Characterization and Applications of Peanut Root Extracts

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Abstract

Lipid oxidation is one of the leading causes of food quality degradation. Manufacturers typically add antioxidants or purge a product's package of oxygen to inhibit oxidation and the resulting off-flavors. Synthetic antioxidants (e.g. BHT, BHA) and some natural antioxidants (e.g. α-tocopherol) have found widespread use in this application. Unfortunately, the public views synthetic additives in a negative light and the current natural antioxidants have been unable to match the protection afforded by the synthetic antioxidants. The search for underutilized and natural antioxidants has led scientists to investigate many different plant-based extracts for use in food and in the treatment and prevention of disease. The objectives of this research were (1) to use ORAChromatography to identify peanut root extract fractions with high antioxidant capacity, (2) identification of compounds in peanut root extracts using HPLC and mass spectrometry, (3) test for the presence of aflatoxins in the extracts, (4) test peanut root extract in food model system for oxidation reduction capabilities, and (5) Testing peanut root extract's ability to decrease protein oxidation in cell culture.

Crude peanut root extracts have high antioxidant activities that do not vary by cultivar. The ORAC activities of the peanut root fractions separated by HPLC with a C18 column varied (600.3 – 6564.4 μM TE/g dry extract), as did the total phenolic contents (23.1 – 79.6 mg GAE/g dry extract). Peanut root fractions had aflatoxins contamination well above the 20 ppb limit. Peanut root extracts and the known antioxidants tested were found to have no significant effect in inhibiting oxidation of peanut paste or HBMEC. Peanut root extracts were not shown to have any positive effects, but further research is necessary to eliminate peanut root extracts as a possible food ingredient and health supplement.

Dedication

I would like to dedicate this to all of those who have passed before me that made the path easier to walk and helped shape who I am.

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LIST OF ABBREVIATIONS AND TERMS

AAPH	2,2’-azobis (2-methylpropionamidine) dichloride
ABAP	2,2’-azobis 2-amidinopropane dichloride
ABTS	2,2’-azinobis 3-ethylbenzothiazoline-6-sulfonic acid
ADI	acceptable daily intake
ANOVA	analysis of variance
BHA	2-tert-butyl-4-hydroxyanisole
BHT	3,5-di(tertiary-butyl)4-hydroxytoluene
CAR	carboxene
CoA	coenzyme A
DAD	diode array
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DPPH	2,2-diphenyl-1-picrylhydrazyl
DVB	divinylbenzene
EPR	electron paramagnetic resonance
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FRAP	ferric reducing antioxidant power
FTIR	Fourier transform infrared spectroscopy
GAE	gallic acid equivalents
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
HBMEC	human brain microvascular endothelial cells
HBSS	Hank’s balanced salt solution
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HSD	honestly significant difference
LDL	low-density lipoprotein

M	molar
MS	mass spectroscopy
MTT	dimethyl thiazolyl diphenyl tetrazolium
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	nuclear magnetic resonance
ORAC	oxygen radical absorbance capacity
PAD	pulsed amperometric detector
PDMS	poly (dimethylsiloxane)
PG	propyl gallate
PGF	prostaglandins
ppb	parts-per-billion
ppm	parts-per-million
RPMI	Roswell Park Memorial Institute medium
SPME	solid phase microextraction
TAA	total antioxidant activity
TBARS	thiobarbituric acid reactive substances
TBHQ	*tert*-butylhydroquinone
TCA	trichloroacetic acid
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TLC	thin layer chromatography
TOF	time of flight
TPTZ	ferric 2,4, 6-tripyridyl-*s*-triazine
TRAP	total radical-trapping antioxidant parameter
TSWV	tomato spotted wilt virus
USDA	United States Department of Agriculture
UV	ultraviolet
v/v	volume/volume
Vis	visible

CHAPTER 1: INTRODUCTION

Oxidation is a necessary evil to living things. Metabolism, cell signaling, destruction of pathogens, and many other cellular processes rely on oxidation and its reactive products. However, when reactive species go unchecked, they can cause numerous health ailments such as atherosclerosis, diabetes, reperfusion injuries, cancer, etc. Endogenous and dietary antioxidants act as the cell's defense against an imbalance of reactive species. Oxidation also plays a primary role in food quality. Lipid oxidation is one of the leading causes of food quality degradation (Nawar 1996). Manufacturers typically add antioxidants or purge a product's package of oxygen to inhibit oxidation and the resulting off-flavors. Synthetic antioxidants (e.g. BHT, BHA) and some natural antioxidants (e.g. α-tocopherol) have found widespread use in this application. Unfortunately, the public views synthetic additives in a negative light and the current natural antioxidants have been unable to match the protection afforded by the synthetic antioxidants (Georgantelis and others 2007). The worry of health implications from synthetic antioxidants is not without some evidence that suggests that BHT and others may be toxic. The search for underutilized and natural antioxidants has led scientists to investigate many different plant-based extracts for use in food and in the treatment and prevention of disease.

Phytoalexins account for much of the health and antioxidant benefits provided by many plants. Phytoalexins are compounds that are produced when a plant cell is under attack and are used to thwart the attack (Purkayastha 1995). The elicitation of phytoalexin synthesis can be induced by numerous factors such as microbes, fungi, UV light, and heavy metal salts (Rao and Strange 1995). These stressors activate the shikimic-polymalonate acid pathway that synthesizes flavonoids and stilbenes (Rao and Strange 1995). The phytoalexins produced then proceed to defend against the threat by attacking the invading cell.

Some of these phytoalexins have been shown in cell culture to have beneficial effects. One of the most well known phytoalexins present in foods is *trans*-resveratrol, which has been found in wine, peanuts, and grapes. *In vitro* experiments with resveratrol have shown it to be a vasodilatory agent and inhibit cancer among other effects in various cell culture lines (Jang and others 1997; Soleas and others 2002; Delmas and others 2003; Signorelli and Ghidoni 2005; Baur and others 2006). Resveratrol may play a role in the "French Paradox" (Kopp 1998). The "French Paradox" is the theory that people living around the Mediterranean Sea consume diets high in saturated fat, but have a lower prevalence of heart disease compared to groups with lower

saturated fat intakes (Renaud and Debord 1992). Their high fat diet is believed at least in part, to be counteracted by their ingestion of red wines high in *trans*-resveratrol (Renaud and Debord 1992; Soleas and others 2002; Pignatelli and others 2006). Resveratrol is certainly not the only phytoalexin shown to have human health implications. Soleas and fellow researchers (2002) investigated the anticarcinogenic effects of catechin, resveratrol, quercetin, and gallic acid. All of these compounds are found in red wine. They applied the compounds topically to mice that had chemically induced skin cancer. The authors discovered that quercetin was the most effective at inhibiting the cancer and gallic acid was the least effective of the listed compounds. Resveratrol is effective at the three different stages of cancer (initiation, promotion, and progression) (Jang and others 1997). The exact mechanism that resveratrol inhibits cancer is unknown, but it is believed to affect the cell signaling pathways (Kundu and Surh 2004). Anderson and colleagues (2004) isolated 3 A-type catechin/epicatechin oligomers from cinnamon that are thought to be responsible for the insulin enhancing activity. These same compounds have also been identified in peanut skins. Anderson *et al*. used the epididymal fat cell assay to determine the insulin enhancing effect. The compounds isolated had insulin enhancing activity. Many of our popular drugs were first extracted from plants (Heinrich and others 2004) and the search for medicine from plants continues to this day.

Peanut farmers, processors and industry produce a large amount of waste including peanut roots, hulls and skins. The roots are usually tilled back into the soil after harvest and the hulls and skins are used for animal feed or burned for energy. These waste products can usually be bought for, at most, pennies a pound. Recent research has shown these waste products to be an excellent source of natural phytochemicals including resveratrol, catechins, and caffeic acid (Yu and others 2006; Medina-Bolivar and others 2007). Little research has explored peanut root extracts and what compounds are present in them. If a commercial use can be developed for the waste products, then peanut roots could add to a farmer's profit per acre and would no longer be "waste."

Specific Aims

I. Use ORAChromatography to identify peanut root extract fractions with high antioxidant capacity.

The crude peanut root extract of different cultivars will be analyzed via Oxygen Radical Absorbance Capacity (ORAC) to test for significant differences in antioxidant activity. Crude extracts exhibiting high ORAC values will then be fractionated with HPLC. ORAC will be used to identify fractions with high antioxidant capabilities. This will allow a focusing of further analysis on the fractions with high antioxidant capacities.

Null hypothesis: There is no difference in antioxidant capacities among cultivars or fractions.

Alternative hypothesis: There is a difference in antioxidant capacities among cultivars or fractions.

II. Identification of compounds in peanut root extracts using HPLC and mass spectrometry.

Fractions identified in Aim I as having high antioxidant capabilities will be further separated. Individual peaks will be collected and then analyzed using mass spectroscopy. This will allow identification of compounds that are responsible for the antioxidant capabilities discovered in Aim I.

III. Test for the presence of aflatoxins in the extracts.

The peanut root extracts will be analyzed for aflatoxins to ensure consumer safety in future applications.

Null hypothesis: There are no aflatoxins in the peanut root extracts.

Alternative hypothesis: There are aflatoxins in peanut root extracts.

IV. Test peanut root extract in food model system for oxidation reduction capabilities.

The peanut root extracts will then be introduced into a food system prone to oxidation in order to test a possible application of these extracts. Peanut butter will be the test model systems. Crude peanut root extracts and active fractions will be utilized.

Null hypothesis: The root extracts have no effect on the oxidation of the model food system.

Alternative hypothesis: The root extracts increase or decrease the rate of oxidation in the model food systems.

V. Testing peanut root extract's ability to decrease protein oxidation in cell culture.

Many diseases are caused or exacerbated by oxidation. Protein oxidation is believed to be a key factor in the progression of Alzheimer's disease. Peanut root extracts will be tested in cell culture for inhibition of protein oxidation. Crude peanut root extracts and active fractions will be utilized. If they are shown to inhibit protein oxidation, then peanut root extracts have possible health applications.

Null hypothesis: The root extracts have no effect on the rate of oxidation in cellular proteins.

Alternative hypothesis: The root extracts increase or decrease the rate of oxidation in cellular proteins.

Rational and Significance

The public and some in scientific community are skeptical of the safety of synthetic antioxidants. This skepticism has led to the search for natural alternatives to BHT, BHA, TBHQ, etc. Very few of the compounds present in peanut roots have been identified and investigated for antioxidant activity. Peanut roots have been largely ignored as a source of natural antioxidants and the extracts have not been tested in a shelf-life model. Aflatoxin concentrations have not been measured in peanut roots or their extracts. Although several other plant extracts have been shown to decrease oxidative markers in cell culture and *in vivo*, no research has examined peanut root extracts' effect on a biological system. Cell culture allows basic research to be undertaken before moving on to animal experiments.

Literature Cited

Anderson RA, Broadhurst CL, Polansky MM, Schmidt WF, Khan A, Flanagan VP, Schoene NW, Graves DJ. 2004. Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. J Agric Food Chem 52(1):65-70.

Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. 2006. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444(7117):337-42.

Delmas D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N, Solary E. 2003. Resveratrol-induced apoptosis is associated with fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. J Biol Chem 278(42):41482-90.

Georgantelis D, Ambrosiadis I, Katikou P, Blekas G, Georgakis SA. 2007. Effect of rosemary extract, chitosan and a-tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4 DegC. Meat Sci 76(1):172-81.

Heinrich M, Barnes J, Gibbons S, Williamson E. 2004. Fundamentals of pharmacognosy and phytotherapy. Edinburgh: Churchill Livingstone.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275(5297):218-20.

Kopp P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur J Endocrinol 138(6):619-20.

Kundu JK, Surh Y-J. 2004. Molecular basis of chemoprevention by resveratrol: NF-kB and AP-1 as potential targets. Mutat Res 555(1-2):65-80.

Medina-Bolivar F, Condori J, Rimando A, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan M. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003.

Nawar W. 1996. Lipids. In: Fennema O, editor. Food Chemistry. 3rd ed. New York: Marcel Dekker, Inc.

Pignatelli P, Ghiselli A, Buchetti B, Carnevale R, Natella F, Germano G, Fimognari F, Di Santo S, Lenti L, Violi F. 2006. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. Atherosclerosis 188:77-83.

Purkayastha RP. 1995. Progress in phytoalexin research during the past 50 years. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 1-39.

Rao PVS, Strange R. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 199-227.

Renaud S, Debord S. 1992. Wine, alcohol, platelets and the french paradox for coronary heart disease. Lancet 339:1523-6.

Signorelli P, Ghidoni R. 2005. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. J Nutr Biochem 16(8):449-66.

Soleas G, Grass L, David Josephy P, Goldberg D, Diamandis E. 2002. A comparison of the anticarcinogenic properties of four red wine polyphenols. Clin Biochem 35:119-24.

Yu J, Ahmedna M, Goktepe I, Dai J. 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. J Food Compos Anal 19:364-71.

CHAPTER 2: REVIEW OF LITERATURE

Plants produce different chemicals in the course of their lives for many different reasons. One class of these compounds is the phytoalexins. Phytoalexins are compounds that are produced when a plant cell is under attack and are used to thwart the attack (Purkayastha 1995). The elicitation of phytoalexins can be induced by numerous factors such as microbes, fungi, UV light, and heavy metal salts. These stressors activate the shikimic-polymalonate acid pathway to create flavonoids and stilbenes (Rao and Strange 1995). The phytoalexins produced then proceed to defend against the threat by attacking the invading cells.

