwell, Texas (CW).

After harvest, about 18 kg of nuts per cultivar were transported to the laboratory and stored in a cold room set at -5 °C. For analysis, 4.5 kg of nuts per cultivar were mechanically cracked and shelled. After removal of rotten and necrotic kernels, healthy pecan halves were stored at -80 °C in freeze-resistant plastic bags. Kernels were chopped using a food processor and then defatted with hexane (1:20 w/v) using an Ultraturrax T25 homogenizer (IKA Works, Wilmington, NC). After homogenizing, samples were filtered with a Buchner funnel and slow filtration rate filter paper. The cake was defatted two more times and the remaining powder was dried at 35 °C under vacuum for 2 h. The powder was flushed with nitrogen and stored in a sealed container at -20 °C until analyses. Oil was obtained after hexane was evaporated using a rotavapor. The oil was flushed with nitrogen and stored at -20 °C until analyses of the lipidic fraction.

Defatted pecan powder (1 g) was placed in 50 mL falcon tubes and homogenized with 20 mL of acetone:water (70:30) solution. Falcon tubes were capped, placed in an oscillatory shaker at 5 °C and shaken overnight. After shaking, slurries were centrifuged at 18,000 g and supernatants were collected, flushed with nitrogen and stored at -20 °C. A similar extraction protocol was followed to determine TP, AC and CT on pecan shells of each cultivar. Another extraction from shells using only water was performed to assess CT leaching from shell to kernel.

Antioxidant Capacity. DPPH free radical was used to measure AC (AC_{DPPH}) as described by Brand-Williams et al. (30) and adapting the methodology to be used in a microplate reader. Extracts were diluted in methanol and 13 μ L was pipetted into each well of a 96 well flat bottom plate (Costar #3595, Corning, Inc., Corning, NY). Using a multichannel pipette, 247 μ L of DPPH aliquot in methanol was added. Plates were then tightly sealed with two layers of parafilm to prevent evaporation and placed in the dark at 20 °C. After 24 h, microplates were read at 515 nm in a plate reader and readings of blanks with water were subtracted from each sample. A standard curve was prepared

using trolox as reference reagent. AC was expressed as micrograms of trolox equivalents per gram of defatted sample ($\mu\text{g TE/g}$).

ORAC Assay. A modification of the procedure described by Wu et al. (9) was used for hydrophilic AC (AC_{ORAC}). All reagents were dissolved in 75 mM phosphate buffer pH 7.4. Clear-bottom 96-well black plates (Costar #3631, Corning, Inc., Corning, NY) were loaded with 25 μL of extracts per well and incubated at 37 °C for 45 min prior analysis. Fluorescein sodium salt (FL) was used as protein probe and AAPH as free radical source. FL stock solution (FL_1) was prepared by diluting 112.5 mg of FL powder in 50 mL of phosphate buffer using a volumetric flask. A second FL solution (FL_2) was made by diluting 100 μL of FL_1 in 10 mL of buffer. After preparation, FL_1 and FL_2 solutions were stored at 2 °C. Prior analysis a third solution (FL_3) was made by dissolving 400 μL of FL_2 in 25 mL of buffer. AAPH solution was prepared after incubation of buffer at 37 °C for 45 min and prompt dissolution of 260 mg of AAPH pellets. After preparation, reagents were placed in plastic containers incubated in an oven at 37 °C and used to prime plate reader injectors. After priming, 200 μL of FL_3 were injected in each well followed by 75 μL aliquots of APPH solution.

Fluorescence readings were done using excitation and emission wavelengths of 485 nm and 520 nm, respectively. Each well was read at ~1 min intervals during 50 min. The loss of fluorescence was recorded and processed by analytical software package KC-4 v. 3.4. Samples were compared against trolox standard and a blank curve. Data were normalized and the area under the curve (AUC) was calculated for each well as follows:

$$\text{AUC} = (f_1 + f_n)/2 + (f_1 + f_2 + \dots + f_{n-2} + f_{n-1})$$

where f_1 and f_2 are the first and second relative fluorescence values of n samples, respectively, and f_n is the last relative value of the well. One blank curve was elaborated for each row and subtracted from each value of the same row. Blank wells AUC values were subtracted from sample and trolox standard AUC values (NetAUC). NetAUC

samples were compared against trolox standard NetAUC from the same plate row. Results were expressed as micromoles of trolox equivalents per gram of defatted kernel ($\mu\text{mol TE/g}$) using the next formula:

$$\mu\text{mol TE} = [(\text{NetAUC}_{\text{sample}})/(\text{NetAUC}_{\text{standard}})] \times \text{Concentration of Standard (40 } \mu\text{M)}$$

Extractable Phenolic and Condensed Tannin Content. TP analysis was performed as explained by Swain and Hillis (31), adapting the methodology to be used in a microplate reader. Pecan extracts were diluted with water and 13 μL was loaded in each well of a 96 well flat bottom plate (Costar #3595, Corning, Inc., Corning, NY). Water was added to six wells to be used as blanks. Using a multi-channel micropipette, 208 μL of nanopure water was added to each well followed by 13 μL of Folin-Ciocalteu reagent. Mixture was allowed to react for 3 min. After time elapsed, 26 μL of 1 N Na_2CO_3 was added. Plates were sealed using two layers of parafilm and allowed to react for 2 h, after which absorbance was read at 725 nm in the microplate reader. A standard curve made with chlorogenic acid was elaborated to express TP as milligrams of chlorogenic acid equivalents per gram of defatted kernel (mg CAE/g def kernel). Six replicates of each sample were analyzed.

Procyanidins or CT were evaluated using the vanillin assay (32). An aliquot of 0.5 g of defatted kernel was placed in a centrifuge tube and 15 mL of 1% HCl in methanol was added to each sample. Each tube was vortexed and placed in a water bath at 30 °C with constant shaking for 20 min and vortexing every 10 min. After incubation, tubes were centrifuged and supernatants were extracted. Aliquots of the supernatants were placed in two separate assay tubes, one for the sample determination and the other for blank determination. Samples and blanks were incubated for exactly 20 min after adding 5 mL of the vanillin reagent (0.5 g of reagent and 200 mL of 4 % HCl methanol) to samples and 4% HCl in methanol to the blanks. After 20 min, absorbance was read at 500 nm from of each sample and blank using a HP 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA). Samples absorbance was rectified

with the blank standard and compared against a standard curve made with (-)catechin. Results were expressed as milligrams of catechin equivalents per gram of defatted sample (mg CE/g def kernel). Six replicates of each sample were used.

Phenolic Hydrolysis and Reverse-Phase HPLC Analysis. To determine phenolic profile, extracts used for AC and TP assays were analyzed by high performance liquid chromatography (HPLC). Acetone from extracts was evaporated under vacuum using a SpeedVac concentrator (Thermo, Marietta, Ohio) and 1 mL of the water residues was transferred to an assay tube and diluted with 1 mL of 8 N NaOH. Samples were flushed with nitrogen, capped and allowed to react for 16 h in the dark. After basic hydrolysis, 1.33 mL of 6 M HCl was added to the tubes and flushed with nitrogen, capped and heated using a block heater (Fisher Scientific, Houston TX) at 85 °C for 45 min. After acid hydrolysis, 1 mL of samples was filtered with a 0.2 µm PTFE filter (Fisher Scientific, Houston, TX) and 20 µL was injected into the HPLC system. The HPLC system was equipped with two Waters 515 gradient pumps (Waters Corp., Milford, MA) and coupled with a Waters 717 autosampler (Waters Corp., Milford, MA). An Atlantis C18 column (5 µm particle size, 4.6 mm x 150 mm; Waters Corp., Milford, MA) coupled with a guard column of the same chemistry was used to separate phenolic compounds. A photodiode array detector was used to scan absorbance from 190 nm to 500 nm. Peak spectra, retention times, and standard spikes were used for determination of compounds. Nanopure water, acidified to pH 2.3 with 2 M HCl (solvent A) and acetonitrile (solvent B) were used as mobile phases. Solvent gradient was used as follows: from 0 to 5 min isocratic 85% A flow, from 5 to 30 min a linear gradient of 85% A to 100% B, and from 35 to 40 min isocratic conditions of 100% B. After termination of the cycle, 30 min of column equilibration (85% A) were allowed prior next injection. Standard curves of the identified compounds were elaborated by dissolving standards in methanol and injecting them into the HPLC. Samples and standard curves were analyzed by triplicate.

Fatty Acid Profile. Fatty acid methyl esters were analyzed in a Varian CP 3800 gas chromatograph (Palo Alto, CA) coupled with a Varian CP-8200 autosampler and a

flame ionization detector (FID). A Varian FAME fused silica capillary column (100 m x 0.25, Varian CP-Select CB) was used to determine lipid profile. Oven temperature was set from 0-30 min at 185 °C and from 30-45 min at 235°C with an increase of 20 °C/min. FID temperature was set at 270 °C, and helium, air and hydrogen flows at 1.6, 300 and 35 mL/min, respectively. Fatty acid derivatization was performed using the protocol described by Misir et al. (33). Approximately 0.15 g of oil was diluted in 3 mL of diethyl ether and 0.2 mL of 20% tetramethyl ammonium hydroxide in water was added and allowed to react for 5 min. After time elapsed, 0.5 mL of methanol was added and vortexed for 1 min. After phase separation, 1 mL of the upper organic phase was transferred to a vial and capped for injection into the gas chromatograph. Fatty acids were identified using standards and expressed as percentage of total fatty acid content.

Tocopherol Content. Tocopherol content was determined as explained by Mendoza et al. (34). Pecan oil was weighted (~0.5 g) and 2 ml of methanol was added. After the sample was vortexed for 1 min, 500 µL of hexane was added and vortexed again. Samples were then centrifuged and 1 mL of the top layer was carefully extracted, filtered using 0.2 µm PTFE filters and 20 µL were injected into an HPLC. The HPLC system was equipped with a Lichrosorb® Spherisorb ODS2 C18 column (5 µm particle size, 4.6 mm x 250 mm) and a guard column of the same chemistry (Waters Corp., Mildford, MA). An isocratic flow of 1 mL/min of methanol 100% was used as mobile phase. Concentration of α -, β -, and γ -tocopherols standard solutions were determined as suggested by AOCS official method Ce 8-89 (35) Peak spectra at 295 nm and retention times were used for identification and quantification. Only γ -tocopherol is reported due to minimal detection of other isomers. Values are reported as micrograms of tocopherol per gram of oil ($\mu\text{g } \gamma\text{-Toc/g}$).

Statistical Analysis. To determine statistical difference between means ($P \leq 0.05$), ANOVA and Tukey's Honestly Significant Differences (HSD) were calculated using SPSS statistical software package v. 11.5 (SPSS Inc., Chicago, IL).

Results and Discussion

Phenolic Constituents and Antioxidant Capacity. TP and AC were significantly affected by cultivar. Table 1 shows mean values of all cultivars for TP and AC_{DPPH} . ‘Kanza’ had the highest TP value followed by ‘Nacono’, ‘Kiowa’, ‘Pawnee’, ‘Shawnee’, ‘Desirable’ BW and ‘Desirable’ CW. Both AC_{DPPH} and AC_{ORAC} showed differences between cultivars. The following trend was found in AC_{ORAC} : ‘Kanza’ \geq ‘Nacono’ \geq ‘Shawnee’ \geq ‘Kiowa’ \geq ‘Pawnee’ \geq ‘Desirable’ CW \geq ‘Desirable’ BW. Strong correlations were found between AC_{ORAC} and TP (0.75) and between AC_{DPPH} and TP (0.89) (Figure 3).

The values obtained for AC_{ORAC} in the present study (373 to 817 $\mu\text{moles TE/g}$) are similar to 583 $\mu\text{moles TE/g}$ reported by Wu et al. (9) (assuming the kernels had 70% fat content). In the same study it was found an ORAC/TP ratio of 8.7, in comparison to the 6.0 to 7.7 found in this study.

In the present study AC and ratio differences between cultivars suggest the presence of phenolic compounds with different antioxidant attributes. Antioxidant compounds such as phenolic acids, catechins and condensed tannins have been reported to be present in pecan kernels and their location in the nut has been suggested to predominate in the outer testa, termed pellicle, of the nut (12, 13, 36).

CT content evaluated with vanillin assay showed differences among cultivars ranging from 0.7 to 1.4 % of kernel weight, similar to the values found by Polles et al. (13). Content of CT followed trends similar to those of AC_{ORAC} , ‘Kanza’ cultivar had the greatest CT values followed by ‘Nacono’, ‘Shawnee’, ‘Kiowa’, ‘Desirable’ CW, ‘Desirable’ BW and ‘Pawnee’ (Table 1). A strong correlation was found for AC_{ORAC} and CT ($R^2 = 0.75$), although a stronger correlation was observed when ‘Pawnee’ values were not included in the correlation ($R^2 = 0.99$) (Figure 4), suggesting the presence of phenolic compounds with different antioxidant activity for ‘Pawnee’ cultivar.

Average TP, AC_{DPPH} and CT values obtained from shells were 6, 4.5 and 18 times, respectively, greater than those found in kernels (Table 2). Forbus et al. (37) suggested that tannins leach from shells to kernels during soaking and preconditioning.

Table 1. Phenolic content, antioxidant capacity, and condensed tannin content of different pecan cultivars.

Cultivar	TP ^a (mg CAE/g)	AC _{DPPH} ^b (mg TE/g)	AC _{ORAC} ^c (μmol TE/g)	CT ^d (mg CE/g)
Kanza	106 a ^e	135 a	817 a	47 a
Nacono	76 b	95 bc	688 ab	43 ab
Kiowa	76 b	100 b	568 bcd	36 bc
Pawnee	72 b	88 cd	562 bcd	23 d
Shawnee	71 bc	89 bcd	623 bc	38 b
Desirable CW	70 bc	90 bcd	449 cd	28 d
Desirable BW	62 c	81 d	373 d	25 d
Average ^f	76 ± 1.9	97 ± 6.7	583 ± 32	34 ± 1.3

^a Total extractable phenolic content (Folin-Coicalteu method), milligrams of chlorogenic acid equivalents per gram of defatted kernel; ^b Antioxidant capacity using DPPH free radical, milligrams of trolox equivalents per gram of defatted kernel; ^c Antioxidant capacity using ORAC assay, micromoles of trolox equivalents per gram of defatted kernel; ^d Condensed tannin content using vanillin-HCl assay; milligrams of catechin equivalents per gram of defatted kernel; ^e Values with similar letters are not significantly different (Tukey HSD, P<0.05); ^f Mean values of cultivars analyzed ± S.E.

