EFFECTS OF PROCESSING ON THE CHEMISTRY AND ANTIOXIDANT CAPACITY OF ENDOGENOUS BIOACTIVE COMPOUNDS IN PEANUT KERNELS AND SKINS

by

BRIAN DAVID CRAFT

(Under the Direction of Ronald Bruce Pegg)

ABSTRACT

The overall aims of this work were to ascertain the primary phenolic constituents in peanut kernels and skins, and determine if there antioxidant content and capacities are conserved through industrial roasting processes. The first study conducted revealed that the predominant phenolic compounds in peanut kernels are free/bound *p*-coumaric acids, which are released from their ester or glycoside forms during thermal treatment. This observed increase in *p*-coumaric acid levels was affected both by peanut type and processing method. A Spanish high-oleic cultivar had the highest naturally occurring levels of free/bound *p*-coumaric acids and a high-oleic Runner yielded an increase of ~785% in free *p*-coumaric acid from raw to oil-roasted sample. The total phenolic contents and antioxidant capacities of processed peanut kernels from the 2007 crop were found to increase in a stepwise fashion from raw < dry-roast < oil-roast for nearly all phytochemical analyses performed. This suggests that although the chemistry of phenolic compounds in peanuts is changing through roasting, their antioxidant potency is preserved and sometimes increased. The second and third studies undertaken involved the chromatographic characterization of low- (LMW) and high-molecular-weight (HMW) phenolic fractions isolated from dry-blanched (DB) and dry-roasted (DR) peanut skins. Catechin,

epicatechin, and *p*-coumaric acid esters were identified in the LMW fraction of DB peanut skins; whereas catechin, free *p*-coumaric and protocatechuic acids, and a protocatechuic acid ester were found in DR skins. Total phenolics and antioxidant capacities were similar for DB and DR skins. Some of the collected LMW fractions exhibited higher antioxidant capacities than their corresponding crude extracts; suggesting that LMW phenolics contribute to peanut skin's total antioxidant capacity. Proanthocyanidin (PAC) analyses showed that DR peanut skin tannins have a higher degree of polymerization than DB skins. A greater incidence of PAC polymerization reactions may be a result of the higher temperatures involved in the dry-roasting process. All three studies indicated that processing is altering the chemistry of the phenolics in peanuts and peanut skins, but their antioxidant efficacy is retained for the consumer.

INDEX WORDS: Peanuts, Peanut Skins, Peanut Industry, Peanut Processing, Roasting, Antioxidants, Phenolics, Proanthocyanidins

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DEDICATION

This work is dedicated to my loving parents, David and Janet Craft, to whom I owe my undying gratitude. Also, to my beautiful puppy Kane ("Bubba"), who taught me patience and selfless love in his lifetime.

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Sincerely,

Brian D. Craft

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CHAPTER 1

INTRODUCTION

1.1 Peanuts and Human Health

Over the past decade, there have been an increasing number of reports on the health benefits attributed to peanuts (*Arachis hypogaea* L.) (Alper and Mattes, 2002; Griel *et al.*, 2004; Li *et al.,* 2009). Epidemiological studies have shown that heart disease risk is reduced with increasing frequency of peanut consumption, such that consuming 1 oz of peanuts or 2 tbsp. of peanut butter five or more times per week reduces risk by up to 50% (Hu *et al.*, 1998). Type-2 diabetes risk can also be reduced by 27 and 21% for consumers of peanut kernels or peanut butters at a daily consumption of one tbsp., respectively (Jiang *et al.*, 2002). In 1999, a human clinical study that fed participants diets including peanuts, peanut butter, or peanut oil resulted in lower total cholesterol, low-density lipoprotein, and triglyceride concentrations while maintaining high-density lipoprotein levels (Kris-Etherton *et al.*, 1999). The positive blood lipid effects and reduced risk of heart disease are believed to be mainly attributed by the mono- and polyunsaturated fats contained in peanuts and peanut products. Emerging research shows that L-arginine (Stephens and Sanders, 2008), phytosterols, Vitamin E, folate, and phytonutrients such as phenolic acids and their esters (Francisco and Resurreccion, 2008; Higgs, 2003; Kris-Etherton *et al.*, 2001) are also contributing to some of the cardioprotective effects experienced by peanut consumers. In 2003, the U.S. Food and Drug Administration affirmed a qualified health claim

for peanut consumption concluding that there was sufficient evidence that peanuts and selected tree nuts reduce the risk of coronary heart disease (FDA, 2003).

1.2 U.S. Peanut Production

The peanut (*Arachis hypogaea* L.) is a major economic agricultural crop of the southern states, specifically the three peanut producing regions: the Southwest (Texas and Oklahoma), the Southeast (Alabama, Georgia, and Florida), and the Virginia/Carolina region (Virginia, North Carolina, and South Carolina). Of the thousands of peanut cultivars grown worldwide, the majority of peanuts belong to one of four common types: Runner, Virginia, Spanish, and Valencia. Edible uses of peanuts account for more than two thirds of the total peanut consumption in the United States (USDA-ERS 2002). The two major categories of food-use peanuts are *shelled* peanuts and roasted *in-shell* peanuts. Shelled peanuts include dry- or oil-roasted snack peanuts, boiled peanuts, peanut butter for sandwiches, peanuts in candy bars, and peanut brittle. Roasted in-shell peanuts are often sold at baseball games and must be de-hulled before consumption. Of the peanuts consumed in the U.S., ~75% undergo the dry-roasting process; whereas, the remaining 25% are oil-roasted or roasted in-shell (Kotz, 2009).

1.3 Significance of Peanut Skins

Currently, peanut skin is a low value by-product of the peanut industry. Peanut skins are removed in dry-blanching operations *via* "split-nut" blanching. Split-nut blanching is a process in which peanuts pass along a belt and through a roller that splits them in half. The skins are then blown off of the halved peanuts and collected in a cyclone, separated, and sent out of the plant (Karn, 2009). Dry-roasted peanut skins are removed after processing by passing the

peanuts over screens that collect the skins. In both operations peanut skins are then gathered, transferred to compactors, and pressed into pellets to be sold as animal feed with a commercial value of only $12 to $20 per ton (Sobolev and Cole, 2004).

1.4 Phenolic Antioxidants in Peanut Kernels

Of the bioactives contained in peanuts, perhaps the least characterized portion is the phenolics. The total phenolic content and antioxidant capacity of peanuts are comparable to other tree nuts (Kornsteiner *et al.*, 2006; Pellegrini *et al.*, 2006). High-oleic and normal Runner peanuts have similar antioxidant profiles (Talcott *et al.*, 2005), though more high-oleic genotypes are being created. High-oleic hybrids of Spanish and Virginia peanuts are now available. Total phenolics and antioxidant capacity values vary between peanut cultivars (Duncan *et al.*, 2006; Talcott *et al.*, 2005), but these samples were collected from the same growing region (southeast FL) and, thus, may differ in their phenolic profiles when compared to peanuts collected from other growing regions. For example, drought stress has been shown to significantly affect peanut quality, including alterations of fatty acid profiles and tocopherol contents (Hashim *et al.*, 1993).

1.5 The Effects of Processing on Peanut Antioxidants

Currently, little is known about the effects of processing on the chemistry of phenolics in peanut kernels. Total phenolics content and antioxidant capacity of peanuts increase significantly upon roasting as determined by HPLC, lipid model systems, *in vitro* radical-scavenging and enzymatic methods (Chukwumah *et al.*, 2007; Hwang *et al.*, 2001; Talcott *et al.*, 2005), but these studies involved experimentation on peanut samples of limited breadth. In fact, two of the three studies purchased in-shell peanuts from the local supermarket. Thermal

processing methods increase the total phenolics content of peanuts according to the following relationship: boiled > oil roast > dry roast > raw (Chukwumah *et al.*, 2007). In short-term frozen storage at -20 °C, sensorial attributes of peanut kernels remain relatively unchanged (Pattee *et al.*, 2002), suggesting that the oxidative stability is good under these conditions.

1.6 Polyphenolic Antioxidants in Processed Peanut Skins

A- and B-type proanthocyanidin (PAC) dimers, trimers, and tetramers have been reported in processed peanut skin extracts (Lazarus *et al.*, 1999; Van Ha *et al.*, 2007; Yu *et al.*, 2006; Yu *et al.*, 2007). However, the effects of processing on the levels and chemistry of these tannins are not well understood. Yu *et al.* (2005) reported that dry roasting increases the total phenolics content of peanut skins, whereas, water blanching yields decreased values. Dry blanching has yet to be reported on. Peanut skin extracts demonstrate high antioxidant potency as measured by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical-scavenging assays (Van Ha *et al.*, 2007; Wang *et al.*, 2007; Yu *et al.*, 2007), ferrous-ion chelating potential (Van Ha *et al.*, 2007; Wang *et al.*, 2007), and various *in vitro* oxygen radical (*e.g.* $HO^{\bullet}$, $O_2^{\bullet-}$) scavenging methods (Wang *et al.*, 2007). Unfortunately, significant variability exists in the literature in the extraction media employed, methods to quantify antioxidant capacity, and results among research groups.

1.7 Peanut Sample Set

While recent research has made nutrient and bioactive composition data more available on peanuts and peanut products, few studies have been completed with proper sampling plans. As part of a larger study on the nutritional components in peanut kernels, a large sample set of peanuts (n_{total} = 309) was gathered over the 2005 to 2007 crop years. This sample set included

Runner, Spanish, and Virginia peanuts as well as high-oleic Runner and Spanish cultivars. The sampling plan was designed to include only the most-used cultivars from the U.S. peanut industry facilitating the applicability of the research. Efforts were made to obtain a representative sample set that would be useful in determining varietal and regional differences of peanut nutrients.

1.8 The Goals of the Work

There are three goals of this research. The first goal is to monitor the effects of processing on the predominant phenolic compounds in peanut kernels and to test their antioxidant and radical-scavenging capacities *in vitro*. The second goal is to test the antioxidant, radical-scavenging, and biological activities of commercially-processed peanut skins. Phenolic extracts of dry-blanched and dry-roasted peanut skins will be prepared. Extracts will then be separated into low-molecular-weight (LMW) and high-molecular-weight (HMW) fractions. Each LMW and HMW fraction will be examined for antioxidant, radical-scavenging, and biological activities *in vitro*, to better understand their contribution to peanut skin's total antioxidant capacity. The third goal of this work is to isolate and characterize the phenolic acids and oligomeric PACs of commercially-processed peanut skins *via* liquid chromatography.

Project I: The Effects of Processing on Peanut Kernel Phenolics and Antioxidant Capacity

The first project of my doctoral research involved a critical examination of the predominant phenolic compounds in raw and processed peanut kernels. Raw peanuts were obtained from a unique sample set collected from the 2005 to 2007 crops. Runner, Virginia, and Spanish peanuts were included in the sampling program as well as high-oleic Runner and

Spanish cultivars. A portion of samples were ground while others were dry- and oil-roasted according to standard industrial practices. Organic solvent extraction techniques were employed to isolate crude phenolic fractions from peanut kernels for further analysis. Antioxidant capacity was measured in raw and roasted peanuts to assess the effects of processing.

The phenolic profiles of raw and roasted peanuts were determined by modern chromatographic techniques including analytical and semi-preparative reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC was carried out on C_{18} (octadecylsilyl-modified silica) columns with ultraviolet-visible diode array detection (UV-Vis DAD). Total phenolics content was measured by a classical colorimetric assay using Folin & Ciocalteu's phenol reagent (Folin-Ciocalteu, 1927; Swain and Hillis, 1959). Antioxidant and radical-scavenging capacities of peanut samples were determined using a hydrophilic oxygen radical absorbance capacity ($ORAC_{FL}$) assay (Prior *et al.*, 2003), a photochemiluminescence technique (Pegg *et al.*, 2007), and the Trolox equivalent antioxidant capacity (TEAC) assay (Re *et al.*, 1999).

Project II: Antioxidant, Radical-Scavenging, and Biological Activities of Commerically-Processed Peanut Skins.

The second project undertaken involved the fractionation of commercially-processed peanut skin phenolics and the measurement of their antioxidant, radical-scavenging, and biological activities. Dry-blanched (DB) and dry-roasted (DR) peanut skins were obtained from local peanut industries. Crude phenolic extractions were made: 80% (v/v) acetonic for HPLC and antioxidant activity measurements, but 50% (v/v) ethanolic for biological activity assays. Acetonic extracts of DB and DR peanut skins were separated on a packed open tubular column

filled with lipophilic Sephadex LH-20 into LMW and HMW fractions. Predominant LMW phenolic fractions were then further separated on a C_{18} RP-HPLC column with a UV-Vis DAD system. Crude DB and DR skin extracts and collected LMW fractions were measured for total phenolics (*via* the Folin-Ciocalteu method) and antioxidant activities (*via* the TEAC assay). Ethanolic extracts of DR peanut skins were screened for their effects on α-amylase activity and fructose-mediated protein glycation *in vitro*; inhibition of these biological activities has been associated with reduced incidence of diabetic complications.

Project III: Elucidation of the Predominant Chemical Forms of Peanut Skin Phenolics

The third project of this research involved the chromatographic isolation of phenolic acids and oligomeric PACs in peanut skins. A rapid procedure for the extraction, purification, and subsequent chromatographic analysis of phenolic acids in peanut skins was employed (Krygier *et al.*, 1982). This method involves the selective separation of phenolic aglycone, ester, and glycoside fractions from samples *via* pH alteration and the use of organic solvent extraction techniques. Once separated the individual phenolic fractions were then separated on an RP-HPLC C_{18} column with UV-Vis DAD. PACs from peanut skins were separated according to their degree of polymerization using a new normal-phase (NP)-HPLC method modified from current procedures in the literature (Adamson *et al.*, 1999; Kelm *et al.*, 2006). The degree of PAC polymerization significantly alters their antioxidant capabilities *in vitro* (Lotito *et al.*, 2000) and, therefore, is a key factor in understanding their antioxidative potential.

Phenolic constituents, whether in aglycone (free) form, esterified, or bound to sugars (glycosides) are processed differently within the human body. Once ingested, phenolic compounds undergo a similar process to that of drug metabolism; therefore, based on their

chemistry, the rate of biotransformation of phenolics can change and differences in the amount absorbed in the gastrointestinal tract may be found. The patterns of intestinal absorption of phenolic acids and flavonoids have been characterized (Das and Sothy, 1971; Day *et al.*, 2000; Scalbert *et al.*, 2002; Scalbert and Williamson, 2000), but the extent to which all phenolic compounds are metabolized in humans has yet to be resolved.

References

Adamson, G. E.; Lazarus, S. A.; Mitchell, A. E.; Prior, R. L.; Cao, G.; Jacobs, P. H.; Kremers, B. G.; Hammerstone, J. F.; Rucker, R. B.; Ritter, K. A.; Schmitz, H. H. 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* **47**:4184-4188.

Alper, C. M.; Mattes, R. D. 2002. Effects of chronic peanut consumption on energy balance and hedonics. *Int. J. Obesity* **26**:1129-1137.

Chukwumah, Y.; Walker, L.; Vogler, B.; Verghese, M. 2007. Changes in the phytochemical composition and profile of raw, boiled and roasted peanuts. *J. Agric. Food Chem.* **55**:9266-9273.

Das, N. P.; Sothy, S. P. 1971. Studies on flavonoid metabolism. *Biochem. J.* **125**:417-423.

Day, A. J.; Cañada, F. J.; Díaz, J. C.; Kroon, P. A.; Mclauchlan, R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R. A.; Williamson, G. 2000. Dietary flavonoid and isoflavone glycosides are hydrolyzed by the lactase site of lactase phlorizin hydrolase. *FEBS Lett.* **468**:166-170.

Duncan, C. E.; Gorbet, D. W.; Talcott, S. T. 2006. Phytochemical content and antioxidant capacity of water-soluble isolates from peanuts (*Arachis hypogaea* L.). *Food Res. Int.* **39**:898-904.

Folin, O.; Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**:627-650.

Francisco, M. L. D. L.; Resurreccion, A. V. A. 2008. Functional components in peanuts. *Crit. Rev. Food Sci. Nutr.* **48**:715-746.

Griel, A. E.; Eissenstat, B.; Juturu, V.; Hsieh, G.; Kris-Etherton, P. M. 2004. Improved diet quality with peanut consumption. *J. Am. Coll. Nutr.* **23**:660-668.

Hashim, I. B.; Koehler, P. E.; Eitenmiller, R. R.; Kvien, C. K. 1993. Fatty acid composition and tocopherol contents of drought stressed Florunner peanuts. *Peanut Sci.* **20**:21-24.

Higgs, J. 2003. The beneficial role of peanuts in the diet-Part 2. *Nutr. Food Sci.* **33**:56-64.

Hu, F. B.; Stampfer, M. J.; Manson, J. E.; Rimm, E. B.; Colditz, G. A.; Rosner, B. A.; Speizer, F. E.; Hennekens, C. H.; Willett, W. C. 1998. Frequent nut consumption and risk of coronary heart disease in women: prospective cohort study. *Brit. Med. J.* **317**:1341-1345.

Hwang J-Y.; Shue, Y-S.; Chang H-M. 2001. Antioxidative activity of roasted and defatted peanut kernels. *Food Res. Int.* **34**:639-647.

Isanga, J.; Zhang, G-N. 2007. Biologically active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.* **23**:123-140.

Jiang, R.; Manson, J. E.; Stampfer, M. J.; Liu, S.; Willett, W. C.; Hu, F. B. 2002. Nut and peanut butter consumption and risk of type 2 diabetes in women. *J. Am. Med. Assoc.* **288**:2554-2560.

Karn, R. 2009. Product Development Manager, American Blanching Company, Fitzgerald, GA. Personal Communication.

Kelm, M. A.; Johnson, J. C.; Robbins, R. J.; Hammerstone, J. F.; Schmitz, H. H. 2006. High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. *J. Agric. Food Chem.* **54**:1571-1576.

Kornsteiner, M.; Wagner, K.-H.; Elmadfa, I. 2006. Tocopherols and total phenolics of 10 different nut types. *Food Chem.* **98**:381-387.

Kotz, B. A. 2009. Vice-President Specialty Products, Golden Peanut Co., Alpharetta, GA. Personal communication.

Kris-Etherton, P. M. Yu-Poth, S.; Sabaté, J.; Ratcliffe, H. E.; Zhao, G.; Etherton, T. D. 1999. Nuts and their bioactive constituents: effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* **70**:504S-511S.

Kris-Etherton, P. M.; Zhao, G.; Binkoski, A. E.; Coval, S. M.; Etherton, T. D. 2001. The effects of nuts on coronary heart disease risk. *Nutr. Rev.* **59**:103-111.

Krygier, K.; Sosulski, F.; Hogge, L. 1982. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem.* **30**:330-334.

Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **47**:3693-3701.

Lotito, S. B.; Actis-Goretta, L.; Renart, M. L.; Caligiuri, M.; Rein, D.; Schmitz, H. H.; Steinberg, F. M.; Keen, C. L.; Fraga, C. G. 2000. Influence of oligomer chain length on the antioxidant activity of procyanidins. *Biochem. Biophys. Res. Commun.* **276**:945-951.

Li, T. Y.; Brennan, A. M.; Wedick, N. M.; Mantzoros, C.; Rifai, N.; Hu, F. B. 2009. Regular consumption of nuts is associated with a lower risk of cardiovascular disease in women with type 2 diabetes. *J. Nutr.* **139**:1333-1338.

Nepote, V.; Grosso, N. R.; Guzman, C. A. 2002. Extraction of antioxidant components from peanut skins. *Grasas Aceites* **53**:391-395.

Pattee, H. E.; Isleib, T. G.; Moore, K. M.; Gorbet, D. W.; Giesbrecht, F. G. 2002. Effect of high-oleic trait and paste storage variables on sensory attribute stability of roasted peanuts. *J. Agric. Food Chem.* **50**:7366-7370.

Pegg, R. B.; Amarowicz, R.; Naczk, M.; Shahidi, F. 2007. PHOTOCHEM® for determination of antioxidant capacity of plant extracts. American Chemical Society Symposium Series 956. Editors: Shahidi, F.; Ho, C-T. In Antioxidant Measurement and Applications. pp.140-158.

Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F. 2006. Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* assays. *Mol. Nutr. Food Res.* **50**:1030-1038.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodhill, M.; Huang, D.; Ou, B.; Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **51**:3273-3279.

Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**:1231-1237.

Scalbert, A.; Morand, C.; Manach, C.; Rémésy, C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother.* **56**:276-282.

Scalbert, A.; Williamson, G. 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**:2073S-2085S.

Sobolev, V. S.; Cole, R. J. 2004. Note on utilisation of peanut seed testa. *J. Sci. Food Agric.* **84**:105-111.

Stephens, A. M.; Sanders, T. H. 2008. Effects of peanuts, peanut oil and fat free peanut flour on cardiovascular disease risk factors in male Syrian golden hampsters. Poster Presentation #136-07, Institute of Food Technologists, 2008.

Swain, T.; Hillis, W. E. 1959. The phenolic constituents of *Prunus domestica*. I.-The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **10**:63-68.

Talcott, S. T.; Passeretti, S.; Duncan, C. E.; Gorbet, D. W. 2005. Polyphenolic content and sensory properties of normal and high oleic acid peanuts. *Food Chem.* **90**:379-388.

U.S. Department of Agriculture, Economic Research Service (USDA-ERS). 2002. Commodity spotlight: Peanut consumption rebounding amidst market uncertainties. Agricultural Outlook 289:2-5. March 2002. <http://www.ers.usda.gov/publications/agoutlook/mar2002/ao289a.pdf>.

U.S. Food and Drug Administration (FDA), Department of Health and Human Services. 2003. Qualified health claims: Letter of enforcement discretion – Nuts and coronary heart disease. (Docket No 02P-0505). July 14, 2003. <www.fda.gov/food/labelingnutrition/labelclaims/qualifiedhealthclaims/ucm072906.htm>.

Van Ha, H.; Pokorný, J.; Sakurai, H. 2007. Peanut skin antioxidants. *J. Food Lipids* **14**:298-314.

Wang, J.; Yuan, X.; Jin, Z.; Tian, Y.; Song, H. 2007. Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem.* **104**:242-250.

Yu, J.; Ahmedna, M.; Goktepe, I. 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem.* **90**:199-206.

Yu, J.; Ahmedna, M.; Goktepe, I. 2007. Peanut Skin Phenolics: Extraction, Identification, Antioxidant Activity, and Potential Applications. ACS Symposium Series 956 Editors F. Shahidi and C-T. Ho. In Antioxidant Measurement and Applications. pp. 226-241.

Yu, J.; Ahmedna, M.; Goktepe, I.; Dai, J. 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. *J. Food Comp. Anal.* **19**:364-371.

CHAPTER 2

LITERATURE REVIEW

2.1 Reactive Oxygen Species (ROS) and the Human Body

Reactive oxygen species (ROS) are of interest in biology and biological chemistry due to strong evidence relating them to the pathogenesis of many degenerative diseases and aging in humans (Barber and Harris, 1994; Halliwell *et al.*, 1992; Halliwell, 1996; Hiramatsu *et al.*, 1997). ROS have even been implicated in the disruption of cellular signaling pathways, and thus, can affect gene expression (Palmer and Paulson, 1997). Perhaps the best known forms of ROS include certain oxygen radicals like the superoxide radical anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), as well as alkoxyl and peroxyl radicals ($RO^{\bullet}$ & $RO_2^{\bullet}$, respectively). In addition to these, there are non-radical oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$), and ozone (O_3) (Halliwell *et al.*, 1995). Reactive nitrogen species (RNS) such as nitroxyl radicals ($NO^{\bullet}$, $NO_2^{\bullet}$) and peroxynitrite ($ONOO^-$) also exist and can have adverse effects on human health and disease (Halliwell *et al.*, 1992; Halliwell 1996).

Though ROS are often viewed as exogenous and deleterious to mankind, these compounds are naturally present within humans and held in check by the body's multiple defense systems including the following: endogenous antioxidant enzymes (*e.g.*, catalase, glutathione reductase, glutathione peroxidase, superoxide dismutase), endogenous factors (*e.g.*, glutathione, co-enzyme Q), metal-ion sequestration systems, and endogenously-generated

primary and secondary antioxidants (*e.g.*, Vitamin E, Vitamin C, and carotenoids) (Halliwell, 1996; Machlin and Bendich, 1987; Sies, 1993). In fact, all vascular cell types produce ROS enzymatically *via* membrane-associated NAD(P)H oxidase. ROS are involved in many stages of vascular function including cell contraction/dilation, cell growth, programmed cell death, and inflammation (Touyz, 2005), various stages of cellular respiration including the mobilization of the electron transport system, oxidative phosphorylation, and consequently to various redox signaling pathways (Adam-Vizi, 2005).

Overproduction of ROS: The Impact of Oxidative Stress

ROS can become dangerous when present in excess in the human body. They result in the overproduction of free radicals that can damage multiple components of cells including deoxyribonucleic acid (DNA), ribonucleic acid (RNA), lipids, proteins, carbohydrates, and enzymes (Aruoma, 1994; Machlin and Bendich, 1987). Furthermore, cellular damage incurred by free radicals can cause further radical production as well as an increased risk of inflammation, cardiovascular disease, cancer, diabetes, Alzheimer's, and age-related functional decline, even though it is perhaps not the primary cause of these diseases (Temple, 2000). Of recent interest is the role of redox modulation in insulin signaling as it pertains to vascular endothelial function and possible links to diabetes (Christon *et al.*, 2005; Stevens, 2005). Although some may think that free-radical research in biological systems is fairly recent, significant breakthroughs in free-radical theory have occurred since the work of D. Harman in the mid 1950s (Niki, 1997).

As a recognized contributing factor to the overproduction of ROS, oxidative stress is thought to be linked to many human degenerative diseases. For example, oxidative processes are involved in the formation of atherosclerotic plaques within arterial walls, and therefore, can lead

to an increased incidence of cardiovascular disease in humans (Steinberg, 1991). Oxidized nucleic acids in DNA can be mutagenic and lead to carcinogenesis (Nakabeppu *et al.*, 2006). Many inflammatory processes can lead to an increased incidence of oxidative stress; this often results from an overproduction of ROS that the body's natural defense systems cannot overcome (Sies, 1993). Greater nutritional intake of pro-oxidant food sources or prolonged nutrient deficiency can also result in oxidative overload within the body (Sies *et al.*, 2005). Although chromatographic and spectrophotometric assays are able to measure the extent of nucleic acid oxidation (Collins, 2005), studies in oxidative/antioxidative research have tended towards *in vitro* antioxidant and radical-scavenging capacity assays, lipid oxidation model systems in foods, and *in vivo* assays of human biological fluids.

2.2 Oxidation, Antioxidation, and Reduction

Before one can truly understand the link between ROS, oxidative stress, and human disease, it is important to know the fundamentals of oxidative/antioxidative relationships. Oxidation can be defined as a chemical reaction involving the transfer of electrons between molecules to an oxidizing agent (*i.e.*, which undergoes a simultaneous reduction). This transfer of electrons between entities can give rise to radical species. In nature, molecules are made of protons, neutrons, and electrons. While protons and neutrons comprise the nucleus of atoms, electrons are left to occupy regions of space outside of the nucleus known as orbitals. In compounds, each molecular orbital can contain a maximum of two paired electrons with opposite spins. Orbital shape (*s*-1, *p*-3, *d*-5, *f*-7) and orientation (x, y, and z dimensions) will differ from molecule to molecule depending on its composition. A free radical is simply an atom or molecule that can exist independently with one or more unpaired electrons in its outermost shell.

These shells can be atomic or compounded. Given that molecules are most stable in the ground state, radicals are highly reactive species that often do not last long in a given form. Once created, radical species propagate with other compounds and beget more radicals. This process continues until the radicals terminate their own existence by covalently bonding to another molecular entity. Antioxidants can interrupt the aforementioned chain reactions by removing reaction intermediates and being oxidized themselves.

Antioxidants Defined

Although the term antioxidant originally referred to molecules that prevent the consumption of oxygen by human tissues, it has evolved to refer to the prevention of oxidative systems as a whole. An antioxidant is a molecule or species that slows or prevents the oxidation of another molecule, and therefore can be considered as a reductant. It is important to note differences in terminology: an antioxidant is a reductant, but not all reductants are antioxidants. In explanation, "reductant" and "oxidant" are chemical (redox) terms, while "antioxidant" and "pro-oxidant" hold a specific reference to biological systems (Prior and Cao, 1999).

The Role of Antioxidants in Humans

Antioxidants are important to humans because of the multiple beneficial interactions they can have within our bodies. Often this protection is case, type, and location dependent. For example, an antioxidant generated to help protect against lipid peroxidation in human tissues may or may not be able to prevent oxidative stress caused to DNA, proteins, or other compounds. In some cases in fact, they can cause more damage than good (Halliwell, 1996). Antioxidant effectiveness *in vitro* may not correlate with effectiveness *in vivo*. The human

digestive tract can degrade or alter the chemical form of antioxidant compounds, as they pass through the stomach, and prevent them from being absorbed in the lower intestines and render them ineffective at preventing oxidation in the body (Scalbert and Williamson, 2000). As a screening process, it is reasonable to assume that if an antioxidant has a poor capability of scavenging free radicals or preventing oxidative reactions *in vitro*, then it likely will also have poor efficacy *in vivo*. Given the cost of animal model systems and human intervention studies, cell culture models to directly assess antioxidant effectiveness *in vivo* have been attempted and hold promise (Liu and Finley, 2005; Wolfe and Liu, 2007).

Assessment of antioxidant profiles in human plasma (Polidori *et al.*, 2001) and other biological fluids is commonplace as an index of oxidative stress. Past research suggests that exogenously-supplemented antioxidants provide relief from multiple oxidative reactions within humans and act as anti-inflammatory, anti-carcinogenic, anti-cancer, and anti-radical agents (Diplock, 1994; Diplock, 1996; Rice-Evans and Diplock, 1993). Whether or not antioxidants are the direct cause for the alleviation of human ailments or can elicit a favorable response in the body, is still under debate (Halliwell *et al.*, 2005). Some *in vivo* studies have suggested that supplementing the human diet with antioxidants may not be warranted given the possibility of pro-oxidative reactions. A pro-oxidant effect of supplemented vitamins C and E was observed in *in vivo* dietary trials (Abudu *et al.*, 2004; Kontush *et al.*, 1996; Paolini *et al.*, 1999). Phenolic antioxidants are also receiving scrutiny in this area (Cao *et al.*, 1997; Fukumoto and Mazza, 2000; Rufián-Henares *et al.*, 2006).

The Role of Antioxidants in Foods

Another important application of antioxidants is their inclusion in food products as natural preservatives. This has led to the attempted correlation of *in vitro* antioxidant capacity data with projected capabilities of antioxidants to perform in food systems. Even though antioxidant capacity assays can gauge the relative capabilities of antioxidant components, antioxidant activity in food systems depends on many factors including the antioxidant's physical location in the food, interaction(s) with other food constituents, and the overall conditions of the food environment (*e.g.,* pH, ionic strength, hydrophilic/lipophilic balance, etc.) (Decker *et al.*, 2005). An antioxidant's effectiveness at scavenging free radicals in the aqueous phase depends on its solubility between the aqueous and lipid layers of a food or beverage. To this end, antioxidant model systems *in vitro* need to be re-evaluated and methods modified to take into account the complex nature of foods.

Some of the most well characterized antioxidant reactions in foods are enzymatic oxidation of polyphenols. This process begins immediately as cell integrity is compromised and can result in significant reduction in food quality unless the proper measures are taken to protect the foodstuff. Many enzymes can be inactivated through the adequate use of thermal processing methods; however, this can also significantly affect the content and chemistry of antioxidants (and other components) contained therein. A recent study (Arts *et al.*, 2000) compared the levels of catechins in fresh and processed fruits & vegetables: a 25-60% decrease in catechin contents of prepared foods and marked decreases in industrial canned foods were noted when compared to their raw counterparts.

2.3 Phenolic and Polyphenolic Antioxidants

One of the most well-known groups of antioxidant compounds in scientific literature is the phenolics. Any compound that contains a hydroxy-substituted aromatic ring is a phenolic compound. Phenolics and polyphenolics (polymeric phenolics) can provide relief from certain physical ailments and degenerative diseases in humans, including the reduction of cardiovascular disease and certain cancers (Arts and Hollman, 2005; Scalbert *et al.*, 2002; Scalbert *et al.*, 2005). Therefore, it is not surprising that the extraction and analysis of phenolics from plants and other food sources have been extensively studied (Naczk and Shahidi, 2004).

Incidence of Phenolics in the Plant Kingdom

In plants, phenolic compounds are metabolized from the amino acid L-phenylalanine and in some cases, L-tyrosine (Shahidi, 2000; Shahidi, 2002). Figure 2.1 is an illustration of the pathways of production of phenylpropanoids including stilbenes, lignans, lignins, suberins, cutins, flavonoids, and tannins. Figures 2.2 and 2.3 are illustrations of the enzymatic reactions undergone in the synthesis of phenolic acids (*trans*-cinnamic and benzoic acids) and flavonoids from phenylalanine. Phenolic compounds exist as a monomeric aglycone or in various bound forms. They are also the building blocks of large polymeric compounds such as tannins (Cheynier, 2005; Shahidi and Naczk, 2004). Figure 2.4 is a summary of the current classification of dietary phenolics, including examples. Many phenolic compounds and mixtures thereof are prevalent in a wide variety of fruits, vegetables, grains, and other plant products (Adom and Liu, 2002; Chu *et al.*, 2002; Madsen and Bertelsen, 1995; Paganga *et al.*, 1999; Pietta *et al.*, 1998; Shan *et al.*, 2005; Stratil *et al.*, 2006; Sun *et al.*, 2002). Research has shown that diets rich in fruits, vegetables, whole grains, and other sources of phenolics can lead to an

increased quanity of antioxidants in the human body (Cao *et al.*, 1998). Also, phenolics may work together synergistically to improve one's total health status (Liu, 2004).

Phenolic Acids

As depicted in Figure 2.2, phenolic acids of the benzoic and *trans*-cinnamic acid families are synthesized from L-phenylalanine (and L-tyrosine) in plants. This process is commonly referred to as phenylpropanoid metabolism. Figures 2.5 and 2.6 are chemical structures of the most predominant phenolic acids of the benzoic and *trans*-cinnamic acid families. Hydroxycinnamic acids are most widely distributed in plant tissues. They are often found in the form of hydroxyacid esters with quinic, shikimic, or tartaric acid residues (Herrmann, 1989).

Phenolic acids have been associated with many aspects of food quality including color, sensory properties, and nutrition (Maga, 1978). Of the many methods available for their selective separation and analysis, RP-HPLC methods with spectrophotometric detection are the overwhelming majority, with gas chromatography (GC) along with derivatization steps being employed to a lesser extent (Robbins, 2003). Figures 2.7 and 2.8 are UV-spectral scans of phenolic acids of the benzoic and *trans*-cinnamic acids families. While benzoic acids typically yield their primary UV-maximum near 260 nm (*i.e.*, especially *p*-hydroxybenzoic, vanillic, and protocatechuic acids), most *trans*-cinnamic acids absorb UV-radiation nearer to 320 nm. The inherent differences in UV-spectra exhibited by the two phenolic acid families provides for their selective chromatographic identification.

Flavonoids

Flavonoids are the most common and widely distributed group of phenolic compounds in plants. As seen in Figure 2.3, their basic makeup is a diphenylpropane core structure that consists of two outer aromatic rings with a three-carbon bridge, that can be closed (*e.g.*, flavones, flavanols, and anthocyanidins) or open (*e.g.*, chalcones). Flavonoids most commonly occur as glycosides in plants, with some classes consisting of up to 380 variations in their chemical structure (Bravo, 1998). In the case of flavonoids, their altered substitution and saturation patterns can result in the production of flavones, flavonols, flavanones, flavanonols, flavanols, and anthocyanidins. The chemical backbones of various flavonoids/isoflavonoids commonly found in plants are depicted in Figure 2.9.

Phenolic Polymers

Phenolic polymers, or tannins, were named because of their capacity to bind to proteins in the transformation of animal hides to leather. Tannins can be subdivided into two classes based on their inherent chemical make-up: hydrolyzable and condensed tannins. Hydrolyzable tannins can be further segregated into gallotannins and ellagitannins. Gallotannins consist of gallic acid subunits esterified to glucose. Ellagitannins are simply polymers of ellagic and gallic acid. Figures 2.10 and 2.11 are examples gallo- and ellagitannins, respectively. Hydrolyzable tannins are so-named because they easily hydrolyze in weak acid or alkali to their individual monomeric units (Bravo, 1998).

Condensed tannins, or proanthocyanidins (PACs), release anthocyanidin monomers when heated in the presence of acid (Cheynier *et al.*, 1999). In foods, PACs are usually classified as procyanidins or prodelphinidins according to the chemistry of their flavan-3-ol subunits.

Procyanidins are comprised of epicatechin monomers, whereas prodelphinidins are comprised of epigallocatechin subunits. PACs are subdivided into A- and B-types according to their interflavonoid linkages. B-type PACs have a C4→D8 or C4→D6 interflavonoid linkage; whereas, A-type PACs have an additional ether linkage from C2→D7 (Ferreira and Li, 2000) as seen in Figure 2.12. PACs can range from dimeric to oligomeric species with many subunits. In fact, decamers with a molecular mass greater than 30 kDa have been reported from cocoa and sorghum (Gu *et al.*, 2002).

Extraction of Phenolics from Plants

In order for proper chromatographic analysis, phenolics must first be extracted from their respective plant or food matrices. Extraction efficiency is influenced by analyte particle size, extraction solvent(s), pH, time, temperature, and agitation as well as the presence of potential interfering substances such as sugars (Naczk and Shahidi, 2004). Solubility of targeted phenolic compounds in the selected extraction solvent is largely dependent on their relative polarities. If one is attempting to extract a wide variety of phenolic and polyphenolic constituents from a single plant or food source, the conditions for extraction should take into account the complex nature of the selected compounds. Often this is accomplished through the use of multiple extraction solvents and sequential liquid partitioning followed by the chromatographic analysis of the components in each fraction. The most common methods of phenolic extraction employed in the current literature involve pH-buffered aqueous/organic mixtures of methanol, ethanol, acetone, and ethyl acetate (Naczk and Shahidi, 2004).

Phenolics in Food

Along with providing health benefits, ingredients rich in phenolics are employed as antioxidants in a variety of food systems (Anderson *et al.*, 2005). More recently polyphenolics have been added to functional foods and nutraceuticals to bestow targeted health benefits to consumers. The inclusion of phenol-rich components in nutritive foods and beverages needs, however, to be intelligently employed to ensure that the phenolics do not adversely affect sensory attributes of the food (Lesschaeve and Noble, 2005) and that they are not significantly biodegraded before reaching their point of absorption in the human body. This is often accomplished by microencapsulation and other stabilization techniques.

Phenolic Bioavailability/Bioactivity Post Consumption

As discussed, the human body contains a very complex system of chemical and enzymatic defense mechanisms. Once antioxidants enter the body, they do not necessarily pass through us unaltered or reach their required absorption site in the gastrointestinal (GI) tract; hence, bioavailability and bioactivity must be considered. The bioavailability of phenolics and polyphenolics has been studied extensively over the past two decades, whether by examining the kinetic patterns of polyphenol absorption in the bodily fluids of healthy volunteers (Manach *et al.*, 2005) or by epidemiological intervention studies in hospitals (Williamson and Manach, 2005). These studies have, however, yielded conflicting results. Though much knowledge has been acquired involving the absorption of phenolic acids and flavonoids in the GI tract (Scalbert and Williamson, 2000), more targeted investigations are warranted.

Dietary origins of polyphenolics have been established including PACs in dark chocolate and ellagitannins in pomegranate, but methods for screening daily intake of these compounds

have only recently been developed (Prior and Gu, 2005). PACs have gained considerable attention as of late and are quickly becoming the most popular ingredient for natural *in vivo* antioxidant therapy (Dixon *et al.*, 2005). Much of this attention is due to their capability of binding to proteins and surviving passage through the human GI tract. Tannins can also survive certain thermal processing and greatly retard lipid oxidation in foods (Amarowicz, 2007; Pegg and Amarowicz, 2004). Whether or not the large-scale addition of phenolics to the American diet in the form of supplements or formulated foods is needed or safe is still under debate (Pokorný, 2007).

2.4 Free-Radical Theory: Basis for Quantification of Antioxidant Capacity

Due to the growing popularity of phenolics over the past decade, new scientific methods have been developed to directly quantify the content of phenolic antioxidants in plants, foods, and food components (Moon and Shibamoto, 2009; Naczk and Shahidi, 2004; Stratil *et al.*, 2006); to determine antioxidant efficacy in lipid and food model systems (Becker *et al.*, 2004; Decker *et al.*, 2005; Laguerre *et al.*, 2007); and to gauge relative antioxidative capacities of phenolic compounds *in vitro* (Apak *et al.*, 2007; Llesuy *et. al*, 2001; Schlesier *et al.*, 2002; Yoo *et al.*, 2007) and *in vivo* (Aruoma, 2003; Cao and Prior, 1998; Prior and Cao, 1999). Perhaps of particular interest is research directed towards the elucidation of structure-activity relationships of phenolic antioxidants (Lemańska *et al.*, 2001; Lien *et al.*, 1999; Nijveldt *et al.*, 2001; Rice-Evans *et al.*, 1996). In this context, it is important to differentiate the terms antioxidant activity and antioxidant capacity, though they are often employed interchangeably. Antioxidant activity refers explicitly to the rate constant of a single antioxidant and free radical within a given system. On the other hand, antioxidant capacity corresponds to the total radical-scavenging

capability of a test solution, independent of individual antioxidant activity constants (Ghiselli *et al.*, 2000). Given the presence of many different antioxidants in biological systems, methods involving the quantification of "total antioxidant capacity" (TAC) are mostly used in today's laboratories.

The mechanisms by which phenolics exert antioxidant activity/capacity hold their basis in free-radical reactions. Free-radical reactions involve the following mechanisms: (i) initiation reactions in which the number of free radicals increases; (ii) propagation reactions in which the total number of radicals remains constant (*i.e.*, the number of radical species can change); and (iii) termination reactions in which the number of free radicals decreases. The following reaction schemes (1-5) illustrate these processes:

$$\mathbf{RH + Initiator \rightarrow R^{\bullet} + H^{\bullet}\ (Initiation)} \qquad \mathbf{(1)}$$

$$\mathbf{R^{\bullet} + O_2 \rightarrow ROO^{\bullet}\ (Propagation)} \qquad \mathbf{(2)}$$

$$\mathbf{ROO^{\bullet} + R'H \rightarrow ROOH + R^{\bullet}\ (Propagation)} \qquad \mathbf{(3)}$$

$$\mathbf{ROO^{\bullet} + R^{\bullet} \rightarrow ROOR\ (Termination)} \qquad \mathbf{(4)}$$

$$\mathbf{R^{\bullet} + R^{\bullet} \rightarrow RR\ (Termination)} \qquad \mathbf{(5)}$$

The generated radical (1) can propagate with molecular oxygen (2) and undergo many subsequent propagation reactions (3) with endogenous or exogenous substrates resulting in a

variety of ROS. It is important to note that $R^{\bullet}$ (1) is relatively unreactive; however, once it propagates with $^{3}O_2$ to form $ROO^{\bullet}$, it becomes highly reactive.

The primary products of lipid autoxidation are lipid hydroperoxides (LOOHs). LOOHs are very unstable and degrade to secondary oxidation products such as aldehydes, ketones, alcohols, and hydrocarbons, which affect food quality. Though many of the *in vitro* radical generation reactions discussed herein are initiated by chemical (metal-ion catalyst), thermal (heat), and electromagnetic (light) means, there are also important enzymatic radical-generation systems (Hodgson and Fridovich, 1976). Most antioxidant capacity assays can be grouped into the following three categories by the chemistry involved therein: (i) hydrogen-atom transfer (HAT); (ii) single-electron transfer (SET); and (iii) mixed-mode methods that contain both HAT and SET chemical processes (Schaich, 2006). HAT and SET mechanisms are two of the main pathways by which antioxidants can reduce the presence of ROS in foods and in the body.

Hydrogen-Atom Transfer (HAT) Mechanism

Antioxidant capacity methods involving the HAT mechanism measure the capability of an antioxidant compound to quench free-radical species by donating hydrogen atoms. These methods usually involve the forced decomposition of azo-initiator compounds in the presence of oxygen, resulting in the production of peroxyl radicals ($RO_2^{\bullet}$), which in turn react with target colorimetric, chemiluminescent, or fluorescent probes. Reaction scheme (6) below illustrates HAT chemistry: an antioxidant component (abbreviated as an aromatic component [Ar] and a hydroxy component [OH]) donates an H-atom to an unstable free radical and in this process becomes a more stable free-radical species, which is less likely to propagate further radical reactions with initiation substrates (Wright *et al.*, 2001):

$$(n)RO_2^{\bullet} + ArOH \rightarrow (n)ROOH + ArO^{\bullet} \text{ (HAT)} \quad (6)$$

Figure 2.13 is an illustration of the conjugated resonance stabilization of phenoxyl radicals. Although the phenoxyl-radical electron (6) initially exists on the highly electronegative oxygen atom, it is likely that the electron is delocalized and shared throughout the aromatic ring. In reaction (6), *n* represents the stoichiometric factor for the reactant free radical and the resultant phenolic compound. Vitamin E has been shown to react with two peroxyl radicals per molecule (Burton and Ingold, 1981). The weaker the hydrogen atom is held to the reactant hydroxy substituent of the antioxidant compound in reaction (6), the more exothermic (*i.e.*, $\Delta H < 0$) the resultant reaction with free-radical species will be, and the more likely and faster it will participate in HAT reactions with free-radical substrates. Therefore, the bond dissociation enthalpy (BDE) of an antioxidant species is a parameter when studying the capacity of a phenolic compound to undergo a HAT in free-radical reactions (Wright *et al.*, 2001).

The Role of Antioxidant Chemistry in HAT Processes

Antioxidant size, chemistry, and polarity play a role in their capacity and speed in HAT reactions (Silva *et al.* 2000). HAT reactions may be hindered by the presence of electron withdrawing groups in the 3- and 5-positions (*meta*) *via* deactivation of the aromatic ring (Streitwieser and Heathcock, 1981). HAT reactions increase with the presence of *t*-butyl groups at the 2- and 5-positions (*ortho*), and methoxy constituents in the 4-position (*para*) by inductive donation of electron density to help in the resonance stabilization of the generated phenoxyl radical (Howard and Ingold, 1963). The *p*-type lone pair orbital of an oxygen-containing substituent located in the 4-position on a phenolic ring is thought to overlap with the semi-

occupied molecular orbital (SOMO) of the generated radical species upon hydrogen abstraction (Burton and Ingold, 1986). If, however, the 4-methoxy substituent is forced out of the plane by neighboring alkyl groups, as in the case of TMMP (4-methoxy-2,3,5,6-tetramethylphenol), its *p*-type lone pair electrons are no longer available to participate in resonance structures with the aromatic ring (Burton and Ingold, 1981). Generally, the presence of large substituents on the aromatic ring reduces the capability of free radicals to dimerize with the phenolic hydroxy group by steric crowding (Mahoney, 1969), thereby increasing the likelihood of HAT. These parameters give a possible explanation for the strong antioxidant activity observed for the food preservatives butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA).

Figure 2.14 is an example of two sequential hydrogen abstractions incurred by peroxyl radicals resulting in the conversion of L-ascorbic acid (Vitamin C) to dehydroascorbic acid, and the resultant creation of two hydroperoxides. Although ascorbic acid lacks phenol chemistry, it does contain two hydroxy functional groups located off of a conjugated furan ring, which renders it sufficiently stable to participate in free-radical redox chemistry (Brand-Williams *et al.*, 1995). Although generated phenoxyl radicals (6) could terminate with each other (dimerization) or with substrate-radical initiators (complexation), the generated phenoxyl radicals are sufficiently stable to readily react with further substrate stoichiometrically until fully oxidized (Blois, 1958). Rice-Evans *et al.* (1996) offer tremendous insight into the structure-activity relationships of phenolic acid and flavonoid HAT reactions. Furthermore, Dangles *et al.* (2000) have assessed the phenomenon exhibited by $DPPH^{\bullet}$ HAT mechanisms with 3′,4′,7-trihydroxyflavylium cation and catechin (*i.e.*, as models for anthocyanins and PACs, respectively).

Single Electron Transfer (SET) Mechanism

SET-based methods involve the capability of an antioxidant to transfer a single electron to aid in the reduction of potential target compounds. SET antioxidant capacity assays often involve an oxidizable substrate that doubles as the colorimetric or fluorometric reaction probe. These reaction probes are often made-up of large nitrogen-containing metal ion-chelating agents that, upon reduction, result in the production of color or emission of electromagnetic (EM) radiation. While the ferric-reducing antioxidant power (Pulido *et al.*, 2000) and cupric-reducing antioxidant capacity assays (Apak *et al.*, 2004) are "in-tube" utilizing reagent kits, other methods involve the binding of chelating agents to solid supports for subsequent drop-and-read spectrophotometric analysis (Zaporozhets *et al.*, 2004).

The Role of Ligand-Metal Ion Complexes

The capability of metal ions (*e.g.,* copper[II], nickel[II], cobalt[II]) to incorporate into nitrogen-containing tridentate and tetradentate closed macrocyclic ligand complexes with organic substrates has long been known (Eichhorn and Latif, 1954; House and Curtis, 1962; Melson and Busch, 1964). Furthermore, chelating agents of this type (especially 2,2′-bipyridine and 1,10-phenanthroline) have long been used as redox indicators (Brandt and Smith, 1949) and chromaphoric reagents in spectrophotometric analyses of inorganic constituents (Zak, 1958). The metal ion-complexes created with bipyridine and phenanthroline derivatives are considered highly ordered and stable due to the presence of strong ligand fields and π-back bonding to metal ions (Pilipenko and Falendysh, 1972). Many *in vitro* SET antioxidant capacity assays, involve the donation of electrons to reduce these conjugated nitrogen-containing metal ion complexes.

The following reaction schemes (7-9) illustrate a SET mechanism, in which an

antioxidant transfers a single electron to a ROS. The resultant radical-cationic antioxidant compound is then deprotonated through interaction with water.

$$(n)RO_2^{\bullet} + ArOH \rightarrow RO_2^{-} + [ArOH]^{\bullet+} \text{ (SET)} \quad (7)$$

$$[ArOH]^{\bullet+} + H_2O \leftrightarrows ArO^{\bullet} + H_3O^{+} \text{ (Deprotonation Equilibrium)} \quad (8)$$

$$RO_2^{-} + H_3O^{+} \leftrightarrows ROOH + H_2O \text{ (Hydroperoxide Formation)} \quad (9)$$

The finality of SET reaction (8) is the same as HAT reaction (6) in terms of radical scavenging; however, the SET reaction (7) can be subject to further radical-propagation reactions with the extended life of $[ArOH]^{\bullet+}$ (Wright *et al.*, 2001). The resultant antioxidant species from reaction (7) $[ArOH]^{\bullet+}$, illustrates that although the radical electron and formal charge do initially exist on the oxygen atom, it is likely that the electron is delocalized and distributed throughout the aromatic ring.