Some of these phytoalexins have been shown in cell culture to have beneficial effects. One of the most well known phytoalexins present in foods is *trans*-resveratrol, which has been found in wine, peanuts, and grapes. *In vitro* experiments with resveratrol have shown it to be a vasodilatory agent and inhibit cancer and oxidation among other effects (Jang and others 1997; Soleas and others 2002; Delmas and others 2003; Signorelli and Ghidoni 2005; Baur and others 2006; Ungvari and others 2007). Resveratrol may play a role in the "French Paradox" (Kopp 1998). The "French Paradox" is the observation that people living around the Mediterranean Sea consume diets high in saturated fat, but have a lower prevalence of heart disease compared to groups with lower saturated fat intakes (Renaud and Debord 1992). Their high fat diet is believed at least in part, to be counteracted by their ingestion of red wines high in *trans*-resveratrol (Renaud and Debord 1992; Soleas and others 2002; Pignatelli and others 2006). Resveratrol is certainly not the only phytoalexin shown to have human health implications. Many of our popular drugs were first extracted from plants (Heinrich and others 2004) and the search for medicine from plants continues to this day.

Why plants produce phytoalexins?

Phytoalexins are produced when the plant experiences stress. This can be elicited by an actual fungal or microbial infection or in the lab with compounds derived from fungi/microbes such as proteins and polysaccharides (Chung and others 2003; Rowland and others 2005; Medina-Bolivar and others 2007). Phytoalexins can also be induced by UV light, heavy metal salts, and other environmental stressors (Barz and others 1990; Chung and others 2003). Rudolf and Resurreccion (2005) investigated abiotic resveratrol elicitors in peanut seeds. They

specifically looked at postharvest techniques such as UV light, size reduction method, and ultrasound. The peanut seeds were reduced in size by various methods and then subjected to one of the abiotic stresses. Rudolf and Resurreccion found that these postharvest abiotic stresses caused an increase in *trans*-resveratrol concentrations over peanut seeds not treated. However, these treatments did not increase the overall phenolic content or antioxidant activity.

The shikimic-polymalonate acid pathway (figure 2.1), involved in phytoalexin synthesis, begins with phenylalanine, which is transformed into cinnamic acid via phenylalanine ammonia-lysase. Cinnamic acid is then transformed into 4-coumaroyl-CoA via cinnamate hydroxylase and 4-coumarate: CoA ligase. The product of this reaction reacts with three molecules of malonyl-CoA. This product can then rearrange itself to form different compounds (Rao and Strange 1995). Once created, the phytoalexins are secreted into the extracellular space. There the phytoalexins can inhibit microbial/fungal invasion. However, the phytoalexins face the threat of being broken down or used in synthesis of polymers by peroxidases (Barz and others 1990). This may be one explanation for the low concentration of phytoalexins in tissue, unless the cells are producing them at a high level induced by stress.

The mechanism by which phytoalexins inhibit fungal growth is not fully understood, but one idea has gained support. Phytoalexins disrupt the cellular membranes and lower the respiration rate of the fungal cells. Pezet and Pont (1995) found that stilbenes, which are a type of phytoalexin, almost immediately slow down the cell's respiration. They believe that stilbenes attacked cellular membranes in the order of mitochondria, nucleus, endoplasmic reticulum, and finally the cytoplasmic membrane. Pezet and Pont conclude that the stilbenes cause their damage by impeding the action of flavin enzymes, which allows lipid peroxidation to proceed unchecked, thereby irreparably damaging the cell membranes.

The level of phytoalexin production can possibly be used to determine the disease resistance of a particular genotype of peanut plant. Sobolev and fellow researchers (2007) looked into this theory and found support for it. Sobolev et al. looked at five different cultivars of peanuts grown in Georgia and found that the cultivars that had a high disease resistance to tomato spotted wilt virus and late leaf spot produced the most phytoalexins in the pods when they were damaged by the lesser cornstalk borer. *trans*-Resveratrol was one of the phytoalexins found in substantial concentrations. They also measured *trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, and *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene. Sobolev's research

group only measured the phytoalexin content in the pods. Another study found that the less resistant plants made less phytoalexins in the initial 24 hours of stress compared to the high resistant plants (Rao and Strange 1995).

Phytoalexins in Peanut Plants

Compounds found in peanut plants include *cis*-resveratrol, *trans*-resveratrol, 4-isopentenylresveratrol, arachidins, medicarpin, *p*-coumaric acid, caffeic acid, ferulic acid, demethylmedicarpin, daidzein, formononetin, isoflavonones, nonyl phenol, and dienols (Keen and Ingham 1976; Aguamah and others 1981; Arora and Strange 1991; Rao and Strange 1995; Sobolev and others 2006a). As mentioned previously, these compounds are typically elicited when the plants experience stress. Tomato spotted wilt virus (TSWV) is one of the major causes of stress to the peanut plant. TSWV is a very detrimental disease that affects many peanut crops, which is usually transferred by thrips. Thrips affect a large percentage of the peanut crop (Herbert and others 2007).

Some of these compounds are found in numerous parts of the peanut plant. Resveratrol has been found in all parts, but is most concentrated in the leaves, hulls, and roots with less being found in the seeds and skins. Chung and fellow researchers (2003) found 1.19 μg/g of resveratrol in peanut roots, 2.05 μg/g in peanut leaves, and 1.34 μg/g in the hulls. They also measured resveratrol in the skins and seeds and found 0.05 and 0.06 μg/g respectively. Chung and colleagues measured these levels in field-grown peanut plants.

Hulls

Lin and fellow researchers (1999) found luteolin in peanut hulls along with 3',4',5,7-tetrahydroxy-8-(3-methylbut-2-enyl)flavone, chrysoeriol, 4',5-dihydroxy-3'-methoxy-2'',2''-dimethylpyrano[5'',6'':7:8]flavone, 4',5,7-trihydroxy-3'-methoxy-8-(3-methylbut-2-enyl)flavone, and eriodictyol (figures 2.2-2.5). Yen and Duh have several publications regarding peanut hulls, antioxidants and their activities. In 1992, Yen and Duh partnered with Yeh and investigated peanut hull extracts (Duh and others 1992). They reduced peanut hulls by pulverizing them in a mill and extracted them with chloroform, acetone, ethanol, methanol, or *n*-hexane (2.5 g hull powder: 50 mL solvent) overnight with shaking. Yen *et al*. utilized the thiocyanate method to measure the antioxidant activity of the hull extracts. They discovered that

methanol hull extracts exhibited the highest antioxidant activity. The methanol hull extracts had higher antioxidant activities then either α-tocopherol or BHA as measured by this system. In another study, Yen and Duh found that between Spanish, Valencia, Runner and Virginia peanut hulls, the Spanish peanut hulls had the highest luteolin content (3.16 mg/g of hulls) and the highest total phenolic compounds (10.2 mg/g of hulls) (Yen and Duh 1995). The Valencia peanut hulls had similar levels of luteolin and total phenolic compounds. Yen and fellow researchers (1993) examined the relationship between peanut hull maturity and antioxidant activity. They also found that total phenolics and luteolin content was positively correlated with hull maturity. Yen *et al*. tested methanolic extracts of the hulls in retarding oxidation of linoleic acid. They discovered that the extracts have a high antioxidant activity and effectively reduced the oxidation of linoleic acid. Daigle and colleagues (1988) investigated the correlation between the flavonoids in the hulls and peanut maturity. They hypothesized the color of the mesocarp of the shell was directly correlated with the flavonoid content (the darker the hull, the higher the flavonoid content). Daigle *et al*. looked at the concentration of 5,7-dihydroxychromone, luteolin, and eriodictyol via HPLC. They found luteolin to have the highest concentration in mature peanut hulls representing about 6 mg/g. The three compounds had increasing concentrations with increasing with hull color, thus confirming their hypothesis. In a follow up study, Yen and Duh (1993) changed the temperature, atmosphere (air or nitrogen), pH, concentration of extract, and added different known antioxidants to test for synergistic effects in their linoleic acid system. They found no synergistic effects or effects from atmosphere and only a small decline in antioxidant power with an increase of temperature. Yen and Duh found that pH positively affected the antioxidant power of the methanolic extracts as did increasing concentrations of the extract. In another study, Yen and Duh (1994) utilized DPPH to test the antioxidant capacity of their hull extracts. They found that the methanolic extracts inhibit oxidation about the same as BHT and catechin. Yen and Duh also used EPR to explore the effect that hull extracts had on hydroxyl radicals. EPR demonstrated that increasing concentrations of hull extract was directly correlated with the percent of hydroxyl radical scavenged. The highest concentration tested in the hydroxyl model system was 24 mg hull extracts, which scavenged approximately 71% of the hydroxyl radicals.

Dean and colleagues (2008) performed a 50% aqueous acetone extraction on peanut hulls, peanut roots, and peanut leaves and then analyzed the extracts by HPLC-TOF-MS and

ORAC. They identified aloin, dihydroxycoumarin, peonidin-3-O-glucoside, syringin, resveratrol, trihydroxyflavone, fustin, 3-O-p-coumaroylquinic acid, luteolin, chlorogenic acid, linolenic acid, linoleic acid, palmitic acid and oleic acid in peanut hulls. The ORAC data revealed the leaves to have the highest ORAC value (510 M Trolox g^{-1}), followed by peanut roots (207 M Trolox g^{-1}), and finally the hulls (86 M Trolox g^{-1}).

Peanut Skins

Peanut skins have been studied for their antioxidant activity and possible applications. The first published study regarding peanut skin extracts performed a methanol extraction and then utilized the extract in sunflower oil (Nepote and others 2000). The extracts were added at 1.8 mL (122.4 mg of phenols) in 150 g of oil. The oil containing the peanut skin extract had significantly less oxidation then the control, but was outperformed by BHT. Nepote and colleagues (2002) compared the antioxidant extraction of methanol, ethanol, acetone, and aqueous extracts of peanut skins. They found that the methanol, ethanol, and acetone extracts extracted the highest levels of phenolics and antioxidants as measured by Folin-Ciocalteu total phenolics method and DPPH respectively. Nepote and fellow researchers also measured the effectiveness of these extracts to combat oxidation with the DPPH assay and a model food system of sunflower oil. The methanol and ethanol extracts had the highest DPPH scavenging activity of 93% at a concentration of 10 μg/mL. There was not a significant difference between these extracts and their defatted counterparts. The sunflower oil portion of this experiment revealed that the peanut skin extracts did not reduce oxidation as much as BHT, but they did reduce oxidation more then the negative control. Nepote and colleagues (2005) performed a follow-up study that looked at optimizing the extraction process by manipulating the concentration of ethanol in an aqueous solution, size of the peanut skin particles, time, ratio of skins to solvent, and number of extractions. They measured the total phenolics content with the Folin-Ciocalteu method and utilized DPPH to assess the antioxidant activity. They found that the optimal extraction occurred with a 70% ethanol aqueous solvent, with uncrushed skins, with 10 minutes of shaking, 20 mL of solvent for every gram of skins, and three extraction stages. This combination resulted in a yield of 0.118 g of phenolic compounds per gram of skins.

Wang and fellow researchers (2007) analyzed 50% aqueous ethanol defatted peanut skin extracts for total phenolics (0.097 g GAE/g of skin) and total flavonoids (0.065 g catechin

equivalents/ g of skin). They also tested the DPPH activity, chelating activity, hydroxyl radical scavenging, superoxide anion scavenging, hydrogen peroxide scavenging, and the ability to protect erythrocytes against peroxyl radicals at an extract concentration of 500 μg/mL. Peanut skin extracts scavenged 97.1% DPPH, 98.6% superoxide anion, 89.1% hydrogen peroxide, 85.3% hydroxyl radical, chelated 76.6% of Fe^{2+}, and inhibited 98.6% of damage to erythrocytes by peroxyl radicals.

As the previous studies have shown, there are many parameters that affect the antioxidant activity of the peanut skin extract collected. Yu and fellow researchers (2005) looked at the skin removal methods and the solvent effects on the total phenolics of peanut skin extract. They tried skins from hand-peeling raw peanuts, hand-peeling blanched peanuts, and hand-peeling skins from peanuts heated at 175°C for 5 minutes. They utilized water, 80% methanol aqueous and 80% ethanol aqueous as solvents. The skins were extracted overnight with 20 mL of solvent and 0.25 g of peanut skins. Yu *et al*. used the Folin-Ciocalteu to determine which method extracted the highest amounts of phenolics and total antioxidant activity (TAA) to measure the antioxidant activity. They concluded that roasting the peanuts prior to peeling the skin and extracting with aqueous ethanol results in the highest extraction of phenolic compounds and the highest antioxidant activity. Yu and colleagues (2006) furthered this research by identifying some compounds by LC-MS and employed DPPH to determine the antioxidant activity of the skin extracts. In this study, they used 80% aqueous ethanol for extraction. They homogenized 30 mL of solvent with 1 g of skin powder for 2 minutes and then centrifuged for 15 minutes at 3,000*g*. Yu *et al*. identified catechins, A-type procyanidins, and B-type procyanidins (figures 2.6 and 2.7). They also observed peaks that they believe to be caffeic acid, chlorogenic acid, resveratrol, and the resveratrol glycoside, piceid. According to the DPPH analysis, the peanut skin extracts from roasted and raw peanuts had a higher antioxidant activity then Trolox or vitamin C. Yu and colleagues also measured total phenolics by the Folin-Ciocalteu method and found that blanching reduces total phenolics by 89%. This work carried out by Yu *et al.* emphasizes the important relationship that processing can have on natural antioxidant extracts.