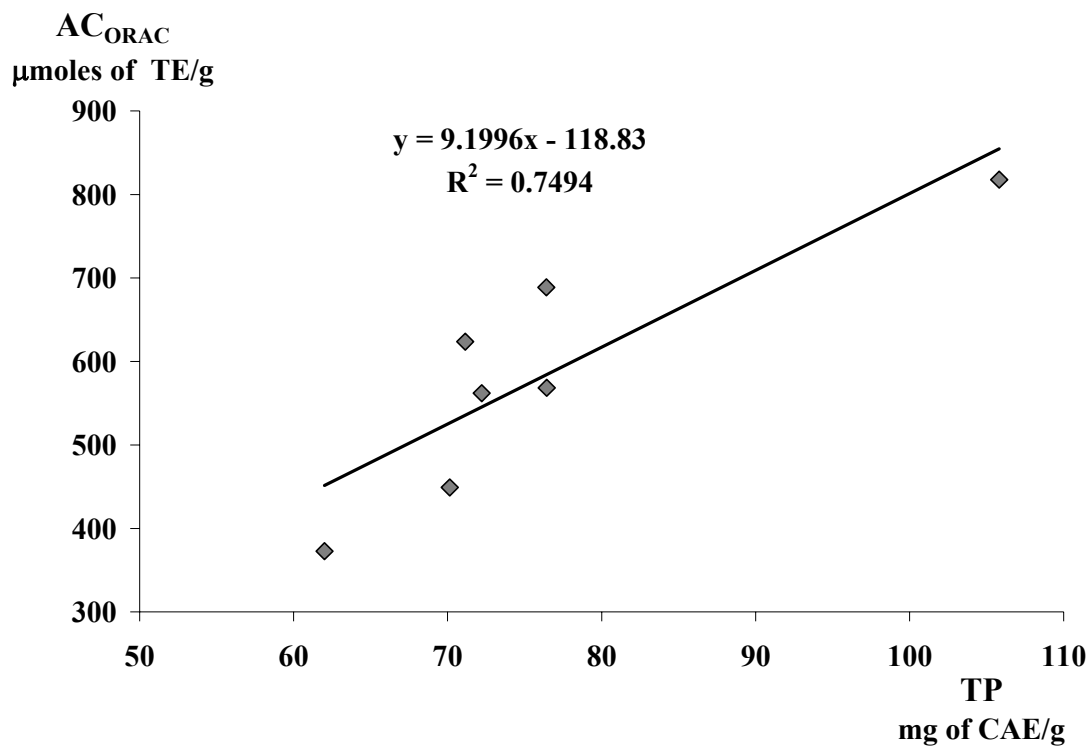


Figure 2. Correlation between antioxidant capacity measured by ORAC assay (AC_{ORAC}) and extractable phenolic content (TP) of pecan kernels. Each point is the average of 6 replicates per cultivar. Vertical axis represented by antioxidant capacity using ORAC assay (AC_{ORAC}), and horizontal axis by total extractable phenolic content (TP), using Folin-Ciocateu reagent.

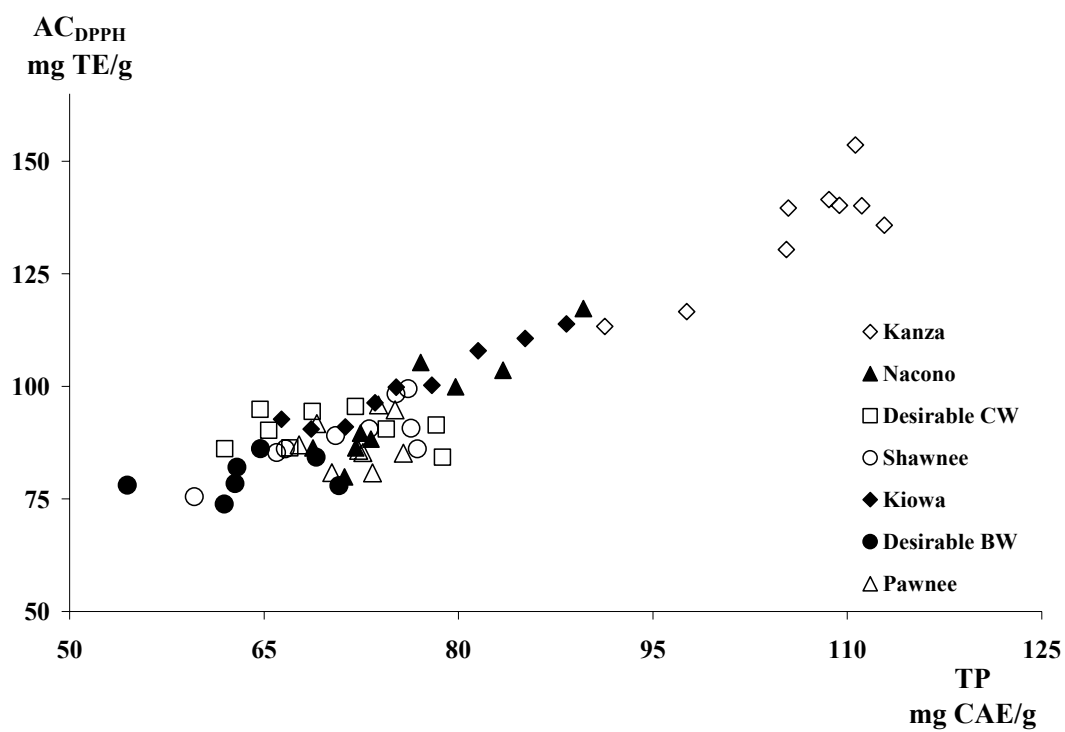


Figure 3. Correlation between antioxidant capacity measured by DPPH free radical (AC_{DPPH}), and total extractable phenolic content (TP).

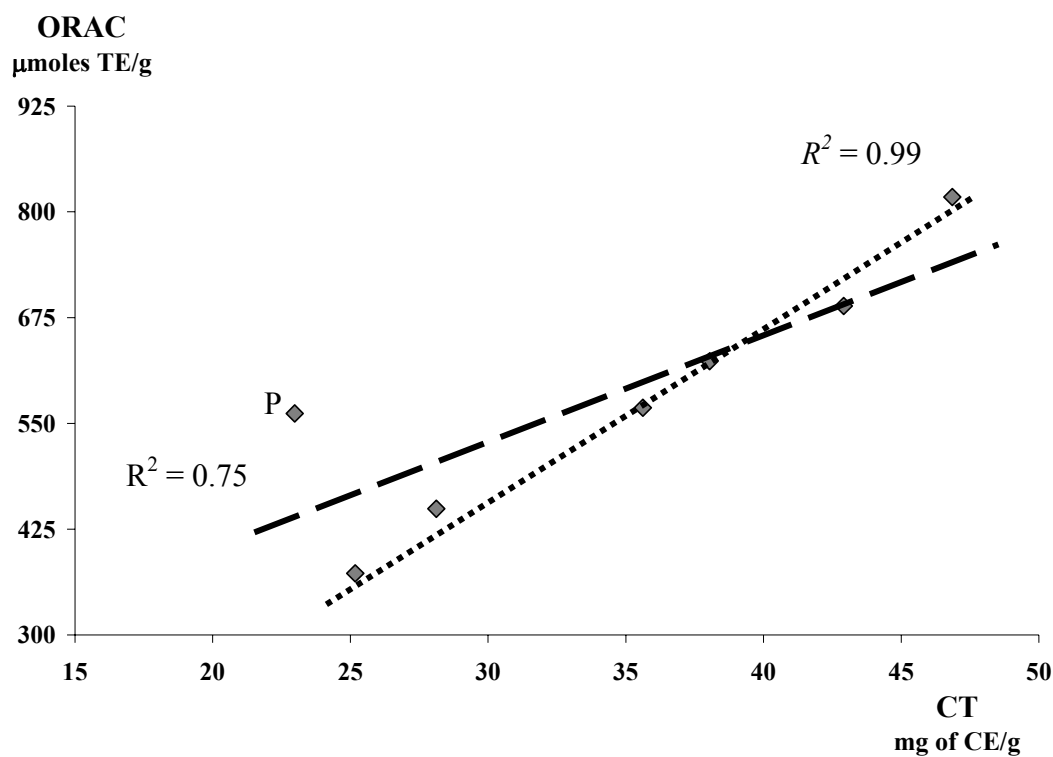


Figure 4. Correlation of AC_{ORAC} and condensed tannin content (CT). Label P assigns value for ‘Pawnee’ cultivar. Stronger correlation was found when ‘Pawnee’ value was excluded from trend line ($R^2 = 0.99$).

Table 2. Phenolic compounds and antioxidant capacity of shells of different pecan cultivars extracted with acetone:water (70:30) or water.

Cultivar	TP ^a (mg CAE/g)	AC _{DPPH} ^b (mg TE/g)	CT ^c (mg CE/g)	CTw ^d (mg CE/g)
Kanza	633 ± 29 a ^e	675 ± 18 a	876 ± 32 a	48 ± 2 a
Nacono	451 ± 6 c	442 ± 7 c	550 ± 23 cde	45 ± 2 a
Kiowa	344 ± 10 de	331 ± 11 d	598 ± 15 e	26 ± 1 b
Pawnee	537 ± 10 b	582 ± 29 b	704 ± 35 bc	49 ± 2 a
Shawnee	506 ± 12 b	444 ± 3 c	827 ± 14 ab	48 ± 2 a
Desirable CW	290 ± 1 e	453 ± 6 c	388 ± 14 ef	29 ± 2 b
Desirable BW	378 ± 17 d	482 ± 30 c	495 ± 21 c	47 ± 1 a
Average ^e	448 ± 45	487 ± 42	634 ± 67	42 ± 1.5

^a Total extractable phenolic content (Folin-Coicalteu method), milligrams of chlorogenic acid equivalents per gram of defatted kernel ± S.E.; ^b Antioxidant capacity using DPPH free radical, milligrams of trolox equivalents per gram of defatted sample ± S.E.; ^c Condensed tannin content of acetone:water (70:30 v/v) extracts using vanillin-HCl assay, milligrams of catechin equivalents per gram of defatted kernel ± S.E.; ^d Condensed tannin content of water extracts, using vanillin-HCl assay, milligrams of catechin equivalents per gram of defatted kernel ± S.E.; ^e Values with similar letters are not significantly different (Tukey HSD, P<0.05).

Samples used in the present study were not soaked in water (as handled in commercial processes). However, nuts were stored in a cold room prior to cracking and shelling, which might have caused water condensation inside nuts, leaching phenolic compounds from shells onto kernels. Water extraction of shells for CT analysis showed values similar to those found in kernel (Table 2). In both extracts, shells of ‘Kanza’ cultivar had the greatest values.

Kernel and shell percentage in pecan nuts vary from 50 to 60 % and 40 to 50%, respectively (6). After processing kernels, shells have to be disposed. It can be used by the landscape industry, but this exchange has no benefit besides disposal expenses for the shelling companies. In this study a high antioxidant capacity of shells was observed providing a potential new source of antioxidants and an alternate use for shells after processing pecan nuts.

HPLC Phenolic Profile. Extracts prior hydrolysis had no identifiable peaks at 280 nm (Figure 5A). After a basic hydrolysis, some phenolic compounds were identified using peak spectra and retention times, yet a better efficiency was found after an additional acid hydrolysis (Figure 5B). Gallic and ellagic acids, catechin and epicatechin were positively identified (Figure 5B). Another peak at 13 min had very similar spectra to that of ellagic acid (Figure 6); nevertheless, retention time differed, suggesting the compound might be an ellagic acid derivative. Qualitatively, hydrolysis was a useful tool; however, high variability was observed when quantifying the content of each compound. Differences in gallic and ellagic acid content were not observed between cultivars (Table 3).

The presence of gentisic, gallic, vanillic, p-hydroxybenzoic, protocatechuic and phenyl acetic acids was observed by Senter et al. (12), who characterized the content of phenolic acids in pecan kernels. The study reports that the content of gallic acid was 130 µg/g of defatted kernel, accounting for 78% of the total phenolic acids; ellagic acid content was not reported. The present study revealed the presence of ellagic and gallic acids in the ranges of 4732 to 2505 and 1300 to 651 µg/g defatted kernel, respectively (Table 3). The difference between the two studies could be explained with the fact that

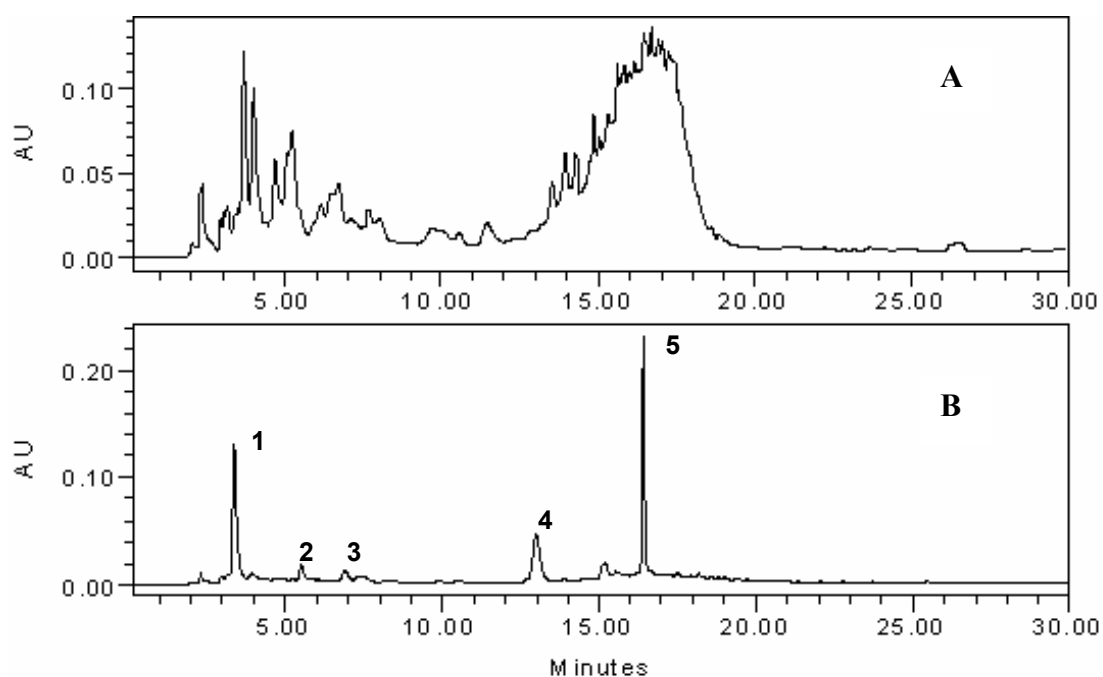


Figure 5. HPLC chromatograms of non-hydrolyzed (A) and hydrolyzed (B) extracts. (1) gallic acid; (2) catechin; (3) epicatechin; (4) tentative ellagic acid derivative; (5) ellagic acid. AU = absorbance units; horizontal axis is retention time in minutes.