The Role of Antioxidant Chemistry and Reaction Medium on SET Processes

Given that reaction (7) involves the creation of ionic species, the ionization potential (IP) of an antioxidant compound becomes a parameter for predicting the capability of a phenolic species to scavenge free radicals *via* SET. The greater the ionization energy required, the more reluctant an antioxidant molecule will be to donate an electron (Wright *et al.*, 2001). IP decreases with increasing pH, so SET reactions are favored in alkaline environments. Given the involvement of metal ions in SET reactions, laboratory environs (*e.g.,* glassware, reagents, and

solvents) must be free of trace inorganics so as not to elevate reaction progress and inflate assay results. Also, SET processes can take long periods of time to reach completion; therefore, monitoring assay progress over time is necessary to elucidate any reaction time-dependence and/or the presence of secondary reaction processes (Schaich, 2006). In terms of phenol structure-activity relationships with SET processes, Mira *et al.* (2002) have made progress in determining the incidence of iron- and copper-ion complexation reactions with respect to flavonoid composition.

Incidence of Mixed HAT and SET Mechanisms

Leopoldini *et al.* (2004) and Wright *et al.* (2001) assert that although many antioxidant reactions are characterized as following either HAT or SET chemical processes, these reaction mechanisms can, and do, simultaneously occur. Migliavacca *et al*. (1997) assert that α-tocopherol undergoes simultaneous HAT and SET mechanisms with radical substrates, and that these processes are interrelated. Zhang and Ji (2006) corroborate this assertion through studies of the interaction of Vitamin E with $DPPH^{\bullet}$ in polar protic media, in which both HAT and sequential proton-loss electron transfer (SPLET), also termed proton-coupled electron transfer (PCET) by Huang *et al.* (2005), were found to be thermodynamically favorable reactions. SPLET reactions represent one of the main mechanistic sources of error in falsely denoting SET reactions as HAT, because they can occur rapidly in certain environs. SET reactions are often slower than HAT ones; therefore, if the reaction kinetics between an antioxidant substrate and free radical are expeditious in a given system, HAT is often assumed to be the predominant mechanism. Figure 2.15 is an example of a SET mechanism between α-tocopherol and 4-methoxybenzoyloxyl radical, as suggested by Evans *et al.* (1992). Although α-tocopherol can

undergo a SET with radical substrates, its radical-scavenging behavior is still thought to be predominantly HAT (Burton and Ingold, 1981; Nakanishi *et al.*, 2002; Zhang and Ji, 2006).

Prior *et al.* (2005) suggest that even though HAT and SET chemical processes result in the same end products, SET reactions can be subject to significant secondary reaction processes and involve more potential interferences than HAT reactions. SET reactions often take long periods of time to reach completion, and interfering substances can exert a great effect on their accuracy. The most prevalent mechanism in any system will depend on antioxidant structure, properties, and medium of interaction (Huang *et al.*, 2005; Prior *et al.*, 2005). If bulky constituents are located adjacent to phenolic hydroxy groups, steric issues may hinder HAT/SET efficacy. Similarly, if antioxidant reactions are carried out in hydrogen bond-accepting solvents, HAT efficiency will be greatly reduced (Barclay *et al.*, 1999; Evans *et al.*, 1992). Therefore, one should not expect the top ten scoring foods in a HAT assay to be identical to those in a SET assay. In fact, it has been reported that they do not directly compare (Bhagwat *et al.*, 2007). Nevertheless, each assay – be it HAT, SET, or mixed – involves the correlation of an antioxidants capability to perform in relation to a standard antioxidant compound.

Carotenoids: A Model for the Explanation of the Dual Functions of Antioxidants

Carotenoids are tetraterpenoids (C40) consisting of a highly conjugated polyene chain with some terminated by substituted-cyclohexene rings. A number of carotenoids are oxygen-containing (xanthophylls), while others are simply polymeric isoprenoids (carotenes). Carotenoids exist in nature as light harvesting and photo-protective pigments in the photosynthetic reaction centers of plants and other organisms such as algae, fungi, and some bacteria. While carotenoids have long held the status of being efficient at quenching $^1O_2$, only in

the past few decades have their other potential mechanisms of antioxidant activity been more understood.

Primarily, carotenoids exert antioxidant activity by catalytically quenching $^1O_2$ through an energy transfer process illustrated by reaction scheme (10) (Garavelli *et al.*, 1998):

$$\mathbf{^1O_2 + {}^1carotenoid \rightarrow {}^3O_2 + {}^3carotenoid \rightarrow {}^3O_2 + {}^1carotenoid (+ heat)} \qquad \mathbf{(10)}$$

Carotenoids interact with $^1O_2$ resulting in the creation of triplet oxygen ($^3O_2$) and a carotenoid triplet (^{3}carotenoid), which then relaxes to the ground state with energy being evolved in the form of heat. The efficiency by which a carotenoid physically quenches $^1O_2$ increases with the greater number of conjugated carbon-carbon double bonds. This is due to the lowering of excitation (triplet) energy as π-conjugation extends in the higher conjugated carotenoid species (Speranza *et al.*, 1990). Furthermore, the reactivity of carotenoids towards $^1O_2$ increases approaching an all-*trans* sterio configuration. Lycopene is the most efficient C40 $^1O_2$ quencher; it is more efficient than all-*trans* β-carotene even though both have $n = 11$ conjugated double bonds (Conn *et al.*, 1991).

Xanthophylls are rendered more reactive toward $^1O_2$ with the presence of an epoxide substituent on the two terminal cyclohexene rings when compared to carbonyl or hydroxyl substituents. Furthermore, kinetic measurements involving the capability of carotenoids to quench $^1O_2$ are often carried out *in vitro*, and they can vary based on the reaction medium (Conn *et al.*, 1991). In examination of this "solvent effect," Speranza *et al.* (1990) observed that in aqueous solutions polyene oxidation is chemical (rather than physical) and undergoes an electron transfer mechanism, characterized by the higher dielectric constant of water and a more negative

oxidation potential (E_o) for carotenoids. The chemical processes by which carotenoids exert antioxidant activity towards $^1O_2$ are regarded as less efficient, and the specific mechanistic processes involved are not yet clearly understood.

Given that carotenoids demonstrate antioxidant activity in model systems where $^1O_2$ is absent (Packer *et al.*, 1981), much of the current research on carotenoids has examined their capability to scavenge a host of generated radical species *in vitro*. Although HAT processes and adduct formation (AF), or carotenoid-radical dimerization, are two viable mechanisms by which carotenoids can exert antioxidant activity towards free radicals, a SET mechanism seems dominant. The following reaction schemes (11-13), illustrate these mechanisms (Galano, 2007):

$$\mathbf{R^{\bullet} + carotenoid(H) \rightarrow RH + carotenoid^{\bullet}\ (HAT)} \qquad \mathbf{(11)}$$

$$\mathbf{R^{\bullet} + carotenoid(H) \rightarrow R^{-} + carotenoid(H)^{\bullet +}\ (SET)} \qquad \mathbf{(12)}$$

$$\mathbf{R^{\bullet} + carotenoid(H) \rightarrow [R\text{-}carotenoid(H)]^{\bullet}\ (AF)} \qquad \mathbf{(13)}$$

In general, the high reactivity of carotenoids toward radical substrates is due to their high electron donating capacity (*i.e.*, nucleophilicity). When the R-groups are electrophilic, they will undergo a SET mechanism with a carotenoid and produce a carotenoid-radical cation and corresponding substrate anion. Mortensen and Skibsted (1997) demonstrated that carotenoids can regenerate phenols by reduction as well as dimerize with phenoxyl radicals (13). Figure 2.16 is a proposed SET mechanism for the reduction of a phenoxyl radical by the carotenoid echinenone resulting in two canonical carotenoid-radical cations.

Burton and Ingold (1984) assert that although not considered conventional chain breaking antioxidants, carotenoids can decrease rates of lipid oxidation by being oxidized themselves. Carotenoids have the capability to form inhibiting carbon-centered radicals through peroxyl-radical dimerization. Figure 2.17 is an illustration of this reaction in which there is a nucleophilic attack by the carotenoid molecule, resulting in addition of the peroxyl radical to the polyene chain. Iannone *et al.* (1998) have also confirmed the quenching of peroxyl radicals by β-carotene. To date, little evidence exists to show the predominance of a HAT mechanism in carotenoid antioxidant capacity.

Example Methodologies for HAT, SET, and Mixed Antioxidant Capacity

Many *in vitro* antioxidant assays can be modified for the production/quenching of several radical species based on the use of a variety of azo-intiator compounds, metal-ion catalysts, and thermal or photo-degradative processes. For example, the ORAC assay (Huang *et al.*, 2002b; Prior *et al.*, 2003) typically measures peroxyl-radical scavenging; yet, it has been successfully modified for hydroxyl-radical production (Ou *et al.*, 2002). The most commonly used azo-initiators are AAPH (also called ABAP, 2,2′-azobis[2-amidinopropane] dihydrochloride) for hydrophilic systems and AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) for hydrophobic systems. For the sake of simplicity, examples of each of the three types of antioxidant assays have been provided in the following sections. They include the following: assay name, acronym, and radical(s) involved. A comprehensive list of *in vitro* antioxidant assays is beyond the scope of this work.

HAT Assays

Examples of HAT assays are as follows: chemiluminescence-based assays such as azo-initiated chemiluminescence (CL) ($RO_2^{\bullet}$) (Alho and Leinonen, 1999), photochemiluminescence (PCL) ($O_2^{\bullet -}$) (Pegg *et al.*, 2007; Popov and Lewin, 1999a) and total antioxidant reactivity (TAR) ($RO_2^{\bullet}$) (Campos *et al.*, 1996; Lissi *et al.*, 1995); fluorescence-based assays such as ORAC ($RO_2^{\bullet}$) (Huang *et al.*, 2002b; Prior *et al.*, 2003) and TRAP ($RO_2^{\bullet}$) (Wayner *et al.*, 1985; Wayner, 1987; Lussignoli *et al.*, 1999); crocin or β-carotene bleaching assays (Kampa *et al.*, 2002; Miller, 1971; Tanizawa *et al.*, 1983; Tubaro *et al.*, 1998); as well as other azo-initiated or metal ion-catalyzed reaction systems such as total oxyradical-scavenging capacity (TOSC) ($RO_2^{\bullet}$ and $HO^{\bullet}$) (Regoli and Winston, 1999; Winston *et al.*, 1998), liposome model systems (Roberts and Gorgon, 2003), and low-density lipoprotein (LDL) oxidation models (Esterbauer *et al.*, 1992; Frankel *et al.*, 1995).

SET Assays

Examples of SET methods include the following: the total phenolics content (TPC) assay with Folin-Ciocalteu's phenol reagent (Mo^{6+}[yellow] → Mo^{5+}[blue]) (Folin and Ciocalteu, 1927; Singleton *et al.*, 1999; Singleton and Rossi, 1965); the cupric reducing antioxidant capacity (CUPRAC) assay (Cu^{2+} → Cu^{+} [complexed]) (Apak *et al.*, 2004; Moffet *et al.*, 1985), and the ferric reducing antioxidant power (FRAP) assay (Fe^{3+}-TPTZ → Fe^{2+}-TPTZ) (Benzie and Strain, 1996; Pulido *et al.*, 2000).

Mixed-Mode Assays

Mixed methods that encompass both HAT and SET chemical processes include the TEAC assay ($ABTS^{\bullet+}$; 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]) (Miller *et al.*, 1993; Re *et al.*, 1999) and the DPPH assay ($DPPH^{\bullet}$; 2,2′-diphenyl-1-picrylhydrazyl radical cation) (Hatano *et al.* 1988, Sánchez-Moreno *et al.* 1998). It is important to note that the TEAC and DPPH assays involve nitro-radicals; therefore, these methods can be used to screen an antioxidant's capacities to scavenge RNS *in vivo* (not ROS). Another, but less common, mixed-mode assay involves the scavenging of *N*,*N*-dimethyl-*p*-phenylenediamine radical cation ($DMPD^{\bullet+}$) (Fogliano *et al.*, 1999).

The Importance of Assay Choice

Given the different chemistries involved in each group of antioxidant/radical-scavenging methods, the assay of choice to be employed is critical. Not all methods and antioxidant sources are compatible, and the same antioxidant species can yield varying results in different assays. Two excellent reviews discussing the strengths and weaknesses of antioxidant methods/ techniques have recently been published (Frankel and Finley, 2008; Schaich, 2006). Though standardization of methods has been suggested (Prior *et al.*, 2005), no official antioxidant capacity assays exist to date.

2.5 *In Vitro* Antioxidant-Screening Methods of Critical Importance

Primary Lipid Oxidation Methods

Peroxide Value

Given that the primary products of lipid oxidation are hydroperoxides (commonly referred to as peroxides), their quantification can provide a suitable measurement of the extent of oxidation present in a lipid sample. Protocols for the quantification of peroxide values (PVs) in foods can be iodometric or colorimetric methods, each with its strong and weak points (*e.g.,* iodometric titration endpoint) (Pegg, 2005). Because the extent of oxidation of a lipid is related to its PV, the capability of an antioxidant compound to perform in a closed system can be gauged by the prevention of peroxide formation over time with respect to a control. Given that autoxidation of lipids is a time-consuming process (Gray, 1978), a radical initiator such as AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) is often used in order to speed up the reaction. The use of such compounds and their applicability to measure the extent of lipid oxidation in foods has, however, come under increased scrutiny as of late. In fact, Frankel and Finley (2008) call for the complete removal of azo-initators from antioxidant screening methods.

The ferrous-oxidation xylenol orange (FOX) assay is a spectrophotometric method for the determination of lipid hydroperoxide activity. FOX is based on the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) under acidic conditions, which then reacts with xylenol orange (XO) complexing dye (*o*-cresolsulfonphthalein-3′,3″-*bis*[methyliminodiacetic acid sodium salt]) in a ratio of 1:1 forming a colored product with a λ_{max} at 550 nm, as seen in reaction scheme (14) (Gay and Gebicki, 2003; Wolff, 1994):

$$\mathbf{LOOH + Fe^{2+} + XO \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+}\text{-}XO\ (Purple\ at\ 550\ nm)\ (SET)} \qquad \mathbf{(14)}$$

Although the assay was initially created for the determination of H_2O_2 in irradiated solutions (Gupta, 1973), it was later applied for lipid and aqueous hydroperoxide measurement (Jiang *et al.*, 1991; Wolff, 1994) and extended to the measurement of protein and lipid hydroperoxides *in vivo* (Gay and Gebicki, 2003). The FOX assay has been modified for use on microtiter plates in 96-well high-throughput format (Waslidge and Hayes, 1995). Recently, it was modified for the determination of lipoxygenase activity in plant extracts (Pinto *et al.*, 2007).

Hydroperoxide values are often reported as mmol LOOH/kg linoleic acid. Percent inhibition of oxidation is compared between different levels of antioxidant extract added and the lipid sample subject to autoxidation (or accelerated oxidation). The assay reaction of iron(III) and xylenol orange follows SET chemistry; therefore, it is subject to interference by trace metal-ion contamination. Furthermore, the assay is only accurate at low LOOH concentrations, so there are problems with measuring highly oxidized samples. The Fe(III)-XO complexing reagent bleaches at LOOH levels normally found in foods and oils, so samples must be diluted extensively along with the preparation of dose-response curves.

Conjugated Dienes

The majority of polyunsaturated fatty acids (PUFAs) in nature have a 1,4-diene structure (*i.e.*, there points of unsaturation are methylene interrupted), so the occurrence/detection of conjugated dienes (CD) is an indication that fatty acids have undergone autoxidation (Corongiu and Banni, 1994). The presence of the two electron-withdrawing double bonds on the 1,4-diene moiety leads the methylene component's two hydrogens to be held rather loosely. Once one of these hydrogens is preferentially abstracted, the two exterior carbons of the 1,4-diene moiety become electron deficient and, thus, preferential targets for addition of $^3O_2$. This then leads to

double bond rearrangement and the formation of a CD. Monohydroperoxides are the primary products of lipid oxidation. The number of positional isomeric peroxides that can result from autoxidation of a lipid depends on the number of double bonds (n) contained, and is equal to 2n-2 (Esterbauer, 1993). The oxidation of linoleic acid results in two monoperoxides formed either at carbon 9 (*i.e.*, 9-OOH) or carbon 13 (*i.e.*, 13-OOH); see Figure 2.18 (Corongiu and Banni, 1994).

The premise of the CD assay, which has been in use before the 1950s (Farmer and Sutton, 1943), is the strong UV absorbance of the CD moiety at λ_{max} = 234 nm. Conjugated dienes are the first indicator of oxidation in model lipid systems and are often retained in many secondary products, even after PVs decrease in later stages of oxidation. Given the lack of complicated reagents and preparative work required, the assay is a very attractive option for a quick assessment of lipid oxidation or the capability of an exogenous antioxidant to inhibit autoxidation. Yet in some cases, absorbance of the diene moiety of an oxidized lipid is not easily related to the full extent of oxidation in a sample. The effects of autoxidation on lipids can vary (Gray, 1978), and results are best explained if the composition of the lipid is known (Holman and Burr, 1946). In explanation, lipid oxidation is a dynamic process and the chemistry of an oxidized sample is constantly changing. Pryor *et al.* (1993) describe a modern linoleic acid model system that follows the same premise of the classical CD assay. When the PUFAs contain three or more double bonds (*e.g.,* α-linolenic acid), conjugated trienes (CTs) can be formed by two sequential double bond rearrangements. CTs yield strong absorbancies at λ_{max} = 268 and 278 nm in the UV-range (Pegg, 2005). There is current development on a standardized conjugated autoxidizable triene (CAT) assay involving the spectral properties of triacylglycerols (TAG) naturally present in tung oil (Laguerre *et al.*, 2008).

The Total Phenolics Content (TPC) with Folin Ciocalteu's Phenol Reagent

Phosphotungstic and phosphomolybdic heteropolyacids have been used as colorimetric reagents since the early 1900s (Folin and Macallum, 1912). In 1912, a breakthrough in colorimetry occurred with the creation of a sensitive chromophoric complexing reagent for the quantification of tyrosine residues in protein hydrolysates (Folin and Denis, 1912a; Folin and Denis, 1912b). The Folin-Denis phenol reagent (as it was named) was again reformulated in 1927 (Folin and Ciocalteu, 1927), with the greater incorporation of molybdenum for increased redox sensitivity. Singleton and Rossi (1965) applied Folin & Ciocalteu's phenol reagent to the assessment of antioxidant contents in wine, resulting in the well-known total phenolics content (TPC) assay. With the increased interest in phenolics over the past two decades, this assay has become a mainstay in antioxidant laboratories the world over.

TPC is often modified based on the antioxidant source under investigation. While the most up-to-date method involves automation on a 96-well microtiter plate reader (Zhang *et al.*, 2006), the original in-tube assay is most prevalent. Despite modifications, the bulk of the total phenol protocol has remained the same since Singleton and Rossi (1965). Briefly, the assay involves the mixture of excess phenol reagent and a diluted sample or standard (gallic acid). The mixture is then treated with alkali till a final pH of 10 to 11 is reached. The resultant color complex is allowed to develop over 30 to 60 min and yields a λ_{max} in the range of 745 to 765 nm, depending on the standard employed.

The mechanism behind the TPC assay involves reduction of the molybdenum component in the phosphotungstic-phosphomolybdic complexing reagent according to the following reaction scheme (15):

$$\mathbf{Mo^{6+}\ (yellow)\ +\ ArOH\ \rightarrow\ Mo^{5+}\ (blue\ at\ 750\ nm)\ +\ [ArOH]^{\bullet+}\ (SET)} \qquad \mathbf{(15)}$$

Reaction (15) is subject to a great many interferences, particularly any readily reducible component present within the assay mixture. Ascorbic acid is the major interference in the case of wine analysis (Singleton *et al.*, 1999) and most fruits.

Although the phenol reagents of Folin-Denis and Folin-Ciocalteu have been around for 75+ years, the chemistry of the reagents and the phenol-reagent reactions are still not well understood. It is possible that the reaction product between phenolate anions and Folin & Ciocalteu's phenol reagent is a group of Keggin clusters: a common form of heteropoly acids comprised of a cage structure of oxygen-containing phosphomolybdic and phosphotungstic repeats bearing the common formula $[XM_{12}O_{40}]^{n-}$, where X is the heteroatom (phosphorus) and M is addenda metal atom (molybdenum or tungsten in this case) (Pope, 1983). The inherent stability of Keggin clusters promotes the reduction of the metal ion contained, and, thus, facilitates the utilization of such reagents for colorimetry. It is generally accepted that in alkaline media, three competitive reactions are proceeding simultaneously including the "destruction" of the yellow Folin-Denis/Ciocalteu phenol reagent, the reduction of the reagent by phenolate anions to produce the characteristic "molybdenum blue," and the destruction of the blue pigment by alkali (fading). Increasing the alkalinity or temperature of the assay promotes the development of the color complex in a shorter period of time. However, precipitation of the phenol reagent can occur. The precipitate is a dense, white, crystalline material that can be formed by excessive heat (above 60 °C), alkalinity (above pH 10-11), or the quantity of reagent in the assay (above 5 mL/100 mL) (Rosenblatt and Peluso, 1941).

HAT Assays

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) Assay

The ORAC assay was developed by Dr. Alexander N. Glazer in the early 1990s for the determination of ROS in biological systems. The assay is based on the fluorescence of photosynthetic phycobiliproteins from cyanobacteria (blue-green algae) and two groups of eukaryotic algae (red algae and cryptomonads) (Glazer, 1990). The B- and R-phycoerythrins were the most stable and suitable for spectrophotometric analysis. The ORAC assay was adapted by Cao *et al.* (1993) for the assessment of antioxidant species in human plasma. It was later automated on the Cobas Fara II centrifugal analyzer (Cao *et al.*, 1995) and used to determine the TRAP of human plasma (Ghiselli *et al.*, 1995). After the application of the phycoerythrin-based assay to tea, vegetables, and biological fluids (Cao *et al.*, 1996; Cao *et al.*, 1998), Dr. Ronald L. Prior and his colleagues modified the method using fluorescein (FL) (3′6′-dihydroxy-spiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) as a more stable and reproducible fluorescent probe (*i.e.*, the $ORAC_{FL}$ assay) (Ou *et al.*, 2001). Over the following years, the $ORAC_{FL}$ assay was adapted to a multi-channel liquid handling system coupled with a microplate fluorescence reader (Huang *et al.*, 2002b), and applied to both hydrophilic and lipophilic systems (Huang *et al.*, 2002a; Prior *et al.*, 2003). Dr. Prior's laboratory further modified the ORAC assay for the controlled generation and scavenging of $HO^{\bullet}$ (Ou *et al.*, 2002). More recently, a derivative of fluorescein (*i.e.*, dichlorofluorescein) (Adom and Liu, 2005) has been applied as the fluorescent probe in the ORAC assay, but fluorescein still remains the probe of choice for the majority of applications.

Despite the series of modifications discussed, the principles of the initial assay remain the same and include the following: azo-initiation of $RO_2^{\bullet}$ *via* thermal degradation of AAPH

followed by the competitive HAT reaction between antioxidant samples (or standard Trolox) and the generated peroxyl radicals with the fluorescent probe. FL gives off a real-time signal registered by the plate reader at an excitation/emission wavelength pair of 493/515 nm and declines rapidly as it undergoes a HAT reaction with the azide-generated peroxyl radicals. The following reaction scheme (16) illustrates this process:

$$\mathbf{2RO_2^{\bullet} + (FL)OH\ (Fluorescence\ at\ 515\ nm) \rightarrow 2ROOH + (FL)O^{\bullet}\ (HAT)} \qquad \mathbf{(16)}$$

Any antioxidant species present in the reaction mixture will undergo HAT with the peroxyl radicals (16) and delay the reduction of the fluorescent signal. Figure 2.19 is a proposed mechanism by which FL (pictured in its free acid form) interacts with peroxyl radicals resulting in the loss of fluorescence at λ_{max} = 515 nm.

Photochemiluminescent (PCL) Detection of Water- and Lipid-Soluble Antioxidants

The capabilities of water- and lipid-soluble antioxidants to scavenge $O_2^{\bullet -}$ can be assessed using a Photochem® unit from Analytik Jena USA (The Woodlands, TX). The initial protocol and system upon which Photochem® was developed is the work of Drs. Igor Popov and Gudrun Lewin from 1987 to 1999. The span of their research covers the photochemiluminescent quantification of ascorbic acid and superoxide dismutase (SOD) in human plasma (*i.e.*, Analytik Jena sells kits for these assays, PCL_{ASC} and PCL_{SOD}, respectively) (Lewin and Popov, 1994; Popov *et al.*, 1987; Popov *et al.*, 2001; Popov and Lewin, 1999b), as well as the measurement of antioxidant capacities of water- and lipid-soluble antioxidants (sold as PCL_{ACW} and PCL_{ACL} kits, respectively) (Popov and Lewin, 1994; Popov and Lewin, 1996; Popov and Lewin, 1999a). Each

assay involves the photo-degradation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and results in the production/quenching of $O_2^{\bullet-}$. From this work, Analytik Jena developed their testing kits for photochemiluminescent measurements as well as the Photochem® system. The simplified radical-generation reaction scheme (17) is as follows (Pegg *et al.*, 2007):

$$\textbf{Luminol} + h\nu_1\textbf{(UV)} \rightarrow \textbf{L*} + {}^3\textbf{O}_2 \rightarrow \textbf{[L* O}_2\textbf{]} \rightarrow \textbf{L}^{\bullet+} + \textbf{O}_2^{\bullet-} \qquad \textbf{(17)}$$

In reaction (17), L* is an intermediate product of the photo-induced luminol and $^3O_2$ is triplet oxygen (no $^1O_2$ is involved in the reaction). Once the $O_2^{\bullet-}$ and luminol radicals are generated, they proceed through a series of reactions resulting in the production of blue luminescence (Merényi *et al.*, 1986; Popov and Lewin, 1994; Schneider, 1970). Though all the steps in the detection reaction are not known, an example of possible chemical intermediates in the chemiluminescence of luminol is illustrated in reaction scheme (18) (Pegg *et al.*, 2007):

$$\textbf{L}^{\bullet+} + \textbf{O}_2^{\bullet-} \rightarrow \textbf{N}_2 + \textbf{AP}^{*2-} \rightarrow \textbf{AP}^{2-} + h\nu_2\textbf{ (Blue at 360 nm)} \qquad \textbf{(18)}$$

In reaction (18), AP^{*2-} is an excited aminophthalate anion, and AP^{2-} is the aminophthalate anion at the ground state. The chemical structure of luminol and the aminophthalate anion are discussed by Schneider (1970).

Once $O_2^{\bullet-}$ radicals are generated, any exogenous antioxidant species present in the reaction mixture will out-compete the luminol radical and halt the production of blue luminescence, until the concentration is exhausted. The resultant lag/log relationships of antioxidant compounds performing in this closed system are then compared to the effectiveness

of standards (*i.e.*, ascorbic acid in ACW, and Trolox in ACL). The antioxidant capacity of compounds in the ACW and ACL assays gauge their relative antioxidant capacities in hydrophilic and lipophilic media.

SET Assays

Ferric Reducing Antioxidant Power (FRAP) Assay

Benzie and Strain (1996) developed an assay to measure the ferric reducing power of human plasma. This method was later adapted to the quantification of ferric reducing antioxidant power (FRAP) of plant extracts (Pulido *et al.*, 2000). Recently, the FRAP assay was adapted to a microtiter plate reader in 96-well format (Dragsted *et al.*, 2004). The assay reaction involves the reduction of Fe^{3+}-TPTZ (*i.e.*, iron[III]-2,4,6-tripyridyl-*s*-triazine) to Fe^{2+}-TPTZ through SET with an antioxidant compound. The result of this reaction is an intense blue color at λ_{max} = 550 nm, as seen in reaction scheme (19):

$$\mathbf{Fe^{3+}\text{-}TPTZ + ArOH \rightarrow Fe^{2+}\text{-}TPTZ\ (Blue\ at\ 595\ nm) + [ArOH]^{\bullet+}\ (SET)} \qquad \mathbf{(19)}$$

Figure 2.20 is a structural representation of the conversion of iron(III)-TPTZ to iron(II)-TPTZ. Reaction (19) can occur with antioxidant compounds with redox potentials lower than 0.7V (the E° of Fe^{3+}-TPTZ) and is, thus, comparable to $ABTS^{\bullet+}$ (E°=0.68 V) based assays (*i.e.*, TEAC) (Prior *et al.*, 2005). Furthermore, reducing power appears to be related to the extent of conjugation in phenols as well as the number of hydroxy constituents (Schaich, 2006). Disadvantages of the FRAP assay include the inability to detect phenols and other compounds that follow traditional hydrogen abstraction mechanisms (especially aromatic amino acids and

sulfhydryl compounds), as well as altered assay results based on the reaction media. The assay reaction must be carried out at acidic pH in order to maintain iron solubility, but this can lower the IP of the reactants and reduce the redox potential of the system.

Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

In comparison with iron, copper has a greater potential to undergo redox reactions with antioxidant components (*i.e.*, E° of copper[I] and [II] spectrophotometric-complexation reactions are generally lower than iron[II] and [III]) (Schaich, 2006). Redox reactions with copper are often faster than with iron, reducing time constraints in the laboratory. Copper has more *3d*-electrons than iron which may lend to its greater capability to coordinate with the π-electrons of incoming ligands during metal-ion chelation (Chatterjee *et al.*, 1983). Just like iron, copper ions coordinate with nitrogen-containing chelating agents such as 2,2′-bipyridine or 1,10-phenanthroline and its derivatives (Pilipenko and Falendysh, 1972). It follows from the reaction of iron(II) with 1,10 phenanthroline (the "ferroin" reaction) that the related copper(I) complexes with 1,10 phenanthroline derivatives began to carry the suffix "cuproine" (Smith and Wilkins, 1953). Tütem and Apak (1991) sought to improve an existing bathocuproine (BC) (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) method for the selective spectrophotometric determination of copper(I) in the presence of copper(II) (Moffett *et al.* 1985), by introducing the use of neocuproine (NC) (2,9-dimethyl-1,10-phenanthroline) as an alternative chelating agent. Later, Apak *et al.* (2004) revised their copper(I)-NC method, applied it to the analysis of dietary polyphenols, and created the CUPRAC assay. The CUPRAC method involves the reduction of free copper(II) to copper(I) in the presence of NC, which results in the coordinated complex Cu(I)-NC at a ratio of 2:1 according to the following reaction scheme (20):

$$\mathbf{Cu^{2+} + ArOH + 2NC \rightarrow Cu^{+}\text{–}(NC)_2\ (Blue\ at\ 450\ nm) + [ArOH]^{\bullet+}\ (SET)} \qquad \mathbf{(20)}$$

Figure 2.21 is a structural representation of reaction (20), including NC and BC complexes. Reagents for the CUPRAC assay include a 0.1 M solution of copper(II) chloride (for free Cu^{2+}), a 7.5 mM NC solution prepared in 95% (v/v) ethanol, ammonium acetate buffer (pH 7) for the reaction medium and diluent of samples, and a standard (usually uric acid). A variation of the old BC (copper[I]-BC, λ_{max} = 490 nm) method for copper is sold as a Bioxytech® AOP-490™ assay kit from OXIS International, Inc. (Portland, OR). This kit consists of a ready-made dilution buffer, cupric sulfate solution, uric acid standard, and stop solution to halt the reaction.

Mixed-Mode Assays

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The Trolox equivalent antioxidant capacity (TEAC) assay is a spectrophotometric method based on the capability of an antioxidant to scavenge the free-radical cation $ABTS^{\bullet+}$. The TEAC assay was originally developed by Miller *et al.* (1993) for the measurement of the antioxidant capacity of human plasma in infants. Re *et al.* (1999) modified the assay for the direct generation of $ABTS^{\bullet+}$ without radical intermediates and applied it to hydrophilic and lipophilic antioxidants. Dragsted *et al.* (2004) adapted the assay to a microplate reader for high-throughput. Though the TEAC assay is generally accepted as a SET assay, $ABTS^{\bullet+}$ can be neutralized by SET and HAT mechanisms. The HAT and SET assay reaction schemes (21-22) are as follows:

$$\mathbf{ABTS^{\bullet+}\ (Green\ at\ 734\ nm)\ +\ ArOH \rightarrow\ ABTS\ (Colorless)\ +\ [ArOH]^{\bullet+}\ (SET)} \qquad \mathbf{(21)}$$

$ABTS^{\bullet+}$ (Green at 734 nm) + ArOH → ABTS(H) (Colorless) + $ArO^{\bullet}$ (HAT) (22)

Figure 2.22 is a structural representation of the $ABTS^{\bullet+}$ decolorization reaction (22).

There are many points for careful consideration in the TEAC assay including the controlled generation of $ABTS^{\bullet+}$, pH, and temperature of the assay media. Also, $ABTS^{\bullet+}$ is a nitro-radical; therefore, it may not correlate well with other antioxidant capacity assays that measure oxyl-radical scavenging. As with the FRAP and CUPRAC assays, the complex nature of $ABTS^{\bullet+}$ may render interaction with polyphenolics time-dependent, so time-curves are often prepared. Given the prevalence of both HAT and SET reactions with $ABTS^{\bullet+}$, the TEAC assay should be considered a mixed-mode assay.

$DPPH^{\bullet}$ (2,2′-Diphenyl-1-picrylhydrazyl radical cation) Assay

$DPPH^{\bullet}$ has been examined for its use as an organic colorimetric reagent since the 1950s (Blois, 1958). Braude *et al.* (1954) made the observation that $DPPH^{\bullet}$ undergoes a HAT mechanism with antioxidant compounds according to the following reaction scheme (23):

$DPPH^{\bullet}$ (Violet at 515 nm) + ArOH → DPPH(H) (Colorless) + $ArO^{\bullet}$ (HAT) (23)

Figure 2.23 is a structural representation of the reaction (23). Blois (1958) determined that if the phenolic compound under analysis contains more than one phenolic hydroxy functional group, the resultant $ArO^{\bullet}$ formed is sufficiently stable to undergo a second simultaneous HAT reaction with another molecule of $DPPH^{\bullet}$; thereby, preserving the stoichiometry of the reaction.

Over the past two decades the DPPH assay has resurfaced as a method for the analysis of

phenols in plants and plant-derived food products (Sánchez-Moreno *et al.*, 1998). The current version of the assay involves adaptation to a high-throughput 96-well microtiter plate system (Fukumoto and Mazza, 2000). The assay is often run in-tube due to relative inexpensiveness. This renewed interest in the DPPH assay has resulted in a re-examination of the kinetics of its reaction with phenolics (Bondet *et al.*, 1997; Brand-Williams *et al.*, 1995; Silva *et al.*, 2000) and possible mechanisms of interaction, whether HAT (Brand-Williams *et al.*, 1995; Dangles *et al.*, 2000; Litwinienko and Ingold, 2003), SET (Foti *et al.*, 2004; Huang *et al.*, 2005), or mixed (Schaich, 2006). The following reaction scheme (24) is an example of a SET mechanism between $DPPH^{\bullet}$ and a phenolic antioxidant:

$$\mathbf{DPPH^{\bullet}\ (Violet\ at\ 515\ nm) + ArOH \rightarrow DPPH^{-}\ (Colorless) + [ArOH]^{\bullet +}\ (SET)} \qquad \mathbf{(24)}$$

As with the TEAC assay, the medium of interaction, size, polarity, and acidity of phenolic hydroxy groups play a role in whether SET or HAT mechanisms dominate. $DPPH^{\bullet}$ is known to react with a variety of compounds including aromatic amino acids, glutathione, α-tocopherol, ascorbic acid, tocopherol, and polyhydroxy aromatics (phenolics); therefore, the amount of potential interferences in the assay's progress is great. A $DPPH^{\bullet}$ variant exists (*i.e.*, abbreviated as $DPPH'^{\bullet}$). The $DPPH'^{\bullet}$ (2,2′-di[4-*tert*-octylphenyl]-1-picrylhydrazyl radical), is depicted in Figure 2.24; it is not typically employed in the DPPH assay.

2.6 Phenolic Source Under Examination: The Peanut (*Arachis hypogaea* L.)

U.S. Peanut Kernel Production

The peanut, or groundnut (*Arachis hypogaea* L.), plant is classified in the *Fabaceae* (or *Leguminosae*) family, which contains dicotyledonous herbs/shrubs having fruit that are legumes/loments and bear nodules on the roots that contain nitrogen-fixing bacteria. Examples of legumes include peanuts, various beans, and peas. Although peanut kernels are legumes, they are often grouped with, or otherwise compared to, tree nuts due to their similar biochemistry and healthful properties. Peanuts are a major economic agricultural crop of the southern states, specifically the three major peanut producing regions: the Southwest (Texas and Oklahoma), the Southeast (Alabama, Georgia, and Florida), and the Virginia/Carolina region (Virginia, North Carolina, and South Carolina). Of the thousands of peanut cultivars grown worldwide, the majority of peanuts belong to one of four common types: Runner, Virginia, Spanish, and Valencia. Edible uses of the peanut account for more than two thirds of the total peanut consumption in the United States and can range from raw or roasted, shelled or unshelled peanuts; boiled peanuts; peanut butter for sandwiches; candy and bakery products; and peanut brittle or other confections (USDA-ERS, 2002). Thus, countless of efforts are made annually to increase crop yield and disease resistance, as well as perform analytical tests to elucidate the beneficial biochemical properties of peanuts. An example of how peanut research can have an economic effect is as follows: a 1% increase in U.S. peanut consumption will have a positive impact on the Georgia-state economy of $16,900,000 (Powell, 2008).

Nutritional Components in Peanuts

Peanut Lipids

Peanuts kernels contain ~52% oil by weight (Holaday and Pearson, 1974; USDA-ARS, 2008), which is rich in monounsaturated fatty acids (MUFA) and PUFAs. Peanuts contain ~80% unsaturates, with 50% oleic (18:1, ω9) and 30% linoleic acids (18:2, ω6) (Mercer *et al.*, 1990). The high-oleic peanut genotype contains ~80% oleic and 5% linoleic acids (Braddock *et al.*, 1995). These lipid profiles are desirable given the current demand for more unsaturated edible oils such as olive oil, which is ~70% MUFA and ~10% PUFA (Mannina *et al.*, 2003). Substitution of diets high in saturated fats with oils low in saturated fats, yet high in MUFA and PUFA (like peanut oil) may lead to lower LDL cholesterol, lower serum TAG, and maintained HDL cholesterol in humans (Kris-Etherton *et al.*, 1999; Kris-Etherton *et al.*, 2001; Yu-Poth *et al.*, 2000). A higher intake of PUFA from peanuts, peanut oil, and peanut butters may improve insulin sensitivity and reduce the risk of Type-2 diabetes (Jiang *et al.*, 2002), as well as promote an increased feeling of satiety in consumers (Alper and Mattes, 2002; Iyer *et al.*, 2006). Peanut consumption can aid in weight management (Bes-Rastrollo *et al.*, 2007; Higgs, 2005; Sabaté, 2003) when substituted for traditional fats. Such effects may be greater in the case of high-oleic peanuts due to the greater presence of MUFA (O'Byrne *et al.*, 1997).

The consumption of healthy oils may indirectly lead to the reduced susceptibility of LDL oxidation *in vivo*, which is a key component in the development of arterial atherosclerotic plaques. Therefore, consuming peanuts, peanut oil, and other peanut-containing products may reduce the incidence of ischemic heart disease (IHD) (Sabaté, 1999), coronary heart disease (CHD) (Hu *et al.*, 1998; Kris-Etherton *et al.*, 2001), and widespread cardiovascular disease (CVD) (Alper and Mattes, 2003). Moreover, peanuts and peanut butter may reduce the

inflammatory process as determined by the reduction of inflammatory markers like C-reactive protein, interleukin-6, and fibrinogen (Jiang *et al.*, 2005). This fact may partially explain the inverse relationship between nut consumption and reduced CVD. The health-promoting effects of peanut consumption are due to their fatty acid profiles as well as other beneficial functional constituents (bioactives). Functional components contained within peanuts include the following: Vitamin E, fatty acids, L-arginine, other organic and inorganic nutrients, soluble and insoluble fiber, phytosterols, as well as water- and lipid-soluble phenolic antioxidants (Francisco and Resurreccion, 2008; Higgs, 2003; Isanga and Zhang, 2007; Kris-Etherton *et al.*, 2001).

Peanut Protein

The nutritional quality of a protein is derived from its indispensable amino acid profile, its digestibility, and amino acid bioavailability. Peanuts are a good source of protein at ~30% (w/w) protein (Holaday and Pearson, 1974; USDA-ARS, 2008) or ~8.5 g/1oz serving size, which is > 10% of the Daily Reference Value for protein (CFR, 2005). However, peanuts are usually deficient in the sulfur-containing amino acids cysteine and methionine, with lysine as the second limiting amino acid (McLarney *et al.*, 1996). Literature values for its protein digestibility corrected amino acid score (PDCAAS) range from 0.5 to 0.7, and express this deficiency. True protein digestibility values for peanuts have been reported from 0.91 to 0.98 (Neucere *et al.*, 1972; Singh and Singh, 1991).

Peanut proteins exhibit altered functional properties post roast including reduced protein solubility, water holding, and oil-binding capacities (Neucere *et al.*, 1969; Yu *et al.*, 2007a). These modifications are due to altered primary and/or secondary protein structure. Although some may think of processing as being detrimental to peanut protein, it has long been considered

beneficial due to improved protein digestibility and reduction in the incidence of anti-nutritional components such as trypsin and chymotrypsin inhibitors contained in legumes (Snyder and Kwon, 1987).

Amino Acid L-Arginine

What sets peanut protein apart from other sources is its abundant L-arginine content. Average L-arginine content in raw peanuts is 3.085 g L-arginine/100g peanuts (USDA-ARS, 2008). Although L-arginine is not considered an indispensable amino acid, it may be synthesized too slowly in infants and, therefore, it is conditionally indispensable. L-Arginine also has an important role in human health: it is a precursor of nitric oxide (NO) in the body, a potent vasodilator and blood pressure regulator (Blum *et al.*, 2000). NO also prevents platelet clumping, reducing the risk of stroke and heart attack (Aji *et al.*, 1997; Feldman, 2002).

Carbohydrates (Dietary Fiber)

About 20% (w/w) of peanut kernels is carbohydrate (Grosso *et al.*, 2000; USDA-ARS, 2008). Nearly half (6-9g/100g) of peanut carbohydrates are non-starch polysaccharides or dietary fiber (Higgs, 2003). Dietary fiber is known to aid in the reduction of coronary artery disease risk (Kushi *et al.*, 1999). Further, of the dietary fiber in peanuts 2/3 is insoluble fiber, while the other 1/3 is soluble fiber. Insoluble fiber is known to aid in the maintenance of intestinal acidity as well as minimize transit time of digested foods through the human GI tract and thereby reduce constipation. Soluble (or fermentable) fiber can be partially digested by intestinal bacteria and result in energy and the production of short-chain fatty acids. Short-chain fatty acids such as acetic, propionic, and butyric acid may aid in the reduced incidence of

gastroenterological disorders, cardiovascular disease, and certain cancers (Wong *et al.*, 2006). Furthermore, soluble fiber is known to reduce LDL cholesterol and improve glycemic response in humans, as well as help in the regulation of blood insulin levels (Higgs, 2003).

Vitamins and Minerals

Peanuts contain an average of 2.5% ash (Grosso *et al.*, 2000; USDA-ARS, 2008). Extracted peanut oil contains an average of 8.3 mg Vitamin E/100g (Kornsteiner *et al.*, 2006; USDA-ARS, 2008). High daily doses of Vitamin E of more than 100 IU/day have been associated with a reduced risk of CHD by the prevention of the oxidation of LDL cholesterol (Rimm and Stampfer, 1997). Vitamin E is also well documented as a chain-breaking antioxidant and radical scavenger of many endogenously- and exogenously-generated radical species (*e.g.,* $O_2^{\bullet-}$ and $HO^{\bullet}$), as discussed.

Along with Vitamin E, peanuts are also a good source of important nutrients such as the B-vitamins thiamin (0.64 mg/100g), niacin (12.066 mg/100g), and folate (240 μg/100g), as well as the minerals manganese (1.934 mg/100g), magnesium (168 mg/100g), phosphorus (376 mg/100g), copper (1.144 mg/100g), and zinc (3.27 mg/100g) (USDA-ARS, 2008). The increased consumption of folic acid is specifically recommended for pregnant women due to its capability to promote healthy cell division and reduce 70% of neural tube defects (NTDs) (Czeizel and Dudas, 1992). Many NTDs in women; however, are due to abnormal folate metabolism, not lack of dietary intake. This metabolic issue involves the reduction of homocysteine levels regulated by the transmethylation and trans-sulfuration pathways (Molloy *et al.*, 1999; Tchantchou, 2006) rather than folate deficiency.

Functional Components in Peanut Kernels

Phytosterols

Dry-roasted peanut kernels contain ~47-94 mg β-sitosterol/100g depending on variety (Awad *et al.*, 2000). Peanuts also consist of other sterols such as campesterol, stigmasterol, and Δ^5-avenasterol (Grosso *et al.*, 2000); as a whole, the phytosterols may help reduce plasma cholesterol levels. This reduction is caused by the inhibition of dietary and bilary cholesterol absorption in humans (Higgs, 2003). Consumption of a diet containing 1.8-2 g plant sterols per day has been associated with a 10-15% reduction of total and LDL cholesterol in various populations (Katan *et al.*, 2003; Ostlund, 2002). Due to the culmination of positive evidence regarding sterol consumption and cholesterol-lowering effects, a U.S. Food and Drug Administration (FDA) qualified health claim was approved in 2005. It states that foods containing at least 0.65 g per serving of plant sterol esters, eaten twice a day with meals for a daily total intake of at least 1.3 g, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease (CFR, 2005).

Phenolic Compounds

Flavonoids/Isoflavonoids

Peanuts contain limited quantities of the isoflavones daidzein (52-1753 μg/100g DW), genistein (13-227 μg/100g), and biochanin A (37-137 μg/100g); the contents found will vary depending on the extraction medium and the sample preparation techniques employed (Chukwumah *et al.*, 2007a; Mazur, 1998; Mazur *et al.*, 1998). Furthermore, isoflavone levels increase with processing (Chukwumah *et al.*, 2007b). Isoflavones have the potential to act as phytoestrogens (*i.e.*, mimic estrogen) in the human body as well as exhibit antioxidant capacity

and aid in the treatment of certain cancers (Horn-Ross, 1995; Yanagihara *et al.*, 1993). The only other flavonoid found in peanuts to date is flavonol dihydroquercetin (taxifolin), which is present in Spanish peanuts in limited amounts (Pratt and Miller, 1984).

Stilbenoids

The stilbenoid, *trans*-resveratrol (3,5,4′-trihydroxystilbene), has been reported in peanut kernels in small quantities (0.01-2 μg/g) (Sanders *et al.*, 2000; Sobolev and Cole, 1999; Tokuşoğlu *et al.*, 2005); however, it is not expected to have a significant effect on the antioxidant capacity of peanuts consumed by humans. Common industry processing methods elicit increased resveratrol production according to the following relationship: boiling > peanut butter processing > dry roasting) (Sobolev and Cole, 1999), but the levels of resveratrol obtained from such processes are minimal. In comparison, peanut roots contain 0.13-1.33 mg/g (Chen *et al.*, 2002), making the roots a much better source of stilbenes.

Free/Bound Phenolic Acids

The total phenolics content and antioxidant capacity of peanuts are comparable to other tree nuts (Kornsteiner *et al.*, 2006; Pellegrini *et al.*, 2006). High-oleic genotype and normal peanuts possess similar antioxidant profiles (Talcott *et al.*, 2005b). Of the little compositional data available on peanut phenolic profiles, bound phenolics are thought to be dominant, as demonstrated by an 86% increase in TRAP values post alkaline hydrolysis (Pellegrini *et al.*, 2006). Phenolic acids and their esters have been tentatively identified in raw and roasted peanuts. These include *p*-coumaric acid, *p*-coumaric acid esters, and possibly *p*-hydroxybenzoic acid esters (Talcott *et al.*, 2005b).

Effects of Storage on Phenolic Profiles

Lower water activity during storage results in an increased incidence of oxidative rancidity in peanuts and decreased sensory quality (*i.e.*, flavor fade) (Reed *et al.*, 2002). The high-oleic genotype has proven to be more stable in accelerated storage studies and subsequent sensory trials, given the greater content of MUFAs (Braddock *et al.*, 1995; Talcott *et al.*, 2005a). In short-term frozen storage at -20 °C, sensory attributes of peanut kernels remain relatively unchanged (Pattee *et al.*, 2002), thereby suggesting that the oxidative stability is good under such conditions. The effects of prolonged frozen storage have yet to be determined.

Effects of Processing on Phenolic Profiles/Antioxidant Capacities

Of the peanuts consumed in the U.S., ~75% undergo dry roasting and ~25% are oil roasted or roasted in-shell (Kotz, 2009). Total phenolics and antioxidant capacity of peanuts increase upon roasting as determined by HPLC, lipid model systems, *in vitro* radical-scavenging, and enzymatic methods (Chukwumah *et al.* 2007b; Hwang *et al.*, 2001; Talcott *et al.*, 2005b). Thermal processing methods increase the total phenolics content of peanut kernels according to the following relationship: boiled > oil roast > dry roast > raw (Chukwumah *et al.* 2007b). Total phenolics content of peanuts vary among cultivars (Duncan *et al.*, 2006; Talcott *et al.*, 2005b), but concrete relationships have yet to be established.

Peanut Skins and Other Plant Parts

By weight, the peanut seed coat (or skin) accounts for an average of 2.6% of peanut production. It has been estimated that over 750,000 tons of peanut skins are produced annually in the world (Sobolev and Cole, 2004). Peanut skin is a low-value by-product of the peanut

industry. Skins are removed in dry-blanching operations *via* "split-nut" blanching. Split-nut blanching is a process in which peanuts pass along a belt and through a roller that splits them in half. The skins are then blown off the halved peanuts and sent to a cyclone, where they are collected (Karn, 2009). Dry-roasted peanut skins are removed after processing by passing the peanuts over screens that trap the skins (Kotz, 2009). In both operations peanut skins are gathered, transferred to compactors, and pressed into pellets to be sold as feed with a commercial value of only $12-20 per ton (Sobolev and Cole, 2004). Their high protein (~17%), fat (~5%), and low cost facilitate their use in this respect (Karchesy and Hemingway, 1986).

Interest in polyphenolics from grape seeds and skins has increased over the past decade. As a result, peanut skins are now being considered for their flavonoids and tannins. At a > 15% (w/w) level of total phenolics (Nepote *et al.*, 2002), peanut skins might be one of the richest sources in nature. Peanut skins and related products are now being screened for insertion into functional foods and nutraceuticals for the promotion of human health and wellness (Isanga and Zhang, 2007).