Lou and colleagues (1999) have also worked in this area and have identified other compounds. In this study, they identified six different A-type proanthocyanidins from peanut skin extracts. They identified epicatechin-(2β→O→7, 4β→6)-catechin, epicatechin-(2β→O→7, 4β→6)-*ent*-catechin, epicatechin-(2β→O→7, 4β→6)-*ent*-epicatechin, proanthocyanidin A-1,

proanthocyanidin A-2, and epicatechin-(2β→O→7, 4β→8)-*ent*-epicatechin. In a following paper, Lou and fellow researchers (2001) identified 8 flavonoids and 2 indole alkaloids (figures 2.8 and 2.9). They found quercetin 3-O-[2-O-β-xylopyranosyl-6-O-α-rhamnopyranosyl]-β-glucopyranoside, isorhamnetin triglycoside, isorhamnetin 3-O-[2-O-β-xylopyranosyl-6-O-α-rhamnopyranosyl]-β-glucopyranoside, rutin, isorhamnetin-3-O-rutinoside, quercetin-3-O-β-glucopyranoside, 3',5,7-trihydroxyisoflavone-4'-methoxy-3'-O-β-glucopyranoside, and luteolin. Most of these compounds showed high antioxidant capacity according to a DPPH assay. In 2004, Lou and colleagues (2004) identified five proanthocyanidins in peanut skins. They discovered epicatechin-(2β→O→7, 4β→6)-[epicatechin-(4β→8)]-catechin, epicatechin-(2β→O→7, 4β→8) epicatechin-(4β→8)-catechin-(4α→8)-epicatechin, procyanidin B2, procyanidin B3, and procyanidin B4.

Seeds

Another part of the peanut plant which has garnered some attention in relation to its antioxidant concentration is the peanut seed. Peanut seeds contain approximately 50% lipids. It is no surprise then that α-tocopherol is found within. Kornsteiner, Wagner, and Elmadfa (2006) compared the tocopherols in 10 different nuts and found that peanuts had an average of 6.1 mg of α-tocopherol in 100 g of extracted oil. In addition to tocopherols, Kornsteiner *et al*. measured the total phenolics of the nuts. They extracted the nuts by chopping, then adding a 75% acetone/ 25% aqueous sodium metabisulfite (526 μmol/L) solution. The total phenolics was then determined with the Folin-Ciocalteu method. The total phenolics contents ranged from 32 mg GAE/ 100 g fresh weight to 1625 mg GAE/ 100 g fresh weight among the different nuts. Peanut seeds had an average of 420 mg GAE/ 100 g fresh weight.

Several phytoalexins have been identified including dihydroquercetin and ethyl protocatechuate. Huang and colleagues (2003) investigated kernel extracts using different solvents and tested them in a linoleic acid system. Methanol and ethanol extracts performed very well at inhibiting oxidation of the linoleic acid with the ethanol extract having the highest antioxidant activity. The extracts listed from highest antioxidant activity to lowest is: ethanol > methanol > acetone > ethyl acetate > hexane. They fractionated the ethanol extract into 17 fractions using silica gel column chromatography. These fractions were then tested in the linoleic acid model system and found that no fractions were as active as the whole at retarding

oxidation. However, Huang *et al*. further investigated the fraction with the highest antioxidant activity and identified ethyl protocatechuate (3,4-dihydroxybenzoic acid ethyl ester), which they attributed as having antioxidant activity as measured by a TLC/linoleic acid method.

Resveratrol has also been found at measurable levels in peanut seeds. Sanders and colleagues (2000) found levels ranging from 0.02-1.70 μg/g of peanut. Another group measured the concentration of resveratrol by HPLC in fourteen commercial peanut butters and found resveratrol levels ranging from 0.265 μg/g to 0.753 μg/g with natural peanut butters containing more resveratrol on average (Ibern-Gomez and others 2000). Ibern-Gomez and colleagues also measured levels of *trans*-piceid (0.067 μg/g to 0.225 μg/g) in the same commercial peanut butters. Chang and colleagues (2006) attempted to increase natural levels of resveratrol found in peanut seeds. Chang *et al*. only found trace amounts of resveratrol prior to incubation in peanut seeds. They sliced Spanish peanut seeds and incubated (23°C to 25°C) them for 3 days with aeration in darkness. They were able to produce 147.3 μg/g of *trans*-resveratrol after 20 hours of incubation.

Chukwumah et al. (2007b) used various extraction methods to measure the level of trans-resveratrol and other phytoalexins in the peanut seed. They measured levels of resveratrol ranging from 0.9 to 19.98 μg/g. Chukwumah and fellow colleagues utilized stirring, sonication, Soxtec, and microwave-assisted sonication as methods to extract phytoalexins. They also performed different treatments on the peanut seeds prior to extraction to determine the effect of fat on phytoalexin extraction. Chukwumah et al. extracted nondefatted peanut seeds, mechanically defatted peanut seeds, and hexane-defatted peanut seeds. They found that microwave-assisted sonication yielded the highest measured levels of resveratrol followed by stirring. Chukwumah et al. also discovered that they were able to extract significantly higher concentrations of phytoalexins from defatted peanuts versus nondefatted peanuts. These results suggest that many of the published levels of resveratrol may be underestimating the true concentration found in foodstuffs and plant materials. The concentration of resveratrol found in peanut roots is much higher then the level found in the peanut seed. Chukwumah and colleagues (2007a) have also investigated the effect that processing methods (boiled, dry-roasted, oil-roasted, raw) has on the phytochemical makeup of peanuts. They found that boiled peanuts had the highest amounts of phytochemicals and were the only peanuts that resveratrol was detectable. Biochanin A and genistein were significantly higher in the boiled peanuts. These results concur

with concentrations of resveratrol that Sobolev and Cole (1999) measured in roasted and boiled peanuts. They found that roasted peanuts contained between 0.018 μg/g to 0.08 μg/g of resveratrol and boiled peanuts contained between 1.779 μg/g to 7.092 μg/g of resveratrol. It is easy to see that processing can greatly effect the concentrations of resveratrol found in peanuts.

Sobolev (2008) investigated which phytoalexins were produced in peanut seeds under duress from different fungal strains. Sobolev infected seeds with different strains of fungi and then sliced the seeds to determine where the phytoalexins were most concentrated. Resveratrol was the dominant phytoalexin after 24 hours of incubation, but after 48 hours arachidin-3 and SB-1 were the major phytoalexins. Sobolev reported that the seeds reacted similarly with different fungal strains and the highest concentrations of phytoalexins were areas of the seed nearest to the infection. Sobolev, Neff, and Gloer (2009) discovered new stilbenes in peanut seeds infected with *Aspergillus caelatus*. They named them arahypin-1 (*trans*-4'-deoxyarachidin-3), arahypin-2 (*trans*-3'-(2'',3''-dihydroxy-3''-methylbutyl) resveratrol), arahypin-3 (*trans*-4-(2'',3''-dihydroxy-3''-methylbutyl) resveratrol), arahypin-4 (*trans*-4-(2'',3''-dihydroxy-3''-methylbutyl)-4'-deoxyresveratrol, arahypin-5, and chiricanine A (*trans*-4'-deoxyarachidin-2).

Roots

The roots of the peanut plant are of great interest because they tend to show a higher infection rate then the rest of the plant. Rowland and fellow colleagues (2005) infected three different cultivars (NC-V11, ANorden, and Georgia Green) with tomato spotted wilt virus (TSWV). They found the roots had a higher rate of infection then the leaves. This higher rate of root infection may cause higher concentrations of phytoalexins in the roots then other parts of the peanut plant. Rowland *et al*. also noticed that root infection coincided with a lower photosynthesis rate (up to a 42% decrease), but root infection had a mixed effect on transpiration of different cultivars. ANorden showed an increase in transpiration, but Georgia Green and NC-V11 showed a decrease in transpiration.

Sobolev and his group (2006c) have recently identified a compound they named SB-1 (figure 1.10). SB-1 is in the stilbene family and a structure has been proposed. They have also found *p*-coumaric acid, caffeic acid and 4-methoxycinnamic acid in peanut roots (Sobolev and others 2006a). The roots had the highest concentration of 4-methoxycinnamic acid (17.5 μg/g)

compared to other parts of the peanut plant. Sobolev, Potter and Horn (2006b) investigated the root mucilage from peanut plants and identified seven prenylated stilbenes. They identified *trans*-resveratrol, medicarpin, *t*-arachidin-1, *t*-arachidin-2, *t*-arachidin-3, *t*-3'-isopentadienyl-3,5,4'-trihydroxystilbene, and 4-(3-methyl-but-1-enyl)-3,5-dimethoxy-4'-hydroxy-*trans*-stilbene, which they named mucilagin A. These compounds were identified using HPLC-Pulsed Amperometric Detector (PAD)-MS. Sobolev and fellow colleagues (2006a) examined seedlings and identified (concentrations in parenthesis) resveratrol (0.06 μg/g wet weight), *p*-coumaric acid (136.8 μg/g wet weight), caffeic acid (56.7 μg/g wet weight), and 4-methoxycinnamic acid (109.6 μg/g wet weight) in the roots.

Dean and colleagues (2008) extracted 0.2 g of peanut roots with 4 mL of 50% aqueous acetone and then analyzed the extract with HPLC-TOF-MS and ORAC. They were able to identify coutaric acid, inositol hexa acetate, 5,6-dimethoxy-3-methyl-7-nitro-1-indanone, tubulosine, saverogenin, resveratrol, sakuranetin, scutellarein, hexamethoxyflavone, 5-hydroxy-4',7'-dimethoxyflavone, 3,7,4'5-methoxy-6-methylflavanone, anthricin, verdolic acid, linolenic acid, linoleic acid, palmitic acid, and oleic acid. Dean and colleagues reported an ORAC value of 207 M Trolox equivlalents per gram of root. They also measured the ORAC activity of leaf and hull extracts at the same time and found that leaf extracts had the highest ORAC value, followed by root extracts, and then hull extracts. Chakraborty and Mandal (2008) identified phenolic acids in acidified aqueous methanol peanut root extracts. They were able to identify *p*-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, caffeic acid, and protocatechuic acid by HPLC analysis and matching UV-Vis spectrum with that of known standards.

Resveratrol was first identified in peanut roots in 1976 (Ingham 1976). Levels of *trans*-resveratrol have been reported as high as 1,330 μg/g in field grown peanut roots (Chen and others 2002). Chung and fellow colleagues (2003) found 1.19 μg/g of resveratrol in peanut roots, with slightly higher amounts found in the leaves and hulls. There has been a wide range of resveratrol concentrations reported in peanut roots. This may be due to variations in analytical procedures, different plant cultivars, growing conditions and locations or yearly variations (Sobolev and others 2007). Each plant experiences different levels of stress depending on location, disease prevalence, insect prevalence, etc. The level of *trans*-resveratrol in peanut roots has not gone unnoticed. Medina-Bolivar and associates (2007) have utilized peanut roots in hairy root cultures to produce resveratrol. They were able to produce 98 μg resveratrol/g of dried

extract elicited by sodium acetate. They tested cellulose, chitosan, laminarin, sodium acetate and copper sulfate as elicitors (stressors). Sodium acetate elicited the highest concentrations of resveratrol and was used at a concentration of 10.2 mM, which this group chose from previous literature. Another group is doing similar research, but is using hydroponic peanut plants. They reported resveratrol concentrations of 0.074 mg/g in hydroponic-grown roots without intentionally stressing the peanut plants (Liu and others 2003). Little work has explored the level of resveratrol found in Virginia-type peanut roots.

Extraction methods

Choice of solvent plays an extremely important role in determining what compounds are extracted and at what concentrations. This will of course affect the antioxidant activity of the extract. Xu and Chang (2007) examined the effects of solvents on total phenolic content, total flavonoids content, condensed tannin content, DPPH activity, FRAP activity, and ORAC activity of legume extracts. They extracted the legumes with 50% aqueous acetone, 80% aqueous acetone, 70% acidified aqueous acetone, 70% aqueous ethanol, 70% aqueous methanol, and 100% ethanol. The highest total phenolics content was given by 50% acetone, followed by 70% ethanol. The highest total flavonoid content and condensed tannin content were both given by 80% acetone, followed by 70% acidic acetone. However, the results of the antioxidant assays lean towards other solvents. The highest DPPH activity was 70% methanol, followed by 70% ethanol and 70% ethanol also gave the highest FRAP and ORAC value. Ballard and fellow researchers (2009) reported that 30% aqueous methanol gave the highest ORAC values from peanut skins when extracted at 52.9°C for 30 minutes. This extraction protocol was estimated by using response surface methodology and gave an estimated ORAC activity of 2149 μM TE/g of dry skins. They tried using methanol, ethanol, and water in 4 different concentrations (0, 30, 60, 90%). Aqueous ethanol was the second best extraction solvent for high ORAC activities. In regards to total phenolics though, 30.8% ethanol is estimated to give the highest total phenolic content with an estimated total phenolics content of 118 mg GAE/g of dry skins.