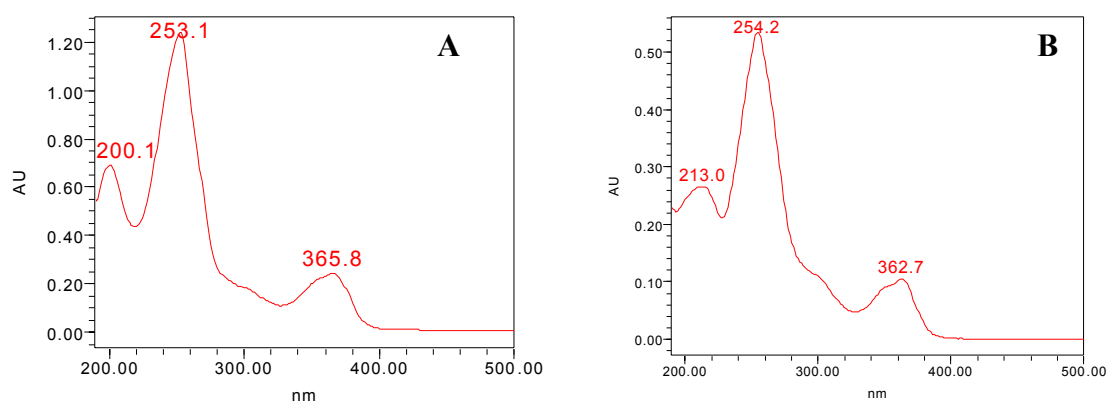


Figure 6. Spectra of ellagic acid (A) and potential ellagic acid derivative (B). Spectra refer to peaks 5 and 4 respectively, found in HPLC chromatograms.

Table 3. Gallic and ellagic acid content of pecan kernels from different cultivars after consecutive basic and acid hydrolyses.

Cultivar	Gallic acid ^a μg GA/g	Ellagic acid ^b μg EA/g
Kanza	1132 ± 321 a ^c	4706 ± 1014 a
Nacono	651 ± 110 a	2505 ± 103 a
Kiowa	1300 ± 112 a	4732 ± 637 a
Pawnee	877 ± 97 a	3008 ± 690 a
Shawnee	748 ± 209 a	2609 ± 475 a
Desirable CW	1056 ± 206 a	4363 ± 699 a
Desirable BW	886 ± 391 a	3981 ± 1345 a
Average ^d	950 ± 87	3701 ± 323

^a Gallic acid content, micrograms of gallic acid per gram of defatted kernel ±S.E.; ^b Ellagic acid content, micrograms of ellagic acid per gram of defatted samples ±S.E.; ^c Mean values followed by the same letter within each column are significantly different (Tukey HSD, $P<0.05$).

ellagic acid is produced in aqueous solutions, where hexahydroxydiphenic acid spontaneously lactonizes to ellagic acid (38). The lack of an aqueous environment and the extreme hydrolysis conditions used by Senter et al. (12) could explain the low amounts of phenolic acids reported and why ellagic acid was not detected. Mattila et al. (39) reported that most phenolic acids are stable under alkaline conditions, and seem to be more resistant to alkaline than to acid hydrolyses.

The presence of ellagic acid in such high levels suggests not only the occurrence of simple phenolic acid esters, but of more complex structures derived from these compounds, such as hydrolyzable tannins. These structures have been found to have antioxidant and chemopreventive activities and to be present in human plasma after consumption (40, 41). The use of hydrolysis treatments and the obtained phenolic compounds suggest the potential presence of compounds such as epigallocatechin, epigallocatechin gallate and other flavonoid derivatives, (11, 42), as well as hydrolyzable tannins. The specific structures of these phenolic acid oligomers need to be investigated to determine the potential health benefits of pecan kernels.

The amounts of catechin and epicatechin in HPLC phenolic profile were minimal when compared to the subunits of hydrolyzable tannins, gallic and ellagic acids (Figure 5B). This could be associated with the alkaline oxidation of these compounds. Jorgensen et al. (43) suggested a degradation of procyanidin subunits during alkaline oxidation.

Liquid and solid phase extractions from raw and hydrolyzed extracts were also analyzed for their HPLC phenolic profile (data not shown). The presence of gallic and ellagic acids, catechin and epicatechin in raw extracts was observed in traces and were amounts extremely low when compared to hydrolyzed extracts. Additional compounds, such as gentisic, vanillic, and protocatechuic acid, previously reported to be present in pecans (12) were not identified.

Fatty Acid Profile. The lipidic content and profile of pecan kernels is well studied and documented (44, 45). In general, lipid content ranges from 65 to 70% and it is moderately affected by variables such as cultivar, environmental effects, and location (44, 45). Fatty acid profile seems to be more dependent on horticultural practices,

environmental conditions, cultivar and maturity (44, 45). In this study, the trend of the main fatty acids content was similar in all cultivars investigated and was oleic > linoleic > palmitic > stearic \geq linolenic. These five fatty acids accounted for 99% of the total fatty acid composition of kernels. There was significant variation in profile between cultivars. Oleic and linoleic acid ranged across cultivars from 53 to 75% and 15 to 36% of lipid composition, respectively (Figure 7). The relative content of palmitic, stearic and linolenic acid did not vary significantly across cultivars. ‘Desirable’ CW and ‘Desirable’ BW had significant differences in their oleic-linoleic composition, despite being the same cultivar. Factors such as horticultural practices, environmental conditions and genetic material have been suggested to influence lipid composition (44, 46, 47). A strong inverse correlation between oleic and linoleic acid, ($R^2 = 0.99$) (Figure 8). Furthermore, it was observed a parallel increase in the content of oleic and linoleic oil during fruit maturation until 7 weeks before harvest. After this time linoleic oil content started to decrease while oleic oil started to increase (44). This additional oleic oil content is due to double bond saturation of existing linoleic oil during last stages of maturation. Late maturation horticultural practices and environmental factors should be addressed in future investigations of oil synthesis and composition in pecan kernels.

When compared to other oils, pecan oil has a high oleic content that suggests greater stability against oxidation as reported by Rudolph et al. (48).

Tocopherol Content. Tocopherol content and profile in pecan kernel oil is well documented. Variables such as genetics, environment, maturity, storage conditions, affect tocopherol content of pecans (14, 44, 49, 50). Content of γ -tocopherol found ranged from 72 to 135 $\mu\text{g } \gamma\text{-Toc/g}$ (Table 4). There is controversy about tocopherol content of pecan oil and values have been reported a number of times with great variation (14, 47, 51). Toro-Vaquez et al. (47) reported similar composition and tocopherol content of olive and pecan oils. However, it was reported that pecan oil was more stable against rancidity than olive oil, suggesting the presence of other antioxidant compounds in addition to tocopherols that may be imparting protection.

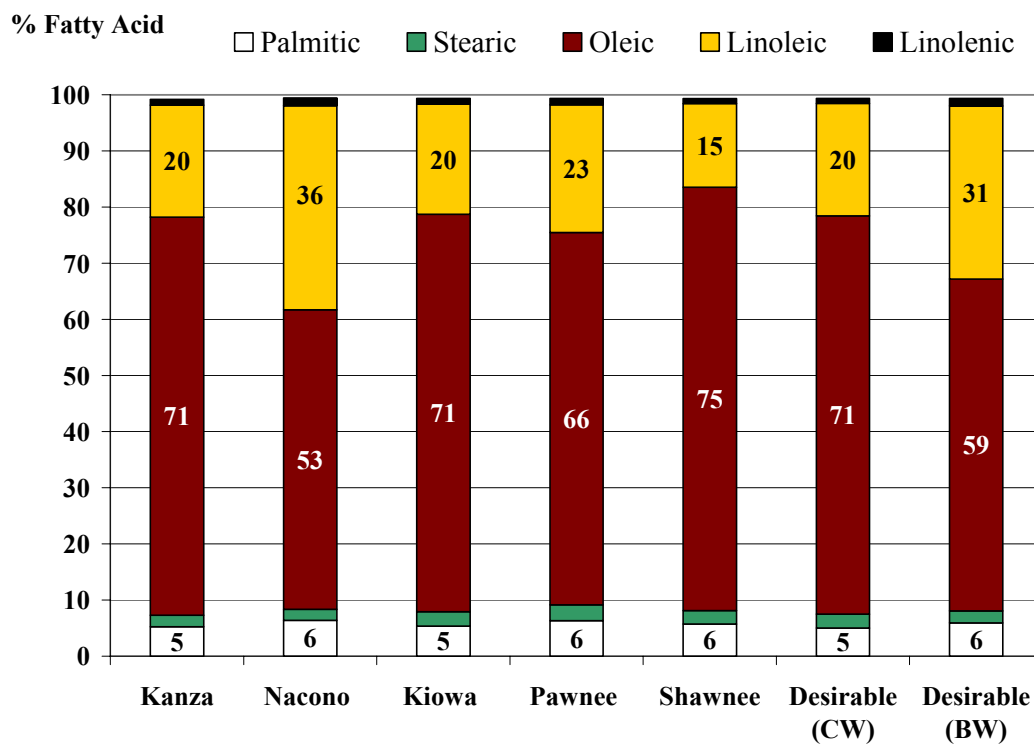


Figure 7. Fatty acid profile of pecan oil extracted with hexane expressed as percentage of total fatty acid content.

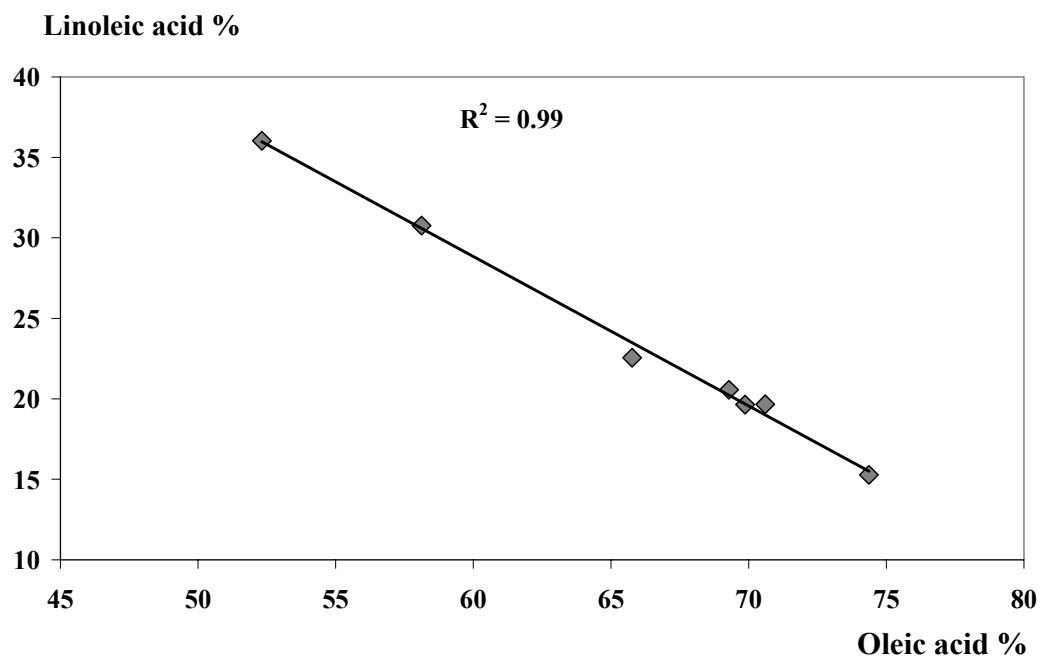


Figure 8. Correlation between linoleic and oleic oil of pecan oil extracted with hexane and expressed as percentage of total fatty acid content.

Table 4. Oleic and linoleic acid composition of pecan oil and content of γ -tocopherol for different cultivars.

Cultivar	Oleic acid %	Linoleic acid %	γ -Toc ^a ($\mu\text{g } \gamma\text{-Toc/g}$)
Kanza	71	20	105 \pm 1 b
Nacono	53	36	135 \pm 4 a
Kiowa	71	20	72 \pm 6 d
Pawnee	66	23	100 \pm 1 bc
Shawnee	75	15	102 \pm 2 b
Desirable CW	71	20	126 \pm 5 cd
Desirable BW	59	31	84 \pm 1 a
Average	66	24	104 \pm 8

^a γ -tocopherol content, grams of γ -tocopherol per gram of oil \pm S.E.; values with similar letters are not significantly different (Tukey HSD, $P < 0.05$).

Horticultural practices, maturity, location and environmental conditions affect pecan physiology and final composition of kernels (44-47, 52). In the present study, pecans of five different cultivars were harvested at the same location (Brownwood, TX) which implicates that the trees grew in very similar conditions and were treated with the same horticultural practices. However, differences between cultivars were found for most of the variables analyzed. Future research should be addressed to determine if horticultural practices have any effects on phytochemical content depending on cultivar genetics. An additional issue to be investigated could be the role of crop load on phytochemical composition of pecan kernels. The tendency of pecan trees to bear fruit in 2-year cycles (i.e., large crops followed by little or no crop), can cause differences in compositional attributes of kernels, such as fatty acid composition and tocopherol content (45).

Health benefits of wine and berries have been linked to the content of gallic and ellagic acids and their oligomeric forms (40, 53, 54). More information is needed about the bioavailability of these phenolic compounds in pecans and the role of storage or decontamination processes on phytochemical composition.

Content of fatty acids makes pecan kernels susceptible to oxidation, as any other food with high lipidic content. This oxidation is accelerated by improper storage conditions. Long storage periods at room temperature in the presence of oxygen, together with other procedures such as sanitizing or roasting can deteriorate the phytochemical profile of kernels. Chapter III will report the effects of irradiation (E-beam) on phytochemical content, antioxidant capacity, color and oxidative status through accelerated storage of pecan kernels.

CHAPTER III

EFFECTS OF E-BEAM IRRADIATION ON ANTIOXIDANT PROFILE OF PECAN KERNELS

Summary

Pecans nuts from ‘Kanza’ and ‘Desirable’ cultivars were irradiated with 0, 1.5 and 3.0 kGy using electron beam (E-Beam) irradiation and stored under accelerated conditions (40 °C and 55-60% R.H.). Antioxidant capacity (AC) using DPPH free radical (AC_{DPPH}) and ORAC assay (AC_{ORAC}), phenolic (TP) and condensed tannin (CT) content, HPLC phenolic profile, tocopherol content, peroxide value (PV) and fatty acid profile were evaluated in kernels after 0, 7, 21, 55 and 134 days of storage. Irradiated kernels had no detrimental effects in AC and TP; however variation was found throughout storage. Tocopherol content decreased after irradiation treatments for both cultivars, but no further decrease was observed through storage. Irradiated ‘Desirable’ samples had greater PV than controls and ‘Kanza’ 1.5 kGy samples had increased PV after 98 days of storage. No change in fatty acid composition was detected for any cultivar. Color determined by Hunter Lab scale had a gradual decrease in lightness and yellowness while redness had an initial increase followed by a decrease after 98 days of storage. No differences for phenolic profile were observed, though no compounds were detected prior hydrolysis. Compounds identified by HPLC in hydrolyzed extracts were gallic and ellagic acid, catechin and epicatechin. In general, besides tocopherol content, no detrimental effects were found in antioxidant composition caused by irradiation treatments. While a faster oxidation rate was seen in irradiated kernels for one cultivar, no other quality attribute was affected by E-Beam irradiation.