Phenolic Compounds in Peanut Skins

In terms of sample preparation, the optimization of phenolic extractions from peanut skins has been assessed, with aqueous/organic solvent mixtures (*e.g.,* 50-100% ethanol) resulting in the highest yield of extractable phenolics (Huang *et al.*, 2003; Nepote *et al.*, 2005; Yu *et al.*, 2005). A- and B-type PAC dimers, trimers, and tetramers have been reported in peanut skin extracts (Lazarus *et al.*, 1999; Van Ha *et al.*, 2007; Yu *et al.*, 2006; Yu *et al.*, 2007b). However, the effects of processing on the levels and chemistry of these tannins are not well understood. Yu *et al.* (2005) reported that dry roasting increases the total phenolics content of peanut skins,

whereas, water blanching yields decreased values; dry blanching has yet to be reported on. Peanut skin extracts demonstrate high antioxidant potency as measured by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical-scavenging (Van Ha *et al.*, 2007; Wang *et al.*, 2007; Yu *et al.*, 2007b), ferrous-ion chelating potential (Van Ha *et al.*, 2007; Wang *et al.*, 2007), and various *in vitro* oxygen radical (*e.g.,* $HO^{\bullet}$, $O_2^{\bullet-}$) scavenging methods (Wang *et al.*, 2007).

Phenolic Compounds in Peanut Plant Parts

A recent study involved phenolic profiling and measurement of the total phenolics content and antioxidant capacities of peanut shells, roots, and leaves (Dean *et al.*, 2008). Among the phytonutrients contained in peanut shells were luteolin, dihydroxycoumarin, resveratrol, and chlorogenic acids. Peanut roots and leaves contained resveratrol and kaempferol, respectively. The study showed that peanut leaves had 4.5 times more total phenols and more than twice the antioxidant capacity of the peanut roots. Peanut shells have a total phenolics content and antioxidant capacity much lower than the roots, but they have demonstrated potent radical-scavenging capacities *in vitro* (Yen and Duh, 1994).

References

Abudu, N.; Miller, J. J.; Attaelmannan, M.; Levinson, S. S. 2004. Vitamins in human arteriosclerosis with emphasis on vitamin C and vitamin E. *Clin. Chim. Acta* **339**:11-25.

Adam-Vizi, V. 2005. Production of reactive oxygen species in brain mitochondria: Contribution by electron transport chain and non-electron transport chain sources. *Antioxid. Redox Signal.* **7**:1140-1149.

Adom, K. K.; Liu, R. H. 2002. Antioxidant activity of grains. *J. Agric. Food Chem.* **50**:6182-6187.

Adom, K. K.; Liu, R. H. 2005. Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. *J. Agric. Food. Chem.* **53**:6572-6580.

Aji, W.; Ravalli, S.; Szabolcs, M.; Jiang, X-C.; Sciacca, R. R.; Michler, R. E.; Cannon, P. J. 1997. L-Arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* **95**:430-437.

Alho, H.; Leinonen, J. 1999. Total antioxidant activity measured by chemiluminescence methods. *Meth. Enzymol.* **299**:3-15.

Alper, C. M.; Mattes, R. D. 2002. Effects of chronic peanut consumption on energy balance and hedonics. *Int. J. of Obes.* **26**:1129-1137.

Alper, C. M.; Mattes, R. D. 2003. Peanut consumption improves indices of cardiovascular disease risk in healthy adults. *J. Am. Coll. Nutr.* **22**:133-141.

Amarowicz, R. 2007. Tannins: the new natural antioxidants? *Eur. J. Lipid Sci. Tech.* **109**:549-551.

Andersen, M. L.; Lauridsen, R. K.; Skibsted, L. H. 2005. Power of phenolic compounds. Functional Foods & Nutraceuticals, March:44-48.

Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S. E.; Bektaşoğlu, B.; Berker, K. I.; Özyurt, D. 2007. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. *Molecules* **12**:1496-1547.

Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. 2004. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J. Agric. Food Chem.* **52**:7970-7981.

Arts, I. C. W.; Hollman, P. C. H. 2005. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **81**:317S-325S.

Arts, I. C. W.; van de Putte, B.; Hollman, P. C. H. 2000. Catechin contents of foods commonly consumed in the Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *J.*

Agric. Food Chem. **48**:1746-1751.

Aruoma, O. I. 1994. Nutrition and health aspects of free radicals and antioxidants. *Food Chem. Toxic.* **32**:671-683.

Aruoma, O. I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutat. Res.* **523-524**:9-20.

Awad, A. B.; Chan, K. C.; Downie, A. C.; Fink, C. S. 2000. Peanuts as a source of β-sitosterol, a sterol with anticancer properties. *Nutr. Cancer* **36**:238-241.

Barber, D. A.; Harris, S. R. 1994. Oxygen free radicals and antioxidants: A review. *Am. Pharm.* **NS34**:26-35.

Barclay, L. R. C.; Edwards, C. E.; Vinqvist, M. R. 1999. Media effects on antioxidant activities of phenols and catechols. *J. Am. Chem. Soc.* **121**:6226-6231.

Becker, E. M.; Nissen, L. R.; Skibsted, L. H. 2004. Antioxidant evaluation protocols: Food quality and health effects. *Eur. Food Res. Technol.* **219**:561-571.

Benzie, I. F. F.; Strain, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **239**:70-76.

Bes-Rastrollo, M.; Sabaté, J.; Gómez-Gracia, E.; Alonso, A.; Martínez, J. A.; Martínez-González, M. A. 2007. Nut consumption and weight gain in a mediterranean cohort: The sun study. *Obes.* **15**:107-116.

Bhagwat, S.; Haytowitz, D. B.; Holden, J. M. 2007. USDA database for the oxygen radical absorbance capacity (ORAC) of selected foods. American Institute for Cancer Research Launch Conference. November 1-2, 2007. Washington, D. C.

Blois, M. S. 1958. Antioxidant determination by the use of a stable free radical. *Nature* **181**:1199-1200.

Blum, A.; Hathaway, L.; Mincemoyer, R.; Schenke, W. H.; Kirby, M.; Csako, G.; Waclawiw, M.

A.; Panza, J. A.; Cannon, R. O. 2000. Effects of oral L-arginine on endothelium-dependent vasodilation and markers of inflammation in healthy postmenopausal women. *J. Am. Coll. Cardiol.* **35**:271-276.

Bondet, V.; Brand-Williams, W.; Berset, C. 1997. Kinetics and mechanisms of antioxidant activity using the DPPH$^{\bullet}$ free radical method. *Lebensm. Wiss. Technol.* **30**:609-615.

Braddock, J. C.; Sims, C. A.; O'Keefe, S. F. 1995. Flavor and oxidative stability of roasted high oleic acid peanuts. *J. Food Sci.* **60**:489-493.

Brand-Williams, W.; Cuvelier, M. E.; Berset, C. 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensm. Wiss. Technol.* **28**:25-30.

Brandt, W. W.; Smith, G. F. 1949. Polysubstituted 1,10-phenanthrolines and bipyridines as multiple range redox indicators. *Anal. Chem.* **21**:1313-1319.

Braude, E. A.; Brook, A. G.; Linstead, R. P. 1954. Hydrogen transfer part V: Dehydrogenation reactions with diphenylpicrylhydrazyl. *J. Chem. Soc.* 3574-3578.

Bravo, L. 1998. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **56**:317-333.

Burton, G. W.; Ingold, K. U. 1981. Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking antioxidants in vitro. *J. Am. Chem. Soc.* **103**:6472-6477.

Burton, G. W.; Ingold, K. U. 1984. β-Carotene: An unusual type of lipid antioxidant. *Science* **224**:569-573.

Burton, G. W.; Ingold, K. U. 1986. Vitamin E: Application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc. Chem. Res.* **19**:194-201.

Campos, A. M.; Escobar, J.; Lissi, E. A. 1996. The total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) of *Ilex paraguayensis* extracts and red wine. *J. Braz. Chem. Soc.* **7**:43-49.

Cao, G.; Alessio, H. M.; Cutler, R. G. 1993. Oxygen-radical absorbance capacity assay for antioxidants. *Free Rad. Biol. Med.* **14**:303-311.

Cao, G.; Booth, S. L.; Sadowski, J. A. Prior, R. L. 1998. Increases in human plasma antioxidant capacity after consumption of controlled diets high in fruit and vegetables. *Am. J. Clin. Nutr.* **68**:1081-1087.

Cao, G.; Prior, R. L. 1998. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **44**:1309-1315.

Cao, G.; Sofic, E.; Prior, R. L. 1996. Antioxidant capacity of tea and common vegetables. *J. Agric. Food Chem.* **44**:3426-3431.

Cao, G.; Sofic, E.; Prior, R. L. 1997. Antioxidant and pro-oxidant behavior of flavonoids: Structure-activity relationships. *Free Rad. Biol. Med.* **22**:749-760.

Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H.; Prior, R. L. 1995. Automated assay of oxygen radical absorbance capacity with the Cobas Fara II. *Clin. Chem.* **41**:1738-1744.

Chatterjee, S. K.; Katyal, A.; Pachauri, L. S. 1983. Physiochemical studies on some phenolic polymer-transition metal complexes in nonaqueous media. *J. Macromol. Sci. Chem.* **A19**:929-936.

Chen, R.-S.; Wu, P.-L.; Chiou, R. Y.-Y. 2002. Peanut roots as a source of resveratrol. *J. Agric. Food Chem.* **50**:1665-1667.

Cheynier, V. 2005. Polyphenols in foods are more complex than often thought. *Am. J. Clin. Nutr.* **81**:223S-229S.

Cheynier, V.; Souquet, J-M.; Le Roux, E.; Guyot, S.; Rigaud, J. 1999. Size separation of condensed tannins by normal-phase high-performance liquid chromatography. *Meth. Enzymol.* **299**:178-184.

Christon, R.; Drouin, O.; Marette, A. 2005. Redox modulation of insulin signaling and endothelial function. *Antiox. Redox Sig.* **7**:1062-1070.

Chu, Y-F.; Sun, J.; Wu, X.; Liu, R. H. 2002. Antioxidant and anti-proliferative activities of common vegetables. *J. Agric. Food Chem.* **50**:6910-6916.

Chukwumah, Y. C.; Walker, L. T.; Verghese, M.; Bokanga, M.; Ogutu, S.; Alphonse, K. 2007a. Comparison of extraction methods for the quantification of selected phytochemicals in peanuts (*Arachis hypogea*). *J. Agric. Food Chem.* **55**:285-290.

Chukwumah, Y.; Walker, L.; Vogler, B.; Verghese, M. 2007b. Changes in the phytochemical composition and profile of raw, boiled and roasted peanuts. *J. Agric. Food Chem.* **55**:9266-9273.

Collins, A. R. 2005. Assays for oxidative stress and antioxidant status: Applications to research into the biological effectiveness of polyphenols. *Am. J. Clin. Nutr.* **81**:261S-267S.

Conn, P. F.; Schalch, W.; Truscott, T. G. 1991. The singlet oxygen and carotenoid interaction. *J. Photochem. Photobiol. B: Biol.* **11**:41-47.

Corongiu, F. P.; Banni, S. 1994. Detection of conjugated dienes by second derivative ultraviolet spectrophotometry. *Methods Enzymol.* **233**:303-310.

Czeizel, A. E.; Dudas, I. 1992. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *New Eng. J. Med.* **327**:1832-1835.

Dangles, O.; Fargeix, G.; Dufour, C. 2000. Antioxidant properties of anthocyanins and tannins: A mechanistic investigation with catechin and the 3′,4′,7-trihydroxyflavylium ion. *J. Chem. Soc. Perk. T.* **2**:1653-1663.

Dean, L. L.; Davis, J. P.; Shofran, B. G.; Sanders, T. H. 2008. Phenolic profiles and antioxidant activity of extracts from peanut plant parts. *Open Nat. Prod. J.* **1**:1-6.

Decker, E. A.; Warner, K.; Richards, M. P.; Shahidi, F. 2005. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **53**:4303-4310.

Diplock, A. T. 1994. Antioxidants and disease prevention. *Mol. Aspects Med.* **15**:293-376.

Diplock, A. T. 1996. The Leon Goldberg memorial lecture: Antioxidants and disease prevention. *Food Chem. Toxicol.* **34**:1013-1023.

Dixon, R. A.; Xie, D-Y.; Sharma, S. B. 2005. Proanthocyanidins-A final frontier in flavonoid research? *New Phytol.* **165**:9-28.

Dragsted, L. O.; Pedersen, A.; Hermetter, A.; Basù, S.; Hansen, M.; Haren, G. R.; Kall, M.; Breinholt, V.; Castenmiller, J. J. M.; Stagsted, J.; Jakobsen, J.; Skibsted, L.; Rasmussen, S. E.; Loft, S.; Sandström, B. 2004. The 6-a-day study: Effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. *Am. J. Clin. Nutr.* **79**:1060-1072.

Duncan, C. E.; Gorbet, D. W.; Talcott, S. T. 2006. Phytochemical content and antioxidant capacity of water-soluble isolates from peanuts (*Arachis hypogaea* L.). *Food Res. Int.* **39**:898-904.

Eichhorn, G. L.; Latif, R. A. 1954. The metal complexes of tris-anhydro-*o*-aminobenzaldehyde. *J. Am. Chem. Soc.* **76**:5180-5182.

Esterbauer, H. 1993. Cytotoxicity and genotoxicity of lipid-oxidation products. *Am. J. Clin. Nutr.* **57**:779S-786S.

Esterbauer, H.; Gebicki, J.; Puhl, H.; Jürgens, G. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Rad. Biol. Med.* **13**:341-390.

Evans, C.; Scaiano, J. C.; Ingold, K. U. 1992. Absolute kinetics of hydrogen abstraction from α-tocopherol by several reactive species including an alkyl radical. *J. Am. Chem. Soc.* **114**:4589-4593.

Farmer, E. H.; Sutton, D. A. 1943. The course of autoxidation reactions in polyisoprenes and allied compounds. Part IV. The isolation and constitution of photochemically-formed methyl oleate peroxide. *J. Chem. Soc.* 119-121.

Feldman, E. B. 2002. The scientific evidence for a beneficial health relationship between walnuts and coronary heart disease. *J. Nutr.* **132**:1062S-1101S.

Ferreira, D.; Li, X-C. 2000. Oligomeric proanthocyanidins: Naturally occurring O-heterocycles. *Nat. Prod. Rep.* **17**:193-212.

Fogliano, V.; Verde, V.; Randazzo, G.; Ritieni, A. 1999. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *J. Agric. Food Chem.* **47**:1035-1040.

Folin, O.; Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**:627-650.

Folin, O.; Denis, W. 1912a. On phosphotungstic-phosphomolybdic compounds as color reagents. *J. Biol. Chem.* **12**:239-243.

Folin, O.; Denis, W. 1912b. Tyrosine in proteins as determined by a new colorimetric method. *J. Biol. Chem.* **12**:245-251.

Folin, O.; Macallum, A. B. 1912. On the blue color reaction of phosphotungstic acid (?) with uric acid and other substances. *J. Biol. Chem.* **11**:265-266.

Foti, M. C.; Daquino, C.; Geraci, C. 2004. Electron-transfer reaction of cinnamic acids and their methyl esters with $DPPH^{\bullet}$ radical in alcoholic solutions. *J. Org. Chem.* **69**:2309-2314.

Francisco, M. L. D. L.; Resurreccion, A. V. A. 2008. Functional components in peanuts. *Crit. Rev. Food Sci. Nutr.* **48**:715-746.

Frankel, E. N.; Finley, J. W. 2008. How to standardize the multiplicity of methods to evaluate natural antioxidants. *J. Agric. Food Chem.* **56**:4901-4908.

Frankel, E. N.; Waterhouse, A. L.; Teissedre, P. L. 1995. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *J. Agric. Food Chem.* **43**:890-894.

Fukumoto, L. R.; Mazza, G. 2000. Assessing antioxidant and pro-oxidant activities of phenolic compounds. *J. Agric. Food Chem.* **48**:3597-3604.

Galano, A. 2007. Relative antioxidant efficacy of a large series of carotenoids in terms of one electron transfer reactions. *J. Phys. Chem. B.* **111**:12898-12908.

Garavelli, M.; Bernardi, F.; Olivucci, M.; Robb, M. A. 1998. DFT study of the reactions between singlet-oxygen and a carotenoid model. *J. Am. Chem. Soc.* **120**:10210-10222.

Gay, C. A.; Gebicki, J. M. 2003. Measurement of protein and lipid hydroperoxides in biological systems by the ferric-xylenol orange method. *Anal. Biochem.* **315**:29-35.

Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferro-Luzzi, A. 1995. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Rad. Biol. Med.* **18**:29-36.

Ghiselli, A.; Serafini, M.; Natella, F.; Scaccini, C. 2000. Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data. *Free Rad. Biol. Med.* **29**:1106-1114.

Glazer, A. N. 1990. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Meth. Enzymol.* **186**:161-168.

Gray, J. I. 1978. Measurement of lipid oxidation: A review. *J. Amer. Oil. Chem. Soc.* **55**:539-546.

Grosso, N. R.; Nepote, V.; Guzmán, C. A. 2000. Chemical composition of some wild peanut species (*Arachis* L.) seeds. *J. Agric. Food Chem.* **48**:806-809.

Gupta, B. L. 1973. Microdetermination techniques for H_2O_2 in irradiated solutions. *Microchem. J.* **18**:363-374.

Gu, L.; Kelm, M.; Hammerstone, J. F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R. L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *J. Agric. Food Chem.* **50**:4852-4860.

Halliwell, B. 1996. Antioxidants in human health and disease. *Annu. Rev. Nutr.* **16**:33-50.

Halliwell, B.; Aeschbach, R.; Löliger, J.; Aruoma O. I. 1995. The characterization of antioxidants. *Food Chem. Toxicol.* **33**:601-617.

Halliwell, B.; Gutteridge, J. M. C.; Cross, C. E. 1992. Free Radicals, antioxidants, and human disease: Where are we now? *J. Lab. Clin. Med.* **119**:598-619.

Halliwell, B.; Rafter, J.; Jenner, A. 2005. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr.* **81**:268S-276S.

Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. 1988. Two new flavonoids and other constituents in licorice root: Their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.* **36**:2090-2097.

Herrmann, K. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Crit. Rev. Food Sci. Nutr.* **28**:315-347.

Higgs, J. 2003. The beneficial role of peanuts in the diet-Part 2. *Nutr. Food Sci.* **33**:56-64.

Higgs, J. 2005. The potential role of peanuts in the prevention of obesity. *Nutr. Food Sci.* **35**:353-358.

Hiramatsu, M.; Yoshikawa, T.; Inoue, M. 1997. Food and Free Radicals. Plenum Press, New York, NY.

Hodgson, E. K.; Fridovich, I. 1976. The accumulation of superoxide radical during the aerobic action of xanthine oxidase: A requiem for $H_2O_4^-$. *Biochim. Biophys. Acta* **430**:182-188.

Holaday, C. E.; Pearson, J. L. 1974. Effects of genotype and production area on the fatty acid composition, total oil and total protein in peanuts. *J. Food Sci.* **39**:1206-1209

Holman, R. T.; Burr, G. O. 1946. Spectrophotometric studies of the oxidation of fats. VI. Oxygen absorption and chromophore production in fatty esters. *J. Am. Chem. Soc.* **68**:562-566.

Horn-Ross, P. L. 1995. Phytoestrogens, body composition, and breast cancer. *Cancer Cause. Control* **6**: 567-573.

House, D. A.; Curtis, N. F. 1962. Transition-metal complexes with aliphatic Schiff bases. IV. Compounds formed by the reaction of copper (II) and nickel (II) triethylenetetramine complexes with acetone. *J. Am. Chem. Soc.* **84**:3248-3250.

Howard, J. A.; Ingold, K. U. 1963. The inhibited autoxidation of styrene: Part III. The relative inhibiting efficiencies of ortho-alkyl phenols. *Can. J. Chem.* **41**:2800-2806.

Hu, F. B.; Stampfer, M. J.; Manson, J. E.; Rimm, E. B.; Colditz, G. A.; Rosner, B. A.; Speizer, F. E.; Hennekens, C. H.; Willett, W. C. 1998. Frequent nut consumption and risk of coronary heart disease in women: Prospective cohort study. *Brit. Med. J.* **317**:1341-1345.

Huang, D.; Ou, B.; Hampsch-Woodhill, M.; Flanagan, J. A.; Deemer, E. K. 2002a. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated *b*-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **50**:1815-1821.

Huang, D.; Ou, B.; Hampsch-Woodhill, M.; Flanagan, J. A.; Prior, R. L. 2002b. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multi-channel liquid handling system coupled with a microplate fluorescence reader in 96 well format. *J. Agric. Food Chem.* **50**:4437-4444.

Huang, D.; Ou, B.; Prior, R. L. 2005. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **53**:1841-1856.

Huang, S. C.; Yen, G-C. ; Chang, L-W. ; Yen, W-J. ; Duh, P-D. 2003. Identification of an antioxidant, ethyl protocatechuate, in peanut seed testa. *J. Agric. Food Chem.* **51**:2380-2383.

Hwang J-Y.; Shue, Y-S.; Chang H-M. 2001. Antioxidative activity of roasted and defatted peanut kernels. *Food Res. Int.* **34**:639-647.

Iannone, A.; Rota, C.; Bergamini, S.; Tomasi, A.; Canfield, L. M. 1998. Antioxidant activity of carotenoids: An electron-spin resonance study on β-carotene and lutein interaction with free radicals generated in a chemical system. *J. Biochem. Mol. Toxic.* **12**:299-304.

Isanga, J.; Zhang, G-N. 2007. Biologically active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.* **23**:123-140.

Iyer, S. S.; Boateng, L. A.; Sales, R. L.; Coelho, S. B.; Lokko, P.; Monteiro, J. B. R.; Costa, N. M. B.; Mattes, R. D. 2006. Effects of peanut oil consumption on appetite and food choice. *Int. J. Obes.* **30**:704-710.

Jiang, R.; Jacobs, D. R. Jr.; Mayer-Davis, E.; Szklo, M.; Herrington, D.; Jenny, N. S.; Kronmal, R.; Barr, R. G. 2005. Nut and seed consumption and inflammatory markers in multi-ethnic study of atherosclerosis. *Am. J. Epidemol.* **163**:222-231.

Jiang, R.; Manson, J. E.; Stampfer, M. J.; Liu, S.; Willett, W. C.; Hu, F. B. 2002. Nut and peanut butter consumption and risk of type 2 diabetes in women. *J. Am. Med. Assoc.* **288**:2554-2560.

Jiang, Z-Y.; Woollard, A. C. S.; Wolff, S. P. 1991. Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. *Lipids* **26**:853-856.

Kampa, M.; Nistikaki, A.; Tsaousis, V.; Maliaraki, N.; Notas, G.; Castanas, E. 2002. A new automated method for the determination of the total antioxidant capacity (TAC) of human plasma, based on the crocin bleaching assay. *BMC Clin. Path.* **2**:3-16.

Karchesy, J. J.; Hemingway, R. W. 1986. Condensed tannins: (4β→8; 2β→O→7)-Linked procyanidins in *Arachis hypogaea* L. *J. Agric. Food Chem.* **34**:966-970.

Karn, R. 2009. Product Development Manager. American Blanching Company, Fitzgerald, GA. Personal Communication.

Katan, M. B.; Grundy, S. M.; Jones, P.; Law, M.; Miettinen, T.; Paoletti, R. 2003. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. *Mayo Clin. Proc.* **78**:965-978.

Kontush, A.; Finckh, B.; Karten, B.; Kohlschütter, A.; Beisiegel, U. 1996. Antioxidant and pro-oxidant activity of α-tocopherol in human plasma and low density lipoprotein. *J. Lipid Res.* **37**:1436-1448.

Kornsteiner, M.; Wagner, K.-H.; Elmadfa, I. 2006. Tocopherols and total phenolics of 10 different nut types. *Food Chem.* **98**:381-387.

Kotz, B. A. 2009. Vice-President of Specialty Products. Golden Peanut Company, Alpharetta, GA. Personal Communication.

Kris-Etherton, P. M. Yu-Poth, S.; Sabaté, J.; Ratcliffe, H. E.; Zhao, G.; Etherton, T. D. 1999. Nuts and their bioactive constituents: Effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* **70**:504S-511S.

Kris-Etherton, P. M.; Zhao, G.; Binkoski, A. E.; Coval, S. M.; Etherton, T. D. 2001. The effects of nuts on coronary heart disease risk. *Nutr. Rev.* **59**:103-111.

Kushi, L. H.; Meyer, K. A.; Jacobs, D. R. Jr. 1999. Cereals, legumes, and chronic disease risk reduction: evidence from epidemiologic studies. *Am. J. Clin. Nutr.* **70**:451S-458S.

Laguerre, M.; Lecomte, J.; Villeneuve, P. 2007. Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Prog. Lipid Res.* **46**:244-282.

Laguerre, M.; López-Giraldo, L. J.; Lecomte, J.; Baréa, B.; Cambon, E.; Tchobo, P. F.; Barouh, N.; Villeneuve, P. 2008. Conjugated autoxidizable triene (CAT) assay: A novel spectrophotometric method for determination of antioxidant capacity using triacylglycerol as ultraviolet probe. *Anal. Biochem.* **380**:282-290.

Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **47**:3693-3701.

Lemańska, K.; Szymusiak, H.; Tyrakowska, B.; Zieliński, R.; Soffers, A. E. M.F.; Rietjens, I. M. C. M. 2001. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Rad. Biol. Med.* **31**:869-881.

Leopoldini, M.; Marino, T.; Russo, N.; Toscano, M. 2004. Antioxidant properties of phenolic compounds: H-atom verses electron transfer mechanism. *J. Phys. Chem. A* **108**:4916-4922.

Lesschaeve, I.; Noble, A. C. 2005. Polyphenols: Factors influencing their sensory properties and their effect on food and beverage preferences. *Am. J. Clin. Nutr.* **81**:330S-335S.

Lewin, G.; Popov, I. 1994. Photochemiluminescent detection of antiradical activity; III: A simple assay of ascorbate in blood plasma. *J. Biochem. Biophys. Meth.* **28**:277-282.

Lien, E. J.; Ren, S.; Bui, H-H.; Wang, R. 1999. Quantitative structure-activity relationship analysis of phenolic antioxidants. *Free Rad. Biol. Med.* **26**:285-294.

Lissi, E.; Salim-Hanna, M.; Pascual, C.; Del Castillo, M. D. 1995. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Rad. Biol. Med.* **18**:153-158.

Litwinienko, G.; Ingold, K. U. 2003. Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) in alcohols. *J. Org. Chem.* **68**:3433-3438.

Liu, R. H. 2004. Potential synergy of phytochemicals in cancer prevention: Mechanism of action. *J. Nutr.* **134**:3479S-3485S.

Liu, R. H.; Finley, J. 2005. Potential cell culture models for antioxidant research. *J. Agric. Food Chem.* **53**:4311-4314.

Llesuy, S.; Evelson, P.; Campos, A. M.; Lissi, E. 2001. Methodologies for evaluation of total antioxidant activities in complex mixtures. A critical review. *Biol. Res.* **34**:51-73.

Lussignoli, S.; Fraccaroli, M.; Andrioli, G.; Brocco, G.; Bellavite, P. 1999. A microplate-based colorimetric assay of the total peroxyl radical trapping capability of human plasma. *Anal. Biochem.* **269**:38-44.

Machlin, L. J.; Bendich, A. 1987. Free radical tissue damage: Protective role of antioxidant nutrients. *FASEB J.* **1**:441-445.

Madsen, H. L.; Bertelsen, G. 1995. Spices as antioxidants. *Trends Food Sci. Tech.* **6**:271-277.

Maga, J. A. 1978. Simple phenol and phenolic compounds in food flavor. *Crit. Rev. Food Sci. Nutr.* **10**:323-372.

Mahoney, L. R. 1969. Antioxidants. *Angew Chem. Int. Ed.* **8**:547-555.

Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy. C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **81**:230S-242S.

Mannina, L.; Dugo, G.; Salvo, F.; Cicero, L.; Ansanelli, G.; Calcagni, C.; Segre, A. 2003. Study of the cultivar-composition relationship in Sicilian olive oils by GC, NMR, and statistical methods. *J. Agric. Food Chem.* **51**:120-127.

Mazur, W. 1998. Phytoestrogen content in foods. *Baillières Clin. Endocrinol. Metab.* **12**:729-742.

Mazur, W. M.; Duke, J. A.; Wähälä, K.; Rasku, S.; Adlercreutz, H. 1998. Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. *Nutr. Biochem.* **9**:193-200.

McLarney, M. J.; Pellett, P. L.; Young, V. R. 1996. Pattern of amino acid requirements in humans: An interspecies comparison using published amino acid requirement recommendations. *J. Nutr.* **126**:1871-1882.

Melson, G. A.; Busch, D. H. 1964. Reactions of coordinated ligands. X. The formation and properties of a tetradentate macrocylic ligand by the self-condensation of *o*-aminobenzaldehyde in the presence of metal ions. *J. Am. Chem. Soc.* **86**:4834-4837.

Mercer, L. C.; Wynne, J. C.; Young, C. T. 1990. Inheritance of fatty acid content in peanut oil. *Peanut Sci.* **17**:17-21.

Merényi, G.; Lind, J.; Eriksen, T. E. 1986. Nucleophilic addition to diazaquinones. Formation and breakdown of tetrahedral intermediates in relation to luminol chemiluminescence. *J. Am. Chem. Soc.* **108**:7716-7726.

Migliavacca, E.; Carrupt, P-A.; Testa, B. 1997. Theoretical parameters to characterize antioxidants: Part I, the case of vitamin E and analogs. *Helv. Chim. Acta* **80**:1613-1626.

Miller, H. E. 1971. A simplified method for the evaluation of antioxidants. *J. Am. Oil Chem. Soc.* **48**:91.

Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **84**:407-412.

Mira, L.; Fernandez, M. T.; Santos, M.; Rocha, R.; Florêncio, M. H.; Jennings, K. R. 2002. Interactions of flavonoids with iron and copper ions: A mechanism for their antioxidant activity. *Free Rad. Res.* **36**:1199-1208.

Moffett, J. W.; Zika, R. G.; Petasne, R. G. 1985. Evaluation of bathocuproine for the spectrophotometric determination of copper(I) in copper redox studies with applications in studies of natural waters. *Anal. Chim. Acta* **175**:171-179.

Molloy, A. M.; Mills, J. L.; Kirke, P. N.; Weir, D. G.; Scott, J. M. 1999. Folate status and neural tube defects. *Biofactors* **10**:291-294.

Moon, J-K; Shibamoto, T. 2009. Antioxidant assays for plant and food components. *J. Agric. Food Chem.* **57**:1655-1666.

Mortensen, A.; Skibsted, L. H. 1997. Importance of carotenoid structure in radical-scavenging reactions. *J. Agric. Food Chem.* **45**:2970-2977.

Naczk, M.; Shahidi, F. 2004. Extraction and analysis of phenolics in food. *J. Chromatogr. A.* **1054**:95-111.

Nakabeppu, Y.; Sakumi, K.; Sakamoto, K.; Tsuchimoto, D.; Tsuzuki, T.; Nakatsu, Y. 2006. Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biol. Chem.* **387**:373-379.

Nakanishi, I.; Fukuhara, K.; Shimada, T.; Ohkubo, K.; Iizuka, Y.; Inami, K.; Mochizuki, M.; Urano, S.; Itoh, S.; Miyata, N.; Fukuzumi, S. 2002. Effects of magnesium ion on kinetic stability and spin distribution of phenoxyl radical derived from a vitamin E analogue: Mechanistic insight into antioxidative hydrogen-transfer reaction of vitamin E. *J. Chem. Soc. Perk. T.* **2**:1520-1524.

Nepote, V.; Grosso, N. R.; Guzmán, C. A. 2002. Extraction of antioxidant components from peanut skins. *Grasas Aceites* **53**:391-395.

Nepote, V.; Grosso, N. R.; Guzmán, C. A. 2005. Optimization of extraction of phenolic antioxidants from peanut skins. *J. Sci. Food Agric.* **85**:33-38.

Neucere, N. J.; Conkerton, E. J.; Booth, A. N. 1972. Effect of heat on peanut proteins. II. Variations in nutritional quality of the meals. *J. Agric. Food Chem.* **20**:256-259.

Neucere, N. J.; Ory, R. L.; Carney, W. B. 1969. Effect of roasting on the stability of peanut proteins. *J. Agric. Food Chem.* **17**:25-28.

Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leeuven, P. A. M. 2001. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **74**:418-425.

Niki, E. 1997. Free radicals in chemistry and biochemistry. Food and Free Radicals. pp.1-10. Plenum Press, New York.

O'Byrne, D. J.; Knauft, D. A.; Shireman, R. B. 1997. Low fat-Monounsaturated rich diets containing high-oleic peanuts improve serum lipoprotein profiles. *Lipids* **32**:687-695.

Ostlund, R. E. Jr. 2002. Phytosterols in human nutrition. *Ann. Rev. Nutr.* **22**:533-549.

Ou, B.; Hampsch-Woodhill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L.; Huang, D. 2002. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J. Agric. Food. Chem.* **50**:2772-2777.

Ou, B.; Hampsch-Woodhill, M.; Prior, R. L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **49**:4619-4626.

Packer, J. E.; Mahood, J. S.; Mora-Arellano, V. O.; Slater, T. F.; Willson, R. L.; Wolfenden, B. S. 1981. Free radicals and singlet oxygen scavengers: Reaction of a peroxy-radical with β-carotene, diphenyl furan and 1,4-diazobicyclo(2,2,2)-octane. *Biochem. Biophys. Res. Commun.* **98**:901-906.

Paganga, G.; Miller, N.; Rice-Evans, C. A. 1999. The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute? *Free Rad. Res.* **30**:153-162.

Palmer, H. J.; Paulson, K. E. 1997. Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr. Rev.* **55**:353-361.

Paolini, M.; Pozzetti, L.; Pedulli, G. F.; Marchesi, E.; Cantelli-Forti, G. 1999. The nature of prooxidant activity of vitamin C. *Life Sci.* **64**:273-278.

Pattee, H. E.; Isleib, T. G.; Moore, K. M.; Gorbet, D. W.; Giesbrecht, F. G. 2002. Effect of high-oleic trait and paste storage variables on sensory attribute stability of roasted peanuts. *J. Agric. Food Chem.* **50**:7366-7370.

Pegg, R. B. 2005. Measurement of primary lipid oxidation products. Handbook of Food Analytical Chemistry: Water, Proteins, Enzymes, Lipids, and Carbohydrates. John Wiley & Sons Inc., Hoboken, NJ.

Pegg, R. B.; Amarowicz, R. 2004. Meat protein-tannin interactions: Observed antioxidant activity and potential health benefits. 50th International Congress of Meat Science and Technology, Helsinki, Finland.

Pegg, R. B.; Amarowicz, R.; Naczk, M.; Shahidi, F. 2007. PHOTOCHEM® method for determination of antioxidant capacity of plant extracts. American Chemical Society Symposium Series 956. Editors F. Shahidi and C-T. Ho. In Antioxidant Measurement and Applications. pp.140-158.

Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F. 2006. Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* assays. *Mol. Nutr. Food Res.* **50**:1030-1038.

Pietta, P.; Simonetti, P.; Mauri, P. 1998. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.* **46**:4487-4490.

Pilipenko, A. T.; Falendysh, E. R. 1972. Analytical chemistry of metal complexes with nitrogen-containing ligands of the 2,2′-bipyridyl type. *Russ. Chem. Rev.* **41**:991-1008.

Pinto, M. del C.; Tejeda, A.; Duque, A. L.; Macias, P. 2007. Determination of lipoxygenase activity in plant extracts using a modified ferrous oxidation-xylenol orange assay. *J. Agric. Food Chem.* **55**:5956-5959.

Pokorný, J. 2007. Are natural antioxidants better – and safer – than synthetic antioxidants? *Eur. J. Lipid Sci. Technol.* **109**:629-642.

Polidori, M. C.; Stahl, W.; Eichler, O.; Niestroj, I.; Sies, H. 2001. Profiles of antioxidants in human plasma. *Free. Rad. Biol. Med.* **30**:456-462.

Pope, M. T. 1983. Heteropoly and isopoly oxometalites. Vol. 8. Springer-Verlag NY, LLC.

Popov, I.; Lewin, G. 1999a. Antioxidative homeostasis: Characterization by means of chemiluminescence technique. *Meth. Enzymol.* **300**:437-456.

Popov, I.; Lewin, G. 1999b. Photochemiluminescent detection of antiradical activity. VI. Antioxidant characteristics of human blood plasma, low density lipoprotein, serum albumin and amino acids during *in vitro* oxidation. *Luminescence* **14**:169-174.

Popov, I. N.; Lewin, G. 1994. Photochemiluminescent detection of antiradical activity: II. Testing of nonenzymic water-soluble antioxidants. *Free Rad. Biol. Med.* **17**:267-271.

Popov, I. N.; Lewin, G. 1996. Photochemiluminescent detection of antiradical activity; IV: testing of lipid soluble antioxidants. *J. Biochem. Biophys. Meth.* **31**:1-8.

Popov, I. N.; Lewin, G.; van Baehr, R. 1987. Photochemiluminescent detection of antiradical activity. I. Assay of superoxide dismutase. *Biomed. Biochim. Acta* **46**:775-779.

Popov, I.; Volker, H.; Lewin, G. 2001. Photochemiluminescent detection of antiradical activity. V. Application in combination with the hydrogen peroxide-initiated chemiluminescence of blood plasma proteins to evaluate antioxidant homeostasis in humans. *Redox Rep.* **6**:43-48.

Powell, J. T. 2008. American Peanut Shellers Association, Albany, GA. Personal communication.

Pratt, D. E.; Miller, E. E. 1984. A flavonoid antioxidant in Spanish peanuts. *J. Am. Oil Chem. Soc.* **61**:1064-1067.

Prior, R. L.; Cao, G. 1999. In vivo total antioxidant capacity: Comparison of different analytical methods. *Free Rad. Biol. Med.* **27**:1173-1181.

Prior, R. L.; Gu, L. 2005. Ocurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* **66**:2264-2280.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocaa, M.; Howard, L.; Hampsch-Woodhill, M.; Huang, D.; Ou, B.; Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity [$ORAC_{FL}$]) of plasma and other biological and food samples. *J. Agric. Food Chem.* **51**:3273-3279.

Prior, R. L.; Wu, X.; Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **53**:4290-4302.

Pryor, W. A.; Cornicelli, J. A.; Devall, L. J.; Tait, B.; Trivedi, B. K.; Witiak, D. T.; Wu, M. 1993. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. *J. Org. Chem.* **58**:3521-3532.

Pulido, R.; Bravo, L.; Saura-Calixto, F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **48**:3396-3402.

Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**:1231-1237.

Reed, K. A.; Sims, C. A.; Gorbet, D. W.; O'Keefe, S. F. 2002. Storage water activity affects flavor fade in high and normal oleic peanuts. *Food Res. Int.* **35**:769-774.

Regoli, F.; Winston, G. W. 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicol. Appl. Pharmacol.* **156**:96-105.

Rice-Evans, C. A.; Diplock, A. T. 1993. Current status of antioxidant therapy. *Free Rad. Biol. Med.* **15**:77-96.

Rice-Evans, C. A.; Miller, N. J.; Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med.* **20**:933-956.

Rimm, E. B.; Stampfer, M. J. 1997. The role of antioxidants in preventive cardiology. *Curr. Opin. Cardiol.* **12**:188-194.

Robbins, R. J. 2003. Phenolic acids in foods: An overview of analytical methodology. *J. Agric. Food Chem.* **51**:2866-2887.

Roberts, W. G.; Gordon, M. H. 2003. Determination of the total antioxidant activity of fruits and vegetables by a liposome assay. *J. Agric. Food Chem.* **51**:1486-1493.

Rosenblatt, M.; Peluso, J. V. 1941. Determination of tannins by photocolorimeter. *J. Assoc. Offic. Agric. Chem.* **24**:170-181.

Rufián-Henares, J. A.; Delgado-Andrade, C.; Morales, F. J. 2006. Assessing the antioxidant and pro-oxidant activity of phenolic compounds by means of their copper reducing activity. *Eur. Food Res. Technol.* **223**:225-231.

Sabaté, J. 2003. Nut consumption and body weight. *Am. J. Clin. Nutr.* **78**:647S-650S.

Sabaté, J. 1999. Nut consumption, vegetarian diets, ischemic heart disease risk, and all-cause mortality: evidence from epidemiologic studies. *Am. J. Clin. Nutr.* **70**:500S-503S.

Sánchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. 1998. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **76**:270-276.

Sanders, T. H.; McMichael, R. W. Jr.; Hendrix, K. W. 2000. Occurrence of resveratrol in edible peanuts. *J. Agric. Food Chem.* **48**:1243-1246.

Scalbert, A.; Johnson, I. T.; Saltmarsh, M. 2005. Polyphenols: Antioxidants and beyond. *Am. J. Clin. Nutr.* **81**:215S-217S.

Scalbert, A.; Morand, C.; Manach, C.; Rémésy, C. 2002. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother.* **56**:276-282.

Scalbert, A.; Williamson, G. 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**:2073S-2085S.

Schaich, K. M. 2006. Developing a rational basis for selection of antioxidant screening and testing methods. Acta Hort. **709**:79-94.

Schlesier, K.; Harwat, M.; Böhm, V.; Bitsch, R. 2002. Assessment of antioxidant activity by using different *in vitro* methods. *Free Rad. Res.* **36**:177-187.

Schneider, H. W. 1970. A new, long-lasting luminol chemiluminescent cold light. *J. Chem. Educ.* **47**:519-522.

Shahidi, F. 2000. Antioxidants in food and food antioxidants. *Nahrung* **44**:158-163.

Shahidi, F. 2002. Antioxidants in plants and oleaginous seeds, in *Free Radicals in Food: Chemistry, Nutrition, and Health Effects,* Morello, M. J.; Shahidi, F.; and Ho, C-T.; Eds., ACS Symposium Series 807. American Chemical Society. Washington, D. C., 162-175.

Shahidi, F.; Naczk, M. 2004. Phenolics in food and nutraceuticals. CRC Press LLC, Boca Raton, Fl.

Shan, B.; Cai, Y. Z.; Sun, M.; Corke, H. 2005. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. Agric. Food Chem.* **53**:7749-7759.

Sies, H. 1993. Strategies of antioxidant defense. *Eur. J. Biochem.* **215**:213-219.

Sies, H.; Stahl, W.; Sevanian, A. 2005. Nutritional, dietary and postprandial oxidative stress. *J. Nutr.* **135**:969-972.

Silva, F. A. M.; Borges, F.; Guimarães, C.; Lima, J. L. F. C.; Matos, C.; Reis, S. 2000. Phenolic acids and derivatives: Studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *J. Agric. Food Chem.* **48**:2122-2126.

Singh, B.; Singh, U. 1991. Peanut as a source of protein for human foods. *Plant Foods Hum. Nutr.* **41**:165-177.

Singleton, V. L.; Rossi, J. A. Jr. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viticult.* **16**:144-158.

Singleton, V. L.; Orthofer, R.; Lamuela-Raventós, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of the Folin-Ciocalteu reagent. *Meth. Enzymol.* **299**:152-178.

Smith, G. F.; Wilkins, D. H. 1953. New colorimetric reagent specific for copper: Determination of copper in iron. *Anal. Chem.* **25**:510-511.

Snyder, H. E.; Kwon, T. W. 1987. Nutritional attributes of soybeans and soybean products. Van Norstrand Reinhold Company Inc., New York, NY.

Sobolev, V. S.; Cole, R. J. 1999. *trans*-Resveratrol content in commercial peanuts and peanut products. *J. Agric. Food Chem.* **47**:1435-1439.

Sobolev, V. S.; Cole, R. J. 2004. Note on utilisation of peanut seed testa. *J. Sci. Food Agric.* **84**:105-111.

Speranza, G.; Manitto, P.; Monti, D. 1990. Interaction between singlet oxygen and biologically active compounds in aqueous solution III. Physical and chemical $^1O_2$-quenching rate constants of 6,6′-diapocarotenoids. *J. Photochem. Photobiol. B.* **8**:51-61.

Steinberg, D. 1991. Antioxidants and atherosclerosis: A current assessment. *Circulation* **84**:1420-1425.

Stevens, M. J. 2005. Redox-based mechanisms in diabetes. *Antiox. Redox Sig.* **7**:1483-1485.

Stratil, P.; Klejdus, B.; Kubáň, V. 2006. Determination of total content of phenolic compounds and their antioxidant activity in vegetables-Evaluation of spectrophotometric methods. *J. Agric. Food Chem.* **54**:607-616.

Streitwieser, A. Jr.; Heathcock, C. H. 1981. Introduction to Organic Chemistry. 2nd Edition. Macmillan Publishing Co., Inc., New York, NY.

Sun, J.; Chu, Y-F.; Wu, X.; Liu, R. H. 2002. Antioxidant and anti-proliferative activities of common fruits. *J. Agric. Food Chem.* **50**:7449-7454.

Talcott, S. T.; Duncan, C. E.; Del Pozo-Insfran, D.; Gorbet, D. W. 2005a. Polyphenolic and antioxidant changes during storage of normal, mid and high oleic acid peanuts. *Food Chem.* **89**:77-84.

Talcott, S. T.; Passeretti, S.; Duncan, C. E.; Gorbet, D. W. 2005b. Polyphenolic content and sensory properties of normal and high oleic acid peanuts. *Food Chem.* **90**:379-388.

Tanizawa, H.; Sazuka, Y.; Komatsu, A.; Toda, S.; Takino, Y. 1983. A new efficacy test of antioxidants based on air-oxidation of linoleic acid. *Chem. Pharm. Bull.* **31**:4139-4143.

Tchantchou, F. 2006. Homocysteine metabolism and various consequences of folate deficiency. *J. Alzheimer's Dis.* **9**:421-427.

Temple, N. J. 2000. Antioxidants and disease: More questions than answers. *Nutr. Res.* **20**:449-459.

Tokuşoğlu, O.; Ünal, M. K.; Yemiş, F. 2005. Determination of the phytoalexin resveratrol (3,5,4′-trihydroxystilbene) in peanuts and pistachios by high-performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS). *J. Agric. Food Chem.* **53**:5003-5009.

Touyz, R. M. 2005. Reactive oxygen species as mediators of calcium signaling by angiotensin II: Implications in vascular physiology and pathophysiology. *Antiox. Redox Sig.* **7**:1302-1314.

Tubaro, F.; Ghiselli, A.; Rapuzzi, P.; Maiorino, M.; Ursini, F. 1998. Analysis of plasma antioxidant capacity by competition kinetics. *Free Rad. Biol. Med.* **24**:1228-1234.

Tütem, E.; Apak, R. 1991. Spectrophotometric determination of trace amounts of copper(I) and reducing agents with neocuproine in the presence of copper(II). *Analyst* **116**:89-94.

U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS). 2008. USDA National Nutrient Database for Standard Reference, Release 21. Nutrient Data Laboratory Home Page, <http://www.ars.usda.gov/ba/bhnrc/ndl>. Search: Nutrients in 100 g raw peanuts (*Arachis hypogea*), all types.

U.S. Department of Agriculture, Economic Research Service (USDA-ERS). 2002. Commodity spotlight: Peanut consumption rebounding amidst market uncertainties. Agricultural Outlook 289:2-5. March 2002. <http://www.ers.usda.gov/publications/agoutlook/mar2002/ao289a.pdf>.

U.S. Food and Drug Administration, Department of Health and Human Services, Code of Federal Regulations (CFR) – Title 21: Food and Drugs. December 2005. Section 101.54, 101.83.

Van Ha, H.; Pokorný, J.; Sakurai, H. 2007. Peanut skin antioxidants. *J. Food Lipids* **14**:298-314.

Wang, J.; Yuan, X.; Jin, Z.; Tian, Y.; Song, H. 2007. Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem.* **104**:242-250.

Waslidge, N. B.; Hayes, D. J. 1995. A colorimetric method for the determination of lipoxygenase activity suitable for use in a high throughput assay format. *Anal. Biochem.* **231**:354-358.

Wayner, D. D. M. 1987. 1049 – Radical-trapping antioxidants in vitro and in vivo. *Bioelectroch. Bioener.* **18**:219-229.

Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Locke, S. 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capability of human blood plasma by controlled peroxidation. *FEBS Lett.* **187**:33-37.

Williamson, G.; Manach, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **81**:243S-255S.

Winston, G. W.; Regoli, F.; Dugas, A. J. Jr.; Fong, J. H.; Blanchard, K. A. 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Rad. Biol. Med.* **24**:480-493.

Wolfe, K. L.; Liu, R. H. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **55**:8896-8907.

Wolff, S. P. 1994. Ferrous ion oxidation in presence of ferric iron indicator xylenol orange for measurement of hydroperoxides. *Meth. Enzymol.* **233**:182-187.

Wong, J. M. W.; de Souza, R.; Kendall, C. W. C.; Emam, A.; Jenkins, D. J. A. 2006. Colonic health: Fermentation and short chain fatty acids. *J. Clin. Gastroenterol.* **40**:235-243.

Wright, J. S.; Johnson, E. R.; Dilabio, G. A. 2001. Predicting the activity of phenolic antioxidants: Theoretical method, analysis of substituent effects, and application to major families of antioxidants. *J. Am. Chem. Soc.* **123**:1173-1183.

Yanagihara, K.; Ito, A.; Toge, T.; Numoto, M. 1993. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Rev.* **53**:5815-5821.

Yen, G-C.; Duh, P-D. 1994. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J. Agric. Food Chem.* **42**:629-632.

Yoo, K. M.; Kim, D.-O.; Lee, C. Y. 2007. Evaluation of different methods of antioxidant measurement. *Food Sci. Biotechnol.* **16**:177-182.

Yu, J.; Ahmedna, M.; Goktepe, I. 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem.* **90**:199-206.

Yu, J.; Ahmedna, M.; Goktepe, I. 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. *J. Food Comp. Anal.* **19**:364-371.

Yu, J.; Ahmedna, M.; Goktepe, I. 2007a. Peanut protein concentrate: Production and functional properties as affected by processing. *Food Chem.* **103**:121-129.

Yu, J.; Ahmedna, M.; Goktepe, I. 2007b. Peanut Skin Phenolics: Extraction, Identification, Antioxidant Activity, and Potential Applications. ACS Symposium Series 956 Editors F. Shahidi and C-T. Ho. In Antioxidant Measurement and Applications. pp. 226-241.

Yu-Poth, S.; Etherton, T. D.; Reddy, C. C.; Pearson, T. A.; Reed, R.; Zhao, G.; Jonnalagadda, S.; Wan, Y.; Kris-Etherton, P. M. 2000. Lowering dietary saturated fat and total fat reduces the oxidative susceptibility of LDL in healthy men and women. *J. Nutr.* **130**:2228-2237.

Zak, B. 1958. Simple procedure for the single sample determination of serum copper and iron. *Clin. Chim. Acta* **3**:328-334.

Zaporozhets, O. A.; Krushynska, O. A.; Lipkovska, N. A.; Barvinchenko, V. N. 2004. A new test method for the evaluation of total antioxidant activity of herbal products. *J. Agric. Food Chem.* **52**:21-25.

Zhang, H-Y.; Ji, H-F. 2006. How vitamin E scavenges DPPH radicals in polar protic media. *New J. Chem.* **30**:503-504.

Zhang, Q.; Zhang, J.; Shen, J.; Silva, A.; Dennis, D. A.; Barrow, C. J. 2006. A simple 96-well microplate method for estimation of total phenol content in seaweeds. *J. Appl. Phycol.* **18**:445-450.