Oxidation

Oxidation has been implicated in numerous diseases and degradation of biological and non-biological systems. Oxidation occurs when a molecule is robbed of an electron, hydrogen

atom or gains an oxygen atom. Oxidation is a self-propagating reaction as free radicals lead to more free radicals as shown in the example below in a free radical chain reaction (Ingold 1961):

(1) Initiation	$RH + O_2 \rightarrow R\bullet + HOO\bullet$
(2) Propagation	$R\bullet + O_2 \rightarrow ROO\bullet$
	$ROO\bullet + RH \rightarrow ROOH + R\bullet$
(3) Termination	$R\bullet + ROO\bullet \rightarrow ROOR$

Another important reaction that generates free radicals is the Fenton reaction. The Fenton reaction requires ferrous iron and hydrogen peroxide, both of which are found in biological systems (such as iron found in hemoglobin and hydrogen peroxide as a metabolic product). The result of iron being reduced with hydrogen peroxide is a hydroxyl anion (OH-) and the highly reactive hydroxyl radical (•OH) (Lloyd and others 1997).

Antioxidants are used to stop the chain reaction and inhibit the formation of highly reactive free radicals. An antioxidant donates a hydrogen atom to the free radical, thus neutralizing the free radical and forming a free radical itself. Although a free radical is formed, an antioxidant free radical is relatively stable due to electron resonance. The antioxidant free radical can then go on to react with another free radical thus leaving no free radicals (Ingold 1961). Polyphenolics are a naturally occurring class of compounds that are well known for their antioxidant activity. They possess easily extractable hydrogens with a self-stabilizing aromatic ring. Polyphenolics have increasing antioxidant effectiveness with increasing hydroxyl groups. Interestingly, many naturally occurring polyphenolics exhibit a prooxidant effect at low concentrations, but synthetic ones do not (Fukumoto and Mazza 2000). These data indicate that potential antioxidants be tested at different concentrations to determine their level of effectiveness and ensure they are not promoting oxidation. Antioxidant activity will also hinge upon solubility of the antioxidant in the targeted oxidizable substance (Montoro and others 2005).

Oxidation products will also depend on the substrate undergoing oxidation. Many foods contain unsaturated fatty acids, which are prone to lipid oxidation. Lipid oxidation leads to off-flavors, loss of nutrition, and loss of shelf-life. It is one of the leading causes of spoilage. One of the initial products of lipid oxidation is lipid hydroperoxides. Hydroperoxides are odorless

and tasteless. These hydroperoxides are unstable and breakdown into a multitude of compounds of various odors and thresholds (Nawar 1996). Different fatty acids have a tendency to produce certain oxidation products. Nonanal, octanal, 2-decenal, and heptane are the most common products formed from free radical-induced oxidation of oleic acid. Linoleic will form hexanal, 2,4-decadienal, pentane, and 2-nonenal from free radical-induced oxidation. There are more compounds formed, but those listed are the main byproducts listed from most to less formed (Przybylski and Eskin 1995).

Antioxidant and oxidation assays

There is not an agreed upon standard method to determine antioxidant capacity, and this causes difficulty when trying to compare one substance to another. Currently there are numerous antioxidant capacity assays available to scientists. Each assay measures the antioxidant capacity differently, which can lead to different results for the same antioxidant. Some of these assays use radicals that are foreign to reactions in the body and in food systems, such as $ABTS^{\bullet+}$ and DPPH•, and this leads to difficulties in interpretation. Llesuy and colleagues (2001) break antioxidant assays into several important groups according to what is measured: assays that measure the reduction in concentration of a free radical, methods that measure the reduction in concentration of the antioxidant, assays that measure rates of reaction with and without the antioxidant present and other miscellaneous assays. The more popular antioxidant assays are Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu total phenolics assay, oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), 2, 4-dinitrophenylhydrazine (DNPH), and thiobarbituric acid reactive substances (TBARS). Many of these assays have found their niche with one product or another. Most of these antioxidant capacity assays can be used either to test a single compound, or to test a biological or food system.

TEAC

The Trolox equivalent antioxidant capacity (TEAC) assay measures an antioxidant's ability to convert $ABTS^{\bullet+}$ to ABTS (figure 2.11). $ABTS^{\bullet+}$ is a colored, long-lived radical and the antioxidants ability to reduce this color is reported. The decrease in absorbance is measured

at 658 nm (Perez-Jimenez and Saura-Calixto 2006). ABTS can be formed from ABTS•$^{+}$ by either single electron transfer or hydrogen atom transfer (Wood and others 2006). This value is reported as Trolox equivalents (Prior and others 2005). Trolox is often used as a reference for antioxidant capacity assays. TEAC is carried out at neutral pH. TEAC measures the reducing power of antioxidants (Huang and others 2005). It is assumed that the reducing power is directly correlated to the antioxidant capacity. One argument against TEAC and any method that utilizes ABTS, is that ABTS is not a natural radical. This leads some to question its validity in natural samples (Wood and others 2006). Perez-Jimenez and Saura-Calixto (2006) investigated the effect of solvent polarity and acidity and discovered that the ABTS value was directly affected by solvent polarity and acidity (the more polar the sample solvent, the higher ABTS value obtained and the lower the pH, the lower the ABTS value). An advantage of TEAC is its low variation within the laboratory and between laboratories. It was found that TEAC results varied 7.5% within laboratories and 11% between laboratories for ascorbic acid (Buenger and others 2006).

FRAP

The ferric reducing antioxidant power (FRAP) method measures the reduction of ferric 2, 4, 6-tripyridyl-*s*-triazine (TPTZ). TPTZ reduces to an intense blue compound that absorbs at 595 nm. The iron molecule in the center of the TPTZ molecule reduces to Fe (II) (figure 2.12). FRAP measures the reducing power of a substance, but not the radical quenching ability. This has led to what some people believe is an underestimation of antioxidant capacity. FRAP is quite similar in mechanism and reaction to TEAC. However FRAP occurs in acidic conditions (Huang and others 2005). The reaction involved in the FRAP assay is electron transfer. Because of this property, FRAP can be used to determine the mechanism responsible for a certain substance's antioxidant ability (Prior and others 2005). FRAP does experience some variation due to the solvent in which the extract is made of, but there is no easily seen pattern (Perez-Jimenez and Saura-Calixto 2006).

DPPH

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method also relies on a chromatically active radical to measure antioxidant capacity. DPPH• is a dark purple. The DPPH radical (figure

2.13) is a long-lived nitrogen radical which does not have to be generated in situ. DPPH concentration can be measured by ESR or by a UV-vis spectrophotometer (Prior and others 2005). The antioxidants placed in the system react with DPPH reducing its absorbance measured at 517nm (Sun and Ho 2005). DPPH is a simple method that is composed of mixing the compounds of interest with DPPH, waiting a given amount of time, and measuring the absorbance. DPPH results can be standardized by comparing them to Trolox or quercetin and reporting them as equivalents (Atoui and others 2005). This is done by running a standard, such as Trolox, with the DPPH method and comparing the results to that of one's sample.

One must be careful when using DPPH because its chemistry is sensitive to the solvent used. Some solvents change the reaction and can thus lead to overestimating or underestimating the true antioxidant capacity of a compound. DPPH is not as greatly affected by solvent as ORAC. Solvent can affect whether the reaction in the DPPH method is single electron transfer or hydrogen atom transfer. In alcoholic solvents, the reaction rate of DPPH is faster, thus leading to an overestimation of antioxidant capacity (Tanko 2005). Also because DPPH does not resemble any naturally occurring radical reaction, its values should be interpreted with caution (Huang and others 2005). DPPH has a fairly low intralaboratory variation of 7.8% and a low interlaboratory variation of 12% for ascorbic acid (Buenger and others 2006).

Folin-Ciocalteu Assay

The Folin-Ciocalteu assay is used to measure the total phenolic content of a substance. Many phenolics possess antioxidant activity; therefore, many researchers measure the total phenolics to gauge the antioxidant potential of a substance. The Folin-Ciocalteu assay is strongly correlated with DPPH scavenging activity (Katsube and others 2004). It is also correlated with ORAC and FRAP (Xu and Chang 2007). It is sometimes difficult to quantitate individual phenols by HPLC or other means and calculate a correct total phenolic concentration. In the Folin-Ciocalteu method, the phenolics react with the Folin-Ciocalteu reagent (a solution of phosphomolybdate and phosphotungstate) to produce a blue color. The Folin-Ciocalteu reagent changes to a blue color with the addition of one or two electrons. The reaction is carried out near pH 10, which is adjusted by the addition of a sodium carbonate solution (Singleton and others 1999). The blue color is measured by absorbance between 745-765 nm. The values are reported as equivalents of a known antioxidant. Compounds that have been used to compare are gallic

acid, caffeic acid, vanillic acid, and others (Prior and others 2005). Gallic acid is the most common (Singleton and others 1999). However, there are problems with compounds interfering with total phenolic determination. Tryptophan, guanine, xanthine, ascorbic acid, and uric acid interfere with the assay. Sugars will also interfere, but only at high levels (25 g/L and above). These interferences can be taken into account by using correction factors and by subjecting standards to the same conditions as samples. Sulfur dioxide (added to most wines as an antimicrobial) will lead to an overestimation of phenolic content, but can be minimized by the addition of acetaldehyde (1000 mg/L), which inhibits the sulfites interfering reactions. The Folin-Ciocalteu method can be modified to determine flavonoid content. Flavonoids will precipitate with formaldehyde. Subtracting the total phenolics content of the sample with the flavonoids removed from the total phenolics of the sample will correspond to the flavonoid content (Singleton and others 1999).

Traditional Folin-Ciocalteu methods require relatively large amounts of reagents and generate much waste. Researchers have been developing methods that require less reagents and less sample. Ainsworth and Gillespie (2007) developed a Folin-Ciocalteu method for plant tissues that required only 100 µL of sample. To this microcentrifuge tube, they added 200 µL of 10% (v/v) Folin-Ciocalteu reagent, vortexed, and added 800 µL of 700 mM sodium carbonate. This resulted in 1.1 mL of waste per sample. After incubating for 2 hours, they transferred 200 µL to a clear 96-well plate and read the absorbance at 765 nm. More recently a research group has developed a method that allows all of the chemistry to be performed on a 96-well plate (Medina-Remon and others 2009). In this method, 15 µL of sample was added to 170 µL of water in a well. Then 12 µL of Folin-Ciocalteu reagent was added, followed by 30 µL of 200 g/L sodium carbonate. After 1 hour of incubation, an additional 73 µL of water was added and the absorbance read at 765 nm. There is no explanation as to why this last addition of water was performed. The total waste per sample in this method is 0.3 mL, which is a vast reduction compared to the Ainsworth-Gillespie method. Improvement of the Folin-Ciocalteu method will provide the scientific community with a simple method to determine total phenolics and less hazardous waste.

Another variation in the Folin-Ciocalteu method that is drawing attention is the use of an automatic flow procedure. This method uses multi-syringe flow injection analysis (Magalhaes Luis and others 2006). This method reduces waste, but does require a flow system. This group

explored different concentrations of the Folin-Ciocalteu reagent and found 10% (v/v) to give the most sensitivity. The authors also performed a batch method on a microplate reader. Their method calls for adding 50 μL of sample, 50 μL of 20% Folin-Ciocalteu reagent (v/v), then adding 100 μL of sodium carbonate (60 g/L). The plate was then read every minute for two hours at 760 nm. This method has reduced the waste produced to 0.2 mL.

ORAC

The oxygen radical absorbance capacity (ORAC) assay measures an antioxidant's ability to quench radicals. The radicals used can be of many varieties such as hydroxyl radicals, peroxyl radicals, etc (Cao and others 1996). This allows one to study many different aspects of one antioxidant and to use naturally occurring radicals. A peroxyl radical generator is commonly used if the author does not have a preference. The antioxidant capacity is measured by the radical's reaction with a probe that becomes non-fluorescent after reaction with the radical. One frequently used combination is fluoroscein's degradation by peroxyl radicals (often generated by AAPH) (Gillespie and others 2007). Fluorescence is measured with a fluorometer. Fluorescein and dichlorofluorescein are both currently in use as fluorescent probes in the ORAC method. ORAC can be used to measure both hydrophilic and lipophilic antioxidants that adds to its attractiveness. Results from ORAC are typically reported as Trolox equivalents and are calculated by area under the curve (figure 2.14) (Prior and others 2005). ORAC does have some disadvantages. Perez-Jimenez and colleagues (2006) found that ORAC was affected the most by the solvent used (water, 30% aqueous methanol, 50% aqueous methanol, 50% acidified aqueous methanol, methanol, and 50% aqueous acetone) compared to DPPH, ABTS, and FRAP. The ORAC value tends to increase inversely with solvent polarity. The effect of proteins were investigated and found to interfere with ORAC and have positive ORAC values. This research group also tested the effect of acidified solvent on ORAC and found that there is no effect of acid (Perez-Jimenez and Saura-Calixto 2006).