Introduction

Pecan [*Carya illinoensis* (Wangenh.) K. Koch] is the only nut cultivated in the U.S. which is native to North America. The U.S. and Mexico are the greatest producers of this nut, with an average of 113,000 and 63,000 tons produced annually, respectively

(16). Mexico is also the principal pecan exporter to the U.S., and, in the last decade, pecan imports from Mexico averaged 27,000 tons. Pecans are not only transported from Mexico to the U.S., Mexico is also the principal importer of U.S. pecans. The U.S. imports pecans from Mexico during the harvest season and are exported back to Mexico during the rest of the year, due to its limited pecan storage capacity.

At harvest, pecan trees are shaken and nuts fall onto the ground. Nuts are then gathered in windrows and harvested by sweeping the orchard floor, thus increasing the risk of contamination with fungi, viruses or bacteria. The risk of bacterial contamination, in particular by *Escherichia coli*, is even higher if cattle are allowed to enter the orchard, which is common practice especially in native pecan orchards. *E. coli* is an extremely dangerous bacterium if it comes in contact with food intended for human consumption. Some *E. coli* strains like 0157:H7 have been responsible for several outbreaks across U.S. causing death of susceptible individuals like children (55). Pecans can also be contaminated with fungi, such as *Aspergillus*, which are capable of producing aflatoxins, some of the most feared microbial toxins for their high carcinogenic potency (levels allowed in cereals are less than 20 ppb). So far, contaminations have not been recorded for pecans, but they are a possible threat, as they are for other nuts (e.g., walnuts and almonds) which are harvested similarly to pecans. In 2004, the Food and Drug Administration (FDA) issued a press release regarding an outbreak of *Salmonella enteritidis* likely due to the consumption of raw almonds. These events lead the almond industry to investigate several decontamination procedures, including irradiation.

Food irradiation is mostly known as a food safety procedure and involves exposure of food to different sources of ionizing energy. Ionizing radiation can kill pathogenic microorganisms by direct or indirect DNA damage. Ionization of water molecules causes loss of one electron, producing reactive species such as hydrogen and hydroxyl radicals, and hydrogen peroxide which can oxidize lipids and produce undesirable compounds (56). Ionizing energy sources include γ -rays, X-rays and electron-beam (E-Beam) beta rays. γ -rays are generated by a radioactive substance (Cobalt 60 or Cesium 137), while to produce X-rays, a beam of electrons is directed at a

thin plate of gold or other metal. Like γ -rays, X-rays can pass through thick foods, and require heavy shielding for safety. Similarly to X-rays, radioactive sources are not required for E-Beam irradiation. Instead, a stream of high energy electrons is propelled out of an electron gun at 99.999% the speed of light. When compared to γ -rays, E-Beam does not produce continuous energy such as γ -rays. However, E-Beam can only penetrate a depth of 3 cm, which is much smaller than γ -rays and X-rays (56), restricting its use to low-depth products. E-Beam has not yet been tested on pecan kernels. However, irradiation with γ -rays up to 1 kGy was used to decrease aflatoxigenesis by *Aspergillus flavus* in pecan kernels. No detrimental effects in oxidative stability or sensorial scores were detected (17). Authors suggested the use of higher levels of ionizing irradiation to increase the inhibition of post-harvest fungi.

γ -irradiation helps inactivate oxidative enzymes such as lipoxygenases. Lipoxygenase has been identified as cause of lipid peroxidation in pecan kernels and is inactivated by different procedures such as steam and dielectric heating (57, 58), which can be used to increase their shelf life. Enzyme inactivation by irradiation technology makes it a potential tool to extend shelf life of foods with high lipid content (59). Pecan kernels could be a good candidate for this technology.

Recently, pecan have been included in the high phenolic content food group (9). Phenolic compounds are present in various food systems and are regarded as antioxidants (12, 60-62). In addition, there is abundant evidence suggesting health benefits and prevention of chronic diseases by these compounds (22, 24).

Pecan kernels high lipid content (~65%) makes them susceptible to oxidation (51, 63). However, the protective effect of phenolic against oxidation may allow the use of technologies such as irradiation. Despite all information about ionizing radiation and its effects on food components, there is no information regarding the effects of E-Beam irradiation on phenolic compounds and other antioxidants present in pecan kernels.

The objective of this study was to evaluate the effects of various E-Beam irradiation doses on antioxidant profile and quality parameters of pecan kernels during accelerated storage conditions.

Materials and Methods

Chemicals and Apparatus. Solvents used were HPLC grade and purchased from Fisher Scientific (Houston, TX). Folin-Ciocalteu reagent, vanillin reagent, 6-hydroxy -2,5,7,8- tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein sodium salt (FL), p-anisidine, and phenolic standards were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2'-Azobis (2-amidino-propane)-dihydrochloride (AAPH) was obtained from Wako Chemicals (Richmond, VA). Nanopure water was used for aliquots and buffers. A Synergy HT plate reader equipped with fluorescence and absorbance and KC-4 v. 3.4 analytical software (Bio-Tek Instruments, Inc., Winooski, VT) were used with different 96-well plates depending on fluorescence or absorbance measurements. A photodiode array spectrophotometer (Hewlett-Packard 8425A, Waldbronn, Germany) was also used to perform assays requiring absorbance measurements with greater volumes.

Sample Preparation and E-Beam Irradiation. Pecan nuts were mechanically harvested during Fall 2004 from trees of 'Kanza' and 'Desirable' cultivars. Cultivars were chosen due to their commercial relevance. 'Kanza' nuts were grown at the USDA Experiment Station located in Brownwood, Texas (BW). 'Desirable' nuts were harvested from a commercial orchard located near Caldwell, Texas (CW). After harvest, about 18 kg of nuts per cultivar were transported to the laboratory and stored in cold room set at -5 °C. Before mechanical cracking and shelling, nuts were equilibrated for 24 h at 5 °C. After removal of rotten and necrotic kernels, pecan halves were stored at -20 °C in freeze-resistant plastic bags.

Prior irradiation, kernels were divided in ~200 g sets and placed in sealable plastic bags. Bags were laid over a cardboard tray, with kernels placed in single layer beds. In each cardboard tray, two alanine dosimeters were inserted in two pecan halves to monitor the amount of energy applied (64). A total of 24 pecan bags were treated with 0, 1.5 or 3.0 kGy using a single electron beam linear accelerator at the National Institute for Electron Beam Food Research (Institute of Food Science and Engineering, College Station, TX). Doses applied were such that the 1.5 and 3.0 kGy absolute minimum were

achieved including the ranged $\pm 7.5\%$ error margin for the material, transport system, accelerator and dosimetry.

After irradiation, pecan bags were perforated and stored in an oven set at 40 °C and a relative humidity of 55-60% using a ReliON humidifier model RWM-975 (Southborough, MA). One bag of each treatment from both cultivars was extracted from the oven at 0, 7, 21, 55, 98 and 134 days of storage and analyzed for its oxidative status, phenolic profile, antioxidant capacity, tocopherol content, and fatty acid profile.

Oils from non-irradiated kernels of both cultivars was extracted, placed inside an open beaker and stored at the same conditions as pecan kernels. Oxidative status by peroxide values was also determined in these oils.

Extraction Methods. Kernels were chopped using a food processor and then defatted by homogenizing samples with hexane (1:20 w/v) using an Ultraturrax T25 homogenizer (IKA Works, Wilmington, NC). After homogenizing, samples were filtered with a Buchner funnel and slow-filtration rate filter paper. The cake was defatted two more times and the remaining powder was dried at 35 °C under vacuum for 2 h. The powder was flushed with nitrogen and stored in a sealed container at -20 °C until analyses. Oil was obtained from filtrates after hexane was evaporated using a rotavapor. The oil was flushed with nitrogen and stored at -20 °C until analyses of the lipidic fraction.

Defatted pecan powders (1 g) were placed in 50 mL falcon tubes and homogenized with 20 mL of acetone:water (70:30) solution. Falcon tubes were capped, placed in an oscillatory shaker at 5 °C and shaken overnight. After shaking, slurries were centrifuged at 18,000 g and supernatants were collected, flushed with nitrogen and stored at -20 °C.

Antioxidant Capacity. DPPH free radical was used to measure antioxidant capacity (AC_{DPPH}) as described by Brand-Williams et al. (30) and adapting the methodology to be used in a microplate reader. Extracts were diluted in methanol and 13 μ L was pipetted into each well of a 96 well flat bottom plate (Costar #3595, Corning, Inc., Corning, NY). Using a multichannel pipette, 247 μ L of DPPH aliquot in methanol

was added. Plates were then tightly sealed with two layers of parafilm to prevent evaporation and placed in the dark at 20 °C. After 24 h, microplates were read at 515 nm in a plate reader and readings of blanks with water were subtracted from samples readings. A standard curve was prepared using trolox as reference reagent. AC was expressed as micrograms of trolox equivalents per gram of defatted sample ($\mu\text{g TE/g}$).

ORAC Assay. A modification of the procedure described by Wu et al. (9) to measure hydrophilic antioxidant capacity (AC_{ORAC}) was used. All reagents were dissolved in 75 mM phosphate buffer pH 7.4. Clear-bottom 96-well black plates (Costar #3631, Corning, Inc., Corning, NY) were loaded with 25 μL of extracts per well and incubated at 37 °C for 45 min prior analysis. Fluorescein sodium salt (FL) was used as protein probe and AAPH as free radical source. FL stock solution (FL_1) was prepared by diluting 112.5 mg of FL powder in 50 mL of phosphate buffer using a volumetric flask. A second FL solution (FL_2) was made by diluting 100 μL of FL_1 in 10 mL of buffer. After preparation, FL_1 and FL_2 solutions were stored at 2 °C. Prior analysis a third solution (FL_3) was made by dissolving 400 μL of FL_2 in 25 mL of buffer. AAPH solution was prepared after incubation of buffer at 37 °C for 45 min and prompt dissolution of 260 mg of AAPH pellets. After preparation, reagents were placed in plastic containers incubated in an oven at 37 °C and used to prime plate reader injectors. After priming, 200 μL of FL_3 were injected in each well followed by 75 μL aliquots of AAPH solution.

Fluorescence readings were done using excitation and emission wavelengths of 485 nm and 520 nm, respectively. Each well was read at ~1 min intervals during 50 min. The loss of fluorescence was recorded and processed by analytical software package KC-4 v. 3.4. Samples were compared against trolox standard and a blank curve. Data were normalized and the area under the curve (AUC) was calculated for each well as follows:

$$\text{AUC} = (f_1 + f_n)/2 + (f_1 + f_2 + \dots + f_{n-2} + f_{n-1})$$

where f_1 and f_2 are the first and second relative fluorescence values of n samples, respectively, and f_n is the last relative value of the well.

One blank curve was elaborated for each row and subtracted from each value of the same row to avoid variability between them. Blank wells AUC values were subtracted from sample and trolox standard AUC values (NetAUC). NetAUC samples were compared against trolox standard NetAUC from the same plate row. Results were expressed as micromoles of trolox equivalents per gram of defatted kernel ($\mu\text{mol TE/g}$) using the next formula:

$$\mu\text{mol TE} = [(\text{NetAUC}_{\text{sample}})/(\text{NetAUC}_{\text{standard}})] \times \text{Concentration of Standard (40 } \mu\text{M)}$$

Extractable Phenolic and Condensed Tannin Content. TP analysis was performed as explained by Swain and Hillis (31), adapting the methodology to be used in a microplate reader. Defatted pecan extracts were diluted with water and 13 μL was loaded in each well of a 96 well flat bottom plate (Costar #3595, Corning, Inc., Corning, NY). Water was added to six wells to be used as blanks. Using a multi-channel micropipette, 208 μL of nanopure water was added to each well followed by 13 μL of Folin-Ciocalteu reagent. Mixture was allowed to react for 3 min. After time elapsed, 26 μL of 1 N Na_2CO_3 was added. Plates were sealed using two layers of parafilm and allowed to react for 2 h, after which absorbance was read at 725 nm in the microplate reader. A standard curve made with chlorogenic acid was elaborated to express TP as milligrams of chlorogenic acid equivalents per gram of defatted kernel (mg CAE/g def kernel). Six replicates of each sample were analyzed.

Procyanidins or CT were evaluated using the vanillin assay (32). An aliquot of 0.5 g of defatted kernel was placed in a centrifuge tube and 15 mL of 1% HCl in methanol was added to each sample. Each tube was vortexed and placed in a water bath at 30 °C with constant shaking for 20 min and vortexing every 10 min. After incubation, tubes were centrifuged and supernatants were extracted. Aliquots of the supernatants were placed in two separate assay tubes, one for the sample determination and the other

for blank determination. Samples and blanks were incubated for exactly 20 min after adding 5 mL of the vanillin reagent to samples and 4% HCl in methanol to the blanks. After 20 min, absorbance was read at 500 nm from of each sample and blank using a HP 8452A diode array spectrophotometer (Hewlett Packard, Palo Alto, CA). Samples absorbance was rectified with the blank standard and compared against a standard curve made with catechin. Results were expressed as milligrams of catechin equivalents per gram of defatted sample (mg CE/g def kernel). Six replicates of each sample were used.

Phenolic Hydrolysis and Reverse-Phase HPLC Analysis. To determine phenolic profile, extracts used for AC and TP assays were analyzed by high performance liquid chromatography (HPLC). Acetone from extracts was evaporated under vacuum using a SpeedVac concentrator (Thermo, Marietta, Ohio) and 1 mL of the water residues was transferred to an assay tube and diluted with 1 mL of 8 N NaOH. Samples were flushed with nitrogen, capped and allowed to react for 16 h in the dark. After basic hydrolysis, 1.33 mL of 6 M HCl was added to the tubes and flushed with nitrogen, capped and heated using a block heater (Fisher Scientific, Houston TX) at 85 °C for 45 min. After acid hydrolysis, 1 mL of samples was filtered with a 0.2 µm PTFE filter (Fisher Scientific, Houston, TX) and 20 µL was injected into the HPLC system. The HPLC system was equipped with two Waters 515 gradient pumps (Waters Corp., Milford, MA) and coupled with a Waters 717 autosampler (Waters Corp., Milford, MA). An Atlantis C18 column (5 µm particle size, 4.6 mm x 150 mm; Waters Corp., Milford, MA) coupled with a guard column of the same chemistry was used to separate phenolic compounds. A photodiode array detector was used to scan absorbance from 190 nm to 500 nm. Peak spectra, retention times, and standard spikes were used for determination of compounds. Nanopure water, acidified to pH 2.3 with 2 M HCl (solvent A) and acetonitrile (solvent B) were used as mobile phases. Solvent gradient was used as follows: from 0 to 5 min isocratic 85% A flow, from 5 to 30 min a linear gradient of 85% A to 100% B, and from 35 to 40 min isocratic conditions of 100% B. After termination of the cycle, 30 min of column equilibration (85% A) were allowed prior next injection. Standard curves of the identified compounds were elaborated by

dissolving standards in methanol and injecting them into the HPLC. Individual phenolic content was evaluated at 0 and 134 days of storage. Samples and standard curves were analyzed by triplicate.