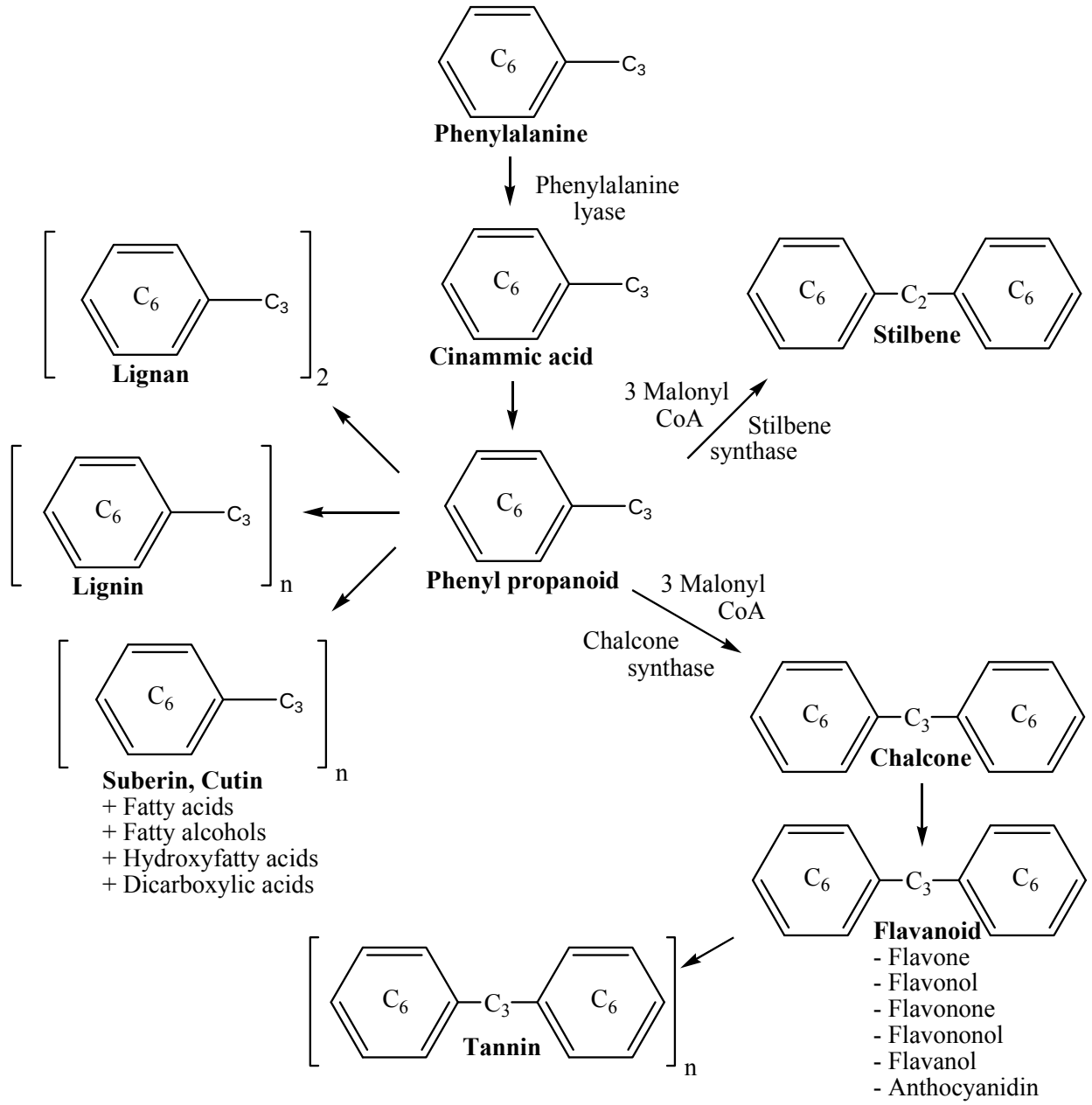


Figure 2.1: Synthesis of phenylpropanoids from phenylalanine, the origin of phenolics

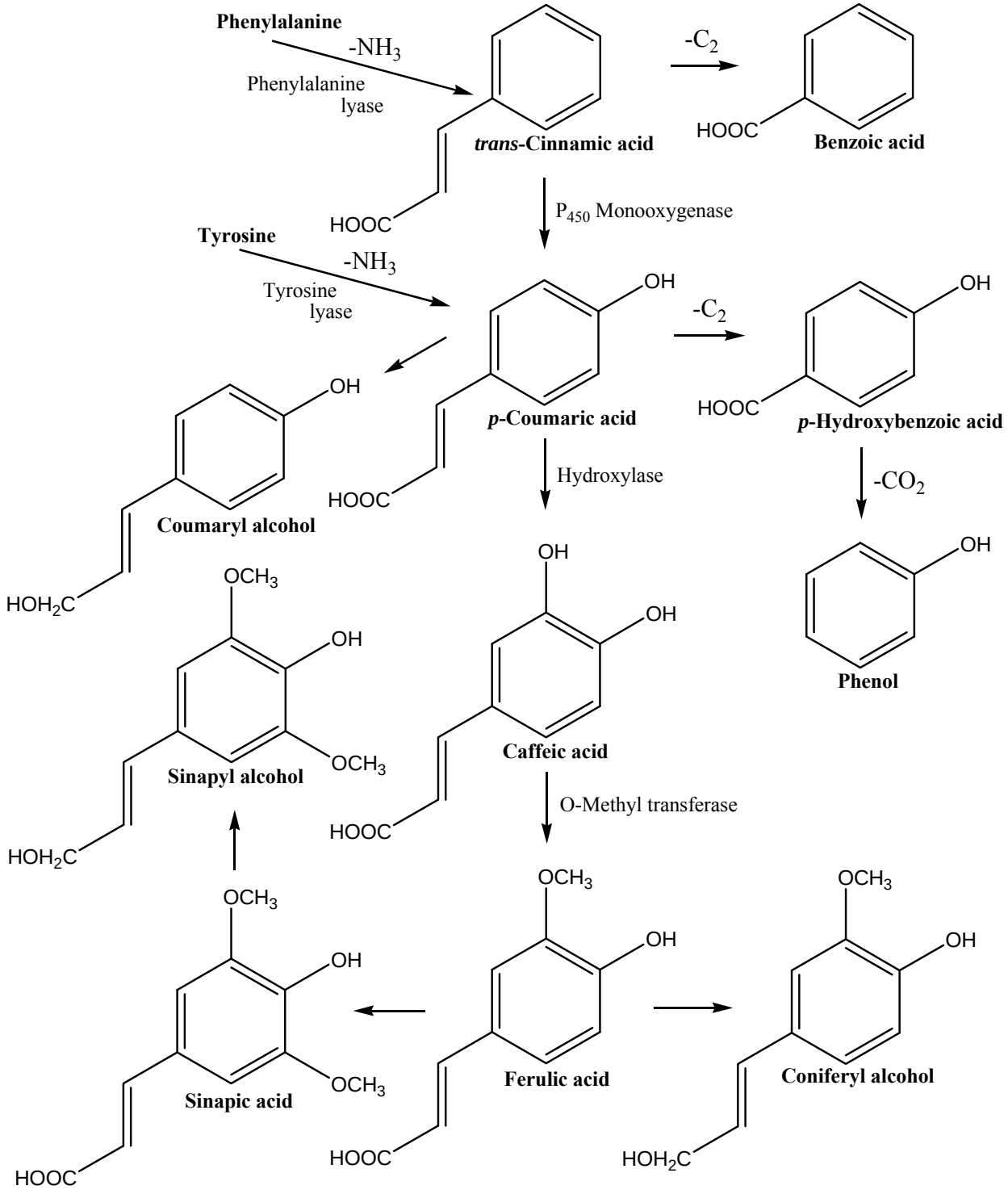


Figure 2.2: Formation of phenylpropanoids from phenylalanine and tyrosine, adapted from Shahidi. 2000. *Nahrung* **44**:158-163

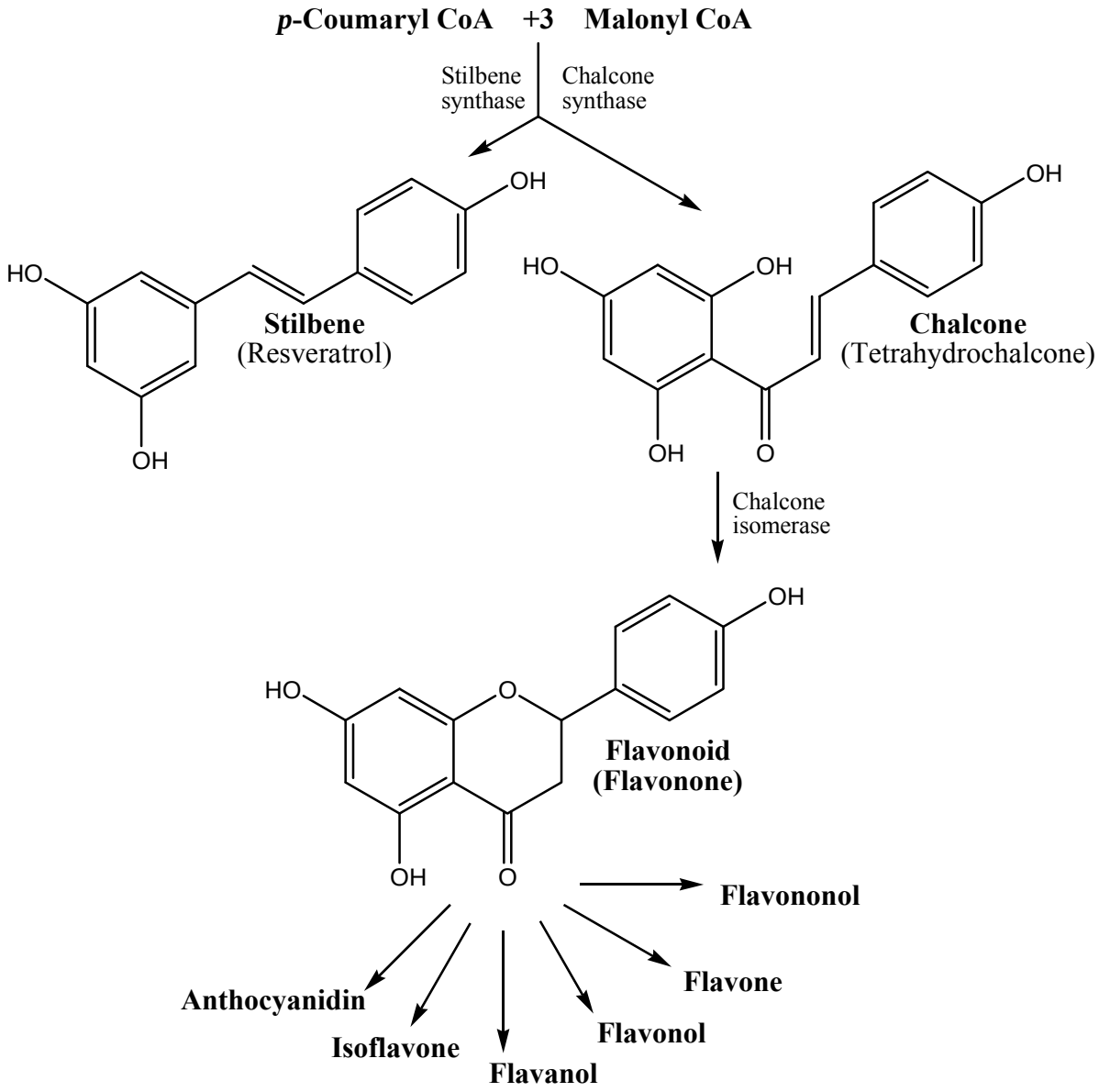


Figure 2.3: Production of flavonoids and stilbenes from phenylpropanoid (*p*-coumaryl CoA) and malonyl CoA, adapted from Shahidi. 2000. *Nahrung* **44**:158-163

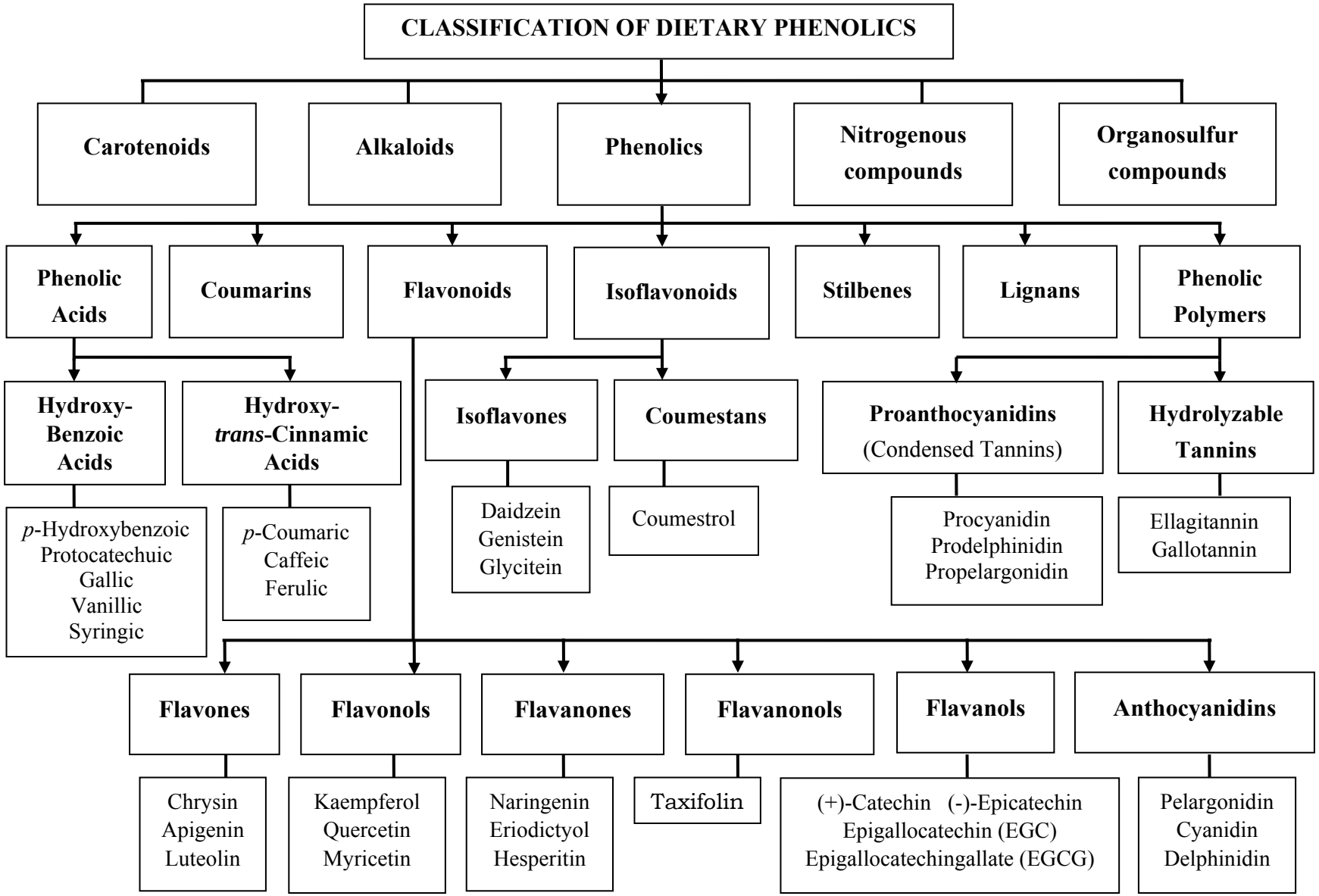
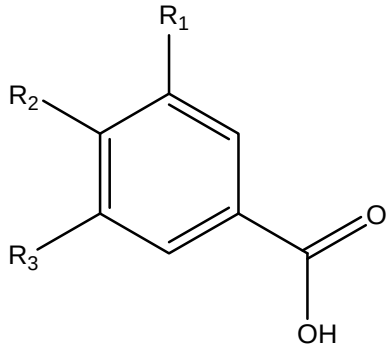
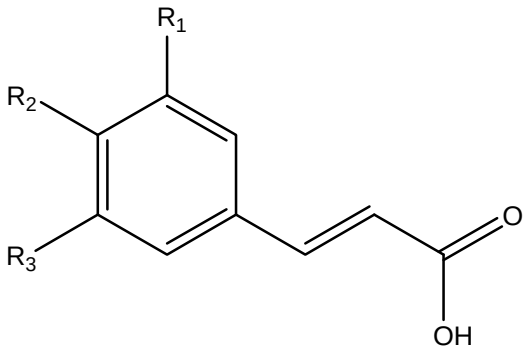


Figure 2.4: Classification of dietary phenolics, adapted from Liu. 2004. *J. Nutr.* **134**:3479S-3485S



Phenolic Acid	**R_1**	**R_2**	**R_3**
p-Hydroxybenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vanillic	OCH3	OH	H
Syringic	OCH3	OH	OCH3
Gallic	OH	OH	OH

Figure 2.5: Phenolic acids of the benzoic acid family



Phenolic Acid	**R_1**	**R_2**	**R_3**
p-Coumaric	H	OH	H
Caffeic	OH	OH	H
Ferulic	OCH3	OH	H
Isoferulic	OH	OCH3	H
Sinapic	OCH3	OH	OCH3

Figure 2.6: Phenolic acids of the *trans*-cinnamic acid family

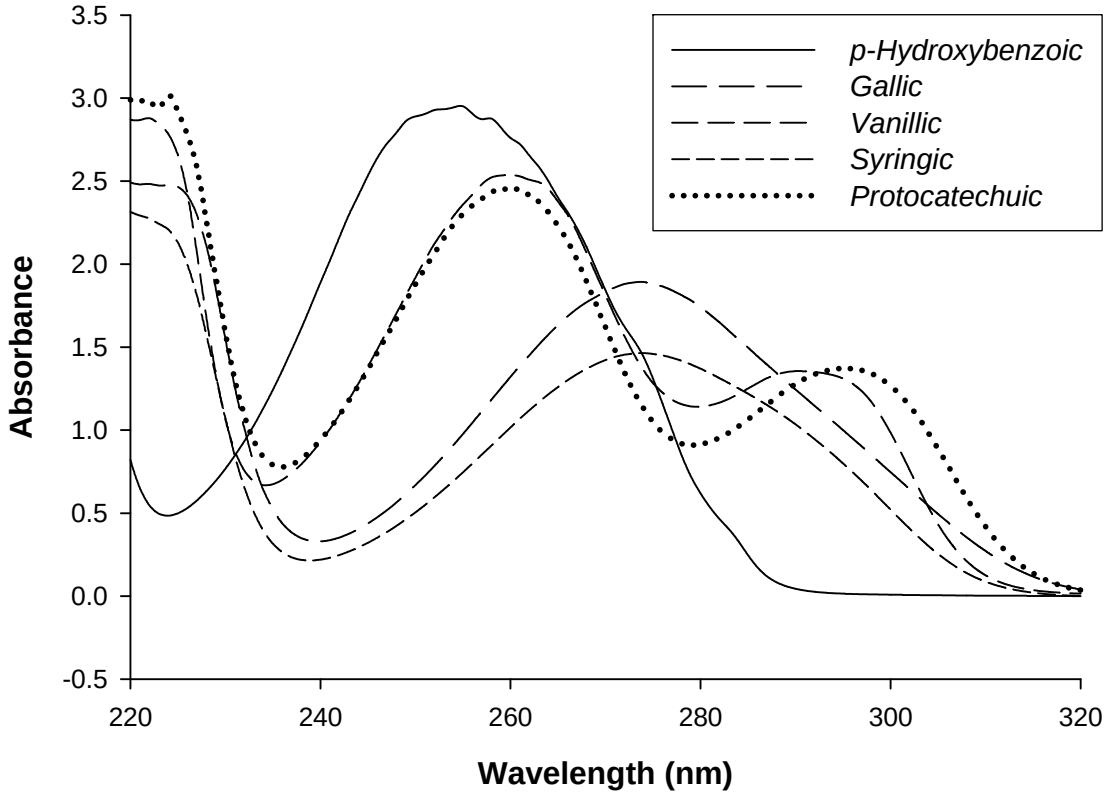


Figure 2.7: UV-spectra of phenolic acids in the benzoic acid family

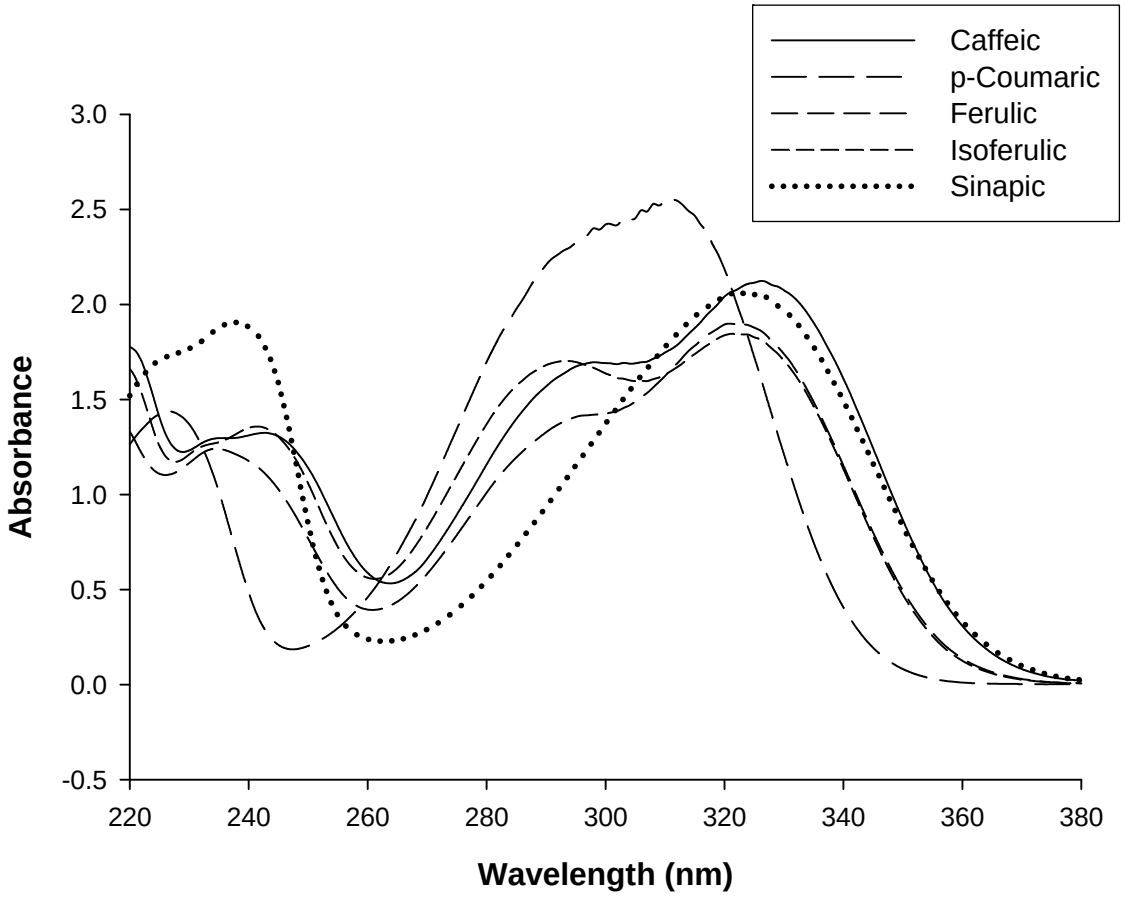


Figure 2.8: UV-spectra of phenolic acids in the *trans*-cinnamic acid family

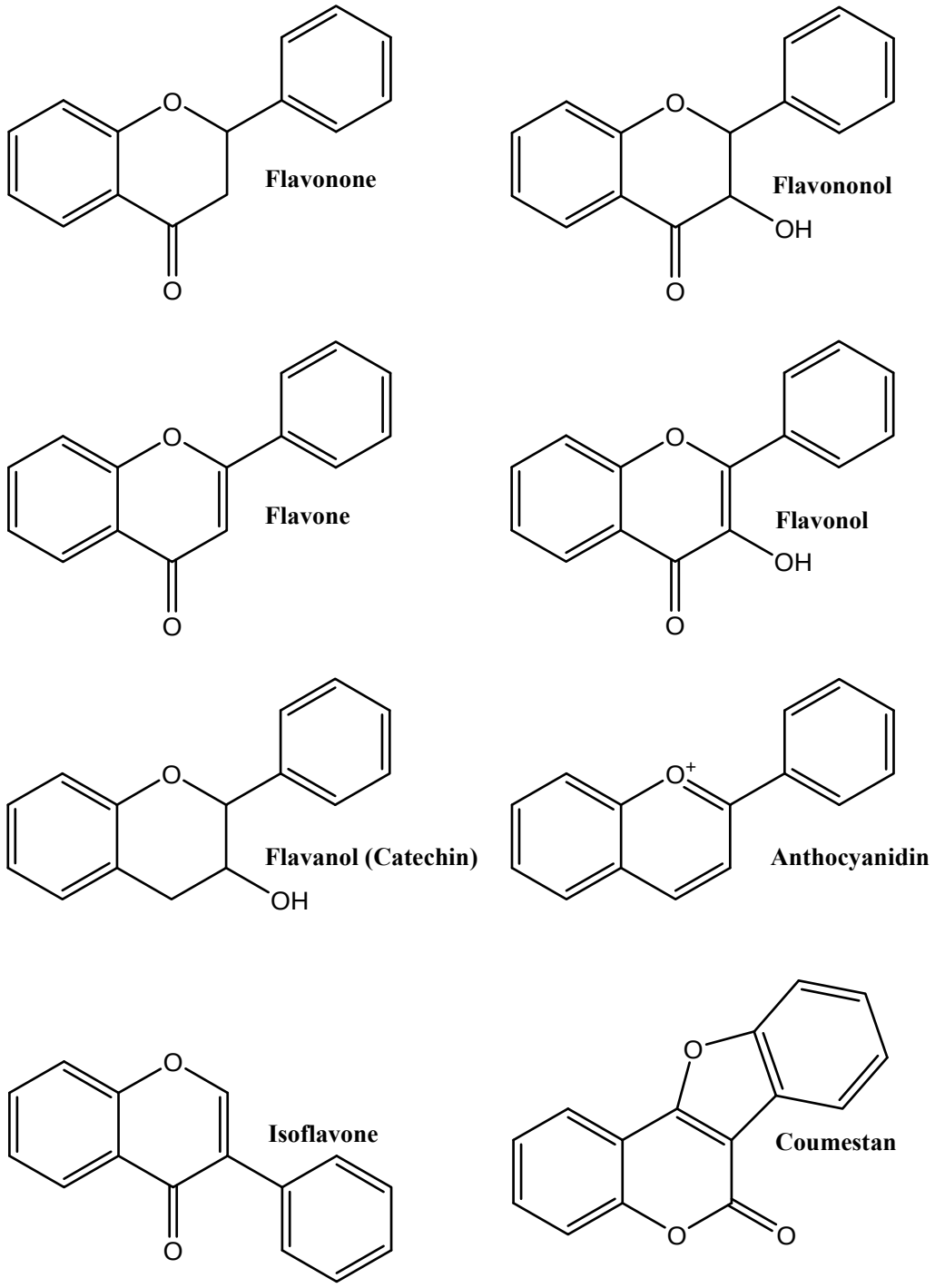


Figure 2.9: Chemical backbone of selected flavonoids and isoflavonoids found in plants

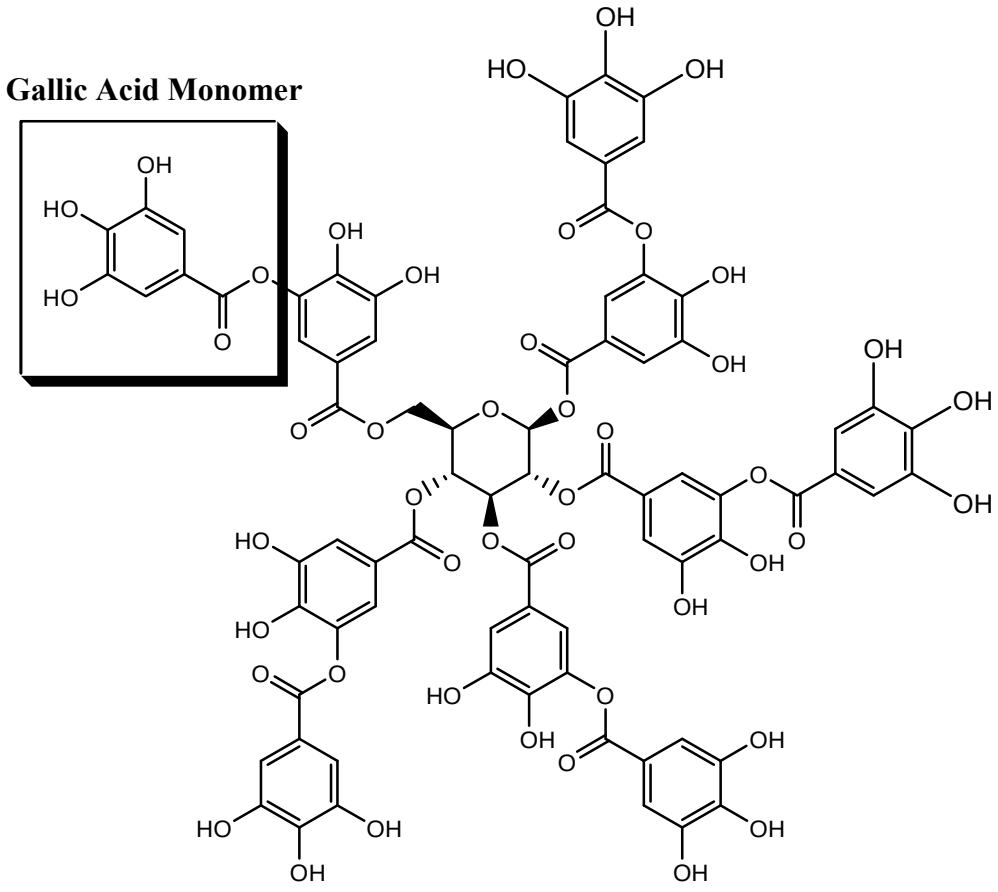


Figure 2.10: A hydrolyzable gallotannin (tannic acid)

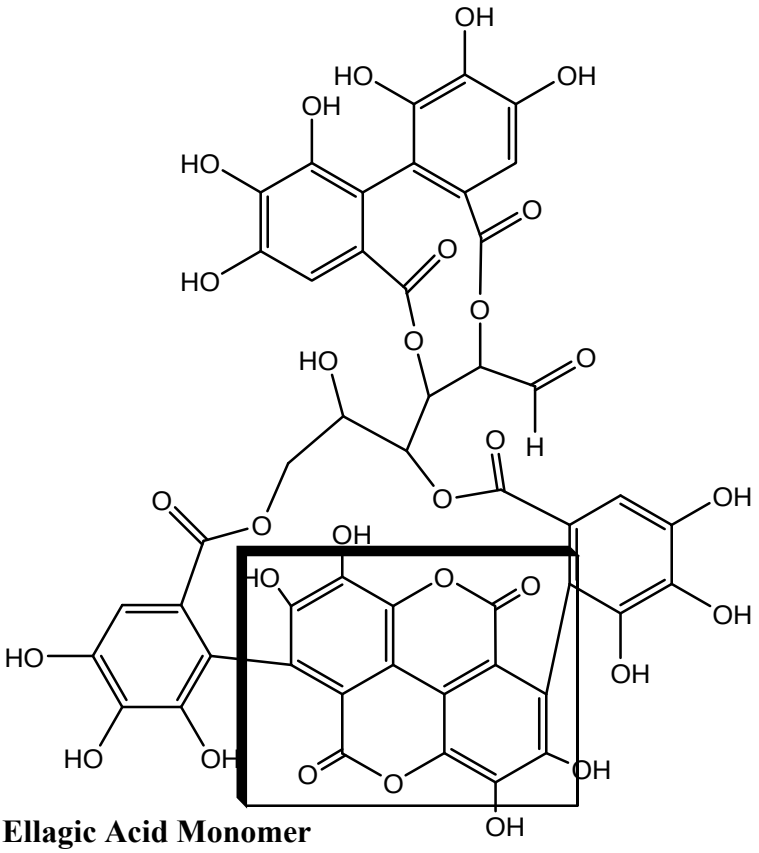


Figure 2.11: A hydrolyzable ellagitannin (punicalagin)

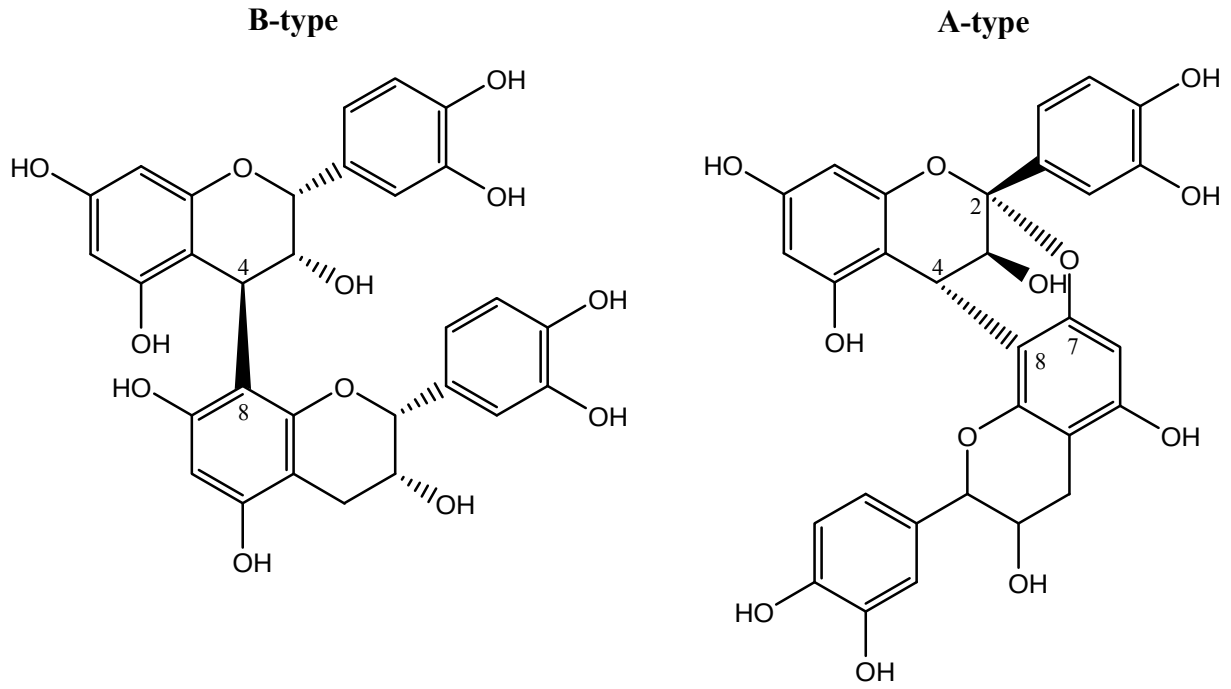


Figure 2.12: Condensed tannins, B-type (4→8) and A-type (4→8, 2→7) procyanidin dimers (B_2 and A_2)

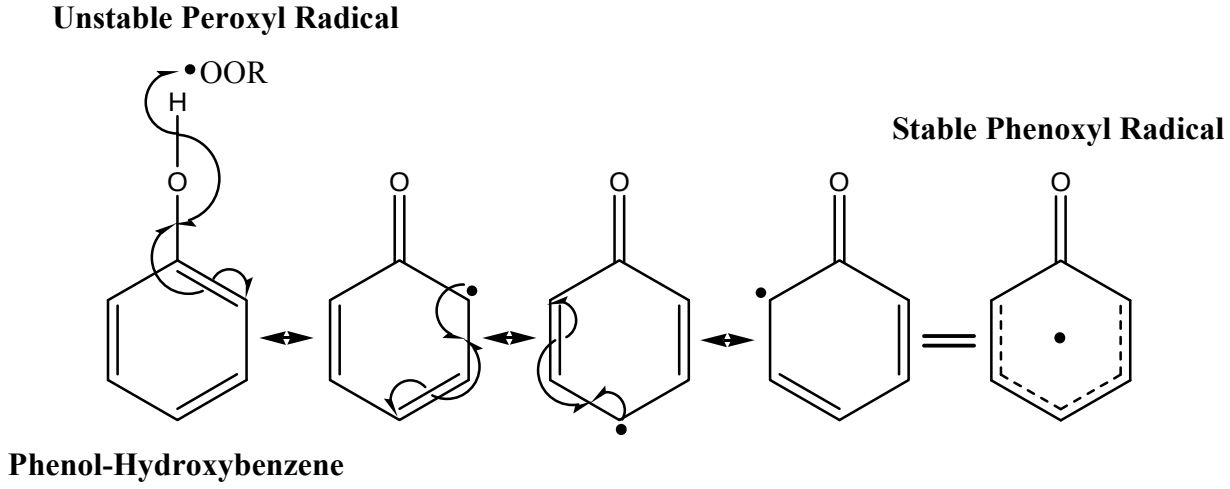


Figure 2.13: A mechanism of phenolic antioxidant efficacy, conjugative resonance stabilization

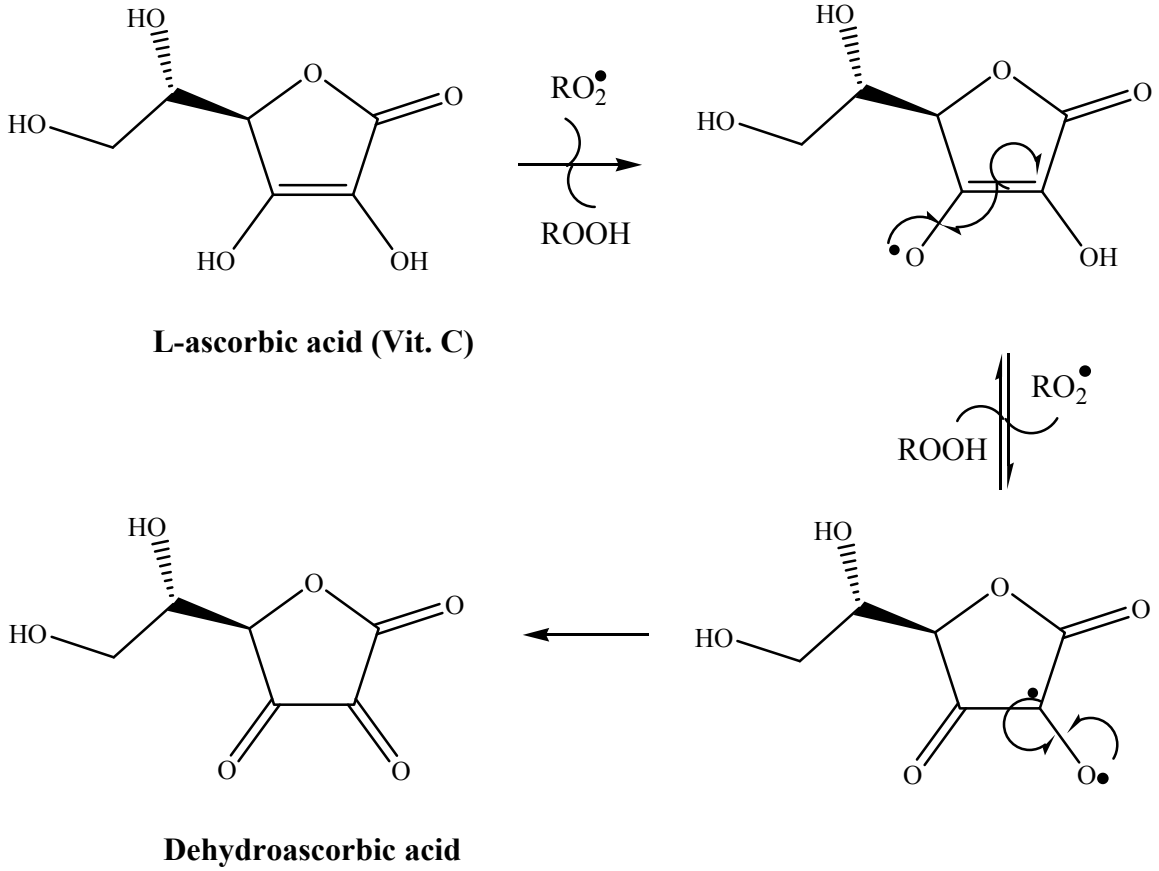


Figure 2.14: HAT conversion of L-ascorbic acid (Vitamin C) to dehydroascorbic acid

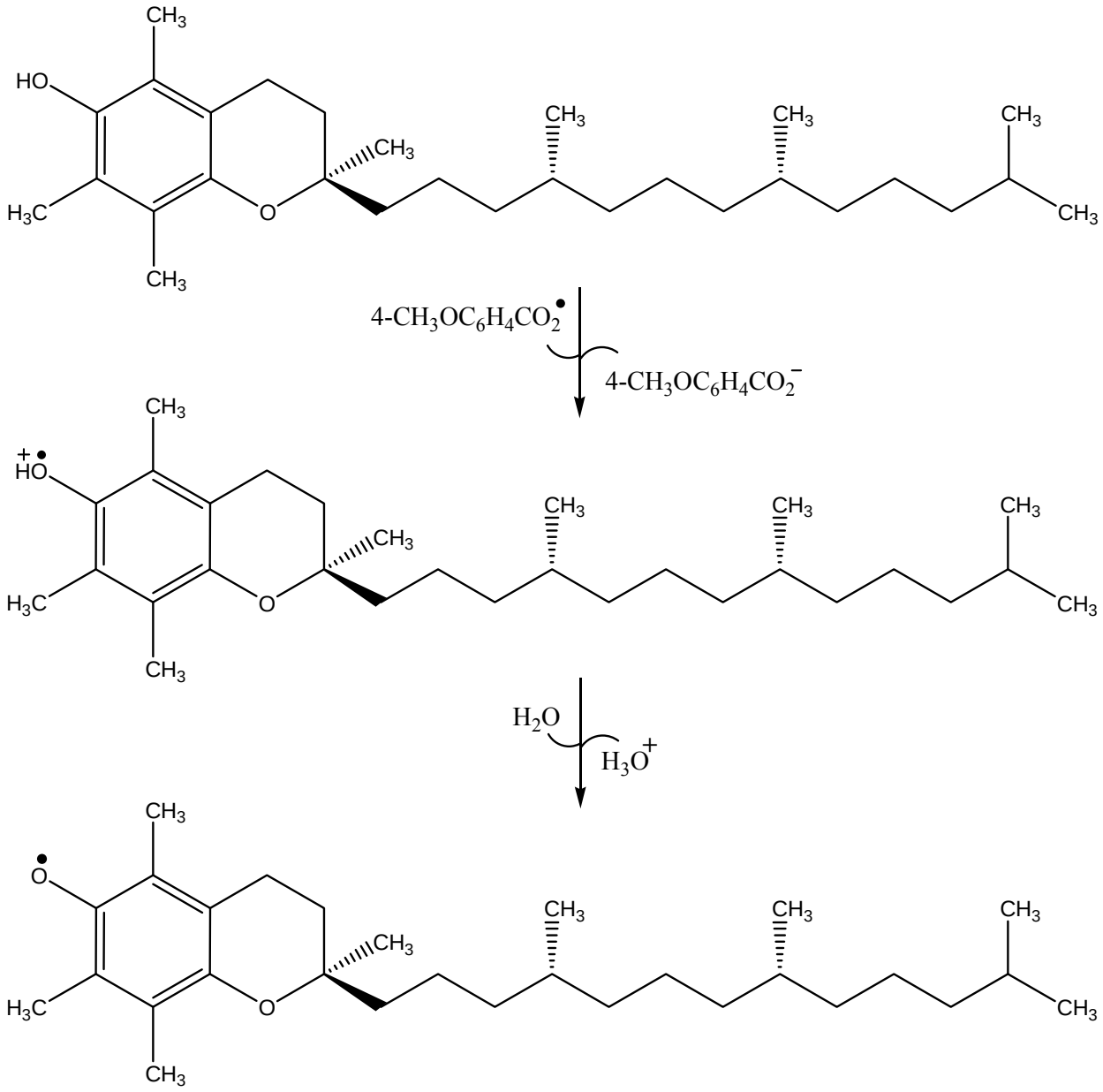


Figure 2.15: A SET mechanism between α-tocopherol (Vitamin E) and 4-methoxybenzoyloxyl radical, adapted from Evans *et al.*, 1992. *J. Am. Chem. Soc.* **114**:4589-4593

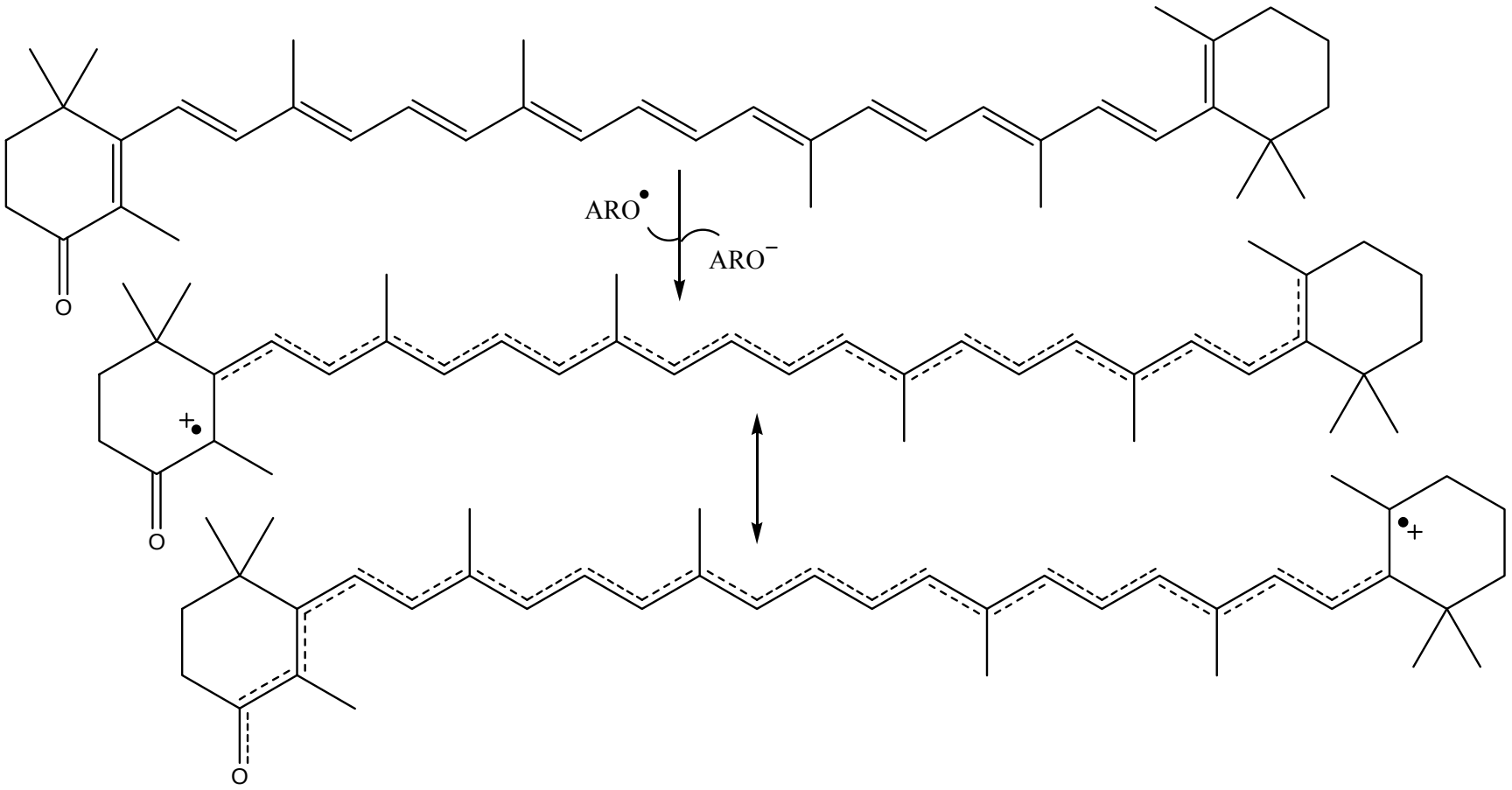


Figure 2.16: A proposed mechanism for the reduction of phenoxyl radical by the carotenoid echinenone through a SET mechanism resulting in two canionical radical cationic carotenoid species, adapted from Mortenson and Skibsted. 1997. *J. Agric. Food Chem.* **45**:2970-2977

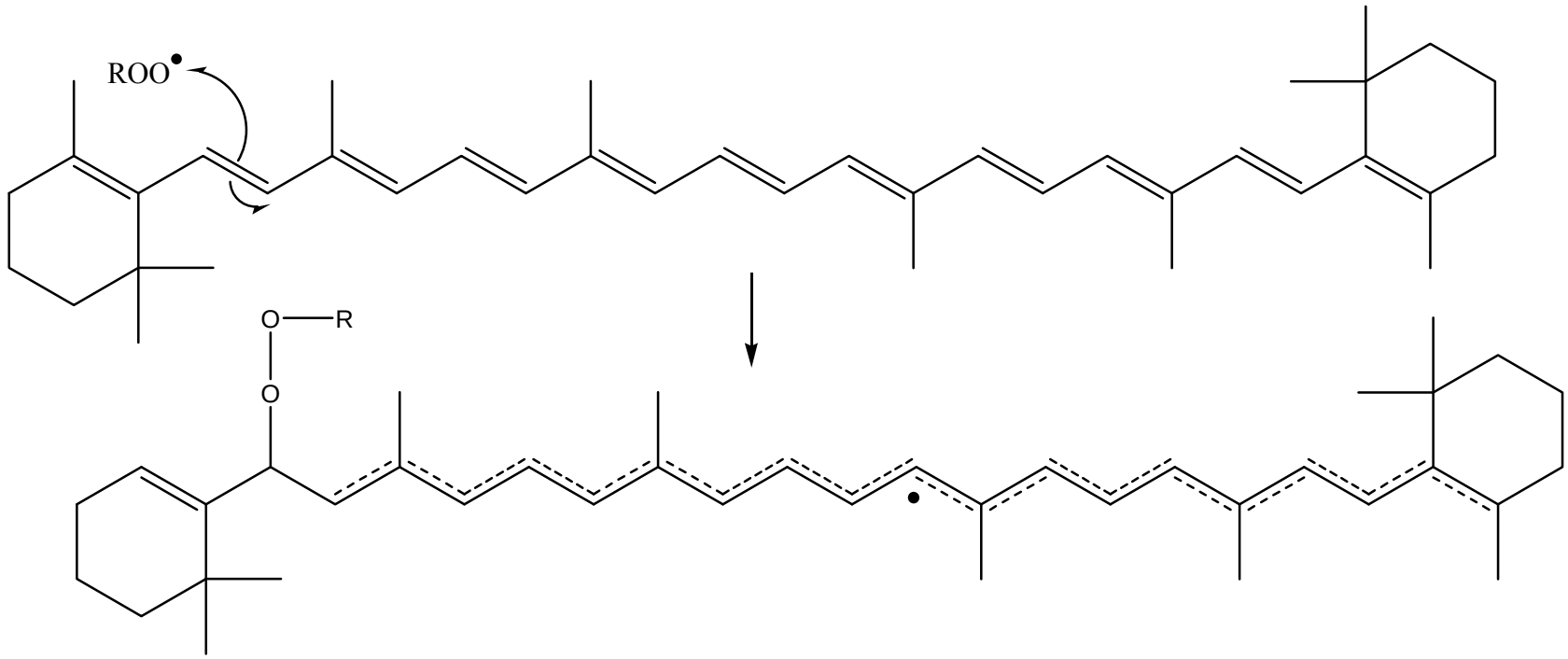


Figure 2.17: A proposed mechanism for the antioxidant preservation of lipids by carotenoids through carotenoid-radical dimerization (*i.e.*, adduct formation [AF]) with peroxyl radicals in food systems, adapted from Burton and Ingold. 1984. *Science* **224**:569-573