ORAC has found widespread use with some manufacturers actually putting the ORAC value on their labels (Prior and others 2005). ORAC has also been used extensively by the USDA (Wu and others 2004a). Wu and fellow colleagues (2004b) have started a database containing the total antioxidant capacity of foods. They calculate the total antioxidant activity by measure both the hydrophilic and lipophilic ORAC values and adding them together. Cao and

Prior (the discoverers of ORAC) (1998) found that ORAC and FRAP did have a weak linear correlation (r=0.349) with one another when used to measure serum antioxidant capacity levels.

TRAP

The total radical-trapping antioxidant parameter (TRAP) has a similar reaction as the ORAC assay. TRAP measures how successful the antioxidant tested is at quenching peroxyl radicals before the radicals react with a probe. The peroxyl radicals are produced by either AAPH or 2, 2'-azobis 2-amidinopropane dichloride (ABAP). The parameter measured can be "oxygen uptake, fluorescence of R-phycoerythrin, or absorbance of 2,2'-azinobis 3-ethylbenzothiazoline-6-suslfonic acid (ABTS)" (Prior and others 2005). TRAP values are typically reported in reaction times compared to reaction times with Trolox. Due to the numerous parameters one can measure, it has been difficult to compare TRAP values with one another if another laboratory used a different parameter. The TRAP assay has found use in measuring antioxidants from in vivo experiments (Prior and others 2005).

Protein Oxidation

When proteins undergo oxidation, they have a tendency to form carbonyls. Protein carbonyls can be formed by several different pathways. Side chains can be directly attacked by a reactive oxygen species or a lipid oxidation product can react with a protein to produce a protein carbonyl (Stadtman and Berlett 1997). The backbone of the protein can undergo carbonylation as well as the side chains. The side chains of histidine, arginine, and lysine are particularly vulnerable to oxidative stress (Ghezzi and Bonetto 2003). These specific amino acids all have a nitrogen moiety in their side chains. This leads to a higher reactivity. The carbonyl content can be measured by the 2, 4-dinitrophenylhydrazine (DNPH) method. This method has found use in measuring the oxidation of meat products (Estevez and Cava 2006) and other products with high protein content. DNPH is quite popular and is available in complete kits. This method cannot provide a certain antioxidant capacity value, but when comparing a sample containing antioxidant versus a sample without the antioxidant, it can be shown how much the antioxidant reduces oxidation. DNPH can be measured with a spectrophotometer or through ELISA (Ghezzi and Bonetto 2003).

Another measure of protein oxidation is the level of 3-nitrotyrosine. It has been shown that 3-nitrotyrosine levels increase in Alzheimer's disease and various other degenerative diseases. It is believed that 3-nitrotyrosine is formed from the reaction of tyrosine with peroxynitrite (Ghezzi and Bonetto 2003). A relatively new method used to measure the oxidized products of proteins (such as 3-nitrotyrosine) is the "EZ: faast" method by Phenomenex. This method takes only 15 minutes and a GC-MS. However due to its recent release, it has found limited exposure thus far.

Lipid Oxidation

The thiobarbituric acid reactive substances (TBARS) assay has found widespread use in the study of meat oxidation and in other lipid containing systems. Specifically, TBARS is used to measure the formation of malondialdehyde primarily from polyunsaturated fatty acids (Buenger and others 2006). Malondialdehyde is a product formed by the breakdown of lipid peroxides. After the sample is mixed with thiobarbituric acid, heated, cooled, and centrifuged, the absorbance is measured at 532nm. Malondialdehyde forms a complex with thiobarbituric acid (Wood and others 2006). The results are reported as nmol malondialdehyde/mg of sample or a similar unit (Lee and others 2005a). This assay also does not provide a definite antioxidant capacity value, but rather the extent of lipid oxidation. There is some concern that other substances can interfere with the TBARS assay, such as hemoglobin and other iron-containing substances. There is also concern that since malondialdehyde represents a small portion of the degradation products, researchers are not getting a true representation of the extent of lipid peroxidation (Wood and others 2006).

Another common method of testing for lipid oxidation is to determine the peroxide value. When lipids undergo oxidation, they form lipid peroxides and hydroperoxides, which are fairly stable. These can then be measured. However, the lipid peroxide level fluctuates as the sample ages because the lipid peroxides further degrade into other products. This can cause some problems interpreting the data gathered. The reaction involves potassium iodide and the sample. The lipid peroxides react with the potassium iodide to produce I_2. This can then be titrated with starch and sodium thiosulfate to determine the milliequivalents of oxygen per kilogram of fat (Nawar 1996).

LDL oxidation can be measured outside of the body. LDL oxidation is believed to be a cause of atherogenesis, thus inhibition of LDL oxidation should impede atherogenesis. In order to test LDL oxidation, LDL must be separated from fresh blood. The antioxidants of interest are then added followed by copper sulfate to accelerate oxidative reactions. Conjugated diene formation is then measured by UV-Vis at 234 nm over 7 hours incubation. From these data, the oxidation lag time is calculated and compared to a standard (Katsube and others 2004).

HPLC and GC

In a complex system, such as a food, if the researcher knows the oxidation products or an antioxidant present in the system, then they can analyze the concentrations of these compounds with chromatographic methods. For example, Nissen and colleagues (2004) were investigating different plant extracts for the ability to reduce oxidation in cooked pork patties. The researchers measured hexanal, a lipid oxidation product, by analyzing the headspace with gas chromatography (GC). The headspace was sampled from a glass vessel containing pork sample. Nissen and associates also measured the concentrations of α-tocopherol with high performance liquid chromatography (HPLC). They found that rosemary offered the best protection against lipid oxidation compared to green tea, coffee, and grape skins. They also found that the level of α-tocopherol was higher in those samples that contained an extract. This was hypothesized to be caused by the extracts protecting the tocopherol (Nissen and others 2004). This would act as an indication as to how much oxidative stress the pork patties without the plant extracts encountered and when compared to the pork patties with the plant extracts would signify if the plant extracts helped to prevent oxidation. HPLC and GC are valuable because they can give the researchers the exact concentrations of a specified compound.

HPLC is a valuable tool for determining concentrations of antioxidant compounds. It is commonly used to investigate plant extracts. HPLC-FTIR was utilized by Robb and colleagues (2002) to measure catechins in green tea. Catechins were identified by matching retention times to known standards as well as utilizing infrared spectra. Another group used HPLC-DAD to investigate flavonoids in different fruits and vegetables. These were identified using known standards (Baranowski and others 2004). Resveratrol and other stilbene levels are often determined using HPLC (Jeandet and others 1994; Romero-Perez and others 2001; Vian and

others 2005). HPLC requires known standards to identify compounds unless a mass spectrometer is used as the detector.

A GC method that has been gaining in popularity for measuring compounds in the headspace is solid phase microextraction (SPME). SPME allows researchers to sample headspace volatiles without the use of solvents with sensitivity (down to parts-per-trillion) and the ability to target classes of compounds (Arthur and Pawliszyn 1990; Zhang and Pawliszyn 1993). Numerous fibers coated with various liquid compounds that impart different polarities are available for SPME. The fiber used is determined by the targeted compound(s). Jelen and fellow colleagues (2000) investigated which fiber would be ideal for measuring headspace above oxidized rapeseed oil and looked specifically at 14 different byproducts of lipid oxidation. The compounds targeted were *n*-pentane, 2-butanone, *n*-butanol, pentanal, pentanol, hexanal, 2-heptanone, heptanal, 2-heptanal, 1-octen-3-ol, octanal, 2-nonanone, nonanal, and *trans*, *trans*-2,4-decadienal. The following four fibers were tested: 85 μm polyacrylate, 100 μm poly (dimethylsiloxane) (PDMS), carbowax/divinylbenzene, and divinylbenzene/ carboxene/PDMS. They concluded that a divinylbenzene/carboxene/PDMS fiber was the optimal fiber for this situation due to the linearity and sensitivity. SPME has been used to measure oxidation products in beef bouillons, infant formula, numerous oils, hazelnuts, peanuts, and walnuts (Mate and others 1996; Steenson and others 2002; Mildner-Szkudlarz and others 2003; Giuffrida and others 2005; Richards and others 2005; Garcia-Llatas and others 2006; Pastorelli and others 2006).

Headspace oxygen

Earth's atmosphere contains approximately 21% oxygen. If the atmosphere of a packaged food is not modified, then the initial headspace oxygen is 21%. However, as oxidative reactions take place, this oxygen is consumed. Measurement of this oxygen can be used as an indicator of degree of oxidation provided the packaging does not allow the transfer of oxygen. Consumption of headspace oxygen can be used to calculate theoretical peroxide values and has been shown to have strong correlations with measured peroxide values and conjugated dienes (Chung and others 2004). In the past, headspace oxygen was measured by puncturing the package and using a gas analyzer or GC, but new instruments allow headspace oxygen to be measured without puncturing the packaging. One such instrument is made by Oxysense®. It

measures the time of fluorescence of oxygen-sensitive films placed inside the packaging (Li and others 2008).

Jensen and fellow researchers (2004) found that headspace oxygen was a good indicator of susceptibility to oxidation. This research investigated the oxidation of pork scratchings, peanuts, oatmeal, and muesli. Pork scratchings consumed little oxygen and muesli consumed approximately 6% over 23 weeks, but peanuts and oatmeal consumed all headspace oxygen by week 12 of the study. All samples were stored at 27°C under fluorescent lights.

How are antioxidant assays applied to biological systems?

Oxidative stress has been linked to numerous diseases such as Alzheimer's, cataracts, cancer, etc (Ciolino and Levine 1997; Aksenov and others 2001). Oxidation has even been linked to the aging process (Harman 1956). The human body produces numerous reactive oxygen species during normal metabolism and produces even more during periods of distress (Valko and others 2007). This leads scientists to look towards antioxidants for prevention and treatment of these diseases and to slow the progression of aging. Oxidation of lipids leads to rancidity and food spoilage. Protein oxidation has been linked to many diseases and can lead to dysfunction within the cell. Oxidation of proteins leads to an increase in surface hydrophobicity and aggregation of proteins (Grune and others 1997). Antioxidant assays carried out with extracts or pure chemicals is just the beginning of finding a new dietary supplement or food ingredient. Antioxidant assays are used as a guide to determine which extracts and chemicals should require further testing and which ones should be discarded. Antioxidant capacity assays tell the researcher nothing on the bioavailability of a compound, if the tested substance is toxic to cells, or if it will be interfered with by some other component.

Cell Culture

Cell culture is the growth of cells for experimentation. The investigator chooses the cells of interest for their research. For example if the researcher was interested in the effect α-tocopherol had on the degree of oxidative stress that vascular epithelial cells experienced, then the researcher should choose a cell line of aortic endothelial cells or something similar. Lu and fellow researchers (2006) chose to use retinal pigmented epithelial cells because they were interested in age-related macular degeneration. The scientist can also choose cells that have a

high sensitivity to oxidative stress if they have no particular cells in mind. Cell culture lines which have a high sensitivity to oxidative stress are the murine NIH 3T3 and L929 (Arrigo and others 2005). The researcher can choose to increase the level of normal oxidative stress by the introduction of free radical generators, hydrogen peroxide, or other factors. Hydrogen peroxide is often used because it can cross cell membranes easily, is naturally occurring, and can be converted to the more reactive hydroxyl radical, which is an important radical in biological systems (Labieniec and Gabryelak 2005). Lipid oxidation and protein oxidation can both be measured in cell culture often by similar means employed with other substances. Lipid peroxidation can be measured by TBARS, conjugated dienes, and peroxide levels. Protein oxidation is usually measured by carbonyl content, but other methods are used. Davies and Goldberg (1987) measured several indicators of lipid and protein oxidation in red blood cells and found protein oxidation to be more sensitive in regards to measuring cell oxidation.

Cell culture demonstrates whether the tested substance is toxic to cells and, if so, at what levels it will become toxic. There are many viability tests for cell culture. The following is a small sampling. To test whether a substance is toxic to cells the scientists can use MTT tests to see whether the cells are alive or dead after exposure to the antioxidant. They can then compare this to their control cells. The researcher can also use Trypan blue to record the number of live cells versus those that are dead. There are several other methods of staining which can be used for this same purpose (Arrigo and others 2005).

A widely used assay to determine the amount of protein oxidative damage that has occurred to the cell is 2, 4-dinitrophenylhydrazine (DNPH). Hydrogen peroxide is produced in cells during respiration. This hydrogen peroxide, if not degraded to harmless products, can go on to form radicals that are more reactive. Hydroxyl radicals can be formed via the Fenton reaction from hydrogen peroxide (Ciolino and Levine 1997). Hydroxyl radicals formed in the cell are not very selective and proteins are often attacked. This leads to the formation of protein carbonyls. In their examination on the relation between different types of stress and age-related macular degeneration, Lu and colleagues (2006) measured protein carbonyl content in their retinal pigmented epithelial cells. They found that carbonyl content increased with added oxidative stress that they felt made this a valid measure. They used ELISA as opposed to a spectrophotometer to measure the results of the DNPH test. ELISA uses light absorbance or fluorescence to discern the results (Lu and others 2006).