Fatty Acid Profile. Fatty acid methyl esters were analyzed in a Varian CP 3800 gas chromatograph (Palo Alto, CA) coupled with a Varian CP-8200 autosampler and a flame ionization detector (FID). A Varian FAME fused silica capillary column (100 m x 0.25 mm, Varian CP-Select) was used to determine lipid profile. Oven temperature was set from 0-30 min at 185 °C and from 30-45 min at 235 °C with an increase of 20 °C/min. FID temperature was set at 270 °C, and helium, air and hydrogen flows at 1.6, 300 and 35 mL/min, respectively. Fatty acid derivatization was performed using the protocol described by Misir et al. (33). Approximately 0.15 g of oil was diluted in 3 mL of diethyl ether and 0.2 mL of 20% tetramethyl ammonium hydroxide in water was added and allowed to react for 5 min. After time elapsed, 0.5 mL of methanol was added and vortexed for 1 min. After phase separation, 1 mL of the upper organic phase was transferred to a vial and capped for injection into the gas chromatograph. Fatty acids were identified and expressed as percentage of total fatty acid content.

Tocopherol Content. Tocopherol content was determined as explained by Mendoza et al. (34). Pecan oil was weighted (~0.5 g) and 2 ml of methanol was added. After the sample was vortexed for 1 min, 500 μ L of hexane was added and vortexed again. Samples were then centrifuged and 1 mL of the top layer was carefully extracted, filtered using 0.2 μ m PTFE filters and 20 μ L were injected into an HPLC. The HPLC system was equipped with a Lichrosorb® Spherisorb ODS2 C18 column (5 μ m particle size, 4.6 mm x 250 mm) and a guard column of the same chemistry (Waters Corp., Mildford, MA). An isocratic flow of 1 mL/min of methanol 100% was used as mobile phase. Concentration of α -, β -, and γ -tocopherols standard solutions were determined as suggested by AOCS official method Ce 8-89 (35). Peak spectra at 295 nm and retention times were used for identification and quantification. Only γ -tocopherol is reported due to minimal detection of other isomers. Values are reported as micrograms of tocopherol per gram of oil (μ g γ -Toc/g).

Oxidative Status. Oxidation of oils was assessed using the peroxide value (PV) and p-anisidine value. The protocol followed for PV was the A.O.C.S. Cd 8b-90 (65) using, isooctane as recommended by the methodology. Values are reported as milliequivalents of peroxide (O_2) per kilogram of oil (mE O_2 /kg oil). P-anisidine value was evaluated using the A.O.C.S. official method Cd 18-90 (66).

Lipoxygenase activity. Enzymatic oxidation by lipoxygenases was evaluated as suggested by Villafuerte et al. (67). Pecan kernels were weighted and homogenized with cold acetone ($-20\text{ }^{\circ}\text{C}$). The slurry was filtered through a Whatman no. 1 filter and this procedure was repeated until filtrate was colorless. The cake was dried with nitrogen flow and powders were used to evaluate enzymatic activity using the methylene blue assay and conjugate diene production (67).

Color. Pecan kernels were evaluated for their color after storage using a LabScan II model SN-12384 equipped with Universal Beta v. 2.4 software (Hunter Associates Laboratories Inc., Reston, VA). Three kernels from each treatment were placed in the color reader and evaluated using the CIELAB $10^{\circ}/D65$ as scale and light source. Values are reported as lightness (L), redness (a) and yellowness (b).

Moisture. Moisture content was evaluated by placing 5 g of chopped pecan kernels in an Isotemp Vacuum Oven Model 285A (Fisher Scientific, Houston, TX) set at $70\text{ }^{\circ}\text{C}$ and -381 mmHg . After 24 h weight difference was recorded and reported as water percentage.

Statistical Analysis. To determine statistical difference between means ($P \leq 0.05$), ANOVA and Tukey's honestly significant difference (HSD) were calculated using SPSS statistical software package v. 11.5 (SPSS Inc., Chicago, IL).

Results and Discussion

Antioxidant Capacity and Total Extractable Phenolics. Pecans were stored under accelerated conditions and evaluated for their antioxidant capacity (AC) and total extractable phenolic content (TP). In general, an increase in AC_{DPPH} and AC_{ORAC} was observed when samples were analyzed after 7 days of storage, except for AC_{ORAC} in

‘Kanza’ (Figure 9 and Figure 10). The greatest initial increase in AC_{DPPH} (42%) was observed for ‘Kanza’ control samples on day 7 (Figure 9A) followed by 1.5 kGy samples (23%). Samples treated with 3.0 kGy had no significant increase in AC_{DPPH} throughout storage (Figure 9A). ‘Desirable’ 1.5 kGy samples had the greatest initial increase (28%) on day 7 followed by kernels treated with 3.0 kGy (18%) (Figure 9B). Control samples for ‘Desirable’ kernels had no substantial increase throughout the experiment. By the end of storage period, final AC_{DPPH} for ‘Kanza’ 1.5 and 3.0 kGy kernels did not differ from initial values ($P \leq 0.05$). AC_{DPPH} for ‘Desirable’ 1.5 and 3.0 kGy samples decreased 11 and 18% respectively by the end of storage (day 134) (Figure 9B).

AC_{ORAC} values for ‘Kanza’ kernels gradually increased from day 7 until day 55 for control and 3.0 kGy samples (27 and 17% respectively) (Figure 10A). ‘Desirable’ samples peaked on day 21 in all treatments (48, 35 and 25 % greater than day 0 values for control, 1.5 and 3.0 kGy samples, respectively), followed by a slow decrease (Figure 10B). As observed for AC_{DPPH} , final AC_{ORAC} values were not different from day 0. In general, values obtained for both AC assays were retained throughout the experiment, suggesting high antioxidant activity of the compounds present at any given time during storage.

These results revealed unexpected responses of non-lipophilic antioxidants to storage conditions. Increases of AC values in both assays suggest the development of compounds with enhanced antioxidant activity in the initial period of storage and with different mechanisms of action. Formation of compounds with increased ability to donate electrons or transfer hydrogen atoms is reflected in the AC_{DPPH} and AC_{ORAC} , respectively (68).

Flavonoids can polymerize if given adequate conditions (36) and it has been suggested that these polymers may be more potent antioxidants than their monomers (69, 70). The oxidation of flavonoids into polymers of varying degrees of polymerization has been reported in pecan kernels stored for 7 days at 70 °C (36). In the same study, authors reported that this oxidation is progressive, which suggests an increase in degree of

polymerization over time. The results in the present study exhibit different sensitivity of AC assays to flavonoids and their degree of polymerization. After 7 days of accelerated storage AC_{DPPH} increased (Figure 9) while AC_{ORAC} values remained stable (Figure 10), thus indicating that AC_{DPPH} is more sensitive to those antioxidants formed in the earlier stages of storage.

TP in ‘Kanza’ samples significantly decreased from day 0 to day 134 of storage. Control, 1.5 and 3.0 kGy samples decreased 14, 20 and 19%, respectively (Figure 11A). The only treatment that had significant decrease for ‘Desirable’ cultivar was the 1.5 kGy set (Figure 11B).

The Folin-Ciocalteu reagent assay is considered to have a similar chemistry to that of AC_{DPPH} and is common to find strong correlations between these two assays (68). However, initial increase in AC_{DPPH} for ‘Kanza’ cultivar (Figure 9A) was not reflected by TP (Figure 11A). An explanation for this event is reported by Yoshida, et al. (71). In the presence of galloylated compounds DPPH produces galloyl radical. This compound (galloyl radical) is highly reactive and can scavenge other DPPH molecules, leaving other antioxidant compounds free to react with other DPPH molecules. Further oxidation of galloylated compounds during storage might have stopped the formation of galloyl radicals with DPPH free radical causing later decrease in AC_{DPPH} (Figure 9A).

CT decreased 19 and 17% in ‘Kanza’ 1.5 and 3.0 kGy samples, respectively, at day 7 of storage, followed by a gradual reduction until day 134 (Figure 12A). ‘Desirable’ had similar behavior but CT for control samples started decreasing at a faster rate after day 21 of storage (Figure 12B). At the end of storage, control samples reached CT levels similar to those of the irradiated kernels, thus indicating that irradiation did not further affect flavonoid content. Reduction of CT in irradiated samples was reported in other studies (72, 73). Breitfellner et al. (73) found that irradiation induced a rapid degradation of catechin and other flavonoids in water solutions. However, such great degradation was not seen after irradiating strawberries with the same doses (0 to 6 kGy) (72), thus implying the presence of a protection mechanism of flavonoids in the fruit

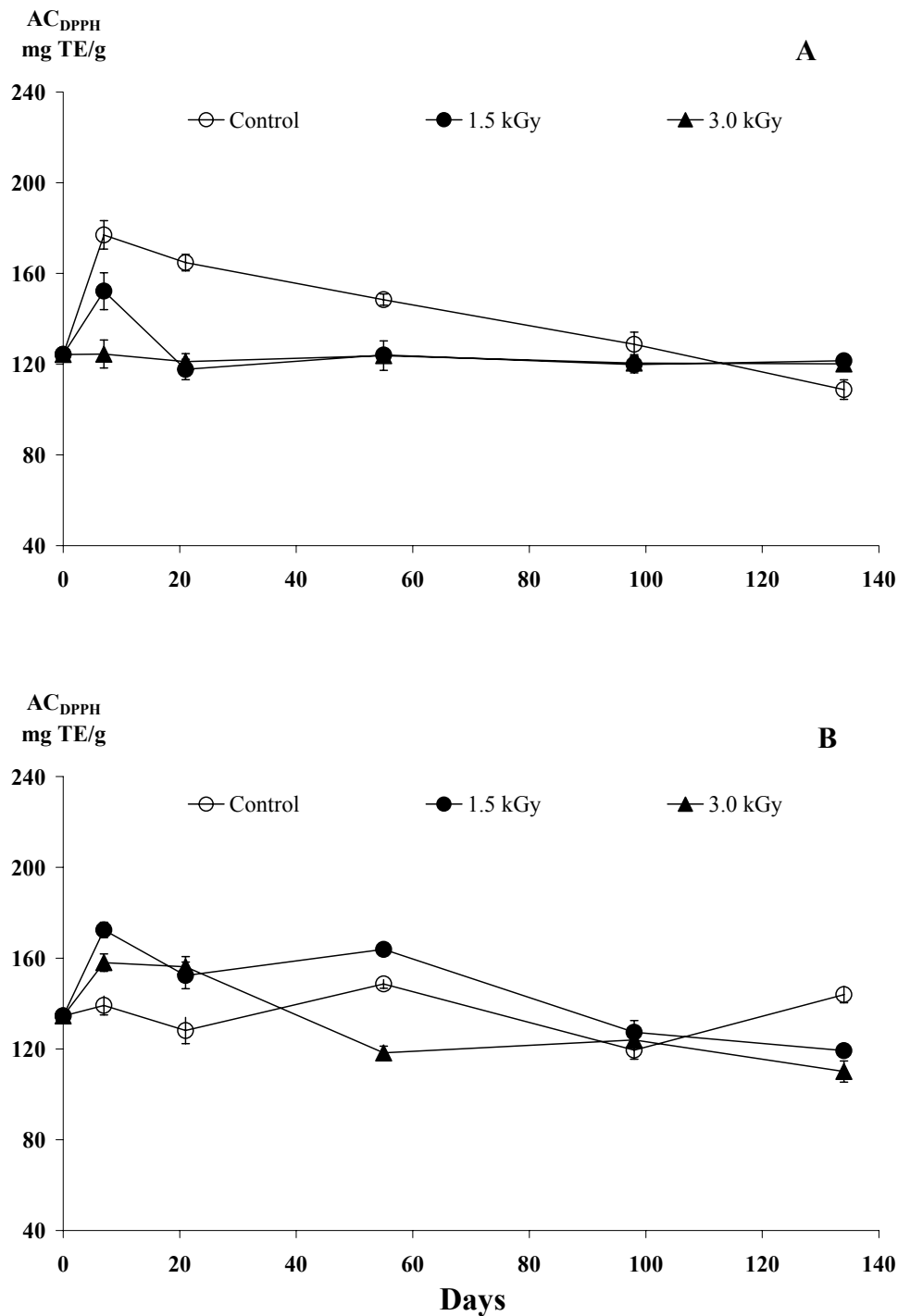


Figure 9. Antioxidant capacity for DPPH assay (AC_{DPPH}) of 'Kanza' (A) and 'Desirable' (B) kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

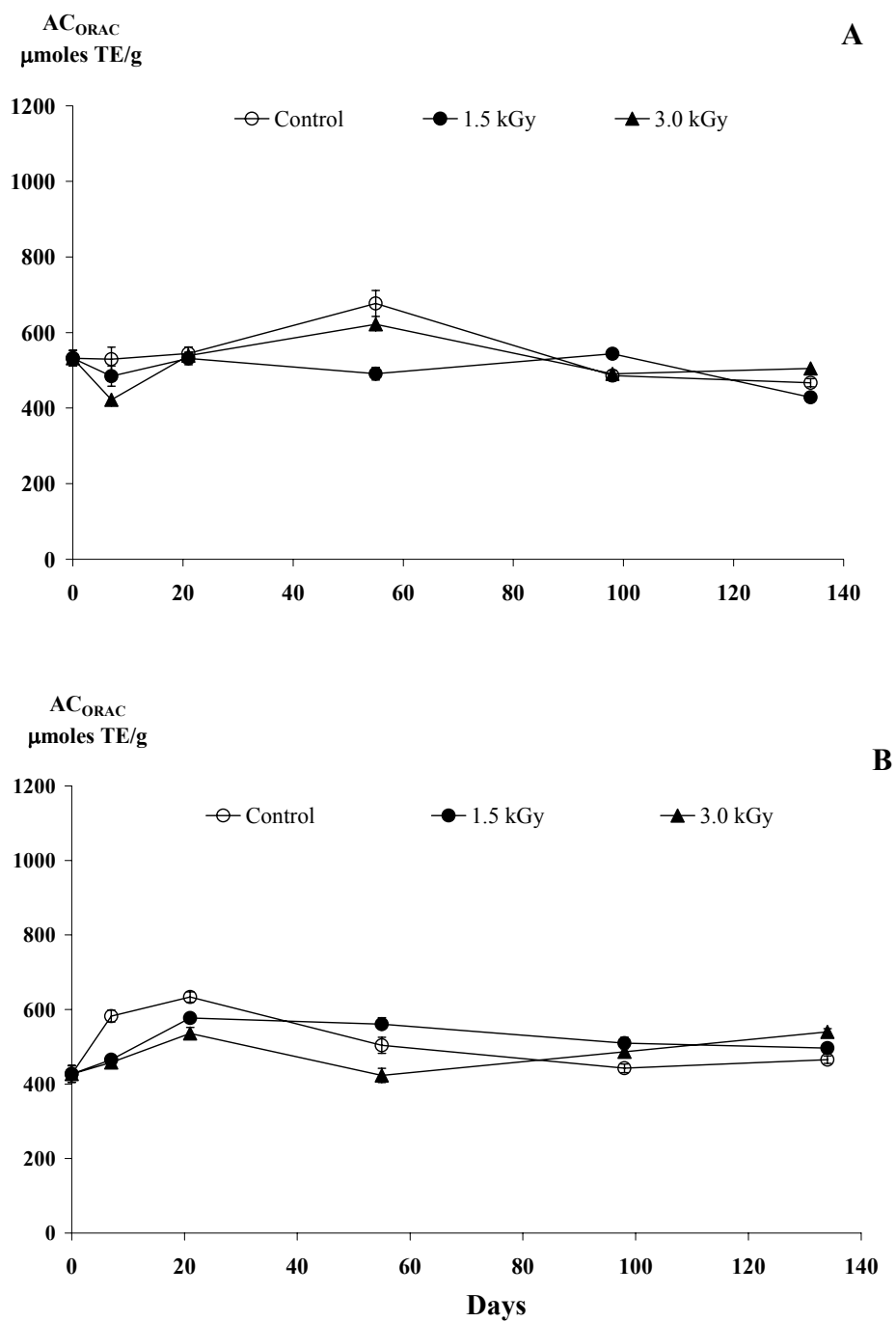


Figure 10. Antioxidant capacity for ORAC assay (AC_{ORAC}) of ‘Kanza’ (A) and ‘Desirable’ (B) kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