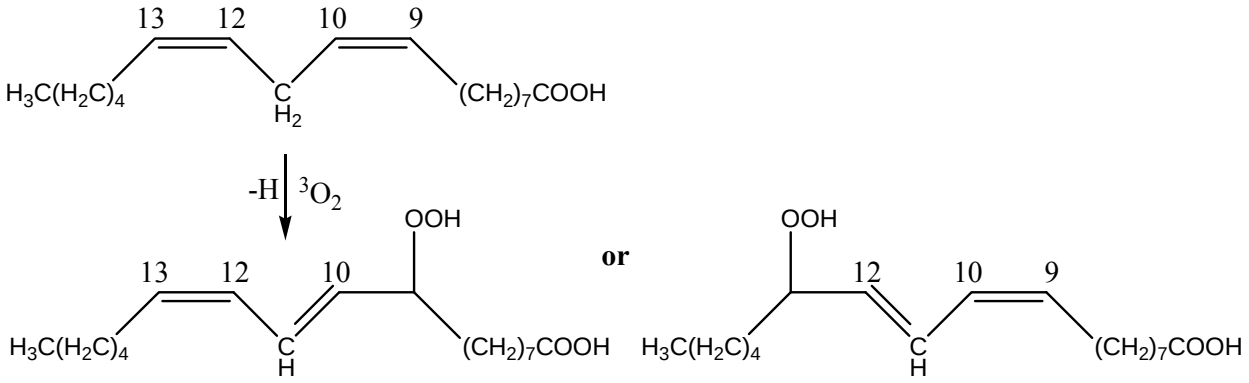


Figure 2.18: An illustration of the oxidation of linoleic acid, which results in the formation of two monoperoxides either at carbon 9 (*i.e.*, 9-OOH) or at carbon 13 (*i.e.*, 13-OOH), adapted from Corongiu and Banni. 1994. *Meth. Enzymol.* **233**:303-310

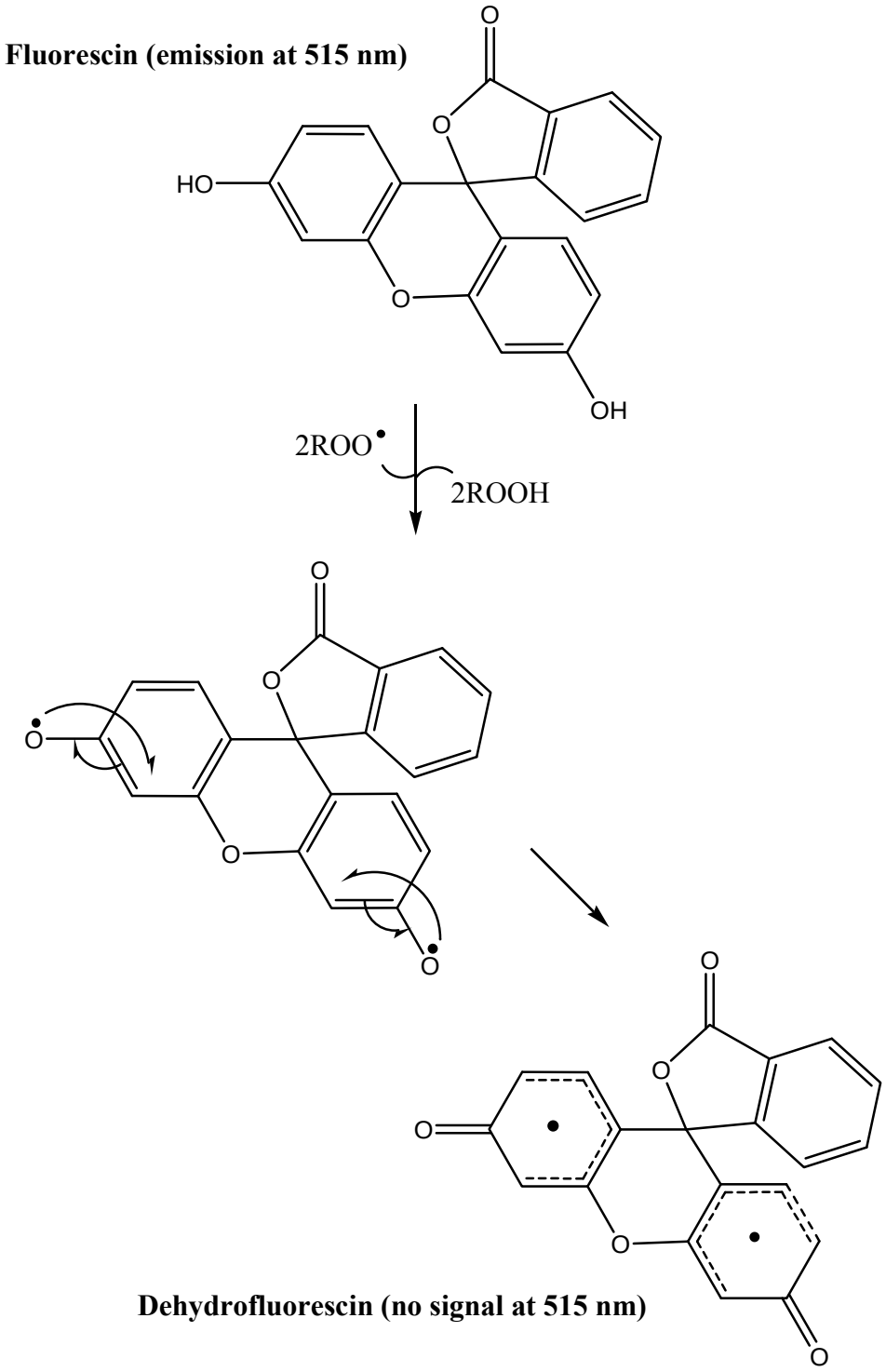


Figure 2.19: Proposed mechanism for the ORAC HAT FL(H) → FL (loss of signal)

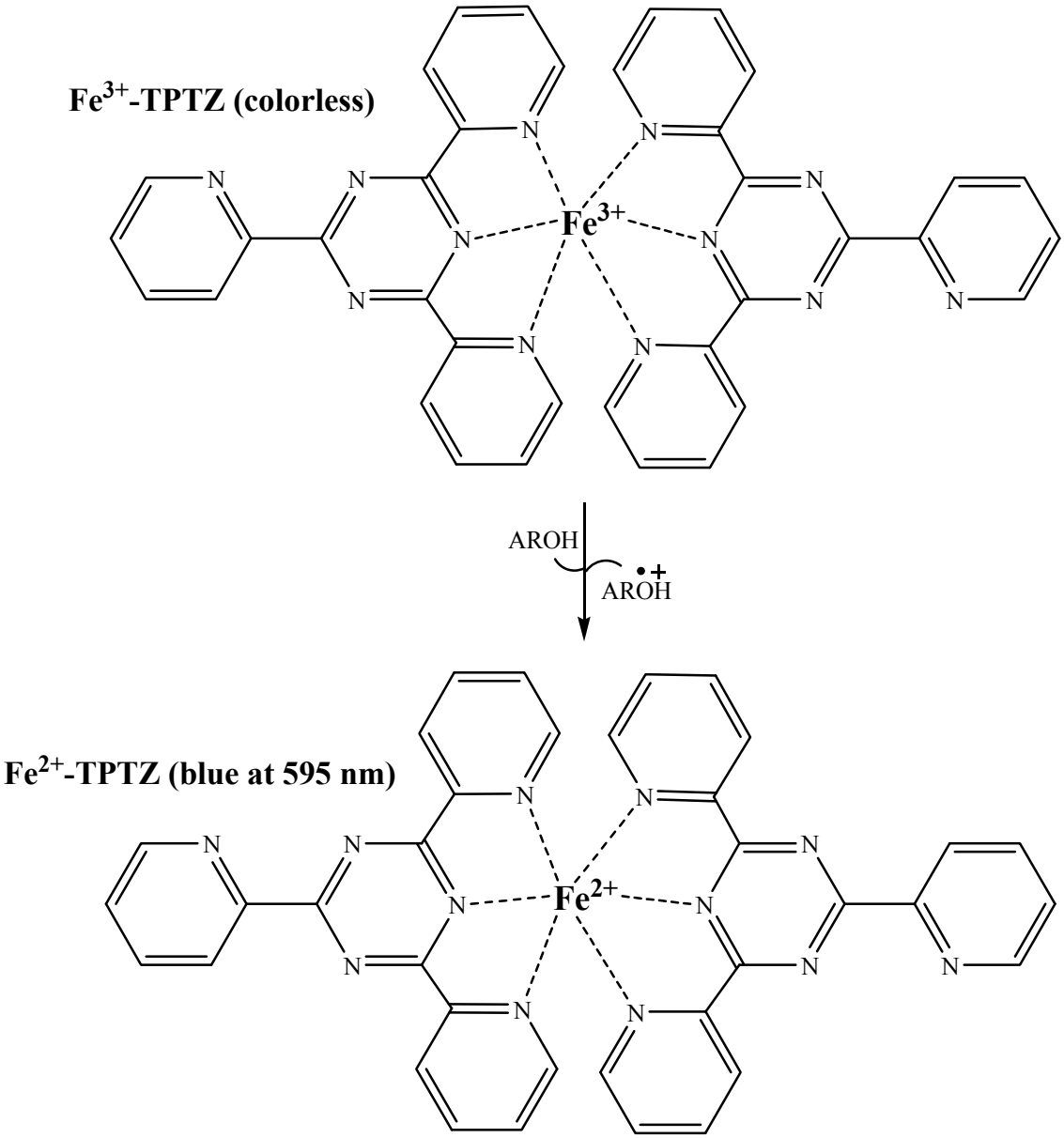


Figure 2.20: FRAP color production reaction: SET reduction of iron(III)-TPTZ to iron(II)-TPTZ (blue at λ_{max} = 595 nm)

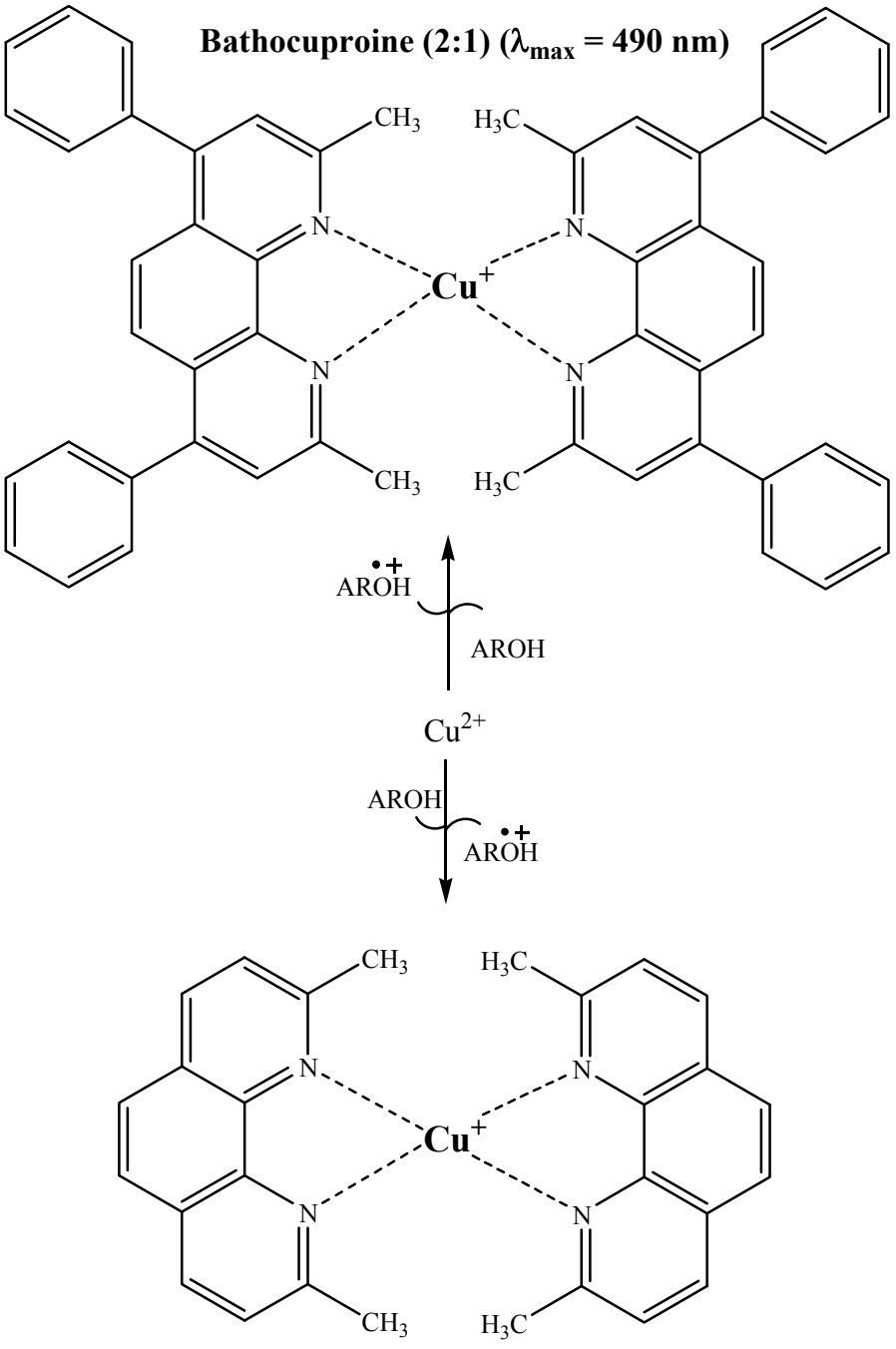


Figure 2.21: CuPRAC color production reaction: SET reduction of copper(II) to copper(I)-BC/NC (blue at λ_{max} = 490 or 450 nm)

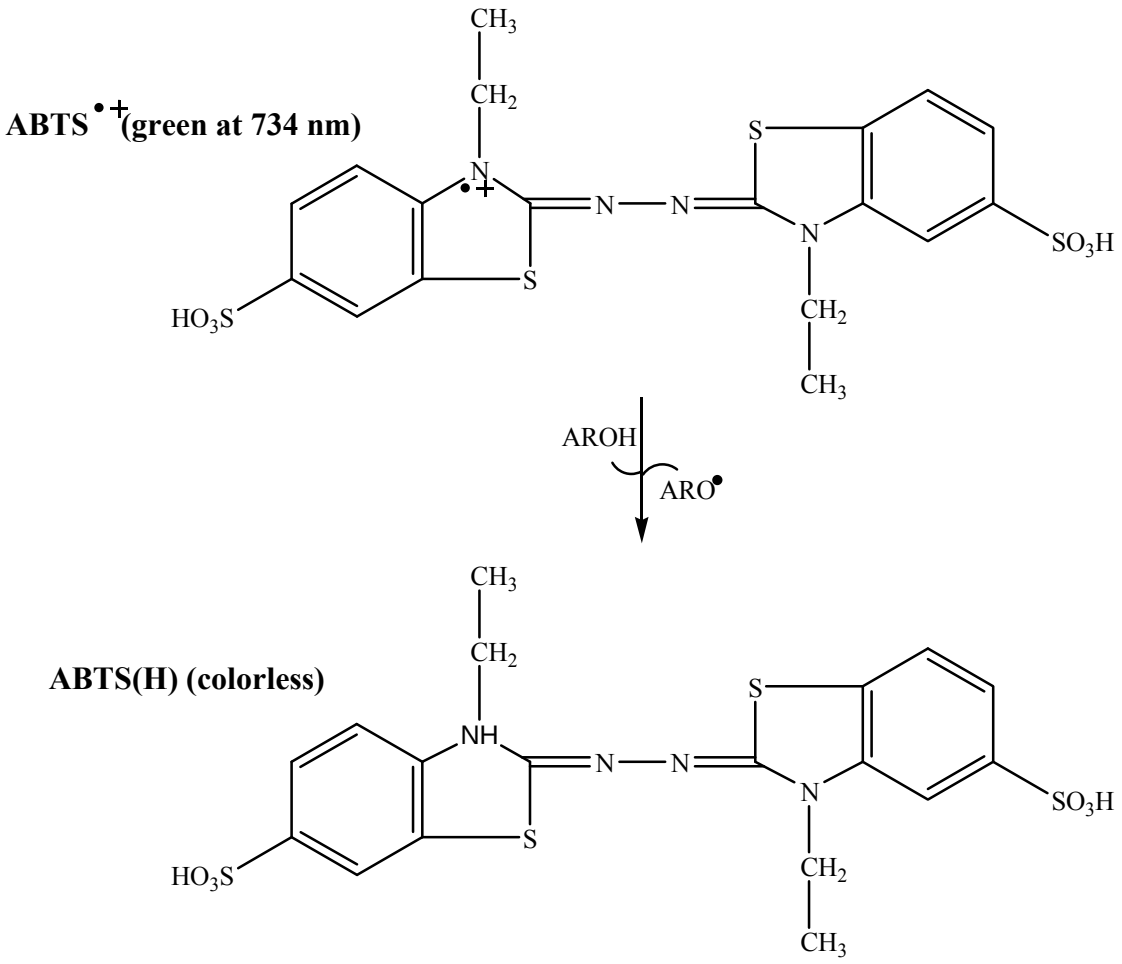


Figure 2.22: Conversion of $ABTS^{\bullet+}$ (green at λ_{max} = 734 nm) to a colorless species ABTS(H) through a HAT mechanism with antioxidant compound ArOH

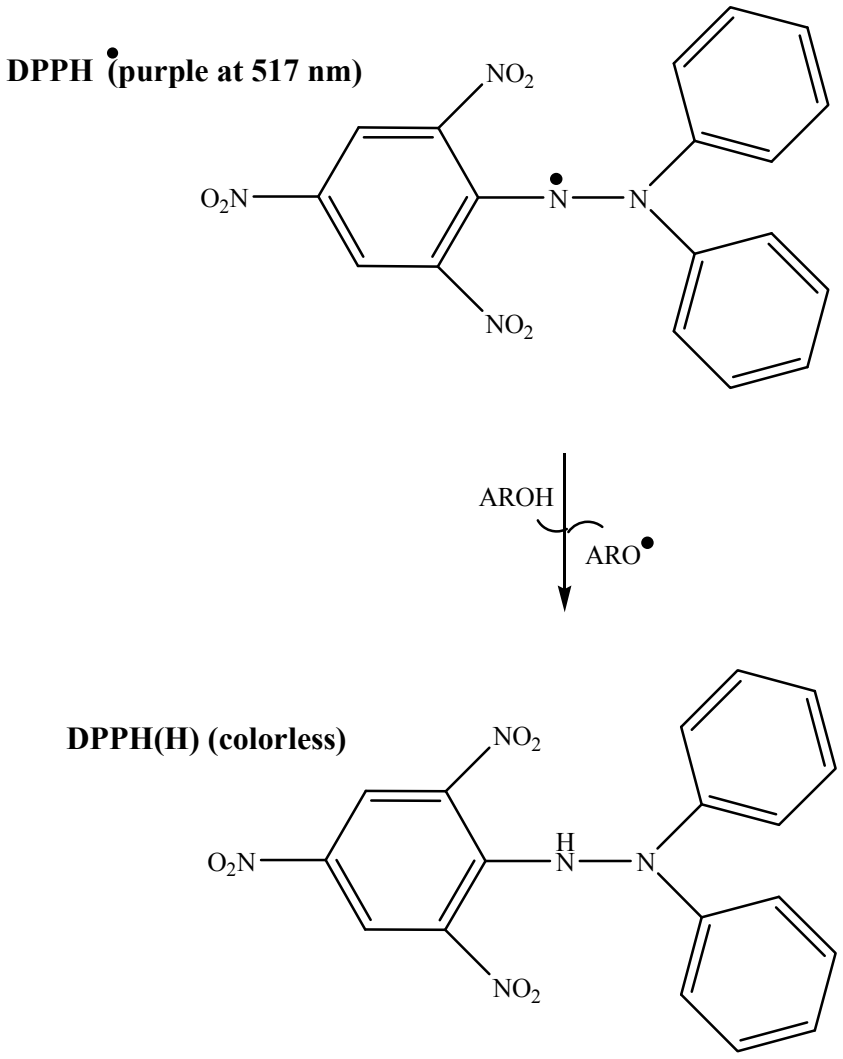


Figure 2.23: HAT conversion of DPPH$^{\bullet}$ (purple at λ_{max} = 517 nm) to colorless species DPPH(H)

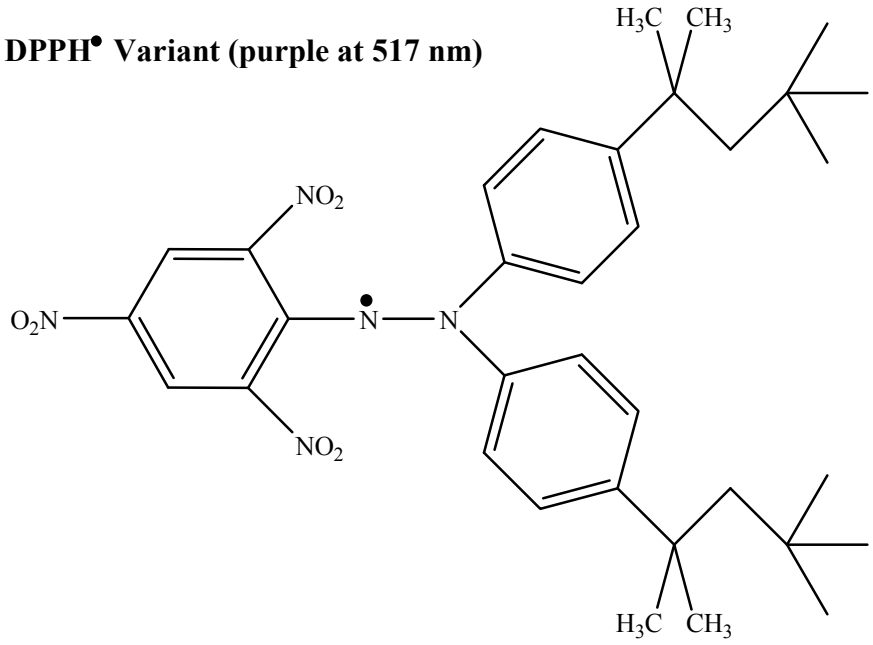


Figure 2.24: The 2,2′-Di(4-*tert*-octylphenyl)-1-picrylhydrazyl free radical

CHAPTER 3

PHENOLIC PROFILES AND ANTIOXIDANT/RADICAL-SCAVENGING CAPACITIES OF RAW, DRY-ROASTED, AND OIL-ROASTED PEANUTS FROM 2005 TO 2007 CROP YEARS[1]

[1]Craft, B. D.; Kosińska, A.; Amarowicz, R.; Pegg, R. B. To be submitted to *Journal of Agricultural and Food Chemistry*, 2009.

Abstract

Raw peanut kernels were dry and oil roasted according to standard industrial practices. The effects of processing on their total phenolics content (TPC) and antioxidant/radical-scavenging capacities were assessed. TPC determinations with Folin & Ciocalteu's phenol reagent as well as $ORAC_{FL}$ ($RO_2^{\bullet}$), photochemiluminescence ($O_2^{\bullet -}$), and TEAC ($ABTS^{\bullet +}$) assays were conducted. Phenolic profiles of peanut kernels were characterized by RP-HPLC on a C_{18} column. HPLC profiles of the cultivars assayed yielded five predominant peaks present at 280 and 320 nm; one peak was identified as free *p*-coumaric acid, while the other four were characterized as *p*-coumaric derivatives by UV-spectra. A Spanish high-oleic peanut (OLIN) was found to have the highest naturally-occurring content of *p*-coumaric acid and derivatives, followed by a high-oleic Runner (TamRun OL02), a Runner (GAGreen), and a Virginia (Gregory) peanut, respectively. *p*-Coumaric acid was liberated at the expense of the derivatives during processing according to the following relationship: oil roasting > dry roasting > raw. A high-oleic Runner (TamRun OL02) had the greatest increase (~785%) in free *p*-coumaric acid levels after oil roasting. The TPC and antioxidant capacities of the 2007 crop increased after processing according to the following relationship: raw < dry roast < oil roast for many of the samples analyzed, but results were cultivar dependent. Photochemiluminescence results were markedly lower for dry-roasted peanuts, compared to raw or oil-roasted ones. This suggests that the superoxide radical-scavenging capacity of peanuts is significantly affected by the dry-roasting process. Overall findings indicate that roasting processes result in the alteration of the predominant chemical forms of phenolics in peanuts. This may alter their antioxidant capacities in certain systems; however, the majority of their beneficial phenolics, such as hydroxybenzoic acids, are not affected.

3.1 Introduction

Peanuts kernels contain approximately 52% oil by weight (Holaday and Pearson, 1974; USDA-ARS, 2008) and are rich in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Mercer *et al.*, 1990). The high-oleic peanut genotype contains upwards of 80% oleic and 5% linoleic acids (Braddock *et al.*, 1995). Substitution of diets high in saturated fats with oils low in saturated fats, but high in MUFA and PUFA (like peanut oil) may lead to lower low-density lipoprotein cholesterol, lower serum triglycerides, and maintained high-density lipoprotein cholesterol in humans (Kris-Etherton *et al.*, 1999; Kris-Etherton *et al.*, 2001; Yu-Poth *et al.*, 2000). A higher intake of PUFA from peanuts, peanut oil, and peanut butters may improve insulin sensitivity and reduce the risk of developing Type-2 diabetes (Jiang *et al.*, 2002). Such effects may be greater in the case of high-oleic peanuts due to the greater presence of MUFA (O'Byrne *et al.*, 1997). Furthermore, the health-promoting effects of peanut consumption are attributed to their fatty acid profiles as well as other beneficial functional constituents. Functional components contained in peanuts include: Vitamin E, fatty acids, L-arginine, other organic and inorganic nutrients, soluble and insoluble fiber, phytosterols, as well as water- and lipid-soluble phenolic antioxidants (Francisco and Resurreccion, 2008; Higgs, 2003; Isanga and Zhang, 2007; Kris-Etherton *et al.*, 2001).

Of the potentially beneficial bioactives found in peanuts, perhaps the least characterized one to date is the phenolics. The limited compositional data available on peanut phenolic profiles suggests that phenolic acids are dominant (Talcott *et al.*, 2005b). Total radical-trapping antioxidant parameter (TRAP) values increase up to 86% post alkaline hydrolysis (Pellegrini *et al.*, 2006), suggesting that ester-bound phenolics are present. High-oleic and normal Runner peanuts possess similar antioxidant profiles (Talcott *et al.*, 2005a), though little is known about

the Virginia and Spanish high-oleic varieties. The total phenolics content (TPC) of peanuts varies among cultivars (Duncan *et al.*, 2006; Talcott *et al.*, 2005b), but concrete relationships have yet to be established.

Additionally, the effects of processing on the chemistry of peanut phenolic profiles are not well understood. TPC and antioxidant capacity of peanuts increase upon processing as determined by HPLC, lipid model systems, *in vitro* radical-scavenging and enzymatic methods (Chukwumah *et al.* 2007b; Hwang *et al.*, 2001; Talcott *et al.*, 2005b). However, these studies involved experimentation on peanut samples of limited breadth. Two of the three noted studies purchased in-shell peanuts from their local supermarkets, thereby limiting the applicability of their experimental conclusions to global peanut populations. The objective of this research was to investigate the effects of dry- and oil-roasting processes on the phenolic profiles and the antioxidant/radical-scavenging capacities of peanuts harvested in the United States. The sample set experimented on included three of the four major peanut types, normal and high-oleic genotypes, and only the dominant commercial cultivars.

3.2 Materials and Methods

Chemicals and Standards

All solvents and reagents were of analytical (ACS) grade or better, unless otherwise specified. Methanol, ethanol (95%), hexanes, water (HPLC grade), glacial acetic acid, mono- and dibasic potassium phosphate, and potassium persulfate were purchased from VWR International (Suwanee, GA). Consumables such as Costar 96-well (Costar #3631) opaque clear-bottom non-sterile non-treated microtiter assay plates, P8 filter paper, cellulose extraction thimbles, Falcon® tubes, amber vials, and glass wool were purchased from Fisher Scientific

(Suwanee, GA). Phenolic acid standards (including *p*-coumaric and gallic acid), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin & Ciocalteu's phenol reagent, fluorescein (3′6′-dihydroxy-spiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) disodium salt, AAPH (2,2′-azobis[2-amidinopropane] dihydrochloride), ABTS (2,2′-azino-bis[3-ethylbenzo-thiazoline-6-sulfonic acid] diammonium salt), and sodium carbonate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Photochemiluminescence kits (*i.e.*, PCL_{ACW}-hydrophilic, for use with the Photochem® system) were purchased from Analytik Jena USA (The Woodlands, TX). Raw peanuts (with skins) were obtained from the National Peanut Research Lab (Dawson, GA). Commercially-refined peanut oil with 100-ppm TBHQ, citric acid, and anti-foaming agent added was a gift from the Golden Peanut Co. (Alpharetta, GA) for oil-roasting experiments.

Peanut Samples/Preparation

Peanuts for this study were taken from a previously collected commercial-sample set (n_{total} = 309) gathered over the 2005 to 2007 crop years by the Peanut Institute (Alexandria, VA), the USDA-ARS National Peanut Research Lab (Dawson, GA), and members of the Georgia peanut industry. Peanut samples, taken from frozen storage in 2008, included: Runners such as GA Green, TamRun96, and C99R; Virginias NC-V11, NC 7, and Gregory; as well as Spanish cultivar TamSpan 90. High-oleic hybrids of Runner and Spanish peanuts were also included (*e.g.*, TamRun OL02 and OLIN, respectively). From this sample group, five cultivars were randomly selected for each of the 2005, 2006, and 2007 harvests. Results of the sampling are as follows: 67% Runners (with 40% high-oleic cultivars), 13% Virginias, and 20% Spanish peanuts. Table 3.1 lists the peanut samples analyzed in this study.

The percentages of each peanut type from this random sampling were expected, given that the overwhelming majority of commercial U.S. peanut cultivars belong to the Runner type (>70%) and with more than half going to peanut butter production (Dixon, 2009). The raw peanut kernels obtained from the various shelling plants (*i.e.*, with skins) were taken from frozen storage (-40 °C) and equilibrated to room temperature before analysis with and without processing. Some peanuts were analyzed raw, while others were dry and oil roasted according to standard industrial practices before analysis. Peanut skins were removed from raw and roasted samples to provide a benchmark for the peanut's antioxidant potential.

Dry-Roasting Protocol

Peanuts (with skins) were split into three lots, weighed, and placed in a single layer on aluminum cooking trays. The peanuts were then dry roasted in a pre-heated Precision Scientific mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL) at ~175 °C for 12 min, then ~150 °C for 15 min, followed immediately by chilling at refrigeration temperatures (4 °C) to halt the roasting process. A thermocouple (Fluke 52II thermometer, Fluke Corp., Everett, WA) was used to ensure temperature stability in the oven. Once dry roasted, peanuts were immediately prepared for extraction. The skins were removed *via* agitation. This protocol was developed to mirror the industrial dry-roasting process with a modification in holding time due to smaller sample size (Kotz, 2009).

Oil-Roasting Protocol

Industrial oil-roasted peanuts are first dry blanched to remove the skins. For this study, however, it was felt that the raw peanuts should not be exposed to any unnecessary thermal

treatment. As a primitive method to remove the skins, agitation of partially-thawed peanuts facilitated the loosening of the skins. Then once fully thawed, the skins were easily slipped off. Raw skinless peanuts were split into lots of 300 g and fried in a Rival high-capacity professional deep fryer (Model C2F725, The Holmes Group, Milford, MA) for 2.5 min along with 375 mL of commercially-refined peanut oil preheated to ~175 °C. Once oil roasted, peanuts were removed from the fryer, drained of oil on baking trays fitted with paper towels, and chilled at refrigeration temperatures (4 °C) to halt the roasting process. Oil-roasted peanuts were then immediately prepared for extraction. This protocol was developed to mimic the industrial oil-roasting process (Kotz, 2009).

Extraction Protocol and Sample Work-up

Crude phenolic extractions from raw and roasted peanut kernels were carried out according to Amarowicz *et al.* (2004) with some modifications. In brief, samples were ground in a coffee mill to the smallest possible particle size. Ground samples were then placed in cellulose extraction thimbles (Whatman single-thickness, 43 mm *i.d.* × 123 mm *e.l.*), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12 h with hexanes as solvent. Defatted peanut meals were placed into 250-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:8 (w/v) for subsequent liquid extraction *via* 80% (v/v) methanol. Crude phenolic extractions were carried out at 45 °C and 150 RPM for three 30-min intervals on a gyratory water bath shaker (New Brunswick Scientific, New Brunswick, NJ). Supernates obtained after each extraction period were gravity filtered through P8 filter paper and collected. Organic solvent fractions were evaporated with a Büchi Rotavapor R-210 (Büchi Corp., New Castle, DE). Remaining aqueous samples were frozen and then lyophilized *in vacuo* in Pyrex

glass crystallization dishes within a Labconco Freezone 2.5 L bench-top freeze dryer (Labconco Corp., Kansas City, MS). Sample extract powders were stored in amber vials at 4 °C until assayed. Figure 3.1 is a flow diagram outlining the extraction, sample work-up, and the analytical assays employed in this research.

Analytical RP-HPLC

RP-18 HPLC fingerprint analyses were performed on raw, dry-roasted, and oil- roasted peanut kernel extracts using an Agilent 1200 series liquid chromatograph with a UV-Vis DAD system (Agilent Technologies, Inc., Wilmington, DE) according to Weidner *et al.* (2001), but with modification of the mobile phase gradient. Conditions for separation entailed a pre-packed Luna C_{18}(II) HPLC column (250 mm × 4.6 mm, 5-μm particle size; Phenomenex, Torrance, CA) equipped with a guard column; gradient elution consisting of mobile phase A (water: acetonitrile:acetic acid – 93:5:2, v/v/v) and phase B (water:acetonitrile:acetic acid – 58:40:2, v/v/v) from 0 to 100% B; a 1 mL/min flow rate; 20 μL injection; with spectral detection set at wavelengths of 280, 320, and 360 nm; and a 50-min run time. Sample concentrations were 5-mg extract/mL in methanol.

Total Phenolics Content (TPC)

The TPC of peanut kernel extracts were determined using Folin & Ciocalteu's phenol reagent (Folin and Ciocalteu, 1927; Singleton and Rossi, 1965) and the colorimetric assay developed by Swain and Hillis (1959), but with a 50% reduction in the assay volume. Briefly, peanut extracts were solubilized in 80% (v/v) methanol. Extracts were diluted as needed with methanol and a 250-μL aliquot was transferred to a clean 10-mL test tube. Then, 3.25 mL of

deionized water were added and the solution was vortexed for 10 s. Next, 250 µL of 2 N Folin & Ciocalteu's phenol reagent were added and the sample was vortexed for 10 s, and allowed to stand 3 min before proceeding. The blank solution should become colorless. Lastly, 500 µL of a saturated sodium carbonate solution (> 30%, w/v) were added to promote the colorimetric reaction, followed by 750-µL deionized water to bring the total assay volume up to 5 mL. The solution was vortexed for 10 s and allowed 1 h for maximal color development. The absorbance of the resulting chromophore was measured at 750 nm with an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc.). TPC values were expressed in mg *p*-coumaric acid equivalents (EQ)/100-g edible peanut (EP) from triplicate samples.

$ORAC_{FL}$: Oxygen Radical Absorbance Capacity

The $ORAC_{FL}$ assay was performed according to Prior *et al.* (2003), with some modifications. Conditions for the assay entailed a BMG FLUOstar Omega (Ω) fluorometer equipped with two internal 500-µL reagent pumps, an external lead system, temperature control set at 37 °C, fluorescent detection set at an excitation/emission pair of 485/520 nm, and a 3-h run time. Reagents included the following: 75 mM phosphate buffer (pH 7.4) as the reaction media and diluent, Trolox standard prepared at concentrations ranging from 3.125 to 100 µM, and a working solution of 0.1 µM fluorescein (FL) as the reaction probe. The peroxyl radical initiator AAPH was prepared in phosphate buffer at a concentration of 80 mM and heated to 37 °C only just before use.

Assay plates including diluted samples and standards were incubated at 37 °C for 10 min before automated addition of FL and AAPH, one full cycle apart. Peanut kernel extracts were solubilized in absolute methanol and diluted with phosphate buffer until they provided a suitable

delay in the FL reduction. Once data was compiled, the Area Under the Curve (AUC) or integral was compared between the samples and standards, to generate equivalence in terms of the standard Trolox. Raw data was averaged and blanks corrected such that the entirety of the resultant signal was sample dependent. Final $ORAC_{FL}$ values were expressed in μmol Trolox EQ/100-g EP from duplicate samples.

Photochemiluminescence

A photochemiluminescence method to determine the scavenging capacity of antioxidant constituents in peanut extracts for the superoxide radical anion ($O_2^{\bullet -}$) was performed according to Pegg *et al.* (2007). Briefly, this assay involved the use of a Photochem® system and reagent kits obtained from Analytik Jena USA. The assay system and reagents are based on the work of Popov and Lewin (1994, 1999). A hydrophilic antioxidant capacity (PCL_{ACW}) reagent kit was used and the four reagents included are as follows: (I) ACW diluent (ultrapure 18 MΩ water); (II) reaction buffer (0.1 M carbonate buffer of pH 10.8, with 0.1 mM EDTA as preservative); (III) photosensitizer and detection reagent (1 mM luminol); and (IV) calibration standard (0.5 to 3 nmol ascorbic acid). Working solutions and samples were prepared according to kit specifications and mixed only briefly before insertion into the assay system. Any exogenous antioxidant species present in the reaction mixture out-competes the photogenerated luminol radicals and halts the production of blue luminescence as measured at 360 nm with the Photochem unit. All peanut kernel extracts were dissolved in absolute methanol and diluted with the carbonate buffer such that they exhibited a suitable delay in luminol chemiluminescence. The antioxidative capacity was calculated by the difference in lag time of blank verses that of the sample, referenced to the calibration curve developed with the ascorbic acid standard, and output

in equivalence. Photochemiluminescence values were reported in μmol ascorbic acid EQ/100-g EP from duplicate samples.

TEAC: Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacities of peanut extracts were determined according to the TEAC assay (Re *et al.*, 1999). Briefly, an ethanolic solution of 7 mM ABTS was mixed with 2.45 mM potassium persulfate and incubated in the dark for 12 to 16 h. The resultant $ABTS^{\bullet+}$ solution (blue-green) was gravity filtered through P8 filter paper. $ABTS^{\bullet+}$ stock was then diluted with 95% (v/v) ethanol until an absorbance of 0.70 was reached at 734 nm with an Agilent 8453 UV/Vis-DAD spectrophotometer. Trolox standards were prepared at concentrations ranging from 0.2 to 2 mM for the development of a standard curve. A 10-μL aliquot of sample or standard was combined with 1 mL of the $ABTS^{\bullet+}$ stock, equilibrated at 30 °C for 5 min, and the absorbance of the resultant solution read at 734 nm. Peanut kernel extracts were diluted in ethanol such that they produced between a 20 to 80% inhibition of the $ABTS^{\bullet+}$ stock. Results were expressed in μmol Trolox EQ/100-g EP from triplicate samples.

Statistical Analysis

Results were summarized with mean and standard deviations reported for each data grouping. TPC, $ORAC_{FL}$, photochemiluminescence, and TEAC data for each peanut kernel sample was analyzed by a 1-way ANOVA statistical model using the statistical analysis system (SAS, version 9.0, SAS Inst Inc., Cary, NC) (O'Rourke *et al.*, 2005) to determine significant differences at the 95% confidence interval ($\alpha = 0.05$). Once significance was determined at ($P < 0.05$), data for each treatment across the processing method was subjected to Fisher's method of

Least Significant Difference (LSD, otherwise known as the *t*-test) in order to segregate treatment means.

3.3 Results and Discussion

Extraction Protocol and Sample Work-up

Extraction results, including lipid content (%) and phenolic extraction yields (%) for all the peanuts examined are in Table 3.2. Lipid content (%) was higher for dry-roasted and oil-roasted peanuts for nearly all samples. This was likely due to water loss of peanut kernels in the dry-roasting and oil-roasting processes. Moreover, frying results in the uptake of oil into peanut kernels. Greater phenolic extraction yields were noted in the 2007 peanuts, when compared to the 2005 and 2006 samples. This phenomenon can be partially explained by the reduction of phenolic antioxidants over storage time due to the inhibition of lipid oxidation. In explanation, peanut kernels may be stored in silos for about a year before they are sent to the sheller (Kotz, 2009). During storage, peanut lipids are subject to autoxidation of their PUFA constituents, which account for ~15% of the kernel mass (Mercer *et al.*, 1990), or ~2.5% for the high-oleic hybrid (Braddock *et al.*, 1995). Furthermore, lypolytic degradation of peanut lipids can occur during storage, depending on the cultivar's lipoxygenase content. Lipoxygenase levels can vary markedly in legumes (Rhee and Watts, 1966; Pinsky *et al.*, 1971). During short-term frozen storage at -20 °C, sensory attributes of peanut kernels remain relatively unchanged (Pattee *et al.*, 2002), thereby suggesting that the oxidative stability is good under these conditions. However, the effects of prolonged frozen storage on the oxidative integrity of peanuts are not known.

Analytical RP-HPLC

RP-HPLC chromatograms of raw, dry-roasted, and oil-roasted peanut extracts from the 2006 crop (*i.e.* S #6 to S #10) shared five predominant peaks with absorbance at 320 nm. Retention times (RT) for these five peaks were variable, but most were near 13.4, 16.0, 17.7, 20.8, and 34.3 min. Figure 3.2 is an RP-HPLC chromatogram of a Runner peanut (S #6) kernel extract at 320 nm. One of the predominant peaks (peak 4, RT = 20.8 min) was tentatively identified as free *p*-coumaric acid by retention time mapping with the commercial standard, while the other four peaks were characterized as *p*-coumaric acid derivatives by UV-Vis spectra. In explanation, phenolic acids of the *trans*-cinnamic acid family typically have primary absorbance maxima at a wavelength of 320 to 330 nm. *p*-Coumaric acid is unique because it absorbs closer to ~315 nm and, thus, it is easily discriminated from other *trans*-cinnamic acids. The naturally-occurring levels of *p*-coumaric acid and its derivatives were highest in the Spanish high-oleic peanut assayed (S #10, OLIN), and lowest in the Virginia peanut (S #9, Gregory), with the Runner (S #6, GaGreen) and Runner high-oleic (S #8, TamRun OL02) falling in between the two. Figure 3.3 depicts an RP-HPLC chromatogram overlay of the Spanish and Virginia cultivars assayed; note the increase in the free *p*-coumaric peak at RT = ~20.8 min. The methyl ester of *p*-coumaric acid has been reported to have antifungal properties (Pant *et al.*, 1988). *Aspergillus* subspecies *flavus* and *parasiticus* are the fungi responsible for the manifestation of aflatoxins in peanuts (Blesa *et al*., 2003; Horn, 2005). It would, therefore, be interesting to determine if a correlation exists between the naturally-occurring levels of *p*-coumaric esters in peanuts and their resistance to *Aspergillus* fungi.

p-Coumaric acid contents of raw and processed peanut kernel extracts were quantified based on the commercial standard and appear in Table 3.3. Free *p*-coumaric acid levels

increased upon dry roasting and more-so on oil roasting for all of the peanut extracts investigated. The most significant increase in free *p*-coumaric levels was found in the high-oleic Runner (S #8): an increase of ~393 and ~785% for the dry- and oil-roasted sample, respectively, was determined. Moreover, as free *p*-coumaric acid levels increased from raw to dry-roasted to oil-roasted samples, all corresponding *p*-coumaric acid derivative peaks declined in intensity. The greater incidence of *p*-coumaric aglycones in the processed samples suggests that thermal treatment results in the liberation of phenolic acids from their parent constituents. Figures 3.4 and 3.5 show the RP-HPLC chromatograms of a Runner peanut (S #8) kernel extract before and after dry-roasting and oil-roasting processes, respectively, illustrating this "processing effect." Similar effects of heat treatment on the increased free/bound phenolic acid ratio of buckwheat (*Fagopyrum esculentum* Moench L.) seeds and citrus (Huyou) peel phenolic extracts have been recently reported in the literature (Zieliński *et al.*¸ 2006; Xu *et al.*, 2007). The marked increase in free *p*-coumaric acid levels in processed peanuts and peanut-containing products may lend to decreased levels of peanut allergenicity given the capability of hydroxycinnamic acids to bind the peanut allergens Ara h 1 and Ara h 2 (Chung and Champagne, 2009).

Total Phenolics Content (TPC)

The TPC data of raw and processed peanut kernel extracts were summarized and appear in Table 3.4. The TPC for the 15 samples analyzed were variable, with a low of 104 and a high of 221 mg *p*-coumaric acid EQ/100-g EP. Processing significantly affected the TPC of all samples assayed except for S #1 and #2. The TPC of S #3, #11, and #12 increased with dry roasting and the TPC of S #11 to #15 all increased with oil roasting. The TPC of others samples, such as S #6 and #10, decreased stepwise from raw > dry-roasted > oil roasted sample.

However, S #14 increased stepwise from raw < dry-roasted < oil-roasted sample. Essentially, TPC values were too variable to make concrete conclusions as to processing or cultivar effects on the phenolics content of peanut kernels. Overall, levels were not altered greatly *via* thermal processing. In correlation to the HPLC results discussed, S #10 (*i.e.*, the cultivar that possessed the highest levels of *p*-coumaric acid and derivatives) yielded the highest quantity of total phenolics. This was not unexpected given that free and bound *p*-coumaric acids are thought to be the predominant phenolic compounds in peanut kernels (Talcott *et al*., 2005).

$ORAC_{FL}$: Oxygen Radical Absorbance Capacity

The $ORAC_{FL}$ results for peanut kernel samples were calculated and appear in Table 3.5. Parallel to the TPC values, $ORAC_{FL}$ values were variable for the cultivars assayed. Sample #10 had the second highest ORAC value of 3490 μmol Trolox EQ/100-g EP, which is in line with the HPLC and TPC data. $ORAC_{FL}$ values ranged from a high of 4016 to a low of 1521 μmol Trolox EQ/100-g EP. The magnitude of these ORAC values is an important result, because they are comparable to known antioxidant-dense foods such as raw blueberries and cranberries (*i.e.*, $ORAC_{FL}$-hydrophilic = ~6500 and ~9500 μmol Trolox EQ/100-g fresh weight, respectively) (ORAC, 2007). $ORAC_{FL}$ values for samples #11 and #15 increased by ~35% and ~19% from the raw to oil-roasted sample, respectively. However S #1, #2, #5, #6, #9, and #10 exhibited decreased $ORAC_{FL}$ values for both dry- and oil-roasted samples. Overall, $ORAC_{FL}$ results suggest that the peroxyl radical ($RO_2^{\bullet}$) scavenging capacities of peanut phenolic extracts were maintained through processing, and in some cases increased.

Photochemiluminescence

Photochemiluminescence (PCL_{ACW}) data are summarized in Table 3.6. All antioxidant capacities of samples were affected by processing except for S #9 and #10. As with the TPC and $ORAC_{FL}$ values, peanut sample antioxidant capacities were variable. The PCL_{ACW} values of S #14 and #15 increased significantly from raw to oil-roasted samples. Sample #15 yielded the highest superoxide anion radical-scavenging capacity at 283 μmol ascorbic acid EQ/100-g EP. In fact, the majority of samples had either preserved or increased antioxidant capacities from raw- to oil-roasted sample. On average, however, dry-roasted samples exhibited decreased antioxidant capacities for nearly all samples. This may suggest that this form of processing is reducing the peanut kernel's capacity to scavenge $O_2^{\bullet -}$, which is an important deleterious free radical in the body.

TEAC: Trolox Equivalent Antioxidant Capacity

The TEAC values of tested peanut samples are summarized in Table 3.7. Parallel to the TPC, $ORAC_{FL}$, and PCL_{ACW} results, TEAC values were variable for the samples analyzed. Sample #15 yielded the highest TEAC at 1199 μmol Trolox EQ/100-g EP. On average, the 2007 peanuts exhibited significantly higher TEAC values than the 2005 and 2006 peanuts assayed. This is an important finding, because the phenolic extraction yields for the 2007 samples were significantly greater than the 2005 and 2006 values. Parallel to PCL_{ACW} results, oil roasting preserved the antioxidant capacity of the peanuts to a greater extent than did dry roasting. In fact, 6 of the 15 samples analyzed had significantly higher mean TEAC values for oil-roasted peanuts than dry-roasted ones.

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References

Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* **84**:551-562.

Bandara, B. M. R.; Hewage, C. M.; Karunaratne, V.; Adikaram, N. K. B. 1988. Methyl ester of *para*-coumaric acid: Antifungal principle of the rhizome of *Costus speciosus*. *Planta Med.* **54**:477-478.

Blesa, J.; Soriano, J. M.; Moltó, J. C.; Marín, R.; Mañes, J. 2003. Determination of aflatoxins in peanuts by matrix solid-phase dispersion and liquid chromatography. *J. Chromatogr. A* **1011**:49-54.

Braddock, J. C.; Sims, C. A.; O'Keefe, S. F. 1995. Flavor and oxidative stability of roasted high oleic acid peanuts. *J. Food Sci.* **60**:489-493.

Chukwumah, Y.; Walker, L.; Vogler, B.; Verghese, M. 2007. Changes in the phytochemical

composition and profile of raw, boiled and roasted peanuts. *J. Agric. Food Chem.* **55**:9266-9273.

Chung, S-Y.; Champagne, E. T. 2009. Reducing allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chem.* **115**:1345-1349.

Dixon, R. Peanut production in Alabama. Encyclopedia of Alabama Online. January 29, 2009. Search: Peanut production in Alabama. <www.encyclopediaofalabama.org/face/ article.jsp?id=h-2016>

Duncan, C. E.; Gorbet, D. W.; Talcott, S. T. 2006. Phytochemical content and antioxidant capacity of water-soluble isolates from peanuts (*Arachis hypogaea* L.). *Food Res. Int.* **39**:898-904.

Folin, O.; Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**:627-650.

Francisco, M. L. D. L.; Resurreccion, A. V. A. 2008. Functional components in peanuts. *Crit. Rev. Food Sci. Nutr.* **48**:715-746.