Another oxidative stress parameter that can be measured is the level of oxidized glutathione versus reduced glutathione. The level of oxidized glutathione rises with the amount of oxidative stress that a cell experiences (Arrigo and others 2005). Levels of other enzymes such as catalase and superoxide dismutase can also indicate the degree of oxidative stress that the cells have been exposed to (Alia and others 2006). As the above examples have shown, DNPH is used in the cell culture experiments to measure protein oxidation and levels of antioxidant enzymes can be measured to gauge the oxidative stress level. Cytotoxicity is gauged by numerous cell-staining methods that separate the dead and live cells.

In vivo

After an antioxidant is shown to have activity in cell culture, the next step will often be an animal model and then eventually, if the substance shows no toxicity, it may be tested in humans. *In vivo* experiments are necessary to gauge the bioavailability of a substance, the antioxidative effect, and if it has any toxic effects on a large scale.

TBARS has found use within *in vivo* experiments to measure the degree of lipid oxidation. Erdogan and colleagues (2006) used TBARS to measure the level of malondialdehyde and similar compounds in tissue samples from rats that were subjected to renal ischemia-reperfusion injury. They also used DNPH to determine the level of protein oxidation in tissue excised from the same rats (Erdogan and others 2006). Sahin and Gumuslu (2004) used TBARS in their study of oxidative stress in the brain. The brain is composed of a large percentage of polyunsaturated fatty acids. These polyunsaturated fatty acids are oxidized at a higher rate then saturated fatty acids. They also used DNPH for their protein oxidation assay (Sahin and Gumuslu 2004).

In another *in vivo* study, the researchers tested the effect of grape seed extract on the blood of volunteers. Simonetti and fellow researchers (2002) used TRAP to determine the antioxidant capacity of the plasma and they utilized HPLC to measure the level of α-tocopherol in the blood. Their thinking was that α-tocopherol might be spared from oxidation by the antioxidants in the blood from the grape seed extract. Their research found that the antioxidant capacity and the α-tocopherol level in the plasma were unchanged with supplementation. However the α-tocopherol level did rise in the red blood cells after supplementation.

One way to determine bioavailability of a phytoalexin is to feed it to an animal or human and then measure the concentration of the substance in the blood. Henning and fellow colleagues (2004) had volunteers ingest green tea, black tea, or a green tea extract. Eight hours following ingestion of the tea treatments, the antioxidant activity of their plasma was measured by TEAC and flavanol concentration in plasma and urine. They found significant increases in plasma antioxidant activity and flavanol concentrations when the subjects ingested the green tea extract. Vitaglione and fellow researchers (2005) investigated the bioavailability of resveratrol in humans by having 25 volunteers drink red wine and varied their meals (fasting, high fat, low fat) and then measured resveratrol serum levels. They found trace amounts of resveratrol 30 minutes after ingestion and found that the meal treatments had no effect.

Common assays in food science

For an extract or chemical to be used as a food ingredient, the FDA must sanction it. If it is a spice extract or something similar, then it can be added because it is a flavoring, even though it has some additional beneficial effects. When looking for an antioxidant to prevent oxidation in food, a scientist may use antioxidant assays as a guide. However, they will probably carry out shelf life studies with taste panels. The antioxidant is not useful if it does not extend the shelf life, which is often judged by taste.

Many researchers choose to use more than one assay when characterizing an antioxidant. Estevez and Cava (2006) used TBARS and DNPH when measuring rosemary oil's ability to prevent oxidation of frankfurters. TBARS measures the oxidative products of lipids, whereas DNPH measures the oxidation products of proteins. The researchers also found a strong correlation between the two assays in this instance (r^2=0.77). Another example of this approach can be found with Nissen and colleagues (2004). They used TBARS and measured the hexanal and α-tocopherol levels in cooked pork patties to follow the rate of oxidation and the usefulness of the plant extracts they were testing. Lee and fellow researchers (2005a) used three different assays in their research with soybean isoflavones. They chose to use TBARS, DPPH, and FRAP. The TBARS was used to determine the amount of LDL oxidation with the addition of different soybean isoflavones. They used DPPH to study the radical scavenging activity of the isoflavones. Finally they used FRAP to again characterize the isoflavones ability to scavenge radicals. Lee and colleagues found that FRAP and DPPH in this study seemed to correlate well

(r^2 not provided), but had some conflicting results when comparing those two assays to TBARS. They believe this difference to be due to steric effects in the different reactions taking place in the assays.

HPLC is useful when an investigator is looking for a particular antioxidant. Kornsteiner and colleagues (2006) looked at α-tocopherol levels in 10 different nut types. They also performed the Folin-Ciocalteu assay which gave them an indication of the total phenolics and the antioxidant capacity of the other compounds found in the nuts. However, in a study which compared the Folin-Ciocalteu assay to a less commonly used method to measure lipid oxidation, the researchers found no correlation between the total phenolics in the plant extracts and the ability to prevent lipid oxidation. In this study, Kahkonen and fellow researchers (1999) looked at the antioxidant activity of 92 different plant extracts. Their lipid oxidation assay consisted of methyl linoleate that was mixed with the plant extract and allowed to oxidize in the dark at 40°C. The mixture was then dissolved in 2, 2, 4-trimethylpentane and the absorption was measured at 234 nm. At 234 nm, conjugated dienes absorb light. The amount of hydroperoxides was then calculated using this value. Some researchers find that the Folin-Ciocalteu assay did correlate well with other antioxidant assays. Karakaya, El, and Tas (2001) found that the Folin-Ciocalteu assay did correlate well with TEAC. They looked at 26 different foods that compose the majority of the Turkish diet. They noted that this depended on their particular testing system(Karakaya and others 2001). From the above studies, it is easy to see that the Folin-Ciocalteu assay is not always an accurate method to determine antioxidant activity. However, it is an easy assay to determine the amount of phenolics present and can be used as a guide.

TBARS has also found use with peanuts. Nepote and fellow investigators (2004) used TBARS to measure the oxidation products of honey roasted peanuts. Each group of peanuts was treated with a different antioxidant (BHT was used with one and a peanut skin extract was used with the other). Often a natural antioxidant or extract will be compared to a synthetic antioxidant such as BHT, BHA, TBHQ, etc. TBARS in this research was well correlated with sensory evaluations. This is important because the consumer typically does not care how their peanuts ranked in a TBARS assay, but they do care about flavor. TBARS has found great use in the study of meat oxidation because it is so closely correlated with sensory data. It has also been noted to be closely correlated with oxidation products in the headspace (Decker and others 2005).

DPPH has found ample usage in the area of food science. Sun and Ho (2005) used DPPH to measure the antioxidant capacity of buckwheat extracts. It has also been used to measure the antioxidant capacity of soybean isoflavones (Lee and others 2005a) and tea/herbal infusions (Atoui and others 2005). Yu and fellow researchers (2006) used DPPH to determine the antioxidant activity of peanut skin extracts. They also used the Folin-Ciocalteu assay to determine the total phenolics of consecutive extractions of peanut skins.

TEAC and TRAP have also found some use in the food science literature. In particular, Pietta, Simonetti, and Mauri (1998) use both of these assays to study the antioxidant capacity of fifteen different medicinal plants. They used TEAC to measure the antioxidant activity of the straight medicinal plant solution. TRAP was used to measure the antioxidant capacity of blood plasma harvested from volunteers in three groups (one received ginkgo extract, another green tea extract, and the third received a placebo). The researchers found that even though the ginkgo extract had a lower TEAC value then the green tea extract, the plasma from the volunteers receiving the ginkgo extract had a higher antioxidant activity then those receiving the green tea extract. This difference seems to suggest that the ginkgo extract contains compounds that possess a higher bioavailability then those contained in the green tea extract.

The USDA has used ORAC to illustrate the different antioxidant capacity levels of various fruits and vegetables. In one study, the USDA used ORAC to characterize over 100 fruits and vegetables (Wu and others 2004a). Wu and fellow researchers from the USDA chose to use ORAC based on "its advantages related to biological systems." They chose not to use FRAP, TEAC, or DPPH because these assays do not have radicals that are naturally found in biological systems. ORAC is also useful because it is often automated which hastens the results. Wu and colleagues measured both lipophilic and hydrophilic fractions with ORAC because they felt that this led to a more accurate measurement. Ou and associates (2002) used ORAC to classify the antioxidant potential of 927 samples of vegetables. They also ran the samples with the FRAP assay. Ou and associates found that ORAC and FRAP did not correlate (r^2= 0.0006 to 0.96) for many of the samples. They also preferred ORAC because of the above reasons proposed by Wu and colleagues. Ou and associates (2002) propose to call the results of ORAC the "peroxyl radical absorption capacity" because it measures the ability of an antioxidant to quench peroxyl radicals. ORAC has appeared to become the preferred antioxidant capacity assay for plant extracts and probably has the largest following.

Scientists interested in studying antioxidants and searching for new ones have numerous antioxidant assays to choose from. Each antioxidant assay has its place in research depending on exactly what one wishes to measure and what composition is their oxidizable substrate. TEAC, FRAP, DPPH, the Folin-Ciocalteu assay, and ORAC have found use in measuring antioxidant capacity in a purely chemical setting. Many researchers are interested in using their antioxidant or extract as a dietary supplement, utilize ORAC because it utilizes naturally occurring radicals. These assays can save money by allowing the researcher to choose only the compounds that appear to have the highest antioxidant capacity to go on and do cell culture research. DNPH is used to measure protein oxidation in cell culture and in food systems. TBARS has found use in measuring the amount of lipid oxidation in cell culture and in food systems. However, there are times when the antioxidant capacity does not correlate well with performance in cell culture, *in vivo* studies, and food systems. Antioxidant capacity tests are difficult to compare even to themselves. This is due to interference from other compounds and solvents, oxidative stress not tested for in the assay, bioavailability, and cytotoxicity. For example, if the antioxidant capacity is promising, but the bioavailability turns out to be nil, then the future of a dietary supplement candidate is dim. It is important to remember that all of these assays are beneficial as guides, but in order to determine if an antioxidant will perform in a certain setting it has to be tested in that exact setting. The search for a standardized assay, which correlates well with the complex natural systems, continues.

Decker and colleagues (2005) propose that food scientists simplify their food oxidation model systems. Typically a researcher will add an antioxidant or extract of interest to a food that easily oxidizable or a food that the researcher has focused on for another reason. Decker *et al*. suggest that researchers utilize bulk oil, oil-in-water emulsions, or muscle foods. This would allow easier comparisons among experiments. They also suggest that storage temperatures be below 60°C due to the nonconformity of reaction mechanisms and rates to those typically experienced at normal storage temperatures. Other recommendations include: starting with low oxidation levels in the model system, matching measured lipid oxidation products with the fatty acids present in the model system, measure the antioxidant of interest against a known antioxidant (i.e. BHT), use one source for the model system lipids to ensure similar levels of naturally occurring antioxidants, measure primary and secondary oxidation products to be certain

that the additive is effective at inhibiting both, and match the pH of the model system to the targeted food.

Human health implications

Many of the compounds found in peanut plants have been shown to have positive health effects. These effects range from insulin-like effects to anticancer properties. It is believed that many of these healthy influences are due to the compounds' antioxidant effects. It is thought that some of these compounds participate in cell signaling pathways that lead to health benefits (Dong 2003; Kundu and Surh 2004; Signorelli and Ghidoni 2005).

Oxidation has been implicated as the causative agent in many diseases. Protein oxidation is thought to be a cause of cataracts and aging. Oxidation is a destructive agent in arteriosclerosis and injuries caused by reperfusion (Vendemiale and others 1999). The free radical theory of aging was first proposed by Denham Harman (1956). The free radical theory of aging proposes that aging is the result of additive damage to cells caused by oxidative species that is not repaired due to repair enzyme failure. This damage can lead to cancer, aging, and disease. Antioxidants would lower the oxidative stress experienced by the body and would subsequently lessen the effect of aging and oxidation related diseases.

Natural sources of antioxidants have shown potential in reducing the oxidative stress experienced by the body. Simonetti and fellow researchers (2002) investigated the effects of procyanidins from grape seeds in ten volunteers. The volunteers took 110 mg of the supplement for 30 days. The researchers found that α-tocopherol levels rose in the subjects' red blood cells and DNA oxidation was reduced. The authors concluded that the procyanidins exhibited a protective effect for α-tocopherol while at the same time reducing DNA oxidation. In another study, Pignatelli and colleagues (2006) explored the "French paradox." They used 30 volunteers. They had ten of them drink 300 mL of white wine per day, ten drink 300 mL of red wine per day, and ten abstain from alcohol. The researchers measured urinary PGF-2α-III as a marker of oxidative stress. The level of PGF-2α-III is directly correlated with oxidative stress levels (Pignatelli and others 2006). They found PGF-2α-III levels decreased in both wine drinking groups and the decrease was highest in the red wine group. Red wine has been shown to have a higher polyphenol content then white wine. Pignatelli and fellow authors conclude that the effect they saw was due to the polyphenolic content found in wine. Frankel and colleagues

(1993) separated ethanol from the wine phenolics and examined the effects they had on retarding *in vitro* oxidation of LDL. They found that wine phenolics were effective at inhibiting LDL oxidation and were more effective then α-tocopherol.