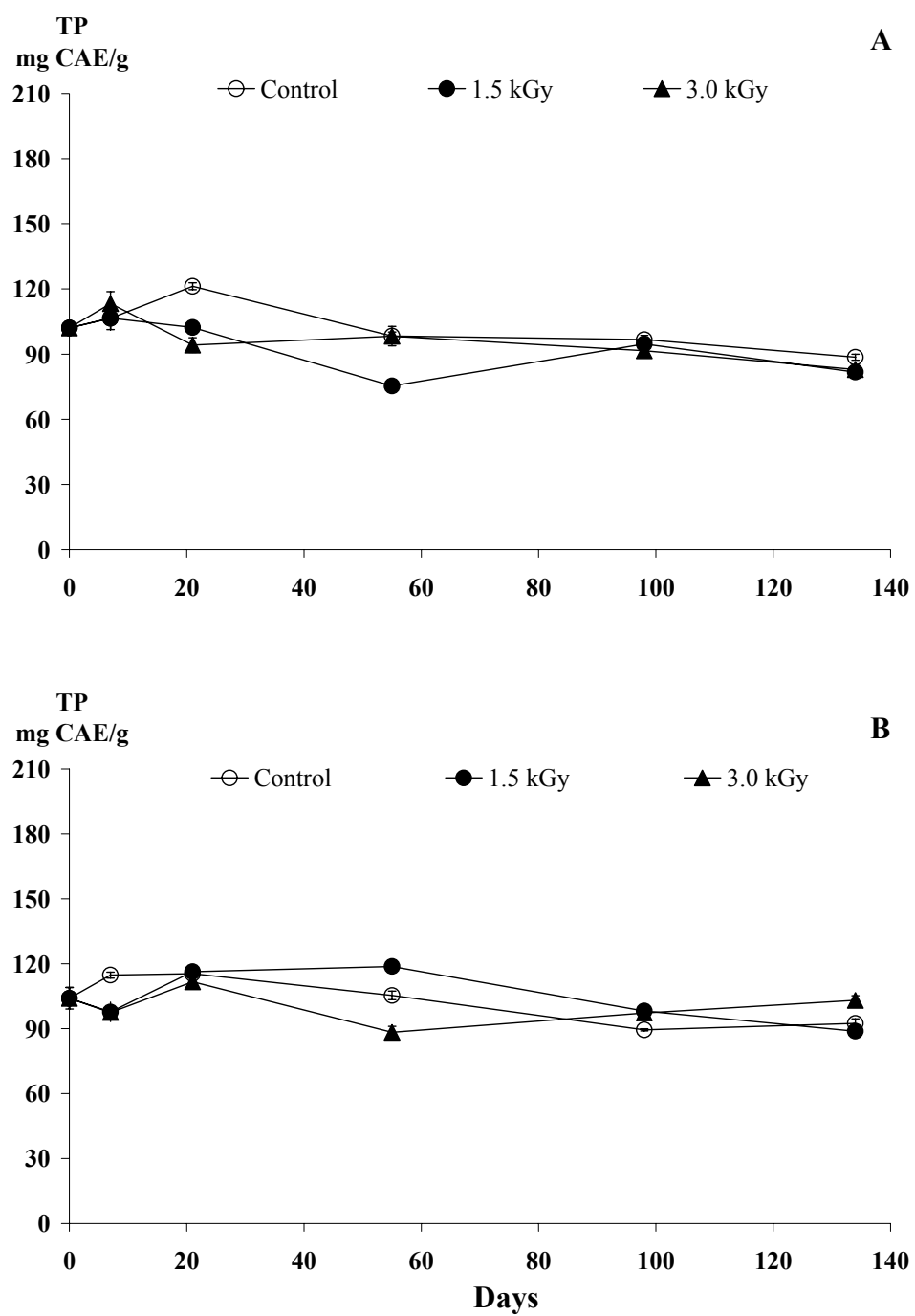


Figure 11. Extractable phenolic content (TP) determined by Folin-Ciocalteu reagent of 'Kanza' (A) and 'Desirable' (B) kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

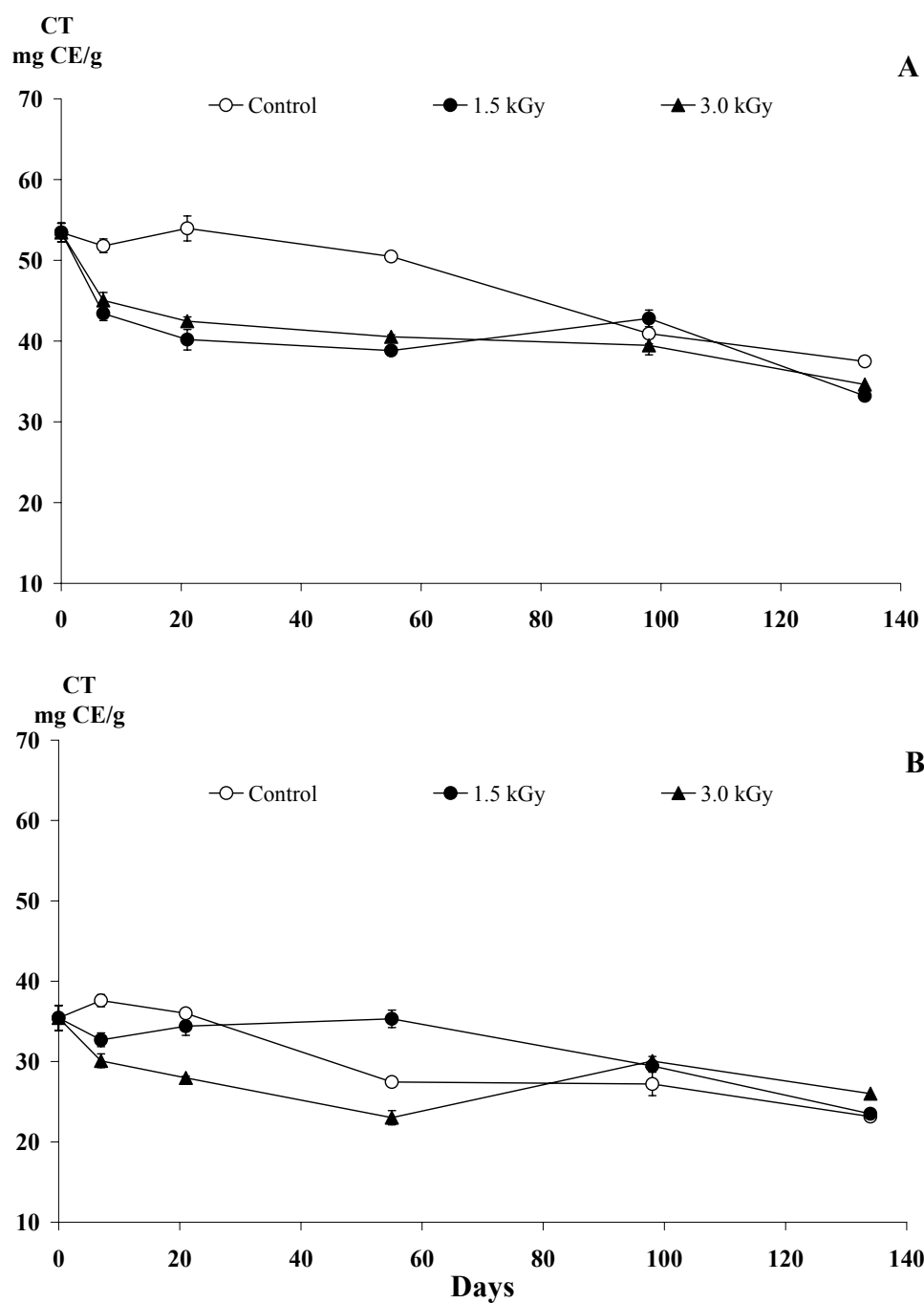


Figure 12. Condensed tannin content (CT) determined with vanillin-HCl assay of 'Kanza' and 'Desirable' kernels irradiated with 0, 1.5 and 3.0 kGy stored at 40 °C and 55% R.H.

matrix. In pecans, low water content of pecans (3-5%) might have avoided greater formation of free radicals, providing greater oxidative stability for these compounds.

Phenolic Acid Profile. Phenolic HPLC profiles for irradiated and non-irradiated samples were similar and no peaks were identified in acetone:water (70:30) extracts (Figure 13). After hydrolyzation with a base (NaOH 4N) followed by an acid (HCl 6M), gallic and ellagic acid were identified and similar chromatograms were observed for all samples. A great variation was found for both compounds (Table 5) and differences or trends could not be confirmed. Gallic acid level for ‘Desirable’ reached 3.2 mg/g of defatted kernel for 1.5 kGy samples (Table 5). Senter et al. (12) found values of 0.13 mg/g of defatted kernel for ‘Stuart’ kernels. A difference between the two studies was the type of hydrolysis to which extracts were subjected: alkaline + acid in the present study, acid in Senter et al. (12) studies. Mattila et al. (39) suggested a higher efficiency of alkaline hydrolysis for phenolic acid extraction from plant-derived foods. Authors also reported great reproducibility for most phenolic acids, although gallic acid presented some variation.

Phenolic compounds in the kernel pellicle are more exposed to environmental agents, such as oxygen and light, and are likely to oxidize at a faster rate than phenolics in the internal matrix. Senter et al. (36) stored pecans at 70 °C for 7 days and studied the changes in their phenolic constituents. Outer layer of kernels produced the oxidation of leucoanthocyanidin and leucodelphinidin, flavan-3,4-diols, to their respective condensed tannins of varying degrees of polymerization. It was suggested that other compounds besides anthocyanidins and condensed tannins were present in the seed testa and markedly influenced oxidative stability of pecan kernels. Jurd et al. (74) studied thoroughly the composition of walnut pellicle and suggested a oxidative protection of kernel oil by the phenolic acids. Authors found that the amount of ellagic acid was up to 4% of pellicle weight, mainly as pyrogallolyl glucose forms (74).

Galloyl groups in epigallocatechin, gallocatechin gallate, and other flavonoids improve their antioxidant properties when compared to their non-galloylated versions (69). The presence of catechin and epicatechin in pecan kernels and the high content of

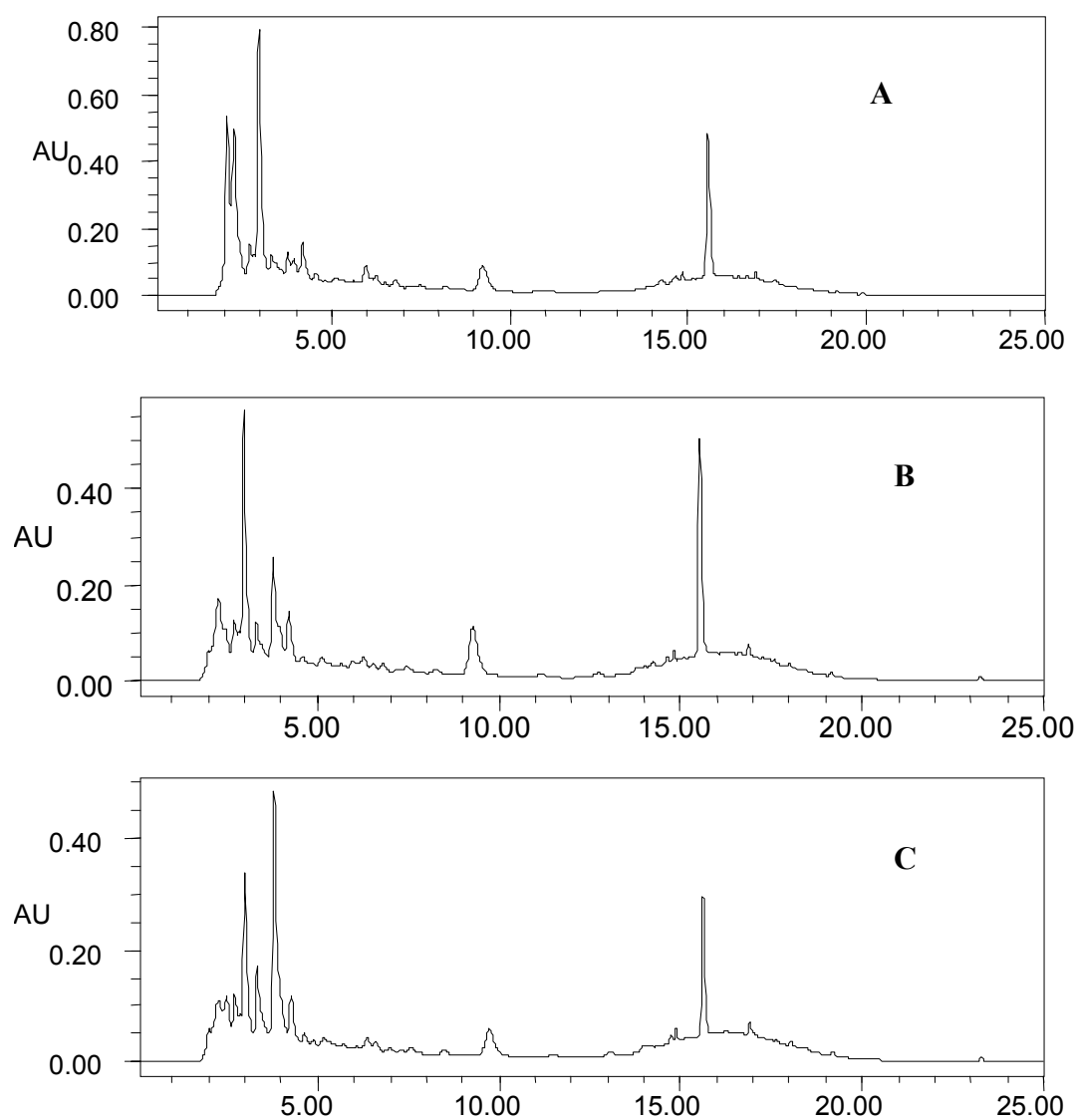


Figure 13. HPLC phenolic profile from hydrolyzed extracts of 'Kanza' kernels irradiated with 0.0 (A), 1.5 (B) and 3.0 kGy (C) after 98 days of storage at 40 °C and 55% R.H.