Higgs, J. 2003. The beneficial role of peanuts in the diet-Part 2. *Nutr. Food Sci.* **33**:56-64.

Holaday, C. E.; Pearson, J. L. 1974. Effects of genotype and production area on the fatty acid composition, total oil and total protein in peanuts. *J. Food Sci.* **39**:1206-1209

Horn, B. W. 2005. Colonization of wounded peanut seeds by soil fungi: Selectivity for species from *Aspergillus* section *Flavi*. *Mycologia* **97**:202-217.

Hwang J-Y.; Shue, Y-S.; Chang H-M. 2001. Antioxidative activity of roasted and defatted peanut kernels. *Food Res. Int.* **34**:639-647.

Isanga, J.; Zhang, G-N. 2007. Biologically active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.* **23**:123-140.

Jiang, R.; Manson, J. E.; Stampfer, M. J.; Liu, S.; Willett, W. C.; Hu, F. B. 2002. Nut and peanut

butter consumption and risk of type 2 diabetes in women. *J. Am. Med. Assoc.* **288**:2554-2560.

Kotz, B. A. 2009. Vice-President of Specialty Products. Golden Peanut Company, Alpharetta, GA. Personal Communication.

Kris-Etherton, P. M. Yu-Poth, S.; Sabaté, J.; Ratcliffe, H. E.; Zhao, G.; Etherton, T. D. 1999. Nuts and their bioactive constituents: Effects on serum lipids and other factors that affect disease risk. *Am. J. Clin. Nutr.* **70**:504S-511S.

Kris-Etherton, P. M.; Zhao, G.; Binkoski, A. E.; Coval, S. M.; Etherton, T. D. 2001. The effects of nuts on coronary heart disease risk. *Nutr. Rev.* **59**:103-111.

Mercer, L. C.; Wynne, J. C.; Young, C. T. 1990. Inheritance of fatty acid content in peanut oil. *Peanut Sci.* **17**:17-21.

O'Byrne, D. J.; Knauft, D. A.; Shireman, R. B. 1997. Low fat-Monounsaturated rich diets containing high-oleic peanuts improve serum lipoprotein profiles. *Lipids* **32**:687-695.

O'Rourke, N.; Hatcher, L.; Stepanski, E. J. 2005. A step-by-step approach to using SAS® for univariate & multivariate statistics. 2nd edition. Cary: SAS Institute Inc.

Oxygen radical absorbance capacity (ORAC) of selected foods - 2007. USDA Nutrient Data Laboratory, Agricultural Research Service (ARS), Beltsville Human Nutrition Research Center (BHNRC), Beltsville, MA, in collaboration with Arkansas Children's Nutrition Center, Little Rock, AR. November 2007.

Pattee, H. E.; Isleib, T. G.; Moore, K. M.; Gorbet, D. W.; Giesbrecht, F. G. 2002. Effect of high-oleic trait and paste storage variables on sensory attribute stability of roasted peanuts. *J. Agric. Food Chem.* **50**:7366-7370.

Pegg, R. B.; Amarowicz, R.; Naczk, M.; Shahidi, F. Photochem® Method for determination of antioxidant capacity of plant extracts. *American Chemical Society Symposium Series* 2007, 956(Antioxidant Measurement and Applications), 140-158.

Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F. 2006. Total

antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* assays. *Mol. Nutr. Food Res.* **50**:1030-1038.

Pinsky, A.; Grossman, S.; Trop, M. 1971. Lipoxygenase content and antioxidant activity of some fruits and vegetables. *J. Food Sci.* **36**:571-572.

Popov, I. N.; Lewin, G. 1994. Photochemiluminescent detection of antiradical activity: II. Testing of nonenzymic water-soluble antioxidants. *Free Rad. Biol. Med.* **17**:267-271.

Popov, I.; Lewin, G. 1999. Antioxidative homeostasis: Characterization by means of chemiluminescence technique. *Methods Enzymol*. **300**:437-456.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocaa, M.; Howard, L.; Hampsch-Woodhill, M.; Huang, D.; Ou, B.; Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **51**:3273-3279.

Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**:1231-1237.

Rhee, K. S.; Watts, B. M. 1966. Evaluation of lipid oxidation in plant tissues. *J. Food Sci.* **31**:664-668.

Swain, T.; Hillis, W. E. 1959. The phenolic constituents of *Prunus domestica*. I.-The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **10**:63-68.

Talcott, S. T.; Duncan, C. E.; Del Pozo-Insfran, D.; Gorbet, D. W. 2005a. Polyphenolic and antioxidant changes during storage of normal, mid and high oleic acid peanuts. *Food Chem.* **89**:77-84.

Talcott, S. T.; Passeretti, S.; Duncan, C. E.; Gorbet, D. W. 2005b. Polyphenolic content and sensory properties of normal and high oleic acid peanuts. *Food Chem.* **90**:379-388.

U.S. Department of Agriculture, Agricultural Research Service (USDA-ARS). 2008. USDA National Nutrient Database for Standard Reference, Release 21. Nutrient Data Laboratory

Home Page, <http://www.ars.usda.gov/ba/bhnrc/ndl>. Search: Nutrients in 100 g raw peanuts (*Arachis hypogea*), all types.

Weidner, S.; Frączek, E.; Amarowicz, R.; Abe, S. 2001. Alternations in phenolic acids content in developing rye grains in normal environment and during enforced dehydration. *Acta Physiol. Plant.* **23**:475-482.

Xu, G.; Ye, X.; Chen, J.; Liu, D. 2007. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract *J. Agric. Food Chem.* **55**:330-335.

Yu-Poth, S.; Etherton, T. D.; Reddy, C. C.; Pearson, T. A.; Reed, R.; Zhao, G.; Jonnalagadda, S.; Wan, Y.; Kris-Etherton, P. M. 2000. Lowering dietary saturated fat and total fat reduces the oxidative susceptibility of LDL in healthy men and women. *J. Nutr.* **130**:2228-2237.

Zieliński, H.; Michalska, A.; Piskula, M. K.; and Kozlowska, H. 2006. Antioxidants in thermally treated buckwheat groats. *Mol. Nutr. Food Res.* **50**:824-832.

Table 3.1: Peanut kernel sample set (n_{total} = 15)

Sample #	Type	Cultivar	Growth Region	Crop Year
1	Runner	GaGreen #2	AL	2005
2	Runner	C99R	GA	2005
3	Runner	C99R	Southwest GA	2005
4	Runner-OL*	TamRun OL02	OK	2005
5	Spanish	TamSpan 90	OK	2005
6	Runner	GaGreen	Southwest GA	2006
7	Runner-OL	TamRun OL02	West TX	2006
8	Runner-OL	TamRun OL02	North FL	2006
9	Virginia	Gregory	TX	2006
10	Spanish-OL	OLIN	West TX	2006
11	Runner	GaGreen	SC/Southern NC	2007
12	Runner	C99R	West AL/MS	2007
13	Runner-OL	TamRun OL02	West TX/NM	2007
14	Virginia	NC-V11	Northern NC	2007
15	Spanish-OL	OLIN	West TX/NM	2007

*OL = High-oleic peanut variety

Table 3.2: Lipid content and (80% [v/v] methanol) yield of raw and processed peanut kernels

	Lipids (%)			Phenolic Extraction Yield (%)		
	Raw	**Dry Roast**	**Oil Roast**	**Raw**	**Dry Roast**	**Oil Roast**
	AVG	*AVG*	*AVG*	*AVG*	*AVG*	*AVG*
1	52.7	53.8	56.5	6.1	5.1	4.8
2	52.7	53.8	55.9	6.1	5.7	5.0
3	53.8	51.3	56.5	5.6	5.4	4.5
4	49.5	46.0	52.5	7.1	6.9	5.8
5	49.9	51.0	53.8	6.6	6.5	5.7
6	49.2	52.9	55.0	6.5	5.9	5.3
7	48.1	50.3	55.3	7.1	4.7	5.4
8	50.6	51.3	52.4	6.0	4.5	5.1
9	51.7	52.8	53.2	7.0	6.0	6.4
10	47.4	49.1	54.3	8.7	6.6	6.8
11	52.3	53.2	53.7	10.1	10.4	10.3
12	53.9	55.5	55.1	10.1	10.6	9.7
13	48.9	51.8	49.7	13.5	14.9	12.8
14	52.2	54.3	52.3	11.3	12.2	11.1
15	51.7	53.3	52.9	13.7	14.1	13.8

Table 3.3: Effects of processing on the quantity of free *p*-coumaric acid and *p*-coumaric derivatives in different peanut kernel types/cultivars from the 2006 crop

	Runner (S #6)			Runner-OL (S #8)			Virginia (S #9)			Spanish-OL (S #10)		
Peak #	**RW**[†]	**DR**	**OR**	**RW**	**DR**	**OR**	**RW**	**DR**	**OR**	**RW**	**DR**	**OR**
1	[*]4.23 ± 0.120	3.90 ± 0.110	3.44 ± 0.100	3.86 ± 0.110	3.65 ± 0.100	3.32 ± 0.100	2.67 ± 0.080	2.13 ± 0.060	2.62 ± 0.070	4.42 ± 0.130	4.22 ± 0.110	3.68 ± 0.110
2	0.82 ± 0.020	0.66 ± 0.020	0.55 ± 0.010	0.72 ± 0.020	0.61 ± 0.020	0.53 ± 0.010	1.00 ± 0.030	0.64 ± 0.020	0.83 ± 0.020	0.80 ± 0.020	0.59 ± 0.010	0.47 ± 0.010
3	0.76 ± 0.020	0.68 ± 0.020	0.59 ± 0.010	0.64 ± 0.020	0.66 ± 0.020	0.58 ± 0.010	0.34 ± 0.010	0.30 ± 0.010	0.46 ± 0.010	0.87 ± 0.020	0.78 ± 0.020	0.71 ± 0.020
4[‡]	0.28 ± 0.007	0.65 ± 0.017	1.01 ± 0.030	0.14 ± 0.004	0.69 ± 0.020	1.24 ± 0.032	0.13 ± 0.003	0.53 ± 0.016	0.76 ± 0.020	0.25 ± 0.003	1.01 ± 0.028	1.24 ± 0.035
5	1.96 ± 0.050	1.66 ± 0.050	1.27 ± 0.030	2.05 ± 0.060	1.78 ± 0.050	1.54 ± 0.040	0.89 ± 0.020	0.75 ± 0.020	0.90 ± 0.020	1.83 ± 0.050	1.54 ± 0.040	1.13 ± 0.030
Totals	*8.05*	*7.55*	*6.86*	*7.41*	*7.39*	*7.21*	*5.03*	*4.35*	*5.57*	*8.17*	*8.14*	*7.23*

[†]Raw (RW), Dry Roast (DR), and Oil Roast (OR) processes were evaluated

[*]Values were quantitated as mean mg *p*-coumaric acid equivalents (EQ) / g dry extract ± standard deviation, from triplicate measurements

[‡]Peak 4 identified as free *p*-coumaric acid by standard

Table 3.4: Total phenolics content (TPC) of raw and processed peanut kernels from 2005 to 2007 crop years

	TPC		
	Raw	**Dry Roast**	**Oil Roast**
	AVG[†] ± *SD*	*AVG* ± *SD*	*AVG* ± *SD*
1	114 ± 8.8[a*]	105 ± 6.4[a]	102 ± 8.8[a]
2	107 ± 10.6[a]	99 ±4.5[a]	93 ± 4.9[a]
3	104 ± 2.9[b]	125 ± 10.2[a]	91 ± 4.8[b]
4	145 ± 8.3[a]	158 ± 7.8[a]	126 ± 6.0[b]
5	134 ± 4.9[a]	143 ± 8.8[a]	119 ± 6.9[b]
6	142 ± 0.7[a]	128 ± 3.8[b]	117 ± 1.0[c]
7	158 ± 2.8[a]	113 ± 0.4[c]	133 ± 1.5[b]
8	118 ± 1.6[a]	92 ± 1.3[c]	106 ± 0.7[b]
9	158 ± 1.5[a]	148 ± 1.3[b]	161 ± 4.1[a]
10	221 ± 1.6[a]	176 ± 3.6[b]	165 ± 3.6[c]
11	110 ± 2.3[b]	123 ± 3.1[a]	128 ± 1.0[a]
12	137 ± 3.5[b]	148 ± 5.6[a]	147 ± 2.4[a]
13	156 ± 2.2[b]	166 ± 4.9[b]	201 ± 15.3[a]
14	147 ± 2.3[c]	152 ± 0.8[b]	162 ± 2.8[a]
15	186 ± 2.0[b]	194 ± 2.2[ab]	202 ± 7.0[a]

[†]TPC values were expressed as mean mg *p*-coumaric acid EQ / 100 g edible peanut (EP) ± standard deviation, from triplicate measurements

[*]Sample means across processing methods sharing a common lower-case letter are not significantly different at P=0.05, as determined by Fisher LSD

Table 3.5: Oxygen radical absorbance capacity ($ORAC_{FL}$-hydrophilic) of raw and processed peanut kernels from 2005 to 2007 crop years

	$ORAC_{FL}$		
	Raw *AVG*† ± *SD*	**Dry Roast** *AVG* ± *SD*	**Oil Roast** *AVG* ± *SD*
1	2160 ± 418	1751 ± 130	1694 ± 334
2	1559 ± 268	1207 ± 147	1103 ± 222
3	1618 ± 260	1683 ± 240	1261 ± 278
4	1898 ± 373	1922 ± 346	1390 ± 177
5	2036 ± 282	1579 ± 273	1350 ± 128
6	2558 ± 425	2164 ± 266	1900 ± 522
7	2657 ± 551	1775 ± 378	1975 ± 697
8	2332 ± 449	1797 ± 479	1951 ± 495
9	1521 ± 285	1415 ± 348	1302 ± 338
10	3490 ± 402	2391 ± 453	2187 ± 606
11	2151 ± 481	2239 ± 552	2905 ± 847
12	3243 ± 500	3353 ± 626	3374 ± 878
13	3253 ± 684	3436 ± 319	3584 ± 996
14	4016 ± 867	4149 ± 1267	4194 ± 742
15	3305 ± 606	3463 ± 936	3920 ± 623

†$ORAC_{FL}$ values were expressed as mean μmol Trolox EQ / 100g EP ± standard deviation, from duplicate measurements

Table 3.6: Photochemiluminescence (PCL_{ACW}-hydrophilic) of raw and processed peanut kernels from 2005 to 2007 crop years

	PCL_{ACW}		
	Raw	**Dry Roast**	**Oil Roast**
	AVG[†] ± *SD*	*AVG* ± *SD*	*AVG* ± *SD*
1	122 ± 28.4[a*]	35 ± 2.1[b]	83 ± 12.2[ab]
2	113 ± 17.2[a]	27 ± 2.2[c]	74 ± 1.0[b]
3	150 ± 6.3[a]	45 ± 5.7[c]	68 ± 3.6[b]
4	147 ± 12.1[a]	35 ± 1.4[c]	74 ± 2.8[b]
5	123 ± 7.9[a]	93 ± 8.2[b]	116 ± 1.6[a]
6	47 ± 8.7[a]	23 ± 0.8[b]	44 ± 2.7[a]
7	120 ± 0.4[a]	40 ± 4.8[c]	82 ± 1.1[b]
8	66 ± 7.2[a]	35 ± 1.2[b]	80 ± 6.0[a]
9	19 ± 6.6[a]	28 ± 1.2[a]	28 ± 0.5[a]
10	66 ± 15.2[a]	70 ± 9.6[a]	80 ± 3.9[a]
11	47 ± 13.3[a]	15 ± 5.9[b]	56 ± 0.2[a]
12	170 ± 3.3[a]	147 ± 2.8[b]	127 ± 7.9[c]
13	92 ± 1.8[ab]	68 ± 15.8[b]	116 ± 12.4[a]
14	213 ± 6.2[b]	194 ± 1.0[c]	283 ± 5.1[a]
15	110 ± 2.3[b]	33 ± 0.9[c]	174 ± 3.5[a]

[†]PCL_{ACW} values were expressed as mean μmol ascorbic acid EQ / 100 g EP ± standard deviation, from duplicate measurements

[*]Sample means across processing methods sharing a common lower-case letter are not significantly different at P=0.05, as determined by Fisher LSD

Table 3.7: Trolox equivalent antioxidant capacity (TEAC) of peanut kernels from 2005 to 2007 crop years

	TEAC		
	Raw	**Dry Roast**	**Oil Roast**
	AVG[†] ± *SD*	*AVG* ± *SD*	*AVG* ± *SD*
1	366 ± 17.0[a*]	279 ± 14.8[b]	297 ± 13.6[b]
2	302 ± 18.2[a]	263 ± 7.3[b]	320 ± 9.7[a]
3	305 ± 11.2[b]	284 ± 15.6[b]	341 ± 1.9[a]
4	382 ± 17.8[a]	338 ± 4.8[b]	371 ± 12.4[a]
5	409 ± 6.6[a]	328 ± 8.2[b]	321 ± 15.4[b]
6	456 ± 19.5[a]	428 ± 6.2[a]	425 ± 13.8[a]
7	665 ± 19.4[a]	411 ± 24.6[c]	505 ± 25.4[b]
8	478 ± 43.7[a]	346 ± 16.8[b]	433 ± 12.9[a]
9	589 ± 19.0[a]	535 ± 1.9[b]	585 ± 0.5[a]
10	746 ± 31.4[a]	607 ± 10.9[b]	626 ± 16.1[b]
11	741 ± 14.3[b]	876 ± 18.0[a]	871 ± 7.8[a]
12	904 ± 42.1[a]	896 ± 4.0[a]	839 ± 50.7[a]
13	1034 ± 24.1[b]	1195 ± 65.3[a]	1022 ± 20.4[b]
14	925 ± 83.0[b]	1023 ± 27.2[ab]	1087 ± 7.8[a]
15	1199 ± 40.0[b]	1377 ± 65.9[a]	1399 ± 62.2[a]

[†]TEAC values were expressed as mean μmol Trolox EQ / 100 g EP ± standard deviation, from triplicate measurements

[*]Sample means across processing methods sharing a common lower-case letter are not significantly different at P=0.05, as determined by Fisher LSD

Figure Captions:

Figure 3.1: Flow diagram for the analysis of phenolic compounds in peanut kernels; including extraction, sample work-up, and analytical assays employed

Figure 3.2: RP-HPLC chromatogram of phenolic compounds from a Runner peanut (S #6) kernel extract

Figure 3.3: RP-HPLC chromatogram of phenolic compounds from Spanish (S #10) and Virginia (S #9) peanut kernel extracts

Figure 3.4: RP-HPLC chromatogram of phenolic compounds from a dry-roasted high-oleic Runner (S #8) peanut kernel extract

Figure 3.5: RP-HPLC chromatogram of phenolic compounds from an oil-roasted high-oleic Runner (S #8) peanut kernel extract

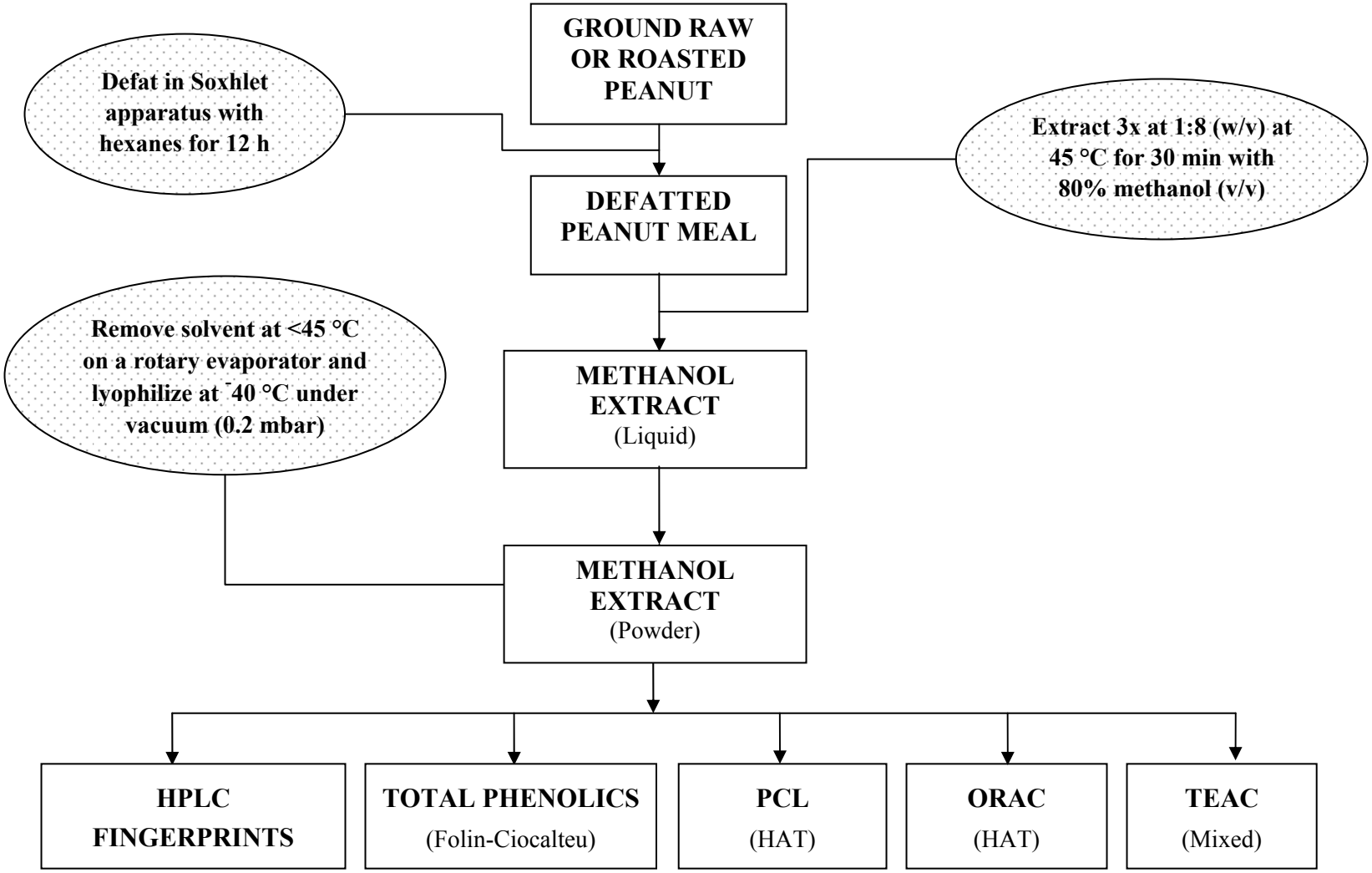
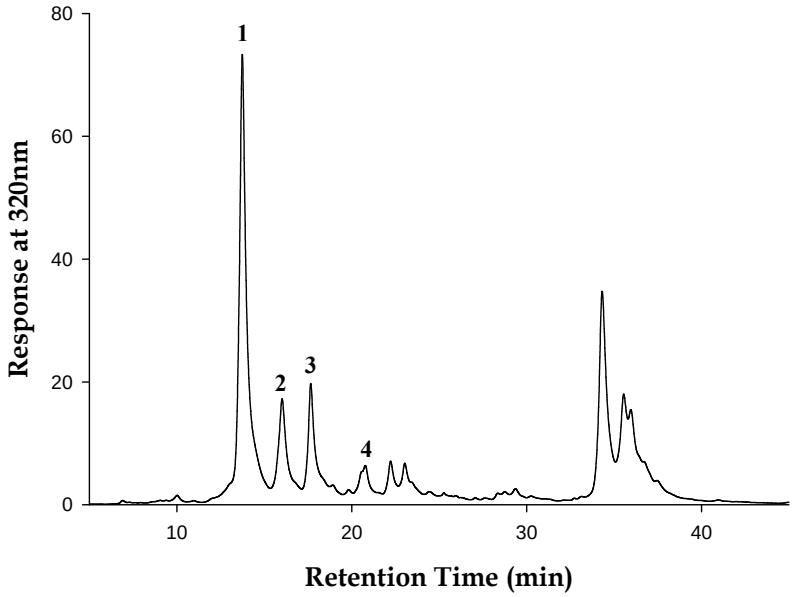

GROUND RAW OR ROASTED PEANUT
Defat in Soxhlet apparatus with hexanes for 12 h
DEFATTED PEANUT MEAL
Extract 3x at 1:8 (w/v) at 45 °C for 30 min with 80% methanol (v/v)
Remove solvent at <45 °C on a rotary evaporator and lyophilize at -40 °C under vacuum (0.2 mbar)
METHANOL EXTRACT (Liquid)
METHANOL EXTRACT (Powder)
HPLC FINGERPRINTS
TOTAL PHENOLICS (Folin-Ciocalteu)
PCL (HAT)
ORAC (HAT)
TEAC (Mixed)

Figure 3.1



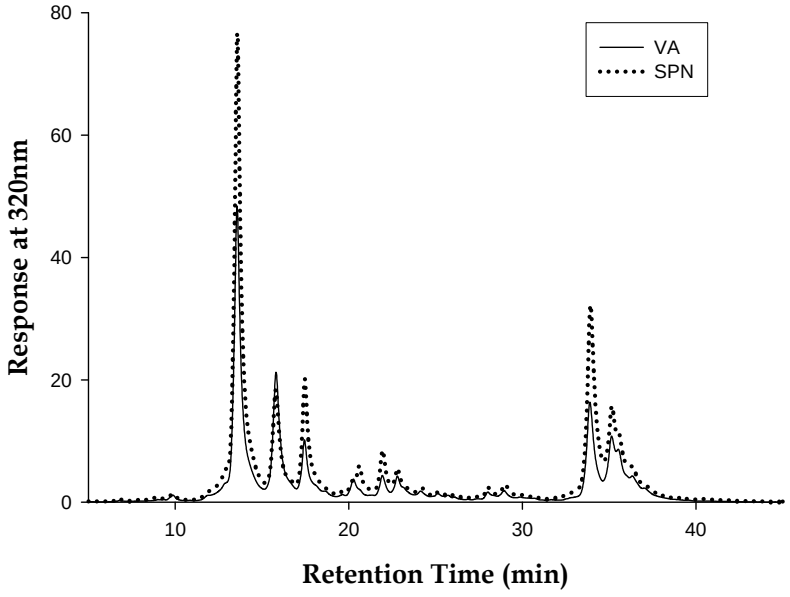


Figure 3.2(left) and 3.3(right)

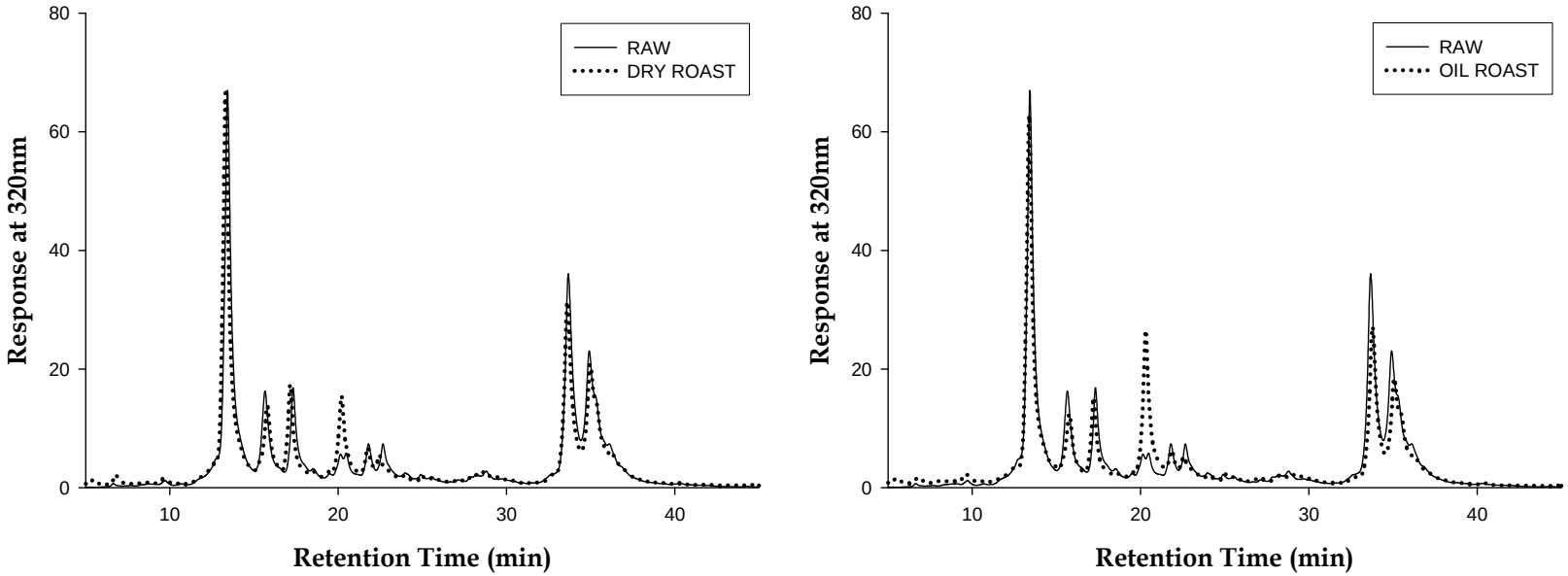


Figure 3.4(left) and 3.5(right)

CHAPTER 4

PHENOLIC PROFILES OF PROCESSED PEANUT SKINS: ANTIOXIDANT, RADICAL-SCAVENGING, AND BIOLOGICAL ACTIVITIES[1]

[1]Craft, B. D.; Kosińska, A.; Hargrove, J. L.; Greenspan, P.; Hartle, D. K.; Amarowicz, R.; Pegg, R. B. To be submitted to *Journal of Agricultural and Food Chemistry*, 2009.

Abstract

80% (v/v) Acetonic extracts of dry-blanched (DB) and dry-roasted (DR) peanut skins were fractionated on an open-tubular column packed with Sephadex LH-20 into low- (LMW) and high-molecular-weight (HMW) subfractions. The LMW subfractions were then pooled and scanned with a UV-Vis DAD spectrophotometer. Predominant LMW fractions were characterized *via* RP-18 HPLC with UV-Vis DAD at 260, 280, 320, and 360 nm. Total phenolics content (TPC) and TEAC assays were conducted on the crude extracts as well as the LMW fractions. The spectral arrays of LMW subfractions yielded significant absorbancies at 260, 280, 320, and 360 nm, which are attributable to benzoic acid derivatives, phenolic rings, *trans*-cinnamic acids, and flavonoids (specifically flavonols and flavones), respectively. (+)-Catechin and (-)-epicatechin as well as *p*-coumaric acid esters were identified in the predominant LMW fraction of DB peanut skin extracts. (+)-Catechin, free protocatechuic and *p*-coumaric acids as well as a protocatechuic acid ester were identified in the predominant LMW fraction of DR peanut skin extracts. The TPC for the crude DB and DR extracts were 404 and 475 mg (-)-epicatechin equivalents (EQ)/g dry extract. TEAC values for the crude DB and DR extracts also increased with processing yielding 3419 and 4495 μmol Trolox EQ/g dry extract, respectively. Marked antioxidant activities were found in the LMW fractions of DB and DR peanut skins (some yielding TEAC values ≥ the crude extracts) and were attributable to free/bound protocatechuic and *p*-coumaric acids, as well as catechins. A 50% (v/v) ethanolic extract of DR peanut skins strongly inhibited α-amylase activity *in vitro*. Furthermore, a 4-μg/mL concentration of ethanolic DR skin extract inhibited fructose-mediated protein glycation by 50%, a critical process in diabetic complications.

4.1 Introduction

Peanut skin is a by-product of the peanut industry yielding a commercial value of only $12 to $20 per ton. Skins are removed in dry-blanching operations (*i.e.*, peanuts are split and the skins are blown off) or in dry-roasting operations (by agitation) and pressed into pellets for use as animal feed (Sobolev and Cole, 2004). Their high protein (~17%), fat (~5%), and low cost facilitate their use in this respect (Karchesy and Hemingway, 1986). Only recently, as interest in polyphenolics from grape seeds and skins has increased, peanut skins are now being considered for their phenolics and tannins. As a result, peanut skins and related products are now being screened for insertion into functional foods and nutraceuticals for the promotion of human health and wellness (Isanga and Zhang, 2007).

At a > 15% (w/w) level of TPC (Nepote *et al.*, 2002); with the majority being condensed tannins, peanut skins are a very strong source of phenolic bioactives. Tannins in peanut skins include A- and B-type proanthocyanidin (PAC) dimers, trimers, and tetramers (Lazarus *et al.*, 1999; Van Ha *et al.*, 2007; Yu *et al.*, 2006; Yu *et al.*, 2007). The optimization of phenolic extractions from peanut skins has been assessed, with aqueous/organic solvent mixtures (*e.g.*, 50-100% ethanol) resulting in the highest yield of extractable phenolics (Huang *et al.*, 2003; Nepote *et al.*, 2005; Yu *et al.*, 2005). While dry roasting increases the TPC of peanut skins, water blanching decreases the total phenolics (Yu *et al.*, 2005); dry blanching has yet to be reported on.

Peanut skin extracts demonstrate high antioxidant potency as measured by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical-scavenging assays (Van Ha *et al.*, 2007; Wang *et al.*, 2007; Yu *et al.*, 2007), ferrous-ion chelating potential (Van Ha *et al.*, 2007; Wang *et al.*, 2007), and various *in vitro* oxygen radical (*e.g.,* $HO^{\bullet}$, $O_2^{\bullet-}$) scavenging methods (Wang *et al.*, 2007). This activity is largely attributed to their flavan-3-ol polymer/PAC constituents. Tannins, especially condensed

tannins, are also known to bind with proteins resulting at times in the precipitation of the complexes so formed (Strumeyer and Malin, 1969; Hagerman and Butler, 1981). These reaction products can not serve as cellular antioxidants, but the protein-tannin complexes have been reported to retain antioxidant activity; they may provide persistent antioxidant activity in the gastrointestinal (GI) tract (Riedl *et al*., 2002).

Pancreatic α-amylase (EC 3.2.1.1) is an interesting pharmacological target because its inhibition will slow the release of glucose from starch and therefore lower the glycemic index of a meal (Yamagishi *et al.*, 2005). α-Amylase inhibitors are sold commercially as "starch blockers," and condensed tannins have been reported to inhibit this enzyme (Strumeyer and Malin, 1969). Moreover, certain PACs have been shown to modulate blood glucose, which is desirable for individuals with poor insulin sensitivity (Anderson, 2008; Anderson *et al.*, 2004). PACs have also been reported to inhibit protein glycation (Peng *et al.*, 2008) and, thus, may limit the accumulation of advanced glycation end products (AGEs) in humans, resulting in a reduced incidence of diabetic complications.

For the purpose of this research, phenolic compounds were extracted from commercially processed dry-blanched (DB) and dry-roasted (DR) peanut skins. Extracts were then chromatographically separated into low-molecular-weight (LMW) and high-molecular-weight (HMW) fractions. Given that the majority of existing studies on peanut skin phenolics have reported on HMW constituents (Lazarus *et al.*, 1999; Yu *et al.*, 2006; Yu *et al.*, 2007), significant efforts were made to better characterize the LMW phenolic profile. Predominant LMW fractions of both skin extracts were examined *via* analytical reversed-phase high-performance liquid chromatography (RP-HPLC) in order to identify the main phenolic species. Lastly, DR peanut skin extracts were screened for their effects on α-amylase activity and fructose-mediated protein

glycation; biological activities that have been associated with a reduced incidence of diabetic complications.

4.2 Materials and Methods

Chemicals and Standards

All solvents and reagents were of analytical (ACS) grade or better, unless otherwise specified. Methanol (ACS and HPLC grades), ethanol (95%), acetone (HPLC grade), hexanes, water (HPLC grade), glacial acetic acid, and potassium persulfate were purchased from VWR International (Suwanee, GA). Consumables such as P8 filter paper, cellulose extraction thimbles, Falcon® tubes, amber vials, and glass wool were obtained from Fisher Scientific Co. (Suwanee, GA). Lipophilic Sephadex LH-20, phenolic acid standards (including *p*-coumaric, protocatechuic, gallic, chlorogenic, and ellagic acid), flavonoid standards (including [+]-catechin hydrate, [-]-epicatechin, quercetin dihydrate, and myricetin), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin & Ciocalteu's phenol reagent, sodium carbonate, ABTS (2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), povidone (PVPP or polyvinyl polypyrrolidone), α-amylase (type 1-A; from porcine pancreas, #A-6255), D-(-)fructose, sodium azide, and bovine serum albumin (BSA; Fraction V, #A-6003) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). An EnzChek Ultra Amylase assay kit (#E33651) was acquired from Invitrogen Corporation (Carlsbad, CA). Dry-blanched (DB) and dry-roasted (DR) peanut skins were a gift from Universal Blanchers (Blakely, GA) and Golden Peanut Co. (Alpharetta, GA), respectively.

Extraction Protocol and Sample Work-up

Crude phenolic extractions from DB and DR peanut skins were carried out according to Amarowicz *et al*. (2004) with slight modification. In brief, samples were ground in a coffee mill to the smallest possible particle size. Ground samples were then placed in cellulose extraction thimbles (Whatman-single thickness, 43 mm *i.d.* × 123 mm *e.l.*), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12 h with hexanes as solvent. Defatted peanut skins were transferred to 250-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:9 (w/v) for subsequent liquid extraction *via* 80% (v/v) acetone for HPLC and antioxidant potency work as well as 50% (v/v) ethanol for biological activity assays. Extractions were carried out at room temperature (~22 °C) and 150 RPM for three 30 min intervals on a gyrotary water bath shaker (New Brunswick Scientific, New Brunswick, NJ). Supernates obtained after each extraction period were gravity filtered through P8 filter paper and collected. Final supernates were subjected to centrifugation at 430 × g for 10 min to precipitate any remaining solid materials, which were removed. Organic solvent fractions were evaporated *in vacuo* with a Büchi Rotavapor R-210 (Büchi Corp., New Castle, DE). Remaining aqueous samples were frozen and then lyophilized in Pyrex glass crystallization dishes in a Labconco Freezone 2.5 L bench-top freeze dryer (Labconco Corp., Kansas City, MS) at -40 °C under reduced pressure (< 0.2 mbar). Sample extract powders were stored in amber vials at 4 °C under a blanket of nitrogen until use for RP-18 HPLC phenolic profiling, TPC, antioxidant capacity, and biological activity screening.

Extract Fractionation and Analytical RP-HPLC

Within a week of each other, 1 g of crude acetonic DB and DR peanut skin extract was solubilized in 10 mL of 95% (v/v) ethanol and fractionated *via* adsorption chromatography on an open-tubular (OT) column (30 mm *i.d.* × 270 mm *e.l.*) packed with lipophilic Sephadex LH-20 fitted at the bottom with a plug of glass wool. The OT column was first washed and then equilibrated with 95% (v/v) ethanol. One liter of 95 % (v/v) ethanol was employed to elute the LMW compounds followed by 750 mL of 50% (v/v) acetone for the HMW species. LMW fractions of DB and DR peanut skin extracts were collected (~8 mL/tube) with a SC100 fraction collector (Beckman Coulter, Inc., Fullerton, CA) and then checked for spectral arrays in the UV region from 280 to 360 nm with an Agilent 8453 UV-Vis diode-array spectrophotometer (Agilent Technologies Inc., Wilmington, DE). Individual tube fractions were combined into larger "pooled" fractions based on absorbancies at 280, 320, and 360 nm. The organic portion of the extract fractions was then evaporated *in vacuo* and the remaining aqueous portions were freeze-dried and stored in refrigerated conditions as previously explained.

Crude extracts and LMW fractions from DB and DR peanut skins were separated *via* RP-HPLC on an Agilent 1200 series liquid chromatograph with a UV/Vis-DAD system (Agilent Technologies Inc.) according to Weidner *et al.* (2001), but with modification to the mobile phase gradient. Conditions for separation entailed a pre-packed Luna C_{18}(II) HPLC column (250 mm × 4.6 mm, 5-µm particle size; Phenomenex, Torrance, CA) equipped with a guard column; gradient elution consisting of mobile phase A (water:acetonitrile:acetic acid – 93:5:2, v/v/v) and phase B (water:acetonitrile: acetic acid – 58:40:2, v/v/v) from 0 to 100% B; a 1 mL/min flow rate; a 20-µL injection volume; with spectral detection set at wavelengths of 260, 280, 320, and 360 nm; and a 50-min run time. Sample concentrations were 5-mg extract/mL for crude peanut skin

extracts, 4-mg extract/mL for DB & DR LMW fractions F1-2, and 2-mg extract/mL for DB LMW fractions F3-7 & DR LMW fractions F3-6; all dissolved samples were dissolved in HPLC-grade methanol. Agilent ChemStation software and a 3D-spectral analysis package were used for chromatogram quantifications.

Total Phenolics Content (TPC)

The TPCs in peanut kernel extracts were measured using Folin & Ciocalteu's phenol reagent (Folin and Ciocalteu, 1927) and the colorimetric assay developed by Swain and Hillis (1959) with a 50% reduction in the assay volume. Briefly, peanut extracts were solubilized in 80% (v/v) methanol. Extracts were diluted as needed with methanol and a 250-µL aliquot was transferred to a clean 10-mL test tube. Then, 3.25 mL of deionized water were added and the solution was vortexed for 10 s. Next, 250 µL of 2 N Folin & Ciocalteu's phenol reagent were added and the sample was vortexed for 10 s, and allowed to stand 3 min before proceeding. The blank solution should become colorless. Lastly, 500 µL of a saturated sodium carbonate solution (> 30%, w/v) were added to promote the colorimetric reaction, followed by 750-µL deionized water to bring the total assay volume up to 5 mL. The solution was vortexed for 10 s and allowed 1 h for maximal color development. The absorbance of the resulting chromophore was measured at 750 nm with an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc.). The TPCs were expressed in mg (-)-epicatechin equivalents (EQ)/g dry extract (DE) from triplicate samples.

Tannin Binding Assay

A stock solution of povidone (PVPP) was prepared by dissolving 1 g in deionized water and adjusting the volume to 10 mL. Next, 50% (v/v) ethanolic DR peanut skin extract was added to 1 mL microcentrifuge tubes to provide 1 mg/mL of peanut skin phenolics and a concentration range of 0 to 1% povidone. For comparison, another set of tubes was prepared with 1 mg/mL of gallic acid and 0 to 1% povidone. The tubes were mixed, placed in an ice bath for 30 min, and centrifuged in a microcentrifuge. Supernatant fractions were assayed for TPC to determine the loss of precipitated tannins by difference.

TEAC: Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacities of crude peanut skin extracts and LMW fractions were determined according to the TEAC assay (Re *et al.*, 1999). Briefly, an ethanolic solution of 7 mM ABTS was mixed with 2.45 mM potassium persulfate and incubated in the dark for 12 to 16 h. The resultant $ABTS^{\bullet+}$ solution (blue-green) was gravity filtered through P8 filter paper. $ABTS^{\bullet+}$ stock was then diluted with 95% (v/v) ethanol until an absorbance of 0.70 was reached at 734 nm with an Agilent 8453 UV/Vis-DAD spectrophotometer. Trolox standards were prepared at concentrations ranging from 0.2 to 2 mM for the development of a standard curve. A 10-µL aliquot of sample or standard was combined with 1 mL of the $ABTS^{\bullet+}$ stock, equilibrated at 30 °C for 5 min, and the absorbance of the resultant solution read at 734 nm. Peanut kernel extracts were diluted in ethanol such that they produced between a 20 to 80% inhibition of $ABTS^{\bullet+}$ stock. Results were expressed in µmol Trolox EQ/g DE from triplicate samples.

α-Amylase Activity Assay

An Invitrogen EnzChek Ultra Amylase assay kit was used to measure α-amylase inhibition caused by the 50% (v/v) ethanolic DR peanut skin extract. Twenty mU of α-amylase from porcine pancreas was allowed to react with a starch:BODIPY-FL conjugate in the presence of various concentrations of peanut skin extracts. The reaction buffer utilized was 50 mM NaCl : 1 mM $CaCl_2$: 50 mM MOPS at pH 6.9. After 30 min at room temperature (~22 °C), fluorescence intensity was measured at an excitation/emission wavelength pair of 485/520 nm using a PerkinElmer LS 55 Luminescence Spectrophotometer (Waltham, MA) with slit width set at 2.5 nm. IC_{50} values were calculated and corrected for any auto-fluorescence of the DR peanut skin extracts.

Albumin Glycation Assay

A fluorescence assay, used to determine the glycation of albumin, was performed as described by McPherson *et al*. (1988). Bovine serum albumin (10 mg/mL) was incubated with D-(-)fructose (250 mM) in potassium phosphate buffer (200 mM: 0.02% [w/v] sodium azide: pH 7.4) in a 5% carbon dioxide incubator at 37 °C for 72 h. The buffer was treated with Chelex 100 prior to use. Various concentrations of a 50% (v/v) ethanolic DR peanut skin extract were added to the 3-mL incubation mixture. Fluorescence intensity was measured at an excitation/emission wavelength pair of 370/440 nm using the PerkinElmer LS 55 Luminescence Spectrometer with slit width set at 3 nm. IC_{50} values were calculated and corrected for any auto-fluorescence of the DR peanut skin extracts.

Statistical Analysis

Results were summarized with mean and standard deviations reported for each data grouping. TPC and TEAC data for crude DB and DR peanut skin extracts and LMW fractions were analyzed by a 1-way ANOVA statistical model using the statistical analysis system (SAS, version 9.0, SAS Inst Inc., Cary, NC) (O'Rourke *et al.*, 2005) to determine significant differences at the 95% confidence interval ($\alpha = 0.05$). Once significance was determined ($P < 0.05$), data were then subjected to Fisher's method of Least Significant Difference (LSD, otherwise known as the *t*-test) in order to segregate treatment means that were significantly different from each other at $\alpha = 0.05$. Estimation of kinetic parameters and linear/non-linear regression curves showing best-fit to the data were calculated using a SigmaStat statistical package (Systat Software, Inc., San Jose, CA).

4.3 Results and Discussion

Extract Fractionation and Analytical RP-HPLC

The open-tubular LH-20 elution profiles of LMW compounds from DB and DR peanut skin extracts are depicted in Figures 4.1 and 4.2, respectively. Significant absorbancies were noted at 280, 320, and 360 nm. Absorbance at these wavelengths may be attributable to the presence of phenolic residues, phenolic acids of the *trans*-cinnamic acid family, and flavonoids (specifically flavonols), respectively. It is apparent from side-by-side comparisons of the elution profiles that increased thermal treatment from the dry-roasting process is causing alterations in the phenolic profile of the DR peanut skins. Typical dry-roasting procedures operate from 150 to 175 °C for 20 to 30 min, whereas dry-blanching procedures operate at much lower temperatures for minimal time periods. Whether or not the DB and DR peanut skins were from the same

peanut types and/or cultivars is unknown; however, both were obtained from South Georgia. The majority (> 90%) of peanuts produced in Georgia are Runners with GAGreen cultivar being the most prevalent.

As illustrated in Figure 4.2, the majority of the LMW species from the DR skin extracts eluted earlier from the OT-LC Sephadex LH-20 column than did the DB skin extracts (Figure 4.1). It is possible that the free phenolic acids present in DR peanut skins are polymerizing with tannin and/or sugar residues more-so in the DR samples with the reaction products possessing a decreased ability to adsorb to the Sephadex LH-20 particles. Amarowicz and Shahidi (1994) observed that glycosylated phenolics from flax eluted earlier from lipophilic Sephadex LH-20 columns than did other LMW phenolic species. Furthermore, glycosylated flavonoids, with higher molecular weights, have been found to undergo gel sieving (*i.e.*, size-exclusion) on Sephadex LH-20 (Johnston *et al.*, 1968), in addition to adsorption chromatography.

Based on the elution profiles, DB peanut skin extracts were pooled into 7 predominant LMW fractions. The tube numbers for the 7 fractions are as follows: 1-7, 8-17, 18-29, 30-43, 44-86, 87-94, 95-125, respectively. DR peanut skin extracts were pooled into 6 predominant fractions with tube numbers as follows: 1-13, 14-20, 21-32, 33-46, 47-76, 77-117, respectively. Each tube was equivalent to ~8 mL of collected fraction. On a dry weight basis, LMW and HMW species accounted for 40.8 & 57.2% and 22.2 & 74.4% for the DB and DR peanut skin extracts, respectively. The greater percentage of HMW species found in the DR peanut skin extracts may be due to the thermal instability of PACs. PACs can undergo oxidative transformation reactions during processing and storage (Santo-Buelga and Scalbert, 2000). This is perhaps further evidence for the greater incidence of polymerization reactions between phenolic aglycones and sugars in the DR extracts, as discussed.

Figures 4.3 and 4.4 are the spectral scans of pooled extract fractions as measured in the UV range from 220 to 420 nm. Each fraction from the DB and DR peanut skin extracts yielded the characteristic wavelength maximum of phenolic rings at 280 nm. DB skin extract fractions had additional wavelength maxima at 260 (F #3), 320 (F #1, F #2, and F #4 to F #7), and 360 nm (F #3 and F #4). For DR peanut skin extract fractions, additional maxima at 260 (F #2 and F #3) and 320 nm (F #1, F #2, and F #4 to F #6) were observed. Absorbance at 260 nm is often attributable to the presence of phenolics of the benzoic acid family such as gallic, protocatechuic, and vanillic acids. Absorbance at 320 and 360 nm may be due to the presence of *trans*-cinnamic acids and flavonoids (specifically flavonols and flavones), respectively, as has been discussed. Specific attention was paid to all of the aforementioned wavelengths in the analytical HPLC work using Agilent's ChemStation 3-D spectral analysis software.

HPLC chromatograms for two of the predominant LMW fractions from DB and DR peanut skin extracts appear in Figures 4.5 and 4.6, respectively. Figure 4.5 is an HPLC chromatogram of the predominant DB peanut skin LMW fraction IV. (+)-Catechin (peak 1, RT = 11.6 min) and (-)-epicatechin (peak 2, RT = 15.5 min) were tentatively identified based on retention time mapping of commercial standards and quantified at 102.25 and 15.82 mg/g dry extract fraction. Karchesy and Hemingway (1986) reported that catechin and epicatechin are present in a 9:1 ratio in peanut skins. This result was in exact concordance with our values. All peaks in the chromatogram denoted with an asterisk were tentatively identified as *p*-coumaric acid derivatives, based on their UV-spectra.

Figure 4.6 is an HPLC chromatogram of the predominant DR peanut skin LMW fraction III. Free protocatechuic acid (peak 1, RT = 8.4 min) was tentatively identified based on retention time mapping of a commercial standard and quantified at 47.9 mg/g dry extract fraction. (+)-

Catechin (peak 2, RT = 11.6 min) and free *p*-coumaric acid (peak 3, RT = 20.8 min) were also identified by commercial standards, and a protocatechuic acid derivative (denoted by an asterisk, RT = 9.73 min) was tentatively identified by UV-spectra. The contents of free protocatechuic and *p*-coumaric acid were highest in the DR peanut skin extract fraction. Ethyl protocatechuate has been identified in peanuts (Huang *et al.*, 2003); therefore, the greater content of free protocatechuic acid in the DR skins may be a result of the release of these phenolic acids from their ester bonds during thermal processing. A similar effect of heat treatment on the increased free/bound phenolic acid ratio of buckwheat (*Fagopyrum esculentum* Moench L.) seeds and citrus (Huyou) peel phenolic extracts was recently noted in the literature (Zieliński *et al.*, 2006; Xu *et al.*, 2007).