Many antioxidants have been shown to inhibit the initiation and spread of cancer. Soleas and fellow researchers (2002) investigated the anticarcinogenic effects of catechin, resveratrol, quercetin, and gallic acid. All of these compounds are found in red wine. They applied the compounds topically to mice that had chemically induced skin cancer. The authors discovered that quercetin was the most effective at inhibiting the cancer and gallic acid was the least effective of the listed compounds. Resveratrol is effective at the three different stages of cancer (initiation, promotion, and progression) (Jang and others 1997). The exact mechanism that resveratrol inhibits cancer is unknown, but it is believed to affect the cell signaling pathways (Kundu and Surh 2004).

Recently some polyphenolic compounds have been gaining attention for their insulin-like activity. The diet is an important factor in controlling diabetes and is believed to be a cause of diabetes. Cinnamon has shown some capability of enhancing insulin activity. Anderson and colleagues (2004) isolated 3 A-type catechin/epicatechin oligomers from cinnamon that are thought to be responsible for the insulin enhancing activity. These same compounds have also been identified in peanut skins (Yu and others 2006). Anderson *et al*. used the epididymal fat cell assay to determine the insulin enhancing effect. The compounds isolated had insulin enhancing activity. Not all studies seem as promising though. Vanschoonbeek and fellow researchers (2006) fed 25 postmenopausal volunteers 1.5 g/day of cinnamon or a placebo. They found no significant difference between groups for insulin sensitivity or glucose tolerance. The researchers conclude that further studies are necessary to have a final conclusion regarding the effects cinnamon has on diabetes or insulin. Resveratrol has also been found to have positive effects on diabetes. In type 2 diabetes, the cells become resistant to insulin. One protein responsible for the reactivity of cells to insulin is SIRT1. SIRT1 is down regulated in insulin resistant cells. Resveratrol acts as an activator of this protein, thus making cells less resistant to insulin and lessening the impact of type 2 diabetes (Sun and others 2007).

Further evidence of resveratrol's healthy attributes has been published in a recent article where the researchers fed mice a high-calorie diet. They treated some of the mice with resveratrol supplements (either 5.2 or 22.4 mg/kg/day), which increased their health and survival

(Baur and others 2006). Baur *et al*. also observed an increase in insulin sensitivity among mice treated with resveratrol compared to the control mice or mice fed just the high calorie diet. They found that the most significant effects were seen in the high dose of resveratrol.

As mentioned previously, some of these compounds not only exhibit their protective effects through their antioxidant capacity, but also participation in cell signaling pathways. Resveratrol has been well researched and would serve as a good example of these dual effects. Resveratrol has been shown to have antioxidant capacity, which in turn protects against low-density lipoprotein (LDL) oxidation (Norata and others 2007; Ungvari and others 2007). LDL oxidation has been shown to be responsible for atherosclerosis (Luc and Fruchart 1991; Alexander 1995). Resveratrol can also protect against blood clots by preventing platelet aggregation and promoting vasodilation (Pace-Asciak and others 1995; Chen and Pace-Asciak 1996). There is some evidence that suggests resveratrol fights cancer through retarding tumor initiation and promotion (King and others 2006). Resveratrol suppresses the NF-κβ pathway that discourages cancer growth and spread (Tsai and others 1999; Holmes-McNary and Baldwin 2000; Cho and others 2002). It also participates in the AP-1 pathway, which is also important in its anticancer properties (Manna and others 2000). How resveratrol is involved in these pathways is still unknown, but is being researched (Aggarwal and Shishodia 2006). The evidence regarding the bioavailability of resveratrol has not been promising thus far (King and others 2006). One team of researchers gave human subjects 25 mg of radiolabeled resveratrol. They found that most of it was absorbed, but very little could be found in the plasma from 0 to 72 hours from ingestion. They concluded that some benefit may still be gained in cells lining the digestive tract (Walle and others 2004). It seems that very little of the compound makes it into the blood stream of humans and further research will need to be done exploring this problem.

Much experimentation has been performed with cell culture and testing antioxidants' effect against oxidation. Ascorbic acid has seen numerous trials and has achieved mixed results. Montecinos and colleagues (2007) found that ascorbic acid was an effective inhibitor of oxidation. They used human vascular endothelial cells and stressed them with the addition of hydrogen peroxide. Not all studies agree with this one. Ek, Strom, and Cotgreave (1995) experimented with human umbilical vein endothelial cells and measured the ability of ascorbic acid to protect them against hydrogen peroxide from xanthine oxidase. They also measured the uptake of ascorbic acid. This group found that endothelial cells actively take up ascorbic acid,

but it weakly protects the cells from hydrogen peroxide. Phenolics have also been investigated. Lee and fellow researchers (2005b) investigated the ability of gallic acid and quercetin to produce hydrogen peroxide in cell media. Gallic acid produced 269 μM of hydrogen peroxide at 100 μg/mL and quercetin produced 59 μM of hydrogen peroxide at the same concentration in 1 hour. Both phenolics were shown to exhibit antiproliferative effects in colon cancer cells and rat liver epithelial cells, but gallic acid was shown to have a stronger effect then quercetin. Another research group examined the protective effects of myricetin, quercetin, and rutin against hydrogen peroxide in HepG2 and Caco-2 cells (O'Brien and others 2000). They found that these antioxidants were protective against hydrogen peroxide (50 μM) as measured by DNA damage by the comet assay up to 200 μM.

As many studies have shown, these polyphenolic compounds can have many health benefits. Not all agree with this view. Lotito and Frei (2006) argue that the antioxidant effects seen after ingestion of foods high in these compounds is due to these compounds increasing the urate concentration found in the plasma. Urate is an endogenous antioxidant. The compounds would have an indirect effect if this is accurate. Our understanding of how the polyphenolic compounds found in many natural sources induce their effects is still under examination. Further research in this area will better our understanding and aid in finding applications for these compounds.

Possible commercial uses

Plant extracts have found some use in the food industry as antioxidants to preserve food quality. The antioxidants currently in widespread use include 3,5-di(tertiary-butyl)4-hydroxytoluene (BHT), 2-tert-butyl-4-hydroxyanisole (BHA), propyl gallate (PG), *tert*-butylhydroquinone (TBHQ), and α-tocopherol. However, BHT, BHA, TBHQ and PG carry the negative connotations associated with synthetic additives and some evidence of detrimental health effects.

Synthetic antioxidants can be added directly to food at a concentration of 200 ppm (in the United States) as specified under Title 21 of the Code of Federal Regulations. The United States allows higher levels of synthetic antioxidants to be added then other countries. Japan does not allow BHA or TBHQ in its food and Canada and much of Europe do not allow TBHQ (Shahidi 2000). It is estimated that Americans ingest 80% (0.4 mg kg^{-1} body weight) of the acceptable

daily intake (ADI) of BHA and 130% (0.39 mg kg^{-1} body weight) of the ADI of BHT (Suh and others 2005). These antioxidants need to be confirmed safe at these levels.

BHT and BHA have been found to be carcinogenic in some organs and inhibit carcinogenesis in other organs. Ito and colleagues (Ito and others 1986) found BHA induced stomach lesions in rats at 109.6 mg kg^{-1} body weight and up. The rats were followed for up to 104 weeks. The researchers also found that BHA concentration was indirectly correlated with body weight. Some of the higher concentrations of BHA caused cancer in the stomach of the rats. Ito and fellow researchers induced colon cancer in some rats and found that BHT decreased the amount of tumors present. BHA and BHT were found to increase the development of bladder cancer. The 3-*tert* isomer of BHA seemed to be more carcinogenic then 2-*tert*-BHA, this is unfortunate because 3-*tert*-BHA is the more potent antioxidant (Ingold 1961). These results illustrate that BHA and BHT have different effects on different organs and that it requires a high dosage to induce these effects over the short term. In monkeys, which are genetically closer to humans than rats, it has been shown that 500 mg kg^{-1} body weight of BHT caused a significant increase in liver size, but BHA at the same level had no ill effects (Branen 1975).

Propyl gallate is used in baked goods and a few other foods. Van Der Heijdan and colleagues (1986) did a metastudy and found that propyl gallate has no carcinogenic effects. They propose a 0.2 mg kg^{-1} body weight ADI for propyl gallates and other gallates. It has been found to cause dermatitis to those who have close contact with it. In rats it was found that no effects occurred at or below 1,000 mg kg^{-1} body weight. This dose is quite high when compared to the ADI, but one must be careful when comparing rats and humans. Humans may metabolize these antioxidants differently then rats or other small mammals.

Rosemary extract is being used in the food industry with positive results. Rosemary extract contains phenolics such as carnosol, rosmanol, and carnosic acid (Georgantelis and others 2007). It is used at levels up to 1000 ppm. Several studies have investigated rosemary extract's effectiveness at retarding oxidation in various products. Estevez and Cava (2006) examined frankfurters and found that rosemary extract was effective at reducing levels of protein and lipid oxidation at 150 ppm, as measured by carbonyl content and TBARS respectively. However at 300 and 600 ppm rosemary extract showed prooxidant qualities in protein oxidation and no inhibition of lipid oxidation. Another study compared rosemary extract to coffee, green tea, and grape skin extract in cooked pork patties store at 4°C in normal atmosphere for 10 days (Nissen

and others 2004). Rosemary was found to be the best natural extract of these at inhibiting lipid oxidation as measured by TBARS. Georgantelis and colleagues (2006) compared rosemary extract to chitosan and α-tocopherol in inhibiting oxidation of beef burgers. Chitosan was the most effective antioxidant and was best when used with rosemary extract. All antioxidant treatments did significantly reduce lipid oxidation when compared to the control. α-Tocopherol has found widespread use in foods with high levels of lipids. Many commercially available oils are protected by α-tocopherol.

Only one study has looked at the effect of peanut root extracts as a food additive. Chen and fellow associates (2002) added peanut root powders (0.6 g) to pork patties (60 g) and then cooked. After the patties cooled, the oil was stored at 60°C and checked for conjugated dienes periodically. The oil from the patties cooked with peanut root powders was less prone to oxidation then the control oil. Another similar study investigated peanut skin extract effects in ground beef (O'Keefe and Wang 2006). O'Keefe and Wang found that peanut skin extract significantly reduced oxidation in ground meat (as measured by TBARS) with no ill effects to microbial growth, texture, aroma, and other sensory attributes. They found that 200 and 400 ppm of peanut skin extracts were effective with little additional effects seen in higher levels. Nepote, Mestrallet, and Grosso (2004) investigated the antioxidant effects of peanut skin extracts in honey-roasted peanuts. They measured peroxide and thiobarbituric acid reactive substance (TBARS) values to gauge the level of oxidation and utilized a sensory panel. Peanut skin extracts inhibited lipid oxidation, but not as well as BHT. The sensory panel determined the extracts did not change the acceptance level of the peanuts.

Van Esch (1986) and Van Der Heijdan *et al.* (1986) both propose long-term studies of synthetic antioxidants, which are still waiting to be done. These studies are needed in order to confirm that the low doses that humans are exposed to everyday do not have negative health effects. The experiments regarding TBHQ shows to it being a carcinogen and an anticarcinogen depending on which study one wishes to put more weight on (Gharavi and others 2007). Until the public receives conclusive evidence of the safety of these additives, they will continue to be skeptical of any label that proclaims their presence. The availability of natural antioxidants is still quite limited with α-tocopherol, ascorbate, and rosemary extract being the most widely used. Both of these antioxidants have their limitations and the search for a better choice continues.

Aflatoxins

Aflatoxins are natural toxins that are most commonly produced by *Aspergillus flavus*, *A. parasiticus*, *A. nomius*. Four different aflatoxins are produced B_1, B_2, G_1, and G_2, so named due to the color of fluorescence given off (B=blue and G=green). B_1 is the most toxic, followed by G_1. Aflatoxins are toxic to liver cells and are highly carcinogenic. In the United States, the level of aflatoxins must be at 20 ng/g or less in food. Aflatoxins cost the peanut industry $25 million annually in the southeast United States (Lamb and Sternitzke 2001). Aflatoxin contamination is most likely to occur 30 °C due to stress experienced by the plant making it more vulnerable to attack by fungi (Scheidegger and Payne 2005). Aflatoxins can be found in numerous foodstuffs including medicinal herbs, peanuts, ginger roots, and corn (Gomez-Catalan and others 2005; D'Ovidio and others 2006; Nakai and others 2007; Fu and others 2008).

One of the most important steps in detecting aflatoxins in foodstuffs is ensuring proper sampling techniques. Aflatoxin contamination is often not uniform throughout the product; so many samples should be taken to ensure that an accurate picture of aflatoxin contamination is grasped. Aflatoxins are extracted from samples with 70% aqueous methanol with little variation. In one study, the variation due to sampling in powdered ginger was found to be 87% (Whitaker and others 2009). Due to the fluorescence nature of aflatoxins, TLC and HPLC with a fluorescent detector are often employed. Enzyme-linked immunosorbent assay (ELISA) kits are also quite common and typically easy to use (Gilbert and Vargas 2005). Some research has looked into using FTIR to detect aflatoxins, but it has not gained widespread use (Mirghani and others 2001).

Literature Cited

Aggarwal B, Shishodia S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol 71:1397-421.

Aguamah G, Langcake P, Leworthy D, Page J, Pryce R, Strange R. 1981. Two novel stilbene phytoalexins from *Arachis hypogaea*. Phytochemistry 20(6):1381-3.

Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2(4):875-7.