Table 5. Content of gallic and ellagic acid of pecan kernels treated with 0, 1.5 and 3.0 kGy after 0 and 134 days of storage at 40 °C and 55% R.H.

	‘Kanza’			‘Desirable’		
	Control	1.5 kGy	3.0 kGy	Control	1.5 kGy	3.0 kGy
Gallic acid ^a						
Day 0	1.9 ± 0.3	1.9 ± 0.3	1.9 ± 0.3	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Day 134	2.3 ± 0.8	1.1 ± 0.0	2.1 ± 0.9	2.2 ± 0.2	3.2 ± 1.0	0.9 ± 0.8
Ellagic acid ^b						
Day 0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Day 134	1.3 ± 1.2	0.3 ± 0.0	0.6 ± 0.1	1.4 ± 0.4	1.1 ± 0.1	1.5 ± 0.5

^a Gallic acid content expressed in milligrams of gallic acid per gram of defatted kernel ± S.E.; ^b Ellagic acid content expressed in milligrams of ellagic acid per gram of defatted kernel ± S.E.

gallic acid greatly increase the probability to find galloylated compounds such as the ones mentioned above. It is important to determine the content of these compounds without procedures that may affect their composition.

Senter et al. (12) found that content of phenolic acids in pecan kernels was correlated to peroxide values (PV) and severely decreased (> 50%) after 12 weeks of storage. Results from the present study could not confirm these high levels of phenolic acid degradation during storage and/or due to irradiation treatments. Other studies suggested that irradiation of strawberries with doses up to 6 kGy neither affected gallic acid content (72) nor resulted in major loss of flavonoids and ellagic acid (73).

It is reasonable to assume that potential health benefits of phytochemicals are still present in irradiated kernels after storage. Further studies in the phytochemical composition after processing kernels need to be investigated.

Peroxide Value and Tocopherol Content. Peroxide values (PV) showed an increase for all samples starting at day 55 of storage (Figure 14). ‘Kanza’ 1.5 kGy samples had the greatest increase after 134 days (Figure 14A). ‘Desirable’ kernels irradiated with 3.0 kGy had the greatest PV after 98 and 134 days of accelerated storage (Figure 14B). When analyzing p-anisidine values, low values were found (data not shown) indicating low production of aldehydes.

Irradiation is known to inactivate lipoxygenase enzymes (59). The presence of lipoxygenases was evaluated according to other authors (46), but no quantification could be made in the pecan kernels. In the present study, PV values for irradiated samples were similar or higher than those of controls (Figure 14), thus, the oil oxidation observed was not enzyme-mediated. Future studies need to determine more in detail this oxidation mechanism.

Another possible explanation for PV increase is the degradation of other antioxidant compounds, such as γ -tocopherol. In the present study, irradiated kernels had lower content of γ -tocopherol throughout storage (Figure 15). A decrease in γ -tocopherol content corresponded to a PV increase in 1.5 kGy ‘Kanza’ kernels after 134 days of storage (Figure 14A and Figure 15A). ‘Desirable’ 3.0 kGy samples showed greater PV

than 1.5 kGy and control on day 98 and 134 of storage. This corresponded to a lower γ -tocopherol value for 3.0 kGy irradiated samples throughout the storage (Figure 15B). Urbain et al. (75) found that irradiation of foods in the presence of oxygen decreased γ -tocopherol content, confirming what observed in the present study. PV increased after 98 days of storage for most samples, but no equivalent decrease in tocopherol content was observed.

Rudolph et al. (48) found a dramatic decrease (about 90%) in tocopherol content for pecan oil extracted with solvent and stored at 70 °C for 9 days. On the other hand, Yao et al. (50) and Fourie et al. (49) found only a slight decrease ($\leq 20\%$) in tocopherol content of pecan kernels after ~1 year of storage at 30 °C.

After 55 days of storage, PV levels for kernels of ~1 mE O₂/kg of oil for most samples were found (Figure 14). Pecan oil extracted from kernels at day 0 and stored at the same conditions as kernels had double amount of peroxides (~2 mE O₂/kg of oil) after only 30 days (Figure 14), thus indicating that protection against lipid oxidation was interrupted in extracted oil. The disruption of kernel pellicle and lipid bodies, and exposure to oxygen are the most reasonable explanations for this expected increased oxidation rate.

In general, several factors in addition to oil composition have to be considered when analyzing kernel oxidative stability of pecan kernels. After enriching a stripped pecan oil (pecan oil without original tocopherols and other antioxidant compounds) with tocopherol, Rudolph et al. (48) observed a reduced oxidative stability than control oils. The presence of other antioxidant compounds besides tocopherols was hypothesized to be the cause of oxidative stability.

The complex oxidation process of kernels should not be assessed considering only lipid oxidation measurements. Erickson et al. (76) found weak correlations between peroxide values and sensory scores, and observed that other physicochemical parameters had stronger correlation to kernel quality. Senter et al. (36) found that oxidation of flavonoids into polymers was strongly correlated to peroxide values for 'Schley' but not for 'Stuart' kernels. Data found in the present study indicates that E-Beam irradiation

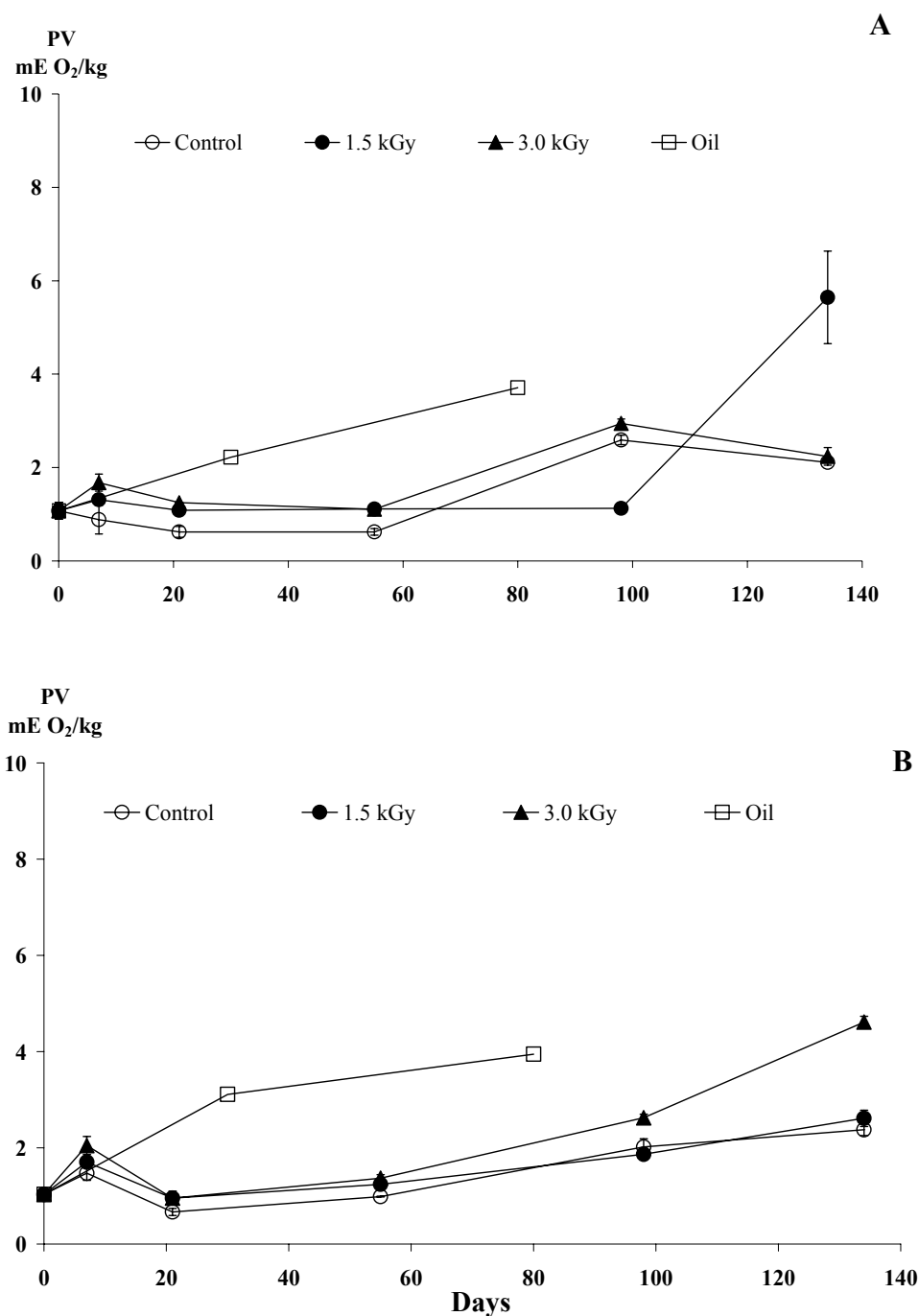


Figure 14. Peroxide values (PV) of ‘Kanza’ (A) and ‘Desirable’ (B) kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H. Oil extracted with hexane from non-irradiated ‘Kanza’ kernels was stored together with pecan kernels.

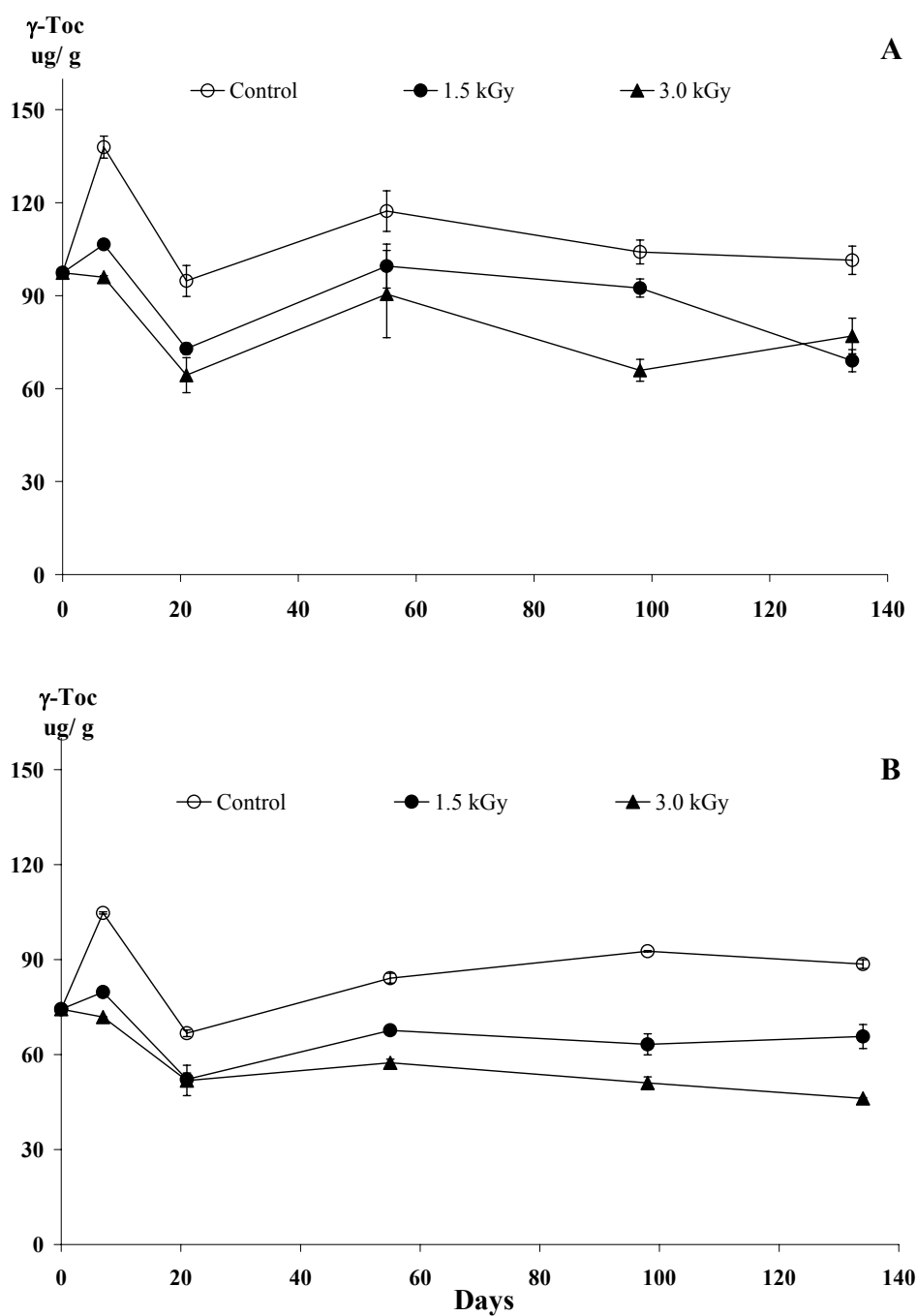


Figure 15. Content of γ -tocopherol (γ -Toc) in oil from 'Kanza' (A) and 'Desirable' (B) kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

treatments had little effect in lipid peroxidation and that lipid oxidation not necessarily diminishes the antioxidant capacity of kernels.

Fatty Acid Profile. Composition of oil was similar for both cultivars investigated. Oleic oil accounted for 70% of total oil composition while linoleic oil was about 20% (Table 6). The rest of components, about 10% of total composition, were linolenic, stearic and palmitic oils (Table 6). At the end of storage period, composition of oil was very similar for all samples. No differences were detected compared to the initial kernel composition even after PV had increased. Increase in PV was probably due to minor fatty acid components such as linolenic and eicosanoic acids, which oxidize faster (77), and have minimum effects on major fatty acid composition. Most of studies relating oxidation and fatty acid composition to pecan kernel quality have been done in oil extracted and stored under accelerated conditions (48, 51). However, as shown in the present study, behavior of extracted oil differs from that of lipids in kernel and caution is recommended when estimating shelf life of pecan nuts by relating whole kernels quality to oil samples.

Color Measurements. ‘Kanza’ had greater initial values for L and greater initial and final values for b when compared to ‘Desirable’ kernels (Table 7 and Table 8). A rapid decrease for L and b (yellowness) in the first 7 days of storage was detected for ‘Kanza’ (Table 7) but not for ‘Desirable’ (Table 8) which had no differences from 0 to 7 days of storage. Values of L and b decreased progressively for both cultivars and all treatments from day 7 until the end of storage. The initial increase of redness was followed by a decrease after 98 days and until the end of storage (Table 7 and Table 8).

Figure 16 shows kernels gradual change from light-golden color to dark-brown confirming the obtained L, a, and b values. These color changes have been previously reported by Senter et al. (36), who found strong correlations between color and polymerization of leucoanthocyanidins (flavan-3,4-diols). Authors found that decrease in lightness and yellowness was correlated with the formation of condensed tannins, and that the rate of formation of these compounds depended on cultivar (12, 36, 78). In the present study this color change may be related to the observed apparent decrease in CT,

Table 6. Fatty acid composition of pecan kernels irradiated with 0, 1.5 and 3.0 kGy and stored for 134 days at 40 °C.