Total Phenolics Content (TPC)

Mean TPCs for crude 80% (v/v) acetonic DB and DR skin extracts and their respective LMW fractions are summarized in Table 4.1. As evident from Table 4.1, nearly all crude extracts and extract fractions yielded significantly different ($P < 0.05$) mean TPC values. The TPC of the last eluting fraction from the OT-LC Sephadex LH-20 column for both DB and DR samples yielded values comparable to the crude fraction (it was slightly higher in the case of the DB samples). Given the increased likelihood of higher molecular-weight phenolics eluting earlier from the Sephadex LH-20 column (*i.e.*, phenolic glycosides, as discussed), TPC values of the later fractions such as DB F #6 and F #7 as well as DR F #5 and F #6 may be due to the presence of phenolic aglycones. This is an interesting result because of the greater incidence of LMW phenolics in the DB samples. Recall, DB skins possessed 40.8% LMW species, whereas the DR samples had 22.2%, as discussed. Even with the greater incidence of LMW species in

the DB skins, the last DR skin fraction's TPC values were still comparable to the DB skins. This may be further evidence for the release of phenolic aglycones from their derivatives post processing. The potential for this phenomenon is greater for the DR skins given the prolonged thermal treatment and higher processing temperatures.

Tannin Binding Assay

The results of the tannin binding assay were plotted and appear in Figure 4.7. Tannins from the ethanolic DR peanut skin extract precipitated as povidone concentration increased from 0 to 0.2%, at which point a maximum of 96% of the PACs settled out of the mixture. Under the same conditions, very little gallic acid precipitated. Correspondingly as tannins were removed from the solution, TPC values of the supernate decreased exponentially. This suggests that the HMW polyphenolics in peanut skin extracts are predominantly tannins. As the concentration of povidone increased beyond 0.25%, greater quantities of peanut skin PACs were recovered in the supernatant fraction, as determined by the TPC assay. The biphasic binding curve shown in Figure 4.7 suggests that there is an optimum ratio for the number of PACs bound per povidone molecule. As more povidone is introduced into the solution, less PACs bind per unit povidone and more of the complex remains soluble. In binding peanut PACs, povidone is acting as a fining agent (commercially defined as a polymeric substance that is capable of binding tannins). The failure of povidone to bind gallic acid indicates that it has a degree of selectivity, and does not bind all phenolic classes to the same degree. This is similar to the finding that tannins bind to proline-rich domains in proteins (Hagerman and Butler, 1981), because the vinyl pyrrolidone backbone in povidone is similar to the proline imino ring.

TEAC: Trolox Equivalent Antioxidant Capacity

TEAC values for crude acetonic DB and DR peanut skin extracts and their respective LMW phenolic fractions were averaged and appear in Table 4.1, alongside the TPC results. TEAC values for the crude DB and DR peanut skin extracts were 3419 and 4495 μmol Trolox EQ/g DE, respectively. These TEAC values are higher than recently published ones. Francisco and Resurreccion (2009) had maximum TEAC values of 2560 μmol Trolox EQ/g DE based on a 70% (v/v) ethanolic extraction of peanut skins. In concordance with TPC results, mean TEAC values were significantly different ($P < 0.05$) for the crude DB and DR peanut skin extracts and their respective LMW fractions. Also in concordance with the TPC values, the TEAC values of the last eluting fraction from Sephadex LH-20 had values comparable to their respective crude fractions. In fact, mean TEAC values for DB F #7 and DR F #6 were both significantly higher ($P < 0.05$) than their respective crude extracts.

The TEAC assay is considered to be a mixed-mode assay, given that $ABTS^{\bullet+}$ has the capability to simultaneously undergo hydrogen-atom-transfer (HAT) and single-electron-transfer (SET) redox mechanisms (Schaich, 2006). These results suggest that LMW phenolic species from peanut skins are potent antioxidant species. The higher TEAC value of crude DR skin extracts, with respect to the DB ones, suggests that increased thermal treatment is affecting peanut skin phenolic antioxidant capacities in a positive manner. Furthermore, protocatechuic acid has recently been proven to have metal-ion chelating capability (*i.e.*, more so with copper) (Psotová *et al.*, 2003). The greater content of free protocatechuic acid in the DR fraction could be contributing to its higher TEAC values.

α-Amylase Activity Assay

The % inhibition of α-amylase activity was plotted with respect to the concentration (μg/mL) of added 50% (v/v) ethanolic DR peanut skin extract, and appears in Figure 4.8. As shown, DR skin extracts inhibited α-amylase activity by 50% at a concentration of 0.8-μg peanut skin extract/mL. Nearly complete inhibition was achieved at a 5-μg peanut skin extract/mL concentration. These results clearly demonstrate that peanut skin phenolics can dramatically alter intestinal enzymatic activity. The rate and extent to which dietary carbohydrate digestion can be slowed by "starch blockers" is still under debate (McCarty, 2005).

Albumin Glycation Assay

The % inhibition of glycation was plotted with respect to the concentration (μg/mL) of added 50% (v/v) ethanolic DR peanut skin extract, and appears in Figure 4.9. Previous studies reported that extracts of green tea, spices, and muscadine grape pomace inhibit protein glycation *in vitro* (Babu *et al.*, 2006; Dearlove *et al.*, 2008; Farrar *et al.*, 2007). In our study, a 4-μg/mL concentration of ethanolic DR peanut skin phenolics extract was found sufficient to block fructose-mediated glycation of albumin by 50%. These results suggest that tannic extracts from peanut skins may help to reduce fructose-mediated protein glycation *in vivo*. Whether or not peanut skin-containing products will have this pharmacological effect, is largely dependent on the absorption of polyphenolic compounds in the GI tract. Though much knowledge has been acquired concerning the absorption of phenolic acids and flavonoids in the GI tract (Scalbert and Williamson, 2000), the bioavailability of tannins in the human GI tract is still largely unknown.

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References

Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* **84**:551-562.

Amarowicz, R.; Shahidi, F. 1994. Application of sephadex LH-20 chromatography for the separation of cyanogenic glycosides and hydrophilic phenolic fraction from flaxseed. *J. Liq. Chromatogr.* **17**:1291-1299.

Anderson, R. A. 2008. Chromium and polyphenols from cinnamon improve insulin sensitivity. *P. Nutr. Soc.* **67**:48-53.

Anderson, R. A. Broadhurst, C. L.; Polansky, M. M.; Schmidt, W. F.; Khan, A.; Flanagan, V. P.; Schoene, N. W.; Graves, D. J. 2004. Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. *J Agric. Food Chem.* **52**:65-70.

Babu, P. V. A.; Sabitha, K. E.; Shyamaladevi, C. S. 2006. Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca2+-ATPase and Na+/K+-ATPase activity in the heart of streptozotocin-diabetic rats. *Chem-Biol Interact.* **162**:157-164.

Dearlove, R. P.; Greenspan, P.; Hartle, D. K.; Swanson, R. B.; Hargrove, J. L. 2008. Inhibition of protein glycation by extracts of culinary herbs and spices. *J. Med. Food* **11**:275-281.

Farrar, J. L.; Hartle, D. K.; Hargrove, J. L.; Greenspan, P. 2007. Inhibition of protein glycation by skins and seeds of the muscadine grape. *BioFactors* **30**:193-200.

Folin, O.; Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**:627-650.

Francisco, M. L. L. D.; Resurreccion, A. V. A. 2009. Total phenolics and antioxidant capacity of heat-treated peanut skins. *J. Food Comp. Anal.* **22**:16-24.

Hagerman, A. E.; Butler, L. G. 1981. The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* **256**:4494-4497.

Huang, S. C.; Yen, G-C., Chang, L-W; Yen, W-J.; Duh, P-D. 2003. Identification of an antioxidant, ethyl protocatechuate, in peanut seed testa. *J. Agric. Food Chem.* **51**:2380-2383.

Isanga, J.; Zhang, G-N. 2007. Biologically active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.* **23**:123-140.

Johnston, K. M.; Stern, D. J.; Waiss, A. C. Jr. 1968. Separation of flavonoid compounds on Sephadex LH-20. *J. Chromatogr. A.* **33**:539-541.

Karchesy, J. J.; Hemingway, R. W. 1986. Condensed tannins: (4β→8; 2β→O→7)-Linked procyanidins in *Arachis hypogaea* L. *J. Agric. Food Chem.* **34**:966-970.

Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **47**:3693-3701.

McCarty, M. F. 2005. Nutraceutical resources for diabetes prevention – an update. *Med. Hypothesis* **64**:151-158.

McPherson, J. D.; Shilton, B. H.; Walton, D. J. 1988. Role of fructose in glycation and cross-linking of proteins. *Biochemistry* **27**:1901-1907.

Nepote, V.; Grosso, N. R.; Guzmán, C. A. 2002. Extraction of antioxidant components from peanut skins. *Grasas Aceites* **53**:391-395.

Nepote, V.; Grosso, N. R.; Guzmán, C. A. 2005. Optimization of extraction of phenolic antioxidants from peanut skins. *J. Sci. Food Agric.* **85**:33-38.

O'Rourke, N.; Hatcher, L.; Stepanski, E. J. 2005. A step-by-step approach to using SAS® for univariate & multivariate statistics. 2nd edition. Cary: SAS Institute Inc.

Peng, X.; Cheng, K-W.; Ma, J.; Chen, B.; Ho, C-T.; Lo, C.; Chen, F.; Wang, M. 2008. Cinnamon bark proanthocyanidins as reactive carbonyl scavengers to prevent the formation of advanced glycation endproducts. *J. Agric. Food Chem.* **56**:1907-1911.

Psotová, J.; Lasovský, J.; Vičar, J. 2003. Metal-chelating properties, electrochemical behavior, scavenging and cytoprotective activities of six natural phenolics. *Biomed. Papers* **147**:147-153.

Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**:1231-1237.

Reidl, K. M.; Carando, S.; Alessio, H. M.; McCarthy, M.; Hagerman, A. E. 2002. Chapter 14. Antioxidant activity of tannins and tannin-protein complexes: Assessment in vitro and in vivo. ACS Symposium Series 807(Free Radicals in Foods: Chemistry, Nutrition, and Health Effects):188-200. Copywright 2002 ACS.

Santos-Buelga, C.; Scalbert, A. 2000. Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **80**:1094-1117.

Scalbert, A.; Williamson, G. 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**:2073S-2085S.

Schaich, K. M. 2006. Developing a rational basis for selection of antioxidant screening and testing methods. *Acta Hort.* **709**:79-94.

Sobolev, V. S.; Cole, R. J. 2004. Note on the utilisation of peanut seed testa. *J. Sci. Food Agric.* **84**:105-111.

Strumeyer, D. H.; Malin, M. J. 1969. Identification of amylase inhibitor from seeds of leoti sorghum. *Biochim. Biophys. Acta* **184**:643-645.

Swain, T.; Hillis, W. E. 1959. The phenolic constituents of *Prunus domestica*. I.-The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **10**:63-68.

Van Ha, H.; Pokorný, J.; Sakurai, H. 2007. Peanut skin antioxidants. *J. Food Lipids* **14**:298-314.

Wang, J.; Yuan, X.; Jin, Z.; Tian, Y.; Song, H. 2007. Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem.* **104**:242-250.

Weidner, S.; Frączek, E.; Amarowicz, R.; Abe, S. 2001. Alternations in phenolic acids content in developing rye grains in normal environment and during enforced dehydration. *Acta. Phys. Plant.* **23**:475-482.

Xu, G.; Ye, X.; Chen, J.; Liu, D. 2007. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract *J. Agric. Food Chem.* **55**:330-335.

Yamagishi, S.; Nakamura, K.; Takeuchi, M. 2005. Inhibition of postprandial hyperglycemia by acarbose is a promising therapeutic strategy for the treatment of patients with the metabolic syndrome. *Med. Hypotheses* **65**:152-154.

Yu, J.; Ahmedna, M.; Goktepe, I. 2007. Chapter 16. Peanut skin phenolics: extraction, identification, antioxidant activity, and potential applications. ACS Syposium Series 956(Antioxidant Measurement and Applications):226-241. Copywright 2007 ACS.

Yu, J.; Ahmedna, M.; Goktepe, I.; Dai, J. 2006. Peanut skin procyanidins: composition and antioxidant activities as affected by processing. *J. Food Comp. Anal.* **19**:364-371.

Yu, J.; Ahmedna, M.; Goktepe, I. 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem.* **90**:199-206.

Zieliński, H.; Michalska, A.; Piskula, M. K.; and Kozlowska, H. 2006. Antioxidants in thermally treated buckwheat groats. *Mol. Nutr. Food Res.* **50**:824-832.

Table 4.1: TPC and TEAC of crude extracts (80% [v/v] acetone) and LMW fractions from DB and DR peanut skins

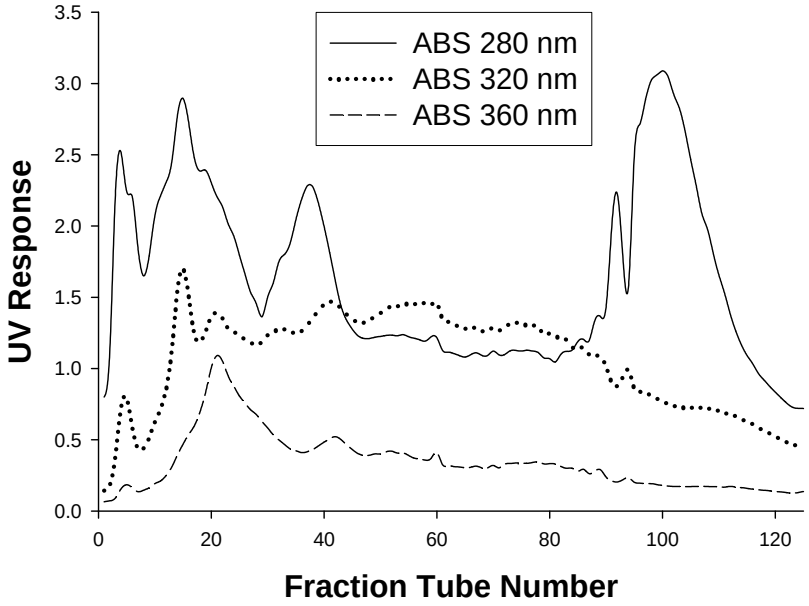
Crude Extract or Fraction	TPC[1]	TEAC[2]
DB-Crude	$404 \pm 1.8^{b\dagger}$	3419 ± 157^{c}
DB-LMW F(#1)	30.1 ± 0.03^{f}	227 ± 4.0^{g}
DB-LMW F(#2)	15.6 ± 0.08^{g}	167 ± 5.1^{g}
DB-LMW F(#3)	102 ± 0.48^{e}	546 ± 12^{f}
DB-LMW F(#4)	151 ± 2.3^{d}	1365 ± 32^{d}
DB-LMW F(#5)	147 ± 5.1^{d}	1100 ± 9.1^{e}
DB-LMW F(#6)	295 ± 7.4^{c}	3680 ± 108^{b}
DB-LMW F(#7)	445 ± 2.1^{a}	4767 ± 40^{a}
DR-Crude	$475 \pm 3.0^{(a)}$	$4495 \pm 90^{(b)}$
DR-LMW F(#1)	$44.5 \pm 0.21^{(f)}$	$289 \pm 3.1^{(f)}$
DR-LMW F(#2)	$29.2 \pm 0.06^{(g)}$	$205 \pm 2.8^{(f)}$
DR-LMW F(#3)	$135 \pm 1.2^{(e)}$	$776 \pm 9.4^{(e)}$
DR-LMW F(#4)	$172 \pm 2.7^{(d)}$	$1378 \pm 42^{(d)}$
DR-LMW F(#5)	$236 \pm 1.3^{(c)}$	$2179 \pm 80^{(c)}$
DR-LMW F(#6)	$405 \pm 3.5^{(b)}$	$4664 \pm 8.7^{(a)}$

†Crude phenolic extracts and their respective LMW fractions within antioxidant assays were compared using Fisher's method of least significant difference (LSD). DB means sharing a common lower-case letter are not significantly different at $\alpha = 0.05$, DR means (within brackets) sharing a common lower-case letter are not significantly different at $\alpha = 0.05$

[1]Total Phenolics Content was expressed in mean mg [-]-epicatechin equivalents (EQ)/g dry extract (DE) or extract fraction (EF) ± standard deviation from triplicate sample measurements

[2]Trolox Equivalent Antioxidant Capacity (TEAC) values were expressed in mean μmol Trolox EQ/g DE or EF ± standard deviation from triplicate sample measurements

Figure Captions



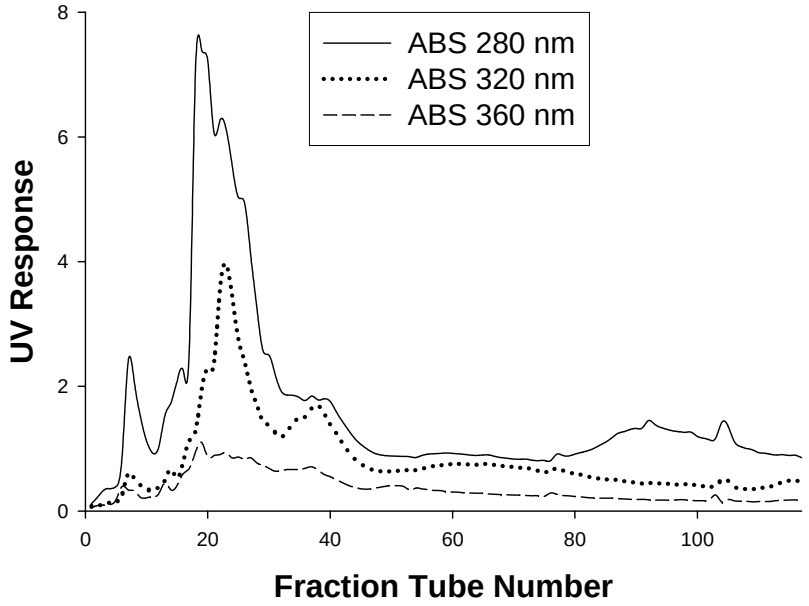
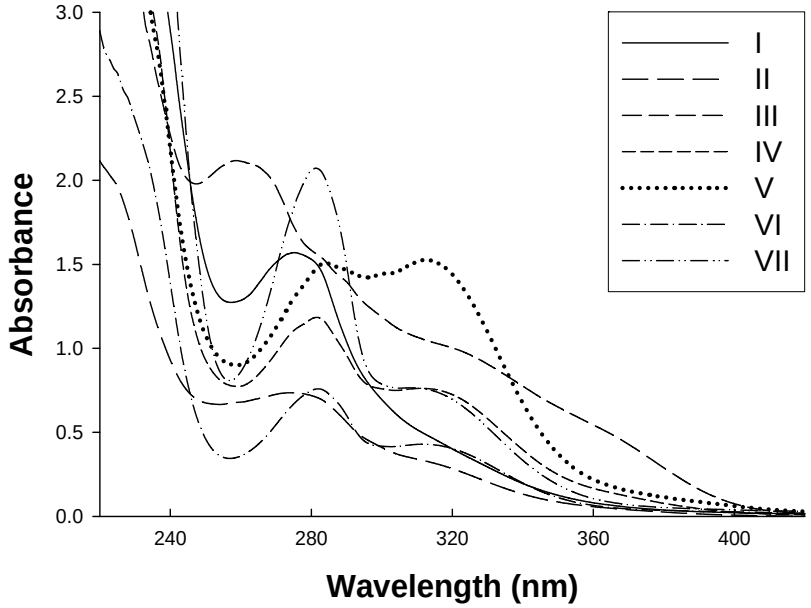


Figure 4.1(left) and 4.2(right)



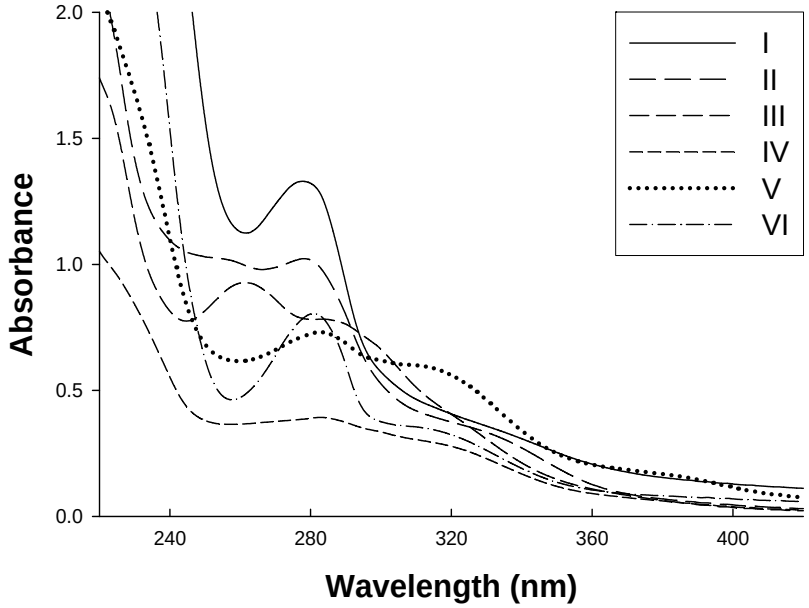


Figure 4.3(left) and 4.4(right)

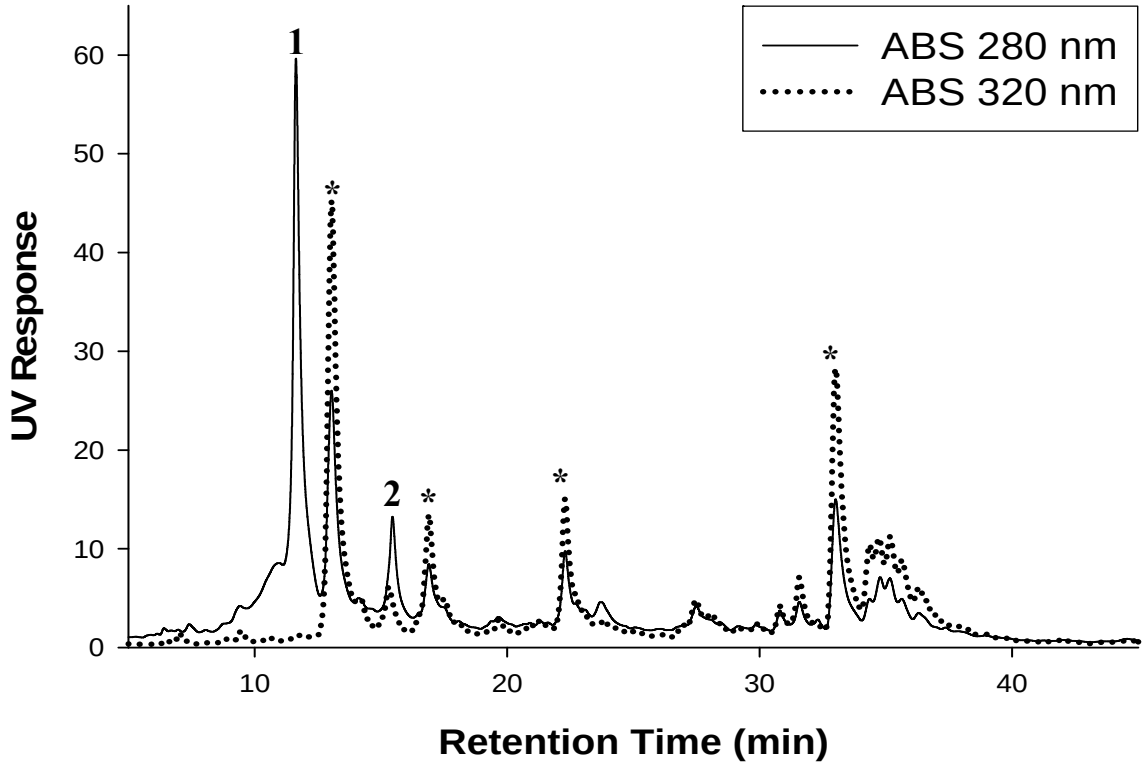

ABS 280 nm
ABS 320 nm
UV Response
Retention Time (min)
1
2
*
0
10
20
30
40
50
60

Figure 4.5

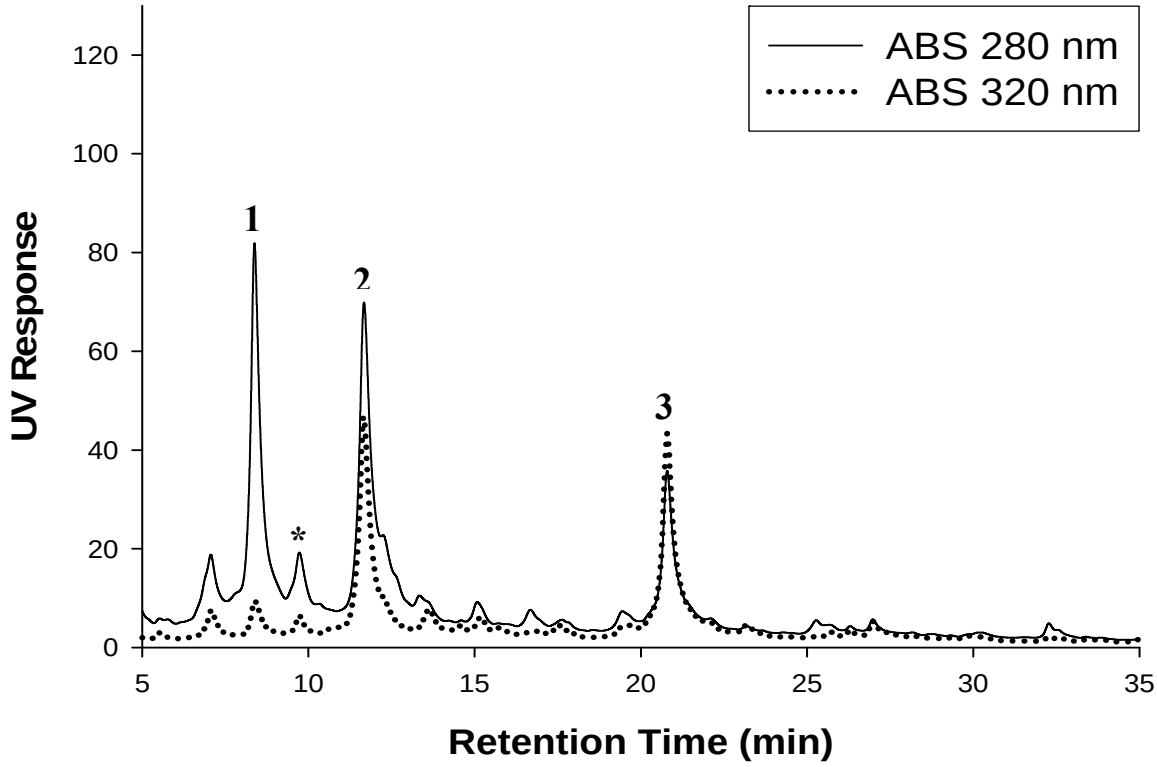

ABS 280 nm
ABS 320 nm
UV Response
Retention Time (min)
1
2
3
*
0
20
40
60
80
100
120
5
10
15
20
25
30
35

Figure 4.6

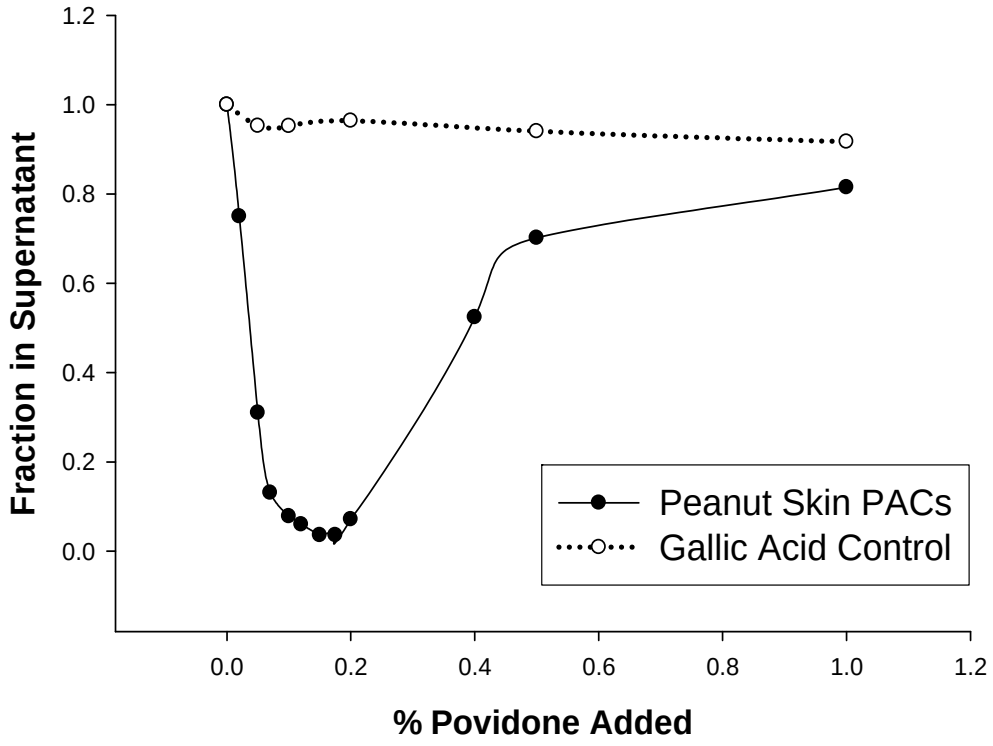

1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fraction in Supernatant
0.0
0.2
0.4
0.6
0.8
1.0
1.2
% Povidone Added
Peanut Skin PACs
Gallic Acid Control

Figure 4.7

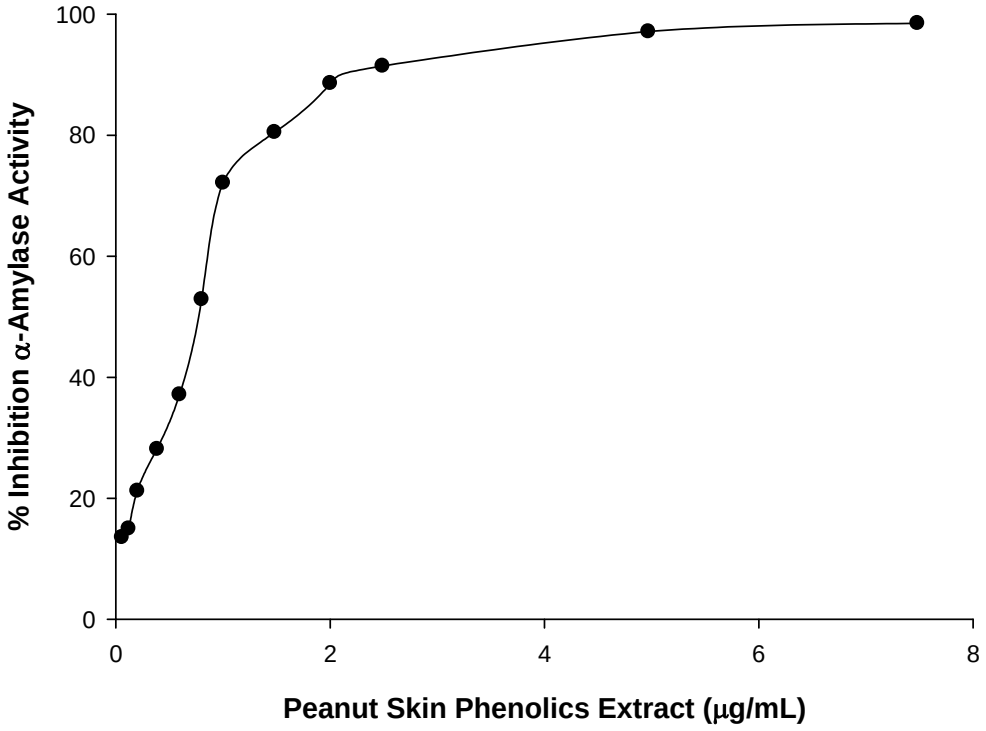

% Inhibition α-Amylase Activity
100
80
60
40
20
0
0
2
4
6
8
Peanut Skin Phenolics Extract (μg/mL)

Figure 4.8

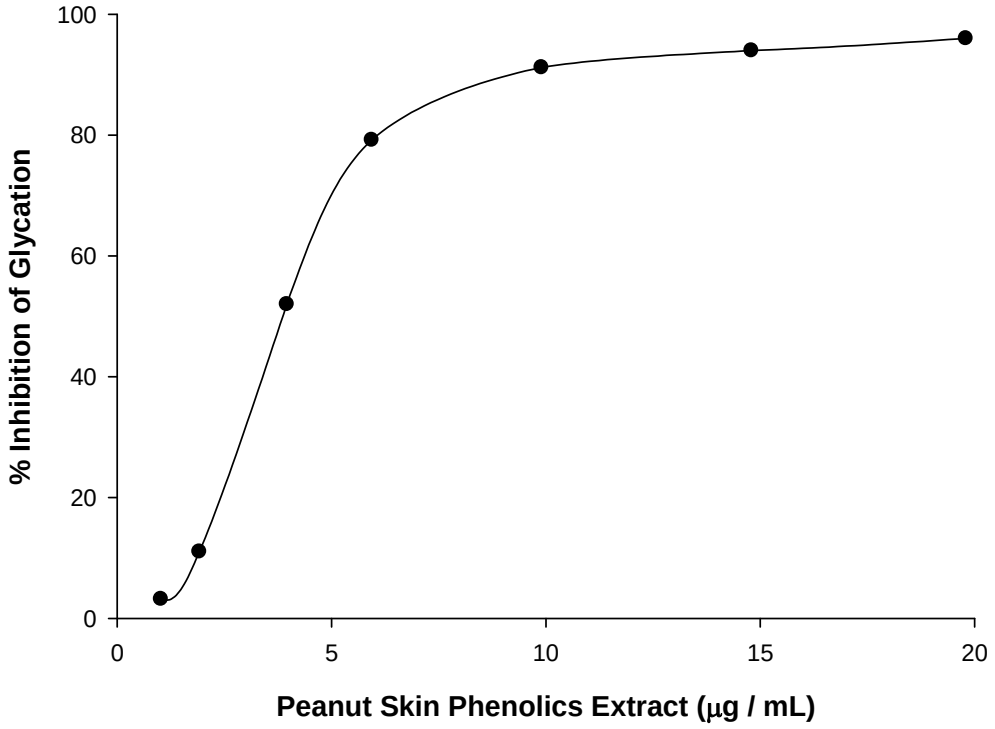

100
80
60
40
20
0
% Inhibition of Glycation
0
5
10
15
20
Peanut Skin Phenolics Extract (μg / mL)

Figure 4.9

CHAPTER 5

CHROMATOGRAPHIC ISOLATION OF PHENOLIC ACIDS AND PROANTHOCYANIDINS FROM DRY-BLANCHED AND DRY-ROASTED PEANUT SKINS[1]

[1]Craft, B. D.; Kosińska, A.; Amarowicz, R.; Pegg, R. B. To be submitted to *Food Chemistry*, 2009.

Abstract

80% (v/v) Acetonic and methanolic extracts of dry-blanched (DB) and dry-roasted (DR) peanut skins were prepared. Methanolic extracts were subjected to a separations procedure for the extraction, isolation, and subsequent quantitative RP_{18} HPLC analysis of free, esterified, and glycosidic-bound phenolic acids. Acetonic extracts were fractionated on an open-tubular column packed with Sephadex LH-20 into their LMW and HMW fractions. The HMW fraction was then further separated *via* NP-HPLC on a diol column, followed by MALDI-MS to characterize its proanthocyanidin (PAC) constituents. Crude extracts and HMW fractions for DB and DR peanut skins were then subjected to total phenolics content (TPC), Trolox equivalent antioxidant capacity (TEAC), and oxygen radical absorbance capacity ($ORAC_{FL}$) assays, for comparison. Protocatechuic acid was identified in its free form (41 μg/g) in DB peanut skin extracts; whereas, protocatechuic, caffeic, and *p*-coumaric acids were identified in the phenolic acid fraction liberated from esters (74, 57, and 716 μg/g, respectively). Free protocatechuic acid (626 μg/g) as well as ester-bound protocatechuic and *p*-coumaric acids (357 and 142 μg/g, respectively) were identified in DR peanut skin extracts; nearly a 15× higher level of free protocatechuic acid was determined compared to that found in the DB skins. No glycoside-bound phenolic acids were detected. NP-HPLC chromatograms of the HMW fractions from the DR skins yielded a greater amount of compounds. Furthermore, the HMW fraction from DR skins exhibited higher fragmentations on MALDI-MS when compared to DB skins, suggesting the existence of PACs of higher polymerization. The TPC and TEAC values for crude DR skin extracts were slightly greater than DB ones at a TPC of 475 & 404 mg (-)-epicatechin EQ/g and a TEAC value of 6022 & 4876 μmol Trolox EQ/g, respectively. In most cases, TPC, $ORAC_{FL}$, and TEAC values for HMW tannin fractions of DB and DR skin extracts were higher than the crude extracts.

5.1 Introduction

Proanthocyanidins (PACs) are subdivided into A- and B-types according to their interflavonoid linkages. B-type PACs are characterized by C4→D8/C4→D6 interflavonoid linkages, where as the A-type are C4→D8 with an additional ether linkage of C2→D7 (Ferreira and Li, 2000). PACs can range from dimeric to oligomeric species with many sub-units. PAC decamers, for example, have been reported in cocoa and sorghum (Gu *et al.*, 2002). Over the past decade, interest in PACs from antioxidant-rich foods such as wine-grape cultivars, tea leaves, cranberries, blueberries, cocoa, and pecans has multiplied (Hammerstone *et al.*, 2000; Gu *et al.*, 2004; Prior and Gu, 2005). Some research findings indicate that PACs from tea and wine may reduce the incidence of cardiovascular disease and gastrointestinal cancer (Santos-Buelga and Scalbert, 2000). Very few foods to-date have been identified as containing A-type PACs (Prior and Gu, 2005). Of these antioxidant-rich foods, peanut skins are perhaps the most interesting source given that they are a by-product of the peanut industry with little commercial value (Sobolev and Cole, 2004). As a consequence of this study and other research initiatives, peanut skins and related products are now being screened for insertion into value-added products and nutraceuticals for the promotion of human health and wellness (Isanga and Zhang, 2007).

A- and B-type PAC dimers, trimers, and tetramers have been reported in processed peanut skin extracts (Lazarus *et al.*, 1999; Van Ha *et al.*, 2007; Yu *et al.*, 2006; Yu *et al.*, 2007). However, the effects of processing on the levels and chemistry of these tannins are not well understood. Yu *et al.* (2005) reported that dry roasting increases the total phenolics content (TPC) of peanut skins; whereas, water blanching yields decreased values. Dry blanching has yet to be reported on. Peanut skin extracts demonstrate high antioxidant potency as measured by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical-scavenging assays (Van Ha *et al.*, 2007; Wang *et al.*, 2007; Yu *et*

al., 2007), ferrous-ion chelating potential (Van Ha *et al.*, 2007; Wang *et al.*, 2007), and various *in vitro* oxygen radical (*e.g.* $HO^{\bullet}$, $O_2^{\bullet -}$) scavenging methods (Wang *et al.*, 2007).

Of the many chromatographic methods used today for the separation of PACs from plant and food matrices, normal-phase high performance liquid-chromatography (NP-HPLC) techniques are dominant. Most NP-HPLC methodologies for the resolution of PAC oligomers are carried out on silica-based columns (*e.g.* LiChrospher) coupled with fluorescence detection (Rigaud *et al.*, 1993; Cheynier *et al.*, 1999; Hammerstone *et al.*, 1999; Natsume *et al.*, 2000; Gu *et al.*, 2002). Pre-fractionation *via* adsorption chromatography on lipophilic Sephadex LH-20 or TSK gel (Toyopearl HW-40F) open-tubular columns is commonplace as a means to isolate the high-molecular-weight (HMW) PAC fractions from the low-molecular-weight (LMW) ones (Sun *et al.*, 1999; Alwerdt *et al.*, 2008), as well as to provide an elution order, in some cases, for the PACs according to their degree of polymerization (Yanagida *et al.*, 2003). Further, it is also important to note that while the tannin fraction of peanut skins has received some attention, very little information exists as to the compositional profiles of LMW phenolic constituents endogenous to peanut skins. To-date, the LMW compounds that have been identified in peanut skins include the following: protocatechuic acid ethyl ester (Huang *et al.*, 2003) and trace quantities of free chlorogenic, caffeic, and ferulic acids (Yu *et al.*, 2005).

For the purposes of this research, 80% (v/v) methanolic and acetonic crude extracts, rich in phenolics, were prepared from dry-blanched (DB) and dry-roasted (DR) peanut skins. To better characterize the LMW compounds endogenous to peanut skins, methanolic extracts were subjected to a phenolic acid analysis in which free-, esterified-, and glycoside-bound phenolic acids were released from their respective matrices, extracted, and then separated and quantified *via* RP-HPLC with a UV-Vis DAD system. 80% (v/v) Acetonic extracts of peanut skins were

separated on a packed open-tubular lipophilic Sephadex LH-20 column in order to isolate the HMW species. HMW tannin fractions were then separated *via* a novel NP-HPLC method on a Develosil diol column with fluorescent detection, followed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy (MS), to ascertain any differences in the patterns of the PAC polymerized constitutents. Crude extracts and HMW tannin fractions of DB and DR peanut skin extracts were then measured for total phenolics content, peroxyl radical ($RO_2^{\bullet}$) scavenging capacities (*via* the $ORAC_{FL}$ assay), and $ABTS^{\bullet+}$ radical-scavenging capacity (*via* the TEAC assay) to better understand the effects of the DB and DR processes on the TPCs and antioxidant/radical-scavenging capacities of peanut skins.

5.2 Materials and Methods

Chemicals and Standards

All solvents and reagents were of analytical (ACS) grade or better, unless otherwise specified. Ethanol (95%), methanol (ACS and HPLC grade), acetone (HPLC grade), diethyl-ether, dichloromethane (HPLC grade), hexanes, water (HPLC grade), glacial acetic acid, hydrochloric acid, sodium hydroxide, mono- and dibasic potassium phosphate, and potassium persulfate were purchased from VWR International (Suwanee, GA). Consumables such as Costar 96-well (Costar #3631) opaque clear bottom microwell assay plates, P8 filter paper, cellulose extraction thimbles, Falcon® tubes, amber vials, and glass wool were purchased from Fisher Scientific Co. (Suwanee, GA). Lipophilic Sephadex (LH-20), phenolic acid standards (including protocatechuic, gallic, caffeic, *p*-coumaric, vanillic, sinapic, ferulic, and syringic acids), flavonoid standards (including [+]-catechin hydrate, [-]-epicatechin, quercetin dihydrate, and myricetin), procyanidin B_2, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic

acid), Folin & Ciocalteu's phenol reagent, sodium carbonate, fluorescein (3′6′-dihydroxy-spiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) disodium salt, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride), and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were purchased from Sigma-Aldrich Chemical Co. (Louis, MO). DB and DR peanut skins were a gift from Universal Blanchers (Blakely, GA) and Golden Peanut Co. (Alpharetta, GA), respectively.

Extraction Protocol and Sample Work-up

Crude phenolic extractions from DB and DR peanut skins were carried out according to Amarowicz *et al*. (2004) with some slight modifications. In brief, samples were ground in a coffee mill to the smallest possible particle size. Ground samples were then placed in cellulose extraction thimbles (Whatman single-thickness, 43 mm *i.d.* × 123 mm *e.l.*), covered with a plug of glass wool and defatted in a Soxhlet extraction apparatus under reflux for 12 h with hexanes as solvent. Defatted peanut skins were transferred to 250-mL Erlenmeyer flasks at a mass-to-solvent ratio of 1:9 (w/v) for subsequent liquid extraction *via* 80% (v/v) acetone for PAC analysis and antioxidant potency work as well as 80% (v/v) methanol for phenolic acids determinations. Extractions were carried out at room temperature (~22 °C) and 150 RPM for three 30 min intervals on a gyrotary water bath shaker (New Brunswick Scientific, New Brunswick, NJ). Supernates obtained after each extraction period were gravity filtered through P8 filter paper and collected. Final supernates were subjected to centrifugation at 430 × g for 10 min to precipitate any remaining solid materials, which were removed. Organic solvent fractions were then evaporated *in vacuo* with a Büchi Rotavapor R-210 (Büchi Corp., New Castle, DE). Remaining aqueous samples were frozen and then lyophilized in Pyrex glass crystallization

dishes in a Labconco Freezone 2.5 L bench-top freeze dryer (Labconco Corp., Kansas City, MS) at -40 °C under reduced pressure (< 0.2 mbar). Sample extract powders were stored in amber vials at 4 °C under a blanket of nitrogen until use for RP-18 HPLC phenolic acids profiling, NP-HPLC PACs separations, TPC determinations, and evaluation of antioxidant capacities.

Analysis of Free, Esterified, and Glycoside-Bound Phenolic Acids

A rapid procedure for the extraction, isolation, and subsequent quantitative RP_{18} HPLC analysis of free, esterified, and glycosidic-bound phenolic acids in crude 80% (v/v) methanolic extracts from DB and DR peanut skins was employed according to Krygier *et al.* (1982). Briefly, an aqueous suspension of the peanut skin extract powders (500 mg in 10 mL) was prepared and adjusted to pH 2 with 6 M HCl. The free phenolic acids were extracted from the mixture 5× with 10-mL portions of diethyl ether *via* a separatory funnel. The collected ether layers were evaporated to dryness *in vacuo* at room temperature (~22 °C) and saved for HPLC analysis. The aqueous portion retained in the separatory funnel was neutralized with 2 M NaOH and then lyophilized. The dried residue was weighed and then dissolved in 10 mL of 2 M NaOH; it was hydrolyzed for 4 h at room temperature under an atmosphere of nitrogen. Following acidification to pH 2 using 6 M HCl, phenolic acids liberated from the soluble esters were extracted from the hydrolyzate 5× with 15-mL portions of diethyl ether *via* a separatory funnel. The collected fraction was evaporated to dryness *in vacuo* and saved for HPLC analysis. To the aqueous portion retained in the separatory funnel, 15 mL of 6 M HCl was added. The solution was placed under a blanket of nitrogen and hydrolyzed for 1 h at 100 °C in an Isotemp convection oven (Fisher). The phenolic acids liberated from the soluble glycosides were then extracted from the hydrolyzate 5× with 10-mL portions of diethyl ether *via* a separatory funnel.

The collected fraction was evaporated to dryness *in vacuo* and saved for HPLC analysis. Before HPLC analysis, the samples containing free phenolic acids and those liberated from the esters or glycosides were dissolved in 2-mL methanol (HPLC grade) and passed *via* a syringe through a 0.45-μm nylon filter. The extracts were analyzed using an Agilent 1200 series HPLC chromatograph (Agilent Technologies Inc., Wilmington, DE) equipped with a quaternary pump, autosampler, thermostated column compartment, and a UV-Vis DAD system. The conditions for separation entailed a pre-packed Luna Phenomenex C_{18}(II) RP-HPLC column (250 mm × 4.6 mm, 5-μm particle size; Phenomenex, Torrance, CA) equipped with a guard column; isocratic elution with water:acetonitrile:acetic acid (88:10:2, v/v/v); a 1 mL/min flow rate; a 20-μL injection volume; and the detector set at λ = 320 nm. Agilent ChemStation software equipped with a 3-D LC Spectral Analysis software package was used for chromatogram quantification and analysis.

Extract Fractionation and Analytical NP-HPLC

Within a week of each other, 1 g of crude acetonic DB and DR peanut skin extract was solubilized in 10 mL of 95% (v/v) ethanol and fractionated *via* adsorption chromatography on a open-tubular (OT) column (30 mm *i.d.* × 270 mm *e.l.*) packed with lipophilic Sephadex LH-20 fitted at the bottom with a plug of glass wool. The OT column was first washed and then equilibrated with 95% (v/v) ethanol. One liter of 95 % (v/v) ethanol was employed to elute the LMW compounds (*i.e.*, phenolic acids, ester- & glycoside-bound phenolics, and some flavonoids) followed by 750 mL of 50% (v/v) acetone for the HMW species (*i.e.,* PACs, and complexes of tannins with LMW compounds [*e.g.* tannin-phenolic acid]). The LMW and HMW fractions were collected. The organic portion of each was evaporated *in vacuo* and the remaining

aqueous portions were freeze-dried and stored in refrigerated conditions as previously explained. Other than % yields, the LMW fractions were not examined further in this study.

The HMW fractions from DB and DR peanut skin extracts were separated *via* NP-HPLC on an Agilent 1200 series liquid chromatograph (Agilent Technologies Inc.) equipped with a quaternary pump, auto-sampler, thermostatted column compartment, a UV-Vis DAD system, and fluorescence detector according to Kelm *et al.* (2006) with some modifications. Briefly, conditions for separation entailed a pre-packed Astec Diol HPLC column (250 mm × 4.6 mm, 5-μm particle size; Supelco, Bellefonte, PA) equipped with a guard column; a gradient elution comprised of dichloromethane: methanol: and 1:1 (v/v) acetic acid:water; 37 °C column temperature; 1.2 mL/min flow rate; a 5 -μL injection volume; a UV-Vis DAD system set at 280 and 320nm; and fluorescence detection set at an excitation/emission pair of 276/316 nm. The starting mobile phase conditions were 82% CH_2Cl_2, 14% CH_3OH, and 4% HOAc:H_2O. Subsequently, CH_3OH was ramped to 28.4% after 30 min, 42.8% after 50 min, and 86% after 51 min, then returned to the starting conditions at 61 min along with a 10 min re-equilibration period between samples. The HOAc:H_2O portion of the mobile phase was held at 4% throughout the chromatographic run. All samples and standards were dissolved in acetone: water:acetic acid (70:29.5:0.5; v/v/v) and passed through a 0.2-μm nylon filter. Injected DB and DR acetonic extract sample concentrations were 2 and 4 mg/mL respectively. Standards (-)-epicatechin and procyanidin B_2 were injected at 2 mg/mL under the same assay conditions.

MALDI-TOF Mass Spectrometry

MALDI-TOF MS measurements were performed on a Bruker Daltonics Autoflex MALDI-TOF mass spectrometer equipped with a pulsed nitrogen laser, a delayed extraction ion

source, and a reflector. Analyses were run in Reflectron positive-ion mode. Briefly, a few crystals of purified HMW DB and DR peanut skin tannin fractions post elution from the lipophilic Sephadex LH-20 were dissolved in 50 μL of methanol. Samples were mixed 1:1 (v/v) with a matrix consisting of saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA): water:acetonitrile:trifluoroacetic acid (50:25:25:0.1, v/v/v/v). A 1-μL aliquot of this solution was spotted onto a target plate, dried in air, and introduced to the mass spectrometer. All mass spectra were collected by averaging the signals of at least 50 to 100 laser shots over a mass-to-charge (*m/z*) range of 120 to 4200. A mixed peptide solution comprised of angiotensin II, bombesin, and oxidized insulin chain B was employed for external calibration.