Aksenov MY, Aksenova MV, Butterfield DA, Geddes JW, Markesbery WR. 2001. Protein oxidation in the brain in Alzheimer's disease. Neuroscience 103(2):373-83.

Alexander RW. 1995. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. Hypertension 25(2):155-61.

Alia M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L, Goya L. 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by *tert*-butyl hydroperoxide. Toxicol Appl Pharm 212:110-8.

Anderson RA, Broadhurst CL, Polansky MM, Schmidt WF, Khan A, Flanagan VP, Schoene NW, Graves DJ. 2004. Isolation and characterization of polyphenol type-A polymers from cinnamon with insulin-like biological activity. J Agric Food Chem 52(1):65-70.

Arora MK, Strange RN. 1991. Phytoalexin accumulation in groundnuts in response to wounding. Plant Sci 78(2):157-63.

Arrigo A-P, Firdaus WJJ, Mellier G, Moulin M, Paul C, Diaz-latoud C, Kretz-remy C. 2005. Cytotoxic effects induced by oxidative stress in cultured mammalian cells and protection provided by Hsp27 expression. Methods 35:126-38.

Arthur CL, Pawliszyn J. 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal Chem 62(19):2145-8.

Atoui AK, Mansouri A, Boskou G, Kefalas P. 2005. Tea and herbal infusions: Their antioxidant activity and phenolic profile. Food Chem 89:27-36.

Ballard TS, Mallikarjunan P, Zhou K, O'Keefe SF. 2009. Optimizing the extraction of phenolic antioxidants from peanut skins using response surface methodology. J Agr Food Chem 57:3064-72.

Baranowski R, Kabut J, Baranowska I. 2004. Analysis of mixture of catechins, flavones, flavanones, flavonols, and anthocyanidins by RP-HPLC. Anal Lett 37(1):157-65.

Barz W, Bless W, Boerger-Papendorf G, Gunia W, Mackenbrock U, Meier D, Otto C, Sueper E. Phytoalexins as part of induced defense reactions in plants: their elicitation, function and metabolism; 1990. p 140-56.

Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. 2006. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444(7117):337-42.

Branen AL. 1975. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J Am Oil Chem Soc 52:59-63.

Buenger J, Ackermann H, Jentzsch A, Mehling A, Pfitzner I, Reiffen KA, Schroeder KR, Wollenweber U. 2006. An interlaboratory comparison of methods used to assess antioxidant potentials. Int J Cosmetic Sci 28:135-46.

Cao G, Prior R. 1998. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clinical Chemistry 44(6):1309-15.
Cao G, Sofic E, Prior R. 1996. Antioxidant capacity of tea and common vegetables. J Agric Food Chem 44:3426-31.
Chakraborty D, Mandal SM. 2008. Fractional changes in phenolic acids composition in root nodules of Arachis hypogaea L. Plant Growth Regul 55(3):159-63.
Chang J-C, Lai Y-H, Djoko B, Wu P-L, Liu C-D, Liu Y-W, Chiou RYY. 2006. Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (Arachis hypogaea L.) arachidin-1, arachidin-3, and isopentadienylresveratrol. J Agric Food Chem 54(26):10281-7.
Chen CK, Pace-Asciak CR. 1996. Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. Gen Pharmacol 27(2):363-6.
Chen R, Wu P, Chiou R. 2002. Peanut roots as a source of resveratrol. J Agric Food Chem 50:1665-7.
Cho D-I, Koo N-Y, Chung WJ, Kim T-S, Ryu SY, Im SY, Kim K-M. 2002. Effects of resveratrol-related hydroxystilbenes on the nitric oxide production in macrophage cells: structural requirements and mechanism of action. Life Sci 71(17):2071-82.
Chukwumah Y, Walker L, Vogler B, Verghese M. 2007a. Changes in the phytochemical composition and profile of raw, boiled, and roasted peanuts. J Agric Food Chem 55(22):9266-73.
Chukwumah YC, Walker LT, Verghese M, Bokanga M, Ogutu S, Alphonse K. 2007b. Comparison of extraction methods for the quantification of selected phytochemicals in peanuts (Arachis hypogaea). J Agric Food Chem 55(2):285-90.
Chung HJ, Colakoglu AS, Min DB. 2004. Relationships among headspace oxygen, peroxide value, and conjugated diene content of soybean oil oxidation. J Food Sci 69(2):FCT83-FCT8.
Chung I-M, Park MR, Chun JC, Yun SJ. 2003. Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. Plant Sci 164(1):103-9.
Ciolino HP, Levine RL. 1997. Modification of proteins in endothelial cell death during oxidative stress. Free Radical Bio Med 22(7):1277-82.
D'Ovidio K, Trucksess M, Weaver C, Horn E, McIntosh M, Bean G. 2006. Aflatoxins in ginseng roots. Food Addit Contam 23(2):174-80.
Daigle DJ, Conkerton EJ, Sanders TH, Mixon AC. 1988. Peanut hull flavonoids: Their relationship with peanut maturity. J Agric Food Chem 36:1179-81.
Davies KJA, Goldberg AL. 1987. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. J Biol Chem 262(17):8220-6.
Dean LL, Davis JP, Shofran BG, Sanders TH. 2008. Phenolic profiles and antioxidant activity of extracts from peanut plant parts. Open Nat Prod J 1:1-6.
Decker EA, Warner K, Richards MP, Shahidi F. 2005. Measuring antioxidant effectiveness in food. J Agric Food Chem 53:4303-10.
Delmas D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N, Solary E. 2003. Resveratrol-induced apoptosis is associated with fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. J Biol Chem 278(42):41482-90.

Dong Z. 2003. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat Res, Fundam Mol Mech Mutagen 523-524:145-50.
Duh P, Yeh D, Yen G. 1992. Extraction and identification of an antioxidative component from peanut hulls. J Am Oil Chem Soc 69(8):814-8.
Ek A, Stroem K, Cotgreave IA. 1995. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. Biochem Pharmacol 50(9):1339-46.
Erdogan H, Fadillioglu E, Yagmurca M, Ucar M, Irmak MK. 2006. Protein oxidation and lipid peroxidation after renal ischemia-reperfusion injury: Protective effects of erdosteine and *N*-acetylcysteine. Urol Res 34:41-6.
Estevez M, Cava R. 2006. Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: Contradictory effects in different types of frankfurters. Meat Sci 72:348-55.
Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. 1993. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341(8843):454-7.
Fu Z, Huang X, Min S. 2008. Rapid determination of aflatoxins in corn and peanuts. J Chromatogr, A 1209(1-2):271-4.
Fukumoto LR, Mazza G. 2000. Assessing antioxidant and prooxidant activities of phenolic compounds. J Agric Food Chem 48(8):3597-604.
Garcia-Llatas G, Lagarda MJ, Romero F, Abellan P, Farre R. 2006. A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: Application to liquid infant foods and powdered infant formulas. Food Chem 101(3):1078-86.
Georgantelis D, Ambrosiadis I, Katikou P, Blekas G, Georgakis SA. 2007. Effect of rosemary extract, chitosan and a-tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4 DegC. Meat Sci 76(1):172-81.
Georgantelis D, Blekas G, Katikou P, Ambrosiadis I, Fletouris DJ. 2006. Effect of rosemary extract, chitosan, alpha -tocopherol on lipid oxidation and colour stability during frozen storage of beef burgers. Meat Sci 75(2):256-64.
Gharavi N, Haggarty S, El-Kadi AOS. 2007. Chemoprotective and carcinogenic effects of tert-butylhydroquinone and its metabolites. Curr Drug Metab 8(1):1-7.
Ghezzi P, Bonetto V. 2003. Redox proteomics: Identification of oxidatively modified proteins. Proteomics 3:1145-53.
Gilbert J, Vargas E. 2005. Advances in sampling and analysis for aflatoxins in food and animal feed. In: Abbas H, editor. Aflatoxins and Food Safety. Boca Raton: CRC Press. p 237-68.
Gillespie KM, Chae JM, Ainsworth EA. 2007. Rapid measurement of total antioxidant capacity in plants. Nat Protoc 2(4):867-70.
Giuffrida F, Golay P-A, Destaillats F, Hug B, Dionisi F. 2005. Accurate determination of hexanal in beef bouillons by headspace solid-phase microextraction gas-chromatography mass-spectrometry. Eur J Lipid Sci Technol 107(11):792-8.
Gomez-Catalan J, Pique E, Falco G, Borrego N, Rodamilans M, Llobet JM. 2005. Determination of aflatoxins in medicinal herbs by HPLC. An efficient method for routine analysis. Phytochem Anal 16(3):196-204.
Grune T, Reinheckel T, Davies KJA. 1997. Degradation of oxidized proteins in mammalian cells. FASEB J 11(7):526-34.
Harman D. 1956. Aging: A theory based on free radical and radiation chemistry. J Gerontol 11:298-300.

Heinrich M, Barnes J, Gibbons S, Williamson E. 2004. Fundamentals of pharmacognosy and phytotherapy. Edinburgh: Churchill Livingstone.
Henning SM, Niu Y, Lee NH, Thames GD, Minutti RR, Wang H, Go VLW, Heber D. 2004. Bioavailability and antioxidant activity of tea flavanols after consumption of green tea, black tea, or a green tea extract supplement. Am J Clin Nutr 80(6):1558-64.
Herbert DA, Jr., Malone S, Aref S, Brandenburg RL, Jordan DL, Royals BM, Johnson PD. 2007. Role of insecticides in reducing thrips injury to plants and incidence of tomato spotted wilt virus in Virginia market-type peanut. J Econ Entomol 100(4):1241-7.
Holmes-McNary M, Baldwin AS, Jr. 2000. Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the IkappaB kinase. Canc Res 60(13):3477-83.
Huang D, Ou B, Prior R. 2005. The chemistry behind antioxidant assays. J Agric Food Chem 53:1841-56.
Huang S, Yen G, Chang L, Yen W, Duh P. 2003. Identification of an antioxidant, ethyl protocatechuate, in peanut seed testa. J Agric Food Chem 51:2380-3.
Ibern-Gomez M, Roig-Perez S, Lamuela-Raventos RM, de la Torre-Boronat MC. 2000. Resveratrol and piceid levels in natural and blended peanut butters. J Agric Food Chem 48(12):6352-4.
Ingham JL. 1976. 3,5,4'-Trihydroxystilbene as a phytoalexin from groundnuts (Arachis hypogaea). Phytochemistry 15(11):1791-3.
Ingold KU. 1961. Inhibition of the autoxidation of organic substances in the liquid phase. Chem Rev 61:563-89.
Ito N, Hirose M, Fukushima S, Tsuda H, Shirai T, Tatematsu M. 1986. Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis. Fd Chem Toxic 24(10/11):1071-82.
Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC, Pezzuto JM. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275(5297):218-20.
Jeandet P, Bessis R, Sbaghi M, Meunier P. 1994. Occurrence of a resveratrol beta -D-glucoside in wine: Preliminary studies. Vitis 33(3):183-4.
Jelen HH, Obuchowska M, Zawirska-Wojtasiak R, Wasowicz E. 2000. Headspace Solid-Phase Microextraction Use for the Characterization of Volatile Compounds in Vegetable Oils of Different Sensory Quality. Journal of Agricultural and Food Chemistry 48(6):2360-7.
Jensen PN, Danielsen B, Bertelsen G, Skibsted LH, Andersen ML. 2004. Storage stabilities of pork scratchings, peanuts, oatmeal and muesli: Comparison of ESR spectroscopy, headspace-GC and sensory evaluation for detection of oxidation in dry foods. Food Chem 91(1):25-38.
Kahkonen MP, Hopia AI, Vuorela HJ, Rauha J-P, Pihlaja K, Kujala TS, Heinonen M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem 47:3954-62.
Karakaya S, El SN, Tas AA. 2001. Antioxidant activity of some foods containing phenolic compounds. Int J Food Sci Nutr 52:501-8.
Katsube T, Tabata H, Ohta Y, Yamasaki Y, Anuurad E, Shiwaku K, Yamane Y. 2004. Screening for antioxidant activity in edible plant products: Comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu Assay. J Agric Food Chem 52:2391-6.

Keen NT, Ingham JL. 1976. New stilbene phytoalexins from American cultivars of Arachis hypogaea. Phytochemistry 15(11):1794-5.
King R, Bomser J, Min D. 2006. Bioactivity of resveratrol. Compr Rev Food Sci Food Saf 5:65-70.
Kopp P. 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the "French paradox"? Eur J Endocrinol 138(6):619-20.
Kornsteiner M, Wagner K-H, Elmadfa I. 2006. Tocopherols and total phenolics in 10 different nut types. Food Chem 98:381-7.
Kundu JK, Surh Y-J. 2004. Molecular basis of chemoprevention by resveratrol: NF-kB and AP-1 as potential targets. Mutat Res 555(1-2):65-80.
Labieniec M, Gabryelak T. 2005. Measurement of DNA damage and protein oxidation after the incubation of B14 Chinese hamster cells with chosen polyphenols. Toxicol Lett 155:15-25.
Lamb MC, Sternitzke DA. 2001. Cost of aflatoxin to the farmer, buying point, and sheller segments of the southeast United States peanut industry. Peanut Sci 28:59-63.
Lee CH, Yang L, Xu JZ, Yeung SYV, Huang Y, Chen Z-Y. 2005a. Relative antioxidant