Fatty acid ^a	‘Kanza’				‘Desirable’			
	Day 0	Day 134			Day 0	Day 134		
	0.0 kGy	0.0 kGy	1.5 kGy	3.0 kGy	0.0 kGy	0.0 kGy	1.5 kGy	3.0 kGy
Palmitic	5 ^b	6	5	5	6	5	5	5
Stearic	2	2	2	2	3	3	3	3
Oleic	71	70	70	71	71	71	71	71
Linoleic	20	21	21	19	20	20	20	20
Linolenic	1	1	1	1	1	1	1	1

^a Oil extracted from pecan kernels using hexane; ^b Percentage of fatty acid in oil composition.

Table 7. Color values^a of ‘Kanza’ kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

Time ^b	Treatment	L ^c	a ^d	b ^e
0	Control	47.7 ± 1.7 ^f	13.4 ± 0.3	35.3 ± 2.2
7	Control	41.5 ± 2.4	14.9 ± 0.5	29.4 ± 3.0
	1.5 kGy	40.6 ± 2.9	15.3 ± 1.1	26.7 ± 1.2
	3.0 kGy	38.7 ± 2.8	15.6 ± 0.9	27.6 ± 3.6
55	Control	33.9 ± 1.0	17.9 ± 0.9	20.5 ± 1.2
	1.5 kGy	31.3 ± 1.6	18.1 ± 0.3	18.8 ± 1.2
	3.0 kGy	36.4 ± 2.1	20.0 ± 1.3	23.1 ± 1.8
98	Control	27.2 ± 1.5	18.8 ± 1.3	13.2 ± 1.3
	1.5 kGy	31.7 ± 0.9	13.7 ± 1.6	11.8 ± 1.3
	3.0 kGy	25.8 ± 0.9	18.1 ± 0.5	12.0 ± 0.8
134	Control	24.0 ± 0.8	15.0 ± 0.8	7.5 ± 0.7
	1.5 kGy	23.6 ± 2.3	15.6 ± 1.3	9.4 ± 1.1
	3.0 kGy	24.2 ± 2.2	13.0 ± 1.9	7.4 ± 2.0

^a CIELAB color values using scale D10°/D65, color measurement was done in middle of the flat side of kernel; ^b Time of storage at 40 °C and 55% relative humidity; ^c (L) Lightness values; ^d (a) Redness values; ^e (b) Yellowness values; ^f Each value is the mean of three kernels ± S.E.

Table 8. Color values^a of ‘Desirable’ kernels irradiated with 0, 1.5 and 3.0 kGy and stored at 40 °C and 55% R.H.

Time ^b	Treatment	L ^c	a ^d	b ^e
0	Control	34.5 ± 5.2	15.2 ± 1.8	25.9 ± 3.5
7	Control	39.3 ± 3.7	13.1 ± 0.8	27.8 ± 0.7
	1.5 kGy	36.9 ± 2.8	12.4 ± 0.9	25.6 ± 2.0
	3.0 kGy	41.0 ± 1.8	15.0 ± 0.8	27.8 ± 1.4
55	Control	31.8 ± 2.3	17.3 ± 0.6	20.1 ± 0.9
	1.5 kGy	35.4 ± 3.0	17.7 ± 0.5	23.5 ± 1.6
	3.0 kGy	36.2 ± 2.8	16.7 ± 0.8	22.5 ± 2.0
98	Control	26.6 ± 1.1	16.5 ± 0.7	15.2 ± 2.2
	1.5 kGy	27.3 ± 1.0	16.5 ± 0.5	15.4 ± 1.3
	3.0 kGy	27.1 ± 1.5	16.7 ± 0.3	14.4 ± 0.6
134	Control	22.4 ± 4.0	14.6 ± 2.5	10.7 ± 2.3
	1.5 kGy	22.4 ± 0.8	14.0 ± 1.5	10.3 ± 0.8
	3.0 kGy	25.9 ± 1.7	14.8 ± 1.1	10.4 ± 1.1

^a CIELAB color values using scale D10°/D65, color measurement was done in middle of the flat side of kernel; ^b Time of storage at 40 °C and 55% relative humidity; ^c (L) Lightness; ^d (a) Redness values; ^e (b) Yellowness values; ^f Each value is the mean of three kernels ± S.E.

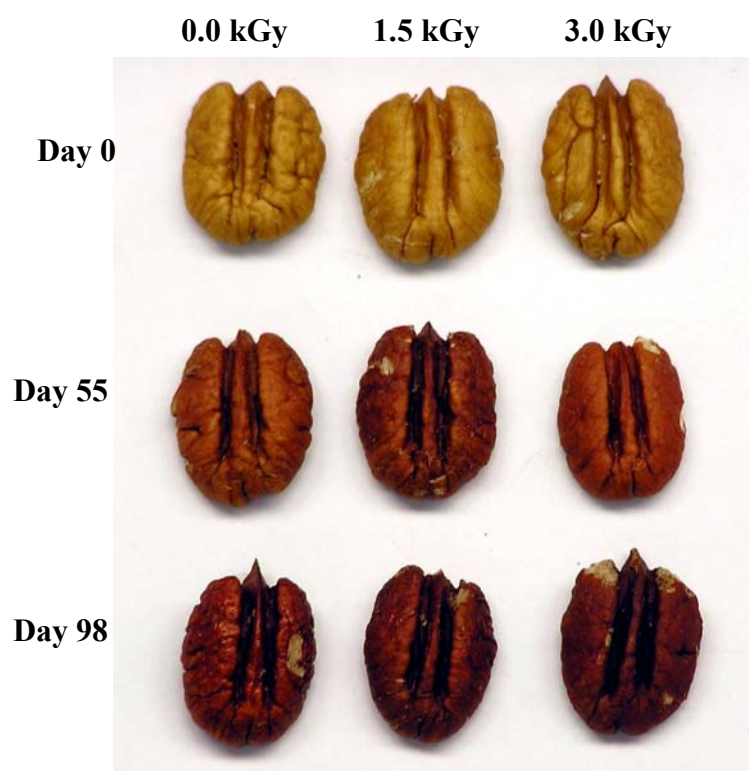


Figure 16. Color of 'Kanza' kernels irradiated with 0, 1.5 and 3.0 kGy and stored for 0, 55 and 98 days at 40 °C and 55% R.H.

which is most likely due to a polymerization process of flavonoids into condensed tannins.

Horticultural practices and processing can increase tannin content in kernels, which will accelerate discoloration during storage (5). A light-brown, golden color is considered to be an attribute of high-quality pecans. Nevertheless, changes in kernel surface color not always parallel sensory scores. Dull et al. (79) found that kernels with the highest sensorial scores were at an intermediate position between the highest and lowest color scores. Therefore, it is recommended to allow a certain degree of color development which will improve flavor and aroma, attributes known to be influenced by tannins (5).

Some methods used for sanitizing pecans in large scale such as soaking in chlorine water and roasting increased darkening rate in kernels (76). In the present study, irradiated kernels had no differences in color when compared to controls, indicating that this technology is suitable for sanitizing kernels without decreasing their visual quality.

Many plants, including pecans, are known to have oil-bearing seeds. Oil accumulation in seeds occurs in many small organelles inside the cell cytoplasm called oil or lipid bodies (80). Lipid bodies in seeds tend to be small (often less than 1 μm in diameter), especially when compared to the ones found in fruit mesocarp (olive, palm, avocado, $\geq 20 \mu\text{m}$) (80). Little information is available about the ultrastructure of pecan kernel parenchyma cells (flesh tissue) and the interface with pellicle tissue. Wakeling et al. (81) studied the microstructure of cells and lipid bodies in opalescent and non-opalescent pecans kernels (opalescence is a non-desirable browning of kernel interior tissue) and found a well-defined and compact cell structure. This tight cell arrangement could provide protection against oxygen transfer through cells and into lipid bodies. A possible oxidation mechanism for kernels is illustrated in Figure 17. The pellicle is exposed to air, causing phenolic compounds therein contained to oxidize and, in some cases, to polymerize (flavonoids). At the same time, the lipid bodies located in parenchyma cells closer to pellicle start oxidizing. Hydrophobic antioxidant compounds inside these lipid bodies, such as tocopherols, start degrading first, thus preventing fatty

acids from oxidation in early stages of storage. However, when measuring total tocopherol content, no changes were observed probably due to the small proportion they represent in whole kernel. (Figure 17). After tocopherol is degraded in the external layers, peroxide values start increasing due to a progressive degradation of fatty acids located in the same cells. Because of the potential low oxygen permeability of kernel tissue, tocopherol content and fatty acids from cells located deeper inside kernels are not affected. This could explain why peroxide values progressively increase with storage without significant effects in total tocopherol content. According to this proposed oxidation mechanism, physical location of fatty acids and tocopherols and oxygen permeability of kernels would play an important role in the oxidation process of pecans.

Oil extracted from both cultivars at day 0 and stored in the same conditions started oxidizing much earlier and in greater rates than kernels. Kanavouras et al. (82) observed that oil oxidized faster with greater area exposed to air. This suggests protection of oil against oxygen permeability, similar to the proposed oxidation mechanism of kernels (Figure 17).

Pecan kernels are difficult to process and sanitize with conventional methods (chlorine and hot water) without decreasing their quality. Irradiation treatments are currently in use to decontaminate different foods across the U.S. The present study showed that E-Beam irradiation had no significant effects on the antioxidant composition of kernels after storage, maintaining some of the nutraceutical attributes analyzed (AC, TP, CT, color). However, some degree of lipid peroxidation was induced by E-Beam irradiation treatments.

Further studies should investigate the specific composition of hydrolyzable and condensed tannins in pecan kernels and how they are affected by processing and storage conditions.

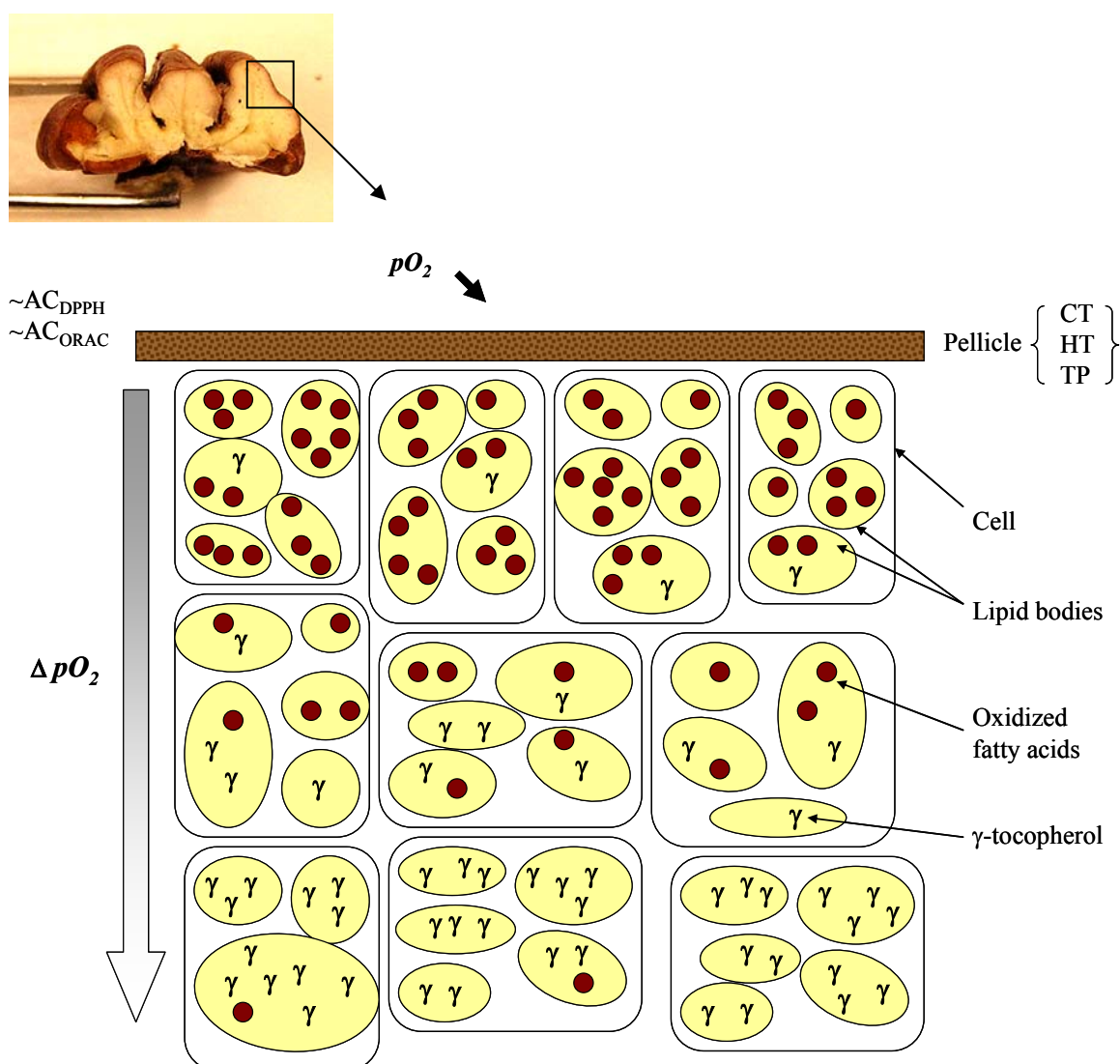


Figure 17. Oxygen transfer and oxidation of lipid bodies in pecan kernel external layers. pO_2 , partial oxygen pressure; ΔpO_2 , partial oxygen pressure gradient; AC_{DPPH} , antioxidant capacity measured by DPPH free radical; AC_{ORAC} , antioxidant capacity measured by ORAC assay; CT, condensed tannins; HT, hydrolyzable tannins; TP, total extractable phenolic content.

CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Compounds found in pecan kernels are known to have different health benefits. Condensed tannins are known to exert antioxidant protection. Gallic and ellagic acid form hydrolyzable tannins and have biological activity after consumption. Pecan kernel unsaturated fatty acids improve human serum lipid profile.

Pecan kernels analyzed in the present study had high phenolic content. A variety of compounds such as catechins, phenolic acids and tocopherols were identified in different amounts depending on cultivar. Differences between cultivars grown at the same location with the same horticultural practices suggest genetic influence on their phytochemical content. Pecan nut shells had greater antioxidant capacity and condensed tannin content than kernels. These antioxidant properties from shells provide new insights for their potential use as dietary antioxidant source.

Irradiation with E-Beam with up to 3 kGy did not induce significant changes in these components indicating to be suitable for sanitizing pecan kernels without decreasing their nutritional and functional properties. In addition, E-Beam irradiation had no significant effects on color and oxidative status, important quality parameters of pecan kernels.

It is recommended to evaluate pecan phenolic composition more in detail to look for the presence of galloylated compounds, such as epigallocatechin gallate. *In vivo* experiments have indicated that these compounds might have anticancer and antioxidant properties. Testing different extracts of pecan kernels for their antimutagenic and anticancer properties could help characterize the compounds with potential health benefits.

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VITA

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