Total Phenolics Content (TPC)

The TPC in crude peanut skin extracts and HMW fractions were measured using Folin & Ciocalteu's phenol reagent (Folin and Ciocalteu, 1927) and the colorimetric assay developed by Swain and Hillis (1959) with a 50% reduction in assay volume. Briefly, peanut extracts were solubilized in 80% (v/v) methanol. Extracts were diluted as needed with methanol and a 250-μL aliquot was transferred to a clean 10-mL test tube. Then, 3.25 mL of deionized water were added and the solution was vortexed for 10 s. Next, 250 μL of 2 N Folin & Ciocalteu's phenol reagent were added and the sample was vortexed for 10 s, and allowed to stand 3 min before proceeding. The blank solution should become colorless. Lastly, 500 μL of a saturated sodium carbonate solution (> 30%, w/v) were added to promote the colorimetric reaction, followed by 750-μL deionized water to bring the total assay volume up to 5 mL. The solution was vortexed for 10 s and allowed 1 h for maximal color development. The absorbance of the resulting chromophore was measured at 750 nm with an Agilent 8453 UV/Vis DAD spectrophotometer

(Agilent Technologies Inc.). TPC values were expressed in mg [-]-epicatechin equivalents (EQ)/g dry extract (DE) from triplicate samples.

$ORAC_{FL}$: Oxygen Radical Absorbance Capacity

The $ORAC_{FL}$ assay was performed according to Prior *et al.* (2003), with some modifications. Conditions for the assay entailed a BMG FLUOstar Omega (Ω) fluorometer equipped with two internal 500-µL reagent pumps, an external lead system, temperature control set at 37 °C, fluorescent detection set at an excitation/emission pair of 485/520 nm, and a 3-h run time. Reagents included the following: 75 mM phosphate buffer (pH 7.4) as the reaction media and diluent, Trolox standard prepared at concentrations ranging from 3.125 to 100 µM, and a working solution of 0.1 µM fluorescein (FL) as the reaction probe. The peroxyl radical initiator AAPH was prepared in phosphate buffer at a concentration of 80 mM and heated to 37 °C only just before use.

Assay plates including diluted samples and standards were incubated at 37 °C for 10 min before automated addition of FL and AAPH, one full cycle apart. Peanut skin extracts/fractions were solubilized in absolute methanol and diluted with phosphate buffer until they provided a suitable delay in the FL reduction. Auto-fluorescence of the extracts was found to be negligible at assay concentrations. Once data was compiled the Area Under the Curve (AUC) or integral was compared between the samples and standards, to generate equivalence in terms of the standard Trolox. Raw data was averaged and blanks corrected such that the entirety of the resultant signal was sample dependent. Final $ORAC_{FL}$ values were expressed in µmol Trolox EQ/g DE from duplicate samples.

TEAC: Trolox Equivalent Antioxidant Capacity

The Trolox equivalent antioxidant capacities of crude acetonic DB and DR peanut skin extracts and the HMW fractions were determined according to the TEAC assay (Re *et al.*, 1999). Briefly, an ethanolic solution of 7 mM ABTS was mixed with 2.45 mM potassium persulfate and incubated in the dark for 12 to 16 h. The resultant $ABTS^{\bullet+}$ solution (blue-green) was gravity filtered through P8 filter paper. $ABTS^{\bullet+}$ stock was then diluted with 95% (v/v) ethanol until an absorbance of 0.70 was reached at 734 nm with an Agilent 8453 UV/Vis-DAD spectrophotometer. Trolox standards were prepared at concentrations ranging from 0.2 to 2 mM for the development of a standard curve. A 10-µL aliquot of sample or standard was combined with 1 mL of the $ABTS^{\bullet+}$ stock, equilibrated at 30 °C for 5 min, and the absorbance of the resultant solution read at 734 nm. DB and DR peanut skin extracts were diluted in ethanol such that they produced between a 20 to 80% inhibition of $ABTS^{\bullet+}$ stock. Results were expressed in µmol Trolox EQ/g DE from triplicate samples.

Statistical Analysis

Results were summarized with mean and standard deviations reported for each data grouping. TPC, $ORAC_{FL}$, and TEAC data for crude DB and DR peanut skin extracts and HMW fractions were analyzed by a 1-way ANOVA statistical model using the statistical analysis system (SAS, version 9.0, SAS Inst Inc., Cary, NC) (O'Rourke *et al.*, 2005) to determine significant differences at the 95% confidence interval ($\alpha = 0.05$). Once significance was determined ($P < 0.05$), data were then subjected to Fisher's method of Least Significant Difference (LSD, otherwise known as the *t*-test) in order to segregate treatment means that were significantly different from each other at $\alpha = 0.05$.

5.3 Results and Discussion

Analysis of Free, Esterified, and Glycoside-Bound Phenolic Acids

Spectral scans of selected phenolic acid standards of the benzoic acid family (*e.g.*, *p*-hydroxybenzoic, gallic, vanillic, syringic, and protocatechuic acids) and the *trans*-cinnamic acid family (*e.g.*, caffeic, *p*-coumaric, *m*-coumaric, *o*-coumaric, ferulic, isoferulic, and sinapic acids), were recorded in the UV-region between 220 to 420 nm to assist with the phenolic acid identification processes; see Figures 5.1 and 5.2. As seen in Figure 5.1, *p*-hydroxybenzoic, vanillic, and protocatechuic acids exhibit a primary maximum at 260 nm, with vanillic and protocatechuic yielding a secondary maximum at 300 nm. On the other hand, gallic and syringic acids possess a primary maximum at 280 nm. Figure 5.2 shows that *p*-coumaric, caffeic, ferulic, isoferulic, and sinapic acids exhibit a primary maximum near 320 nm, with a secondary one at ~295 nm; whereas, *m*- and *o*-coumaric acids show a primary maximum at 280 nm. Chromatograms for the free phenolic acids and those liberated from soluble esters of DB peanut skins are reported in Figures 5.3 and 5.4, respectively. Protocatechuic acid (peak 1, retention time [RT] = 6.5 min) was identified in the free fraction of the DB peanut skin methanolic extracts (Figure 5.3) and quantified at 41 μg/g dry extract. Another predominant phenolic species was present (*i.e.*, denoted by an asterisk, RT = 13.49 min); however, this sample was unidentifiable from the commercial standards run. It is possible that this component is a derivative of the *trans*-cinnamic acid family, given that it possesses a marked absorbance near 320 nm (*i.e.*, it exhibited a spectrum similar to *p*-coumaric acid, but with a broad maximum at ~314 nm and a secondary one at ~232 nm). Perhaps more likely, this compound is an ester or glycoside of *p*-coumaric acid that was initially soluble in the acidified diethyl-ether (*i.e.*, the initial medium employed for free phenol acid extraction). Protocatechuic (peak 1, RT = 6.53

min), caffeic (peak 2, RT = 14.26 min), and *p*-coumaric (peak 3, RT = 27.76 min) acids were all identified and quantified at 74, 57, and 716 μg/g dry extract of the DB peanut skin phenolic acids released from esters (Figure 5.4). This indicates that the majority of the LMW phenolic acids contained in the DB peanut skin extracts is esterified, and possesses a very high content of *p*-coumaric acid esters. No glycoside-bound phenolics were detected in DB peanut skin extracts.

Chromatograms of the free phenolic acids and the phenolic acids liberated from the esters of 80% (v/v) methanolic DR peanut skin extracts are presented in Figures 5.5 and 5.6, respectively. Protocatechuic acid (peak 1, RT = 6.51 min) was tentatively identified based on retention time mapping of the standard and quantified at 626 μg/g dry extract of the free phenolic acids of DR peanut skin extracts, as seen in Figure 5.5. That is nearly a 15 fold increase in free protocatechuic acid levels present in the aglycone fraction of DR extracts when compared to the DB skin extracts. This may suggest that the higher thermal temperatures/holding times exhibited in typical dry-roasting procedures (when compared to dry-blanching) are causing a significant alteration in the phenolic profiles of roasted peanut skins. A similar effect of heat treatment on the increased free/bound phenolic acid ratio of buckwheat (*Fagopyrum esculentum* Moench L.) seeds and citrus (Huyou) peel phenolic extracts was recently noted in the literature (Zieliński *et al.*, 2006; Xu *et al.*, 2007). However, the type/cultivars from which these peanut skins originate are unknown so comparisons between them are limited. Given that both of the peanut skin samples were collected from south GA processors, it is highly likely they are from Runner peanuts of predominant Georgia varieties such as GAGreen. Another predominant phenolic species was present in the free phenolic acids from DR peanut skin extracts (*i.e.*, denoted by an asterisk, RT = 13.63 min); however, this sample was unidentifiable from the commercial standards employed. As with the unidentified compound in DB skin extracts, this compound

exhibited a spectrum characteristic of a *trans*-cinnamic acid; it is, therefore, possibly a derivative.

Figure 5.6 is an HPLC chromatogram of phenolic acids released from esters of DR peanut skin extracts. Both protocatechuic (peak 1, RT = 6.49 min) and *p*-coumaric (peak 2, RT = 27.77 min) acids were identified in the phenolic acids liberated from esters of DR peanut skin extracts and quantified at 357 and 142 μg/g dry extract, respectively. There is not only a greater presence of free protocatechuic acid in the DR skin extracts (when compared to the DB ones), but also there exists a greater presence of protocatechuic esters. Perhaps this increase is due to the release of protocatechuic acid from its ethyl ester (Huang *et al.*, 2003). No glycoside-bound phenolic acids were detected in DR peanut skin extracts.

Extract Fractionation, Analytical NP-HPLC, and MALDI-TOF MS

LMW and HMW species accounted for 40.83 & 57.16% and 22.22 & 74.44% for the DB and DR peanut skin extracts, respectively. Karchesy and Hemingway (1986) previously reported peanut skins to contain ~17% PACs by weight with ~50% as LMW species oligomers. The larger percentage of HMW species encountered in the DR peanut skin extracts, when compared to the DB extracts, may be partially attributable to the thermal instability of PACs. PACs can undergo oxidative transformation during processing and storage (Santos-Buelga and Scalbert, 2000). The results of the NP-HPLC PAC separations on the Develosil diol (Phenomenex) bonded-phase for the DB and DR peanut skin HMW fractions were overlaid in a single chromatogram and are presented in Figure 5.7. All lower PAC oligomers (*i.e.* possibly dimers, trimers, and/or tetramers, as discussed) eluted from the NP-HPLC column within the first 15 min. Procyanidin B_2 (a B-type dimer, epicatechin4β→8βepicatechin) eluted at a RT of 7.3 min,

whereas the HMW DB peanut skin PACs eluted at 9.6, 10.6, and 11.7 min. HMW peanut skin PACs of DR skin extracts shared fluorescence maxima at 9.6, 10.6, and 11.7 min; however, the DR extracts exhibited opposite ratios for the species eluting at RT = 9.6 and 11.7 min, suggesting higher levels of the more highly-bound PAC species (11.74 min). Furthermore, injected sample concentrations of DR skins were twice as high as the DB extracts, suggesting that the more severe thermal processing conditions are causing a greater instance of polymerization reactions. DR samples also exhibited two additional peaks in the chromatogram at later RTs than the procyanidin B_2 standard (*i.e.* at 6.86 and 6.55 min, respectively). Given the conditions for separation and that PAC monomers up to decamers have been resolved in this manner in related methods (Adamson *et al.*, 1999), it is possible that the HMW tannin fractions are eluting according to their size/molecular weight. The fact that the DB peanut skins have a greater number of components eluting at lower RTs than the DB samples could mean that the DR PACs have undergone more extensive oxidative degradation, resulting in a greater array of HMW species.

MALDI-TOF mass spectra for the DB and DR HMW peanut skin tannin fractions post separation on Sephadex LH-20 are shown in Figures 5.8 and 5.9, respectively. Both DB and DR HMW extract fractions share fragmentations at 279.1, 441.2, and 624.1 *m/z* in the positive-ion mode, which are equivalent molecular masses (MM) of 278.1, 440.2 and 623.1 g/mol. Catechin and epicatechin are the flavan-3-ol monomers of PACs; they have a MM of ~289, which is quite close to 278. There was a greater instance of *m/z* signals at 440 and 623 in the DR extracts and parent ions of higher masses, as seen in Figure 5.9. This strongest *m/z* signal encountered was at 963.1 in the case of the DR skin HMW fraction, and this is equivalent to a MM of 962.1g/mol, which is between the MM of a PAC trimer (~865 g/mol) and tetramer (~1151 g/mol) (Yu *et al.*,

2007). However, the *m/z* signal at 963.1 on the DR mass spectrum could be a daughter ion, rather than the parent and, thus, may be part of an even larger molecule. As a reference, Figure 5.10 is the MALDI mass spectrum of procyanidin B_2, a dimer. Upon correlation of the spectrum of procyanidin B_2 with the DB and DR spectra, one notices that three fragmentation ions are preserved in the DB skins (*i.e.*, *m/z* = 190.0, 294.1, and 379.1). None of the three shared daughter ions from procyanidins B_2 are shared with the DR skin HMW fraction. In fact the DR samples exhibited fragments with higher *m/z*, which further suggests that these tannins have undergone a greater extent of oxidative polymerization reactions through the dry roasting process. Yu *et al.* (2006) determined A-type procyanidin dimers, trimers, and tetramers accounting for *m/z* ratios of 575, 863, and 1149, as well as B-types with *m/z* of 577, 865, and 1151.

Total Phenolics Content and Antioxidant Capacities ($ORAC_{FL}$ and TEAC)

Mean TPC, $ORAC_{FL}$ and TEAC data for the crude acetonic extracts and the HMW tannin fractions of DB and DR peanut skins are given in Table 5.1. As evident from Table 5.1, the TPC and TEAC values of the extracts were quite similar for both processing methods (*i.e.*, 404 & 475 mg [-]-epicatechin EQ/g and 3419 & 4495 μmol Trolox EQ/g for the DB and DR extracts, respectively. Given the greater presence of protocatechuic acids and esters in the DR skins, it is not surprising that the extract yielded higher TPC levels. Furthermore, protocatechuic acid and other phenolic acids (*e.g.* caffeic and ferulic acids) have recently been shown to possess good metal-ion chelating capabilities (Psotová *et al.*, 2003). This appears to be greater in the case of the *trans*-cinnamic acids than the benzoic acids and the incidence of bound species of higher-molecular weight. Therefore, the DR extracts might have a greater potential than the DB to

scavenge $ABTS^{\bullet+}$ *via* electron reduction in the TEAC assay, based on their higher phenolic acid contents. $ORAC_{FL}$ values for the crude DR skin extracts, however, were quite lower than the DB ones (4876 < 6022 μmol Trolox EQ/g dry extract), but results were variable. TPC, $ORAC_{FL}$, and TEAC results for the HMW tannin fractions of both DB and DR peanut skin extracts yielded greater values than the crude extracts in every case except for the DR extracts examined in the $ORAC_{FL}$ assay. These results suggest that the tannin fraction of peanut skin extracts is a considerable factor in their exhibition of marked antioxidant activities *in vitro*. The DB peanut skins had nearly twice as much LMW species as obtained post Sephadex LH-20 fractionation (*i.e.* ~41% for the DB, vs. ~22% for the DR skin LMW fractions), and crude DB fractions demonstrated the greatest $ORAC_{FL}$ activity of all extracts examined. From these results one may deduce that the marked antioxidant activities of peanut skins are due to a combination of both their high PAC content and the presence of other LMW phenolic species, such as *p*-coumaric acid, caffeic acid, and free/bound protocatechuic acid contained therein.

Acknowledgments

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References

Adamson, G. E.; Lazarus, S. A.; Mitchell, A. E.; Prior, R. L.; Cao, G.; Jacobs, P. H.; Kremers, B. G.; Hammerstone, J. F.; Rucker, R. B.; Ritter, K. A.; Schmitz, H. H. 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* **47**:4184-4188.

Alwerdt, J. L.; Seigler, D. S.; De Mejia, E. G.; Yousef, G. G.; Lila, M. A. 2008. Influence of alternative liquid chromatography techniques on the chemical complexity and bioactivity of isolated proanthocyanidin mixtures. *J. Agric. Food Chem.* **56**:1896-1906.

Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. 2004. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* **84**:551-562.

Cheynier, V.; Souquet, J-M.; Le Roux, E.; Guyot, S.; Rigaud, J. 1999. Size separation of condensed tannins by normal-phase high-performance liquid chromatography. *Meth. Enzymol.* **299**:178-184.

Ferreira, D.; Li, X-C. 2000. Oligomeric proanthocyanidins: Naturally occurring O-heterocycles. *Nat. Prod. Rep.* **17**:193-212.

Folin, O.; Ciocalteu, V. 1927. On tyrosine and tryptophan determinations in proteins. *J. Biol. Chem.* **73**:627-650.

Gu, L.; Kelm, M.; Hammerstone, J. F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R. L. 2002. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *J. Agric. Food Chem.* **50**:4852-4860.

Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Gebhardt, S.; Prior, R. L. 2004. Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *J. Nutr.* **134**:613-617.

Hammerstone, J. F.; Lazarus, S. A.; Mitchell, A. E.; Rucker, R.; Schmitz, H. H. 1999. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography / mass spectrometry. *J. Agric. Food Chem.* **47**:490-496.

Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. 2000. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **130**:2086S-2092S.

Huang, S. C.; Yen, G-C., Chang, L-W; Yen, W-J.; Duh, P-D. 2003. Identification of an antioxidant, ethyl protocatechuate, in peanut seed testa. *J. Agric. Food Chem.* **51**:2380-2383.

Isanga, J.; Zhang, G-N. 2007. Biologically active components and nutraceuticals in peanuts and related products: Review. *Food Rev. Int.* **23**:123-140.

Karchesy, J. J.; Hemingway, R. W. 1986. Condensed tannins: (4β→8; 2β→O→7)-Linked procyanidins in *Arachis hypogea* L. *J. Agric. Food Chem.* **34**:966-970.

Kelm, M. A.; Johnson, J. C.; Robbins, R. J.; Hammerstone, J. F.; Schmitz, H. H. 2006. High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. *J. Agric. Food Chem.* **54**:1571-1576.

Krygier, K.; Sosulski, F.; Hogge, L. 1982. Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure. *J. Agric. Food Chem.* **30**:330-334.

Lazarus, S. A.; Adamson, G. E.; Hammerstone, J. F.; Schmitz, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* **47**:3693-3701.

Natsume, M.; Osakabe, N.; Yamagishi, M.; Takizawa, T.; Nakamura, T.; Miyatake, H.; Hatano, T.; Yoshida, T. 2000. Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal-phase and reversed-phase HPLC. *Biosci. Biotechnol. Biochem.* **64**:2581-2587.

O'Rourke, N.; Hatcher, L.; Stepanski, E. J. 2005. A step-by-step approach to using SAS® for univariate & multivariate statistics. 2nd edition. Cary: SAS Institute Inc.

Prior, R. L.; Gu, L. 2005. Ocurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* **66**:2264-2280.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocaa, M.; Howard, L.; Hampsch-Woodhill, M.;

Huang, D.; Ou, B.; Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **51**:3273-3279.

Psotová, J.; Lasovský, J.; Vičar, J. 2003. Metal-chelating properties, electrochemical behavior, scavenging and cytoprotective activities of six natural phenolics. *Biomed. Papers* **147**:147-153.

Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **26**:1231-1237.

Rigaud, J.; Escribano-Bailon, M. T.; Prieur, C.; Souquet, J.-M.; Cheynier, V. 1993. Normal-phase high-performance liquid chromatographic separation of procyanidins from cacao beans and grape seeds. *J. Chromatogr. A.* **654**:255-260.

Santos-Buelga, C.; Scalbert, A. 2000. Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **80**:1094-1117.

Sobolev, V. S.; Cole, R. J. 2004. Note on the utilization of peanut seed testa. *J. Sci. Food Agric.* **84**:105-111.

Sun, B.; Belchior, G. P.; Ricardo-da-Silva, J. M.; Spranger, M. I. 1999. Isolation and purification of dimeric and trimer procyanidins from grape seeds. *J. Chromatogr. A.* **841**:115-121.

Swain, T.; Hillis, W. E. 1959. The phenolic constituents of *Prunus domestica*. I.-The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **10**:63-68.

Van Ha, H.; Pokorný, J.; Sakurai, H. 2007. Peanut skin antioxidants. *J. Food Lipids* **14**:298-314.

Wang, J.; Yuan, X.; Jin, Z.; Tian, Y.; Song, H. 2007. Free radical and reactive oxygen species scavenging activities of peanut skins extract. *Food Chem.* **104**:242-250.

Xu, G.; Ye, X.; Chen, J.; Liu, D. 2007. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract *J. Agric. Food Chem.* **55**:330-335.

Yanagida, A.; Shoji, T.; Shibusawa, Y. 2003. Separation of proanthocyanidins by degree of polymerization by means of size-exclusion chromatography and related techniques. *J. Biochem. Biophys. Methods* **56**:311-322.

Yu, J.; Ahmedna, M.; Goktepe, I. Chapter 16. Peanut skin phenolics: extraction, identification, antioxidant activity, and potential applications. ACS Symposium Series 956(Antioxidant Measurement and Applications):226-241. Copyright 2007 ACS.

Yu, J.; Ahmedna, M.; Goktepe, I.; Dai, J. 2006. Peanut skin procyanidins: composition and antioxidant activities as affected by processing. *J. Food Comp. Anal.* **19**:364-371.

Yu, J.; Ahmedna, M.; Goktepe, I. 2005. Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem.* **90**:199-206.

Zieliński, H.; Michalska, A.; Piskula, M. K.; and Kozlowska, H. 2006. Antioxidants in thermally treated buckwheat groats. *Mol. Nutr. Food Res.* **50**:824-832.

Table 5.1: TP, $ORAC_{FL}$, and TEAC values for crude extracts (80% [v/v] acetone) and HMW tannin fractions DB and DR peanut skins

Sample	**TPC[1]**	**$ORAC_{FL}$[2]**	**TEAC[3]**
DB-Crude Extract	$404 \pm 1.8^{b\dagger}$	6022 ± 215	3419 ± 157^{b}
DB-HMW Tannin Fraction	520 ± 7.1^{a}	7204 ± 684	5946 ± 75^{a}
DR-Crude Extract	$475 \pm 3.0^{(b)}$	4876 ± 264	$4495 \pm 90^{(b)}$
DR-HMW Tannin Fraction	$503 \pm 8.0^{(a)}$	3743 ± 617	$5561 \pm 266^{(a)}$

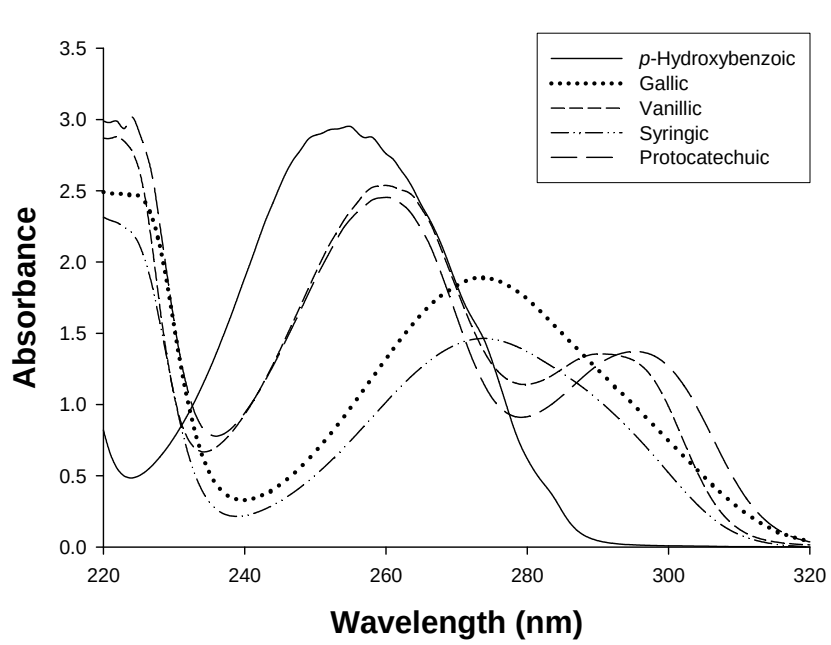
†Crude DB and DR peanut skin extracts and their respective HMW fractions within antioxidant assay were compared using Fisher's LSD. DB means sharing a common lower-case letter are not significantly different at $\alpha = 0.05$, DR means sharing a common lower-case letter (within brackets) are not significantly different at $\alpha = 0.05$.

[1]Total Phenolics values were expressed as mean mg [-]-epicatechin equivalents (EQ)/g dry extract (DE) ± standard deviation from triplicate sample measurements.

[2]Oxygen Radical Absorbance Capacity ($ORAC_{FL}$-hydrophilic) values were expressed as mean μmol Trolox EQ/g DE ± standard deviation from duplicate sample measurements. ORAC 1-way ANOVA was not significant at $\alpha = 0.05$

[3]Trolox Equivalent Antioxidant Capacity (TEAC) values were expressed as mean μmol Trolox EQ/g DE ± standard deviation from triplicate sample measurements.

Figure Captions



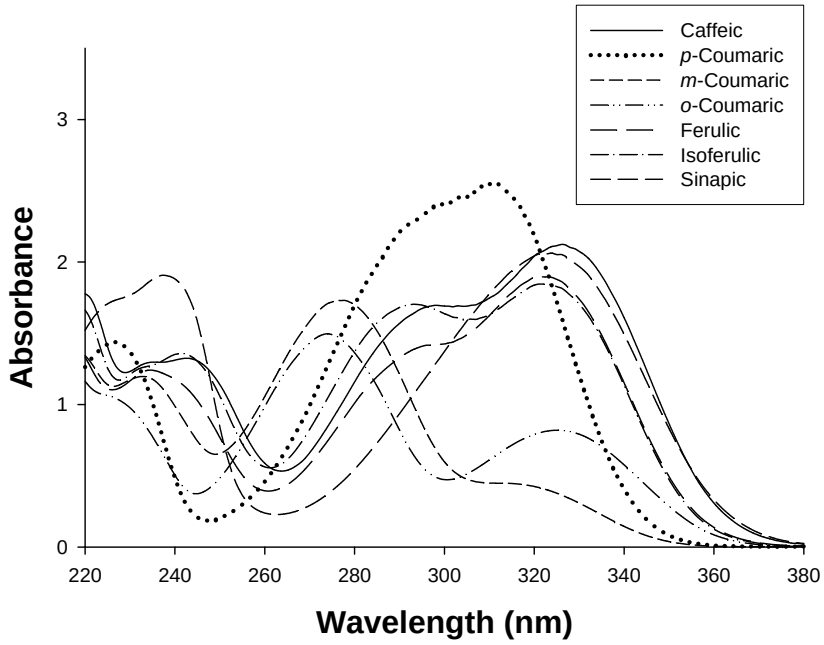
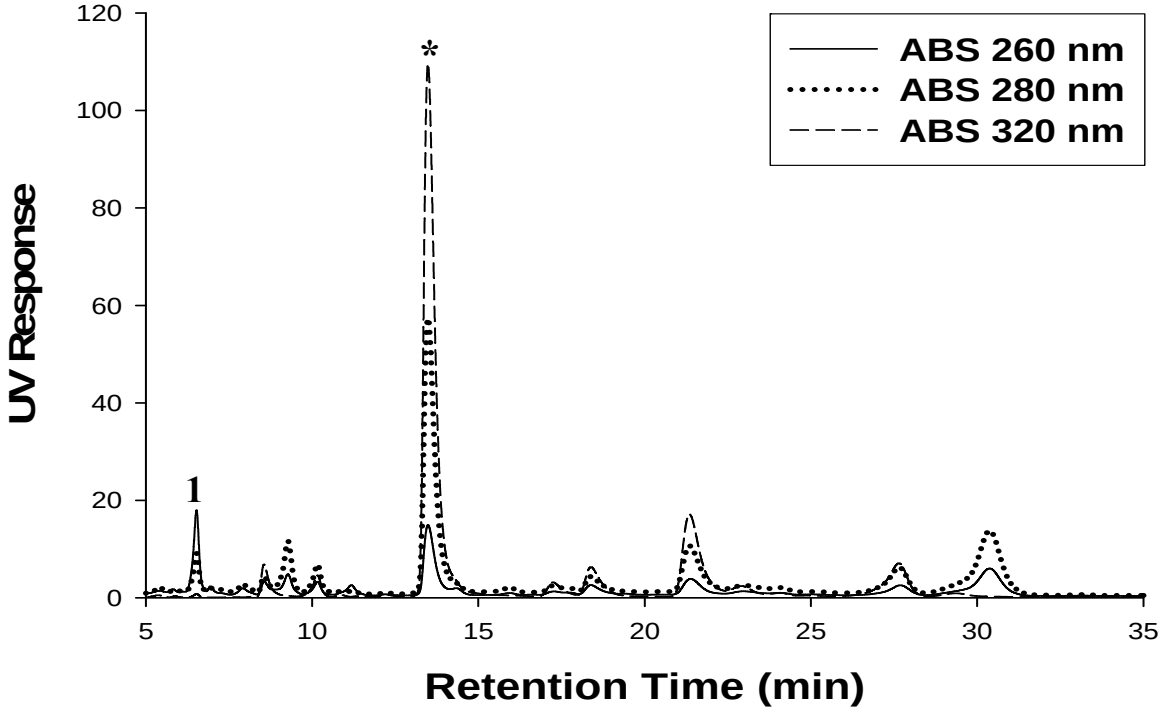


Figure 5.1(left) and 5.2(right)



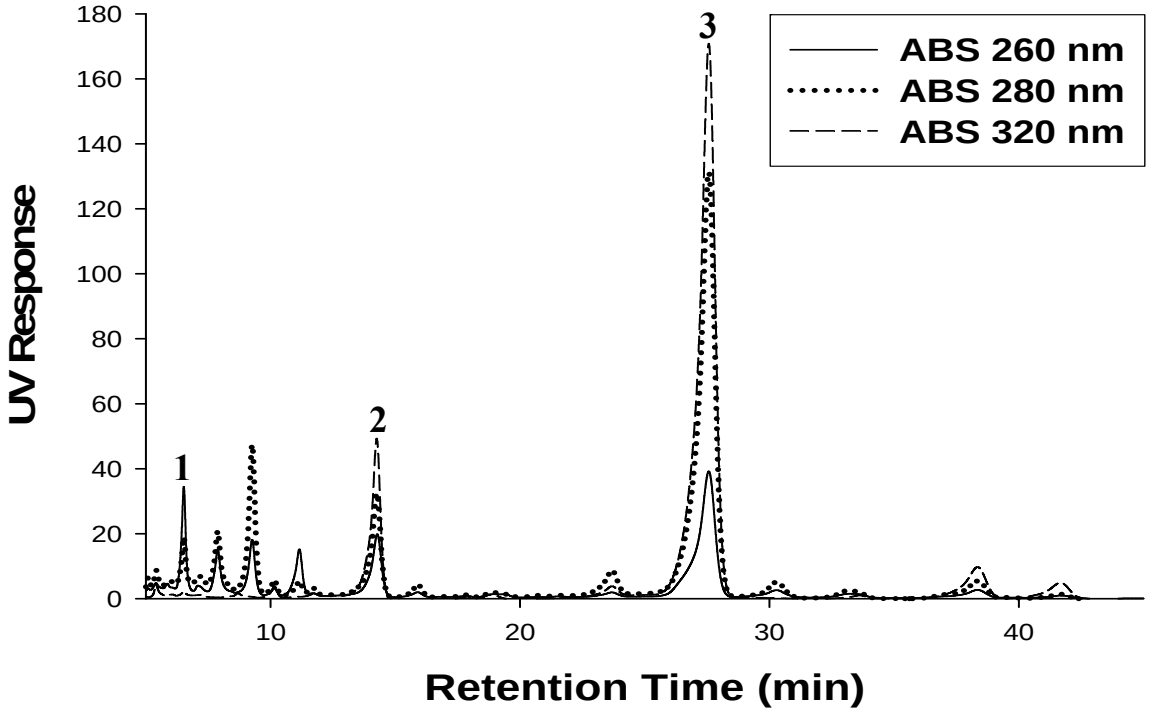
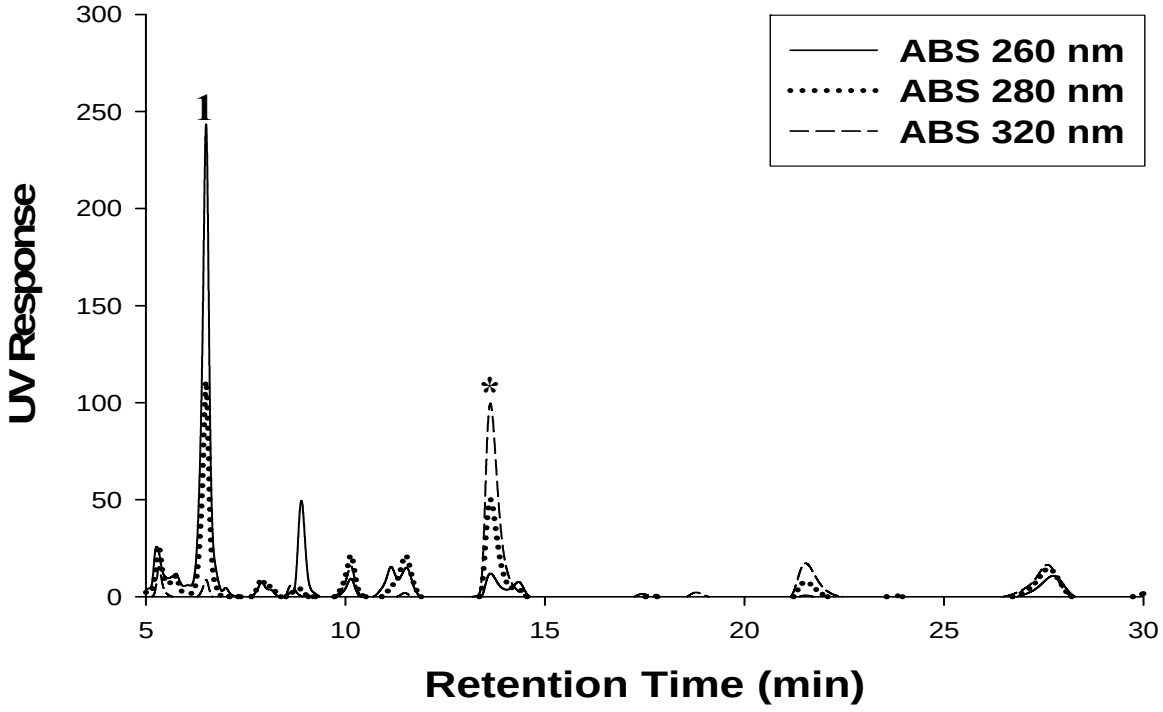


Figure 5.3(top) and 5.4(bottom)



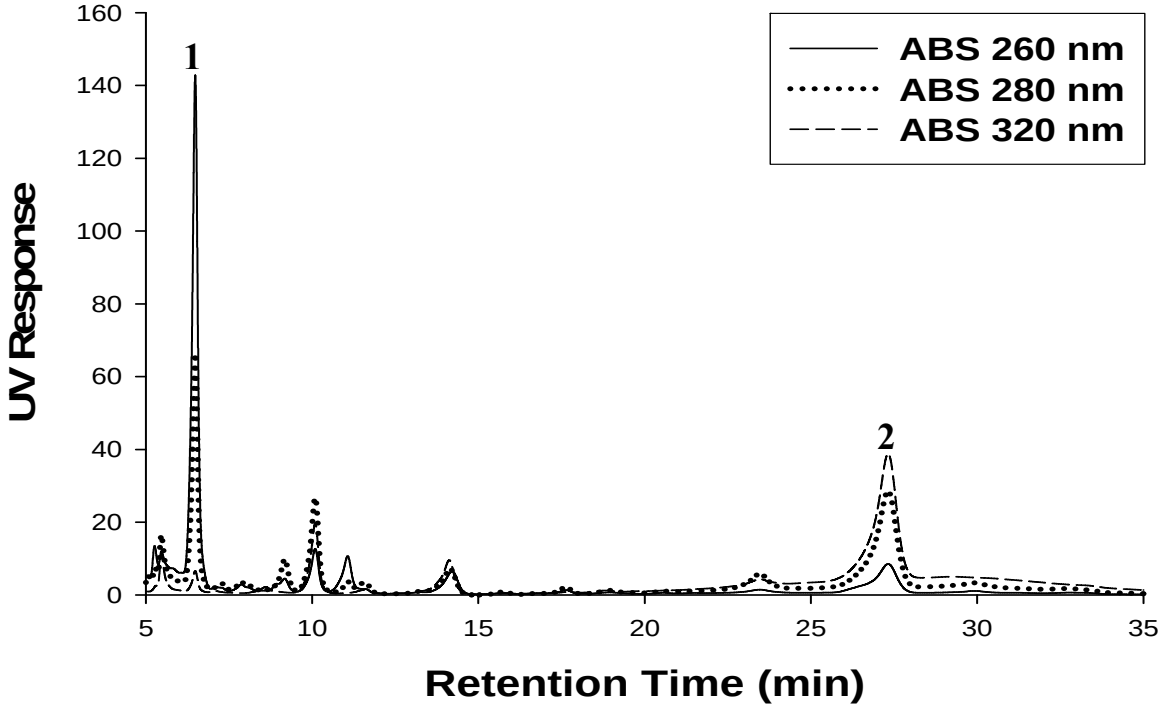
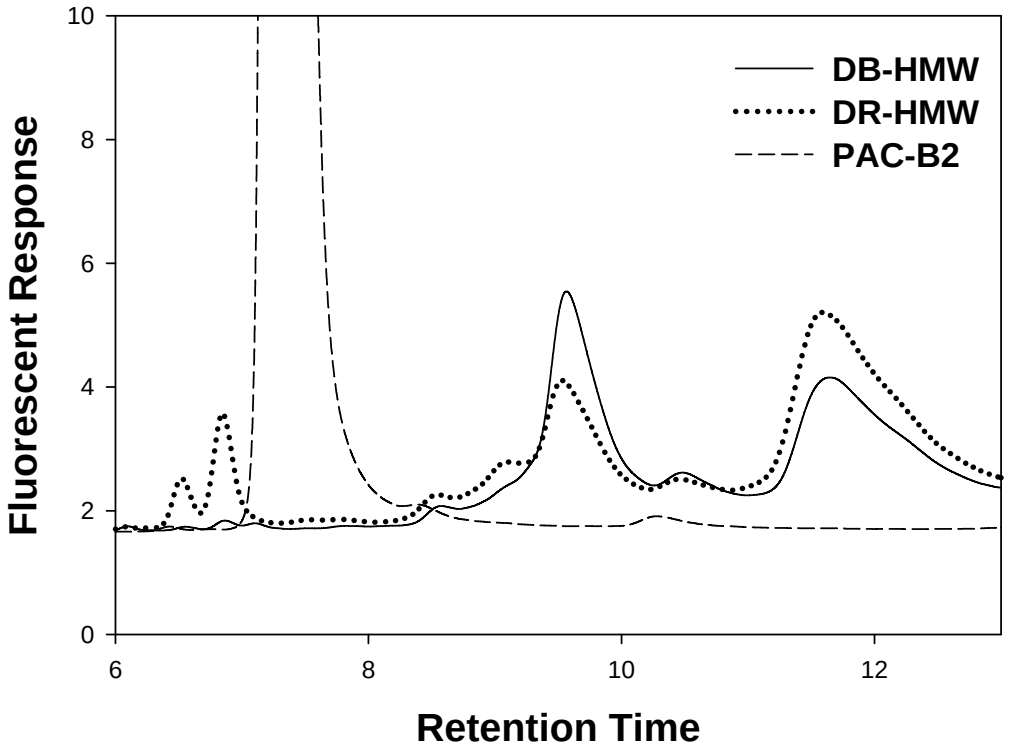


Figure 5.5(top) and 5.6(bottom)


Fluorescent Response
Retention Time
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2
4
6
8
10
6
8
10
12

Figure 5.7

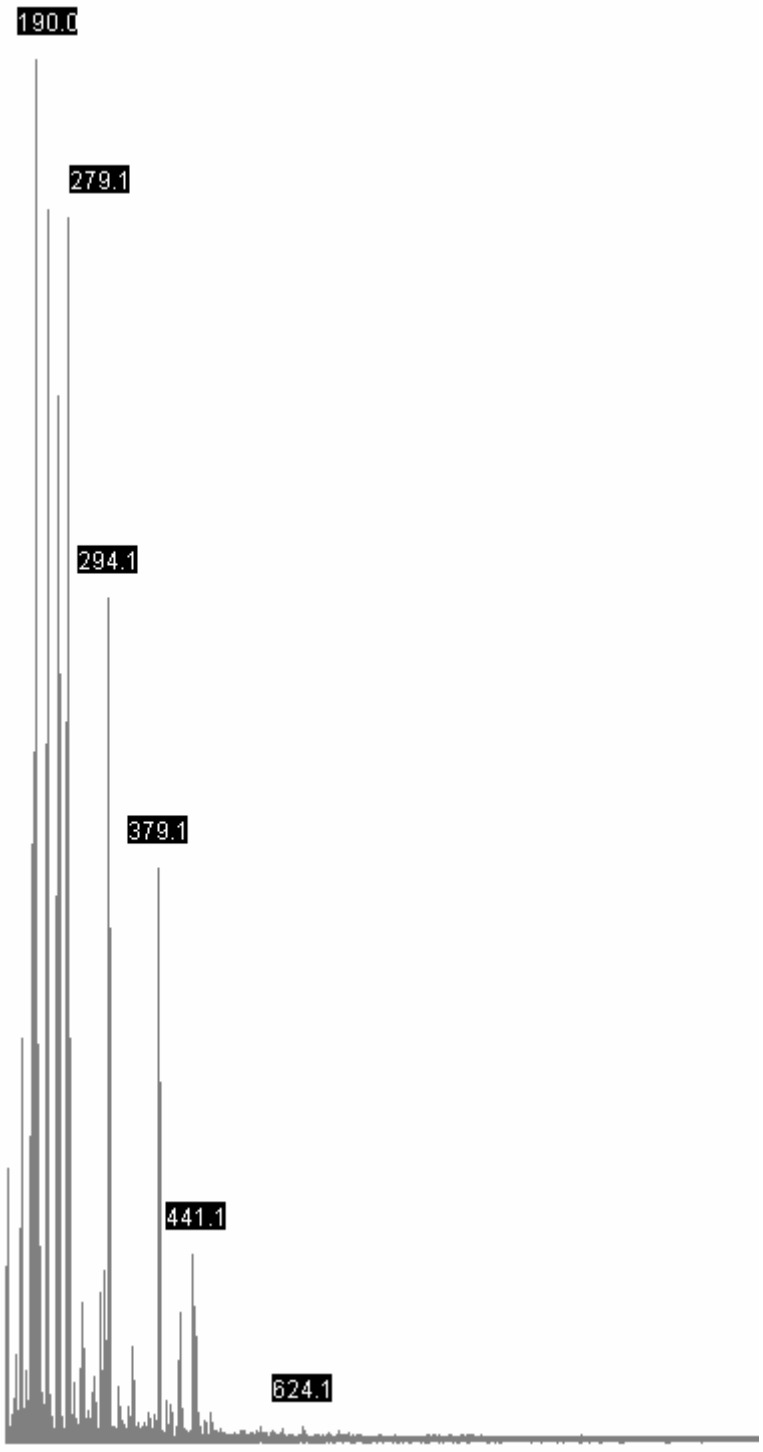

Figure 5.8

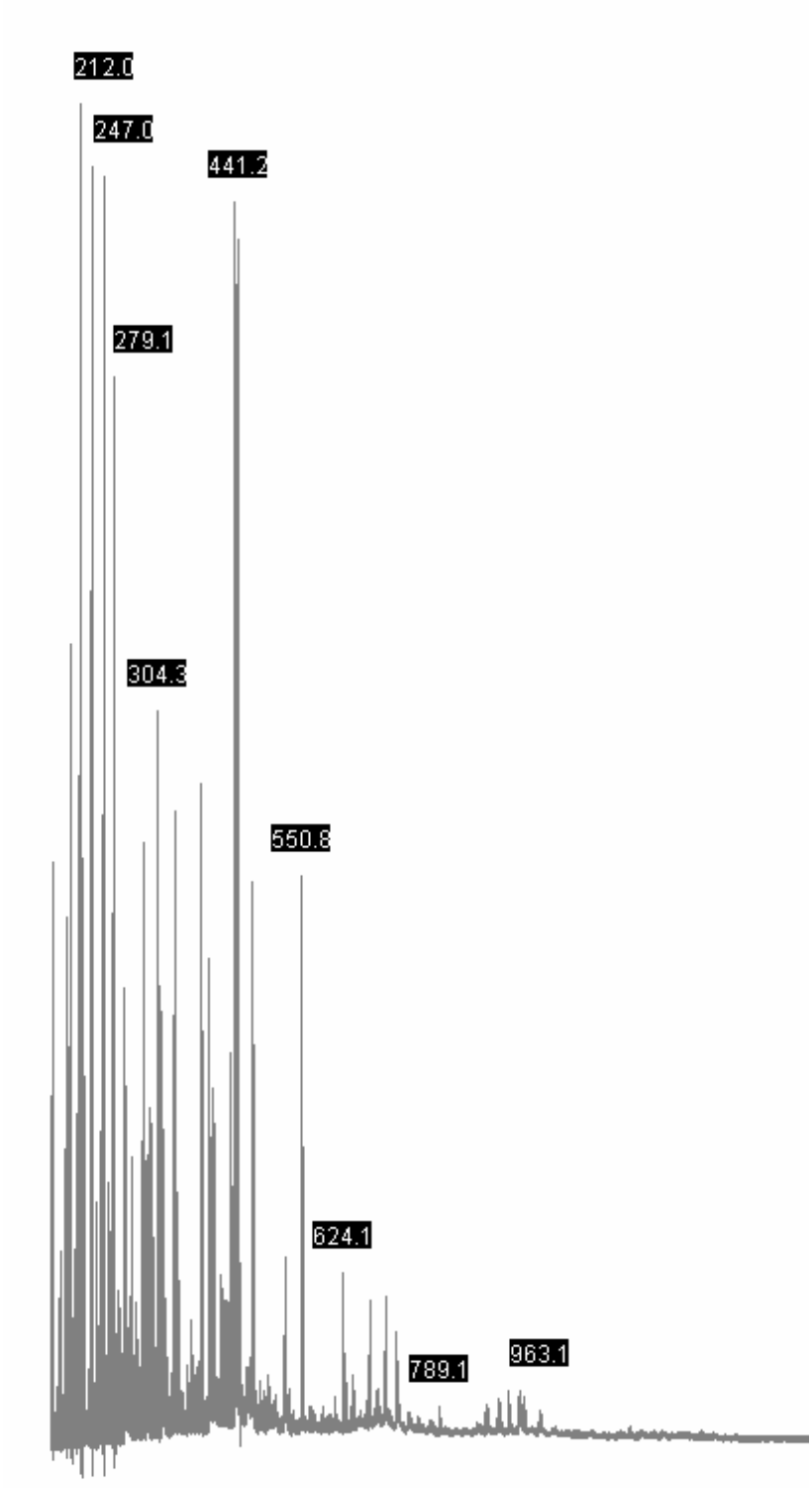

Figure 5.9

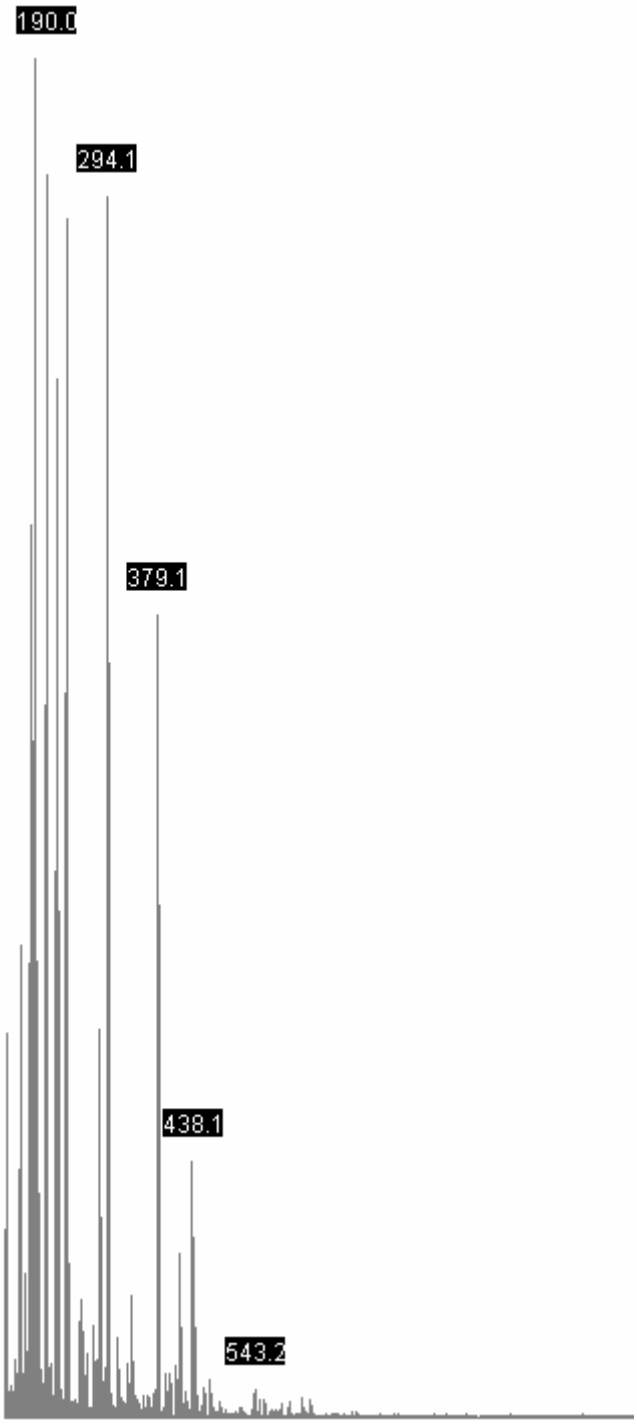

Figure 5.10

CHAPTER 6

SUMMARY AND CONCLUSIONS

This research provided new insight as to the contents and diversity of phenolic compounds in peanut kernels and skins, as well as the fate of phenolics through processing methods such as dry-blanching, dry-roasting, and oil-roasting. Included in the phenolic profiles of peanut kernels and skins are a wide variety of phenolic species ranging from low-molecular-weight (LMW) phenolic acids to high-molecular-weight (HMW) tannins. The overall trend observed was that phenolic acid aglycones (in the case of LMW fractions) and catechin monomers (in the case of the tannins) are being released from their parent compounds through thermal processing. Although their chemistry is altered; however, they still maintain much of their antioxidant/radical-scavenging capacities as demonstrated by various *in vitro* antioxidant, radical-scavenging, and biological activity assays. With the knowledge gained from this research, the peanut industry can maximize the efficiency of production by utilizing peanut skins as well as optimize processing conditions for the preservation of beneficial phytochemicals in peanuts. This will then translate toward beneficial marketing strategies for peanut consumption, advance the marketability of the crop, and provide a definitive case for peanut inclusion into functional foods for health.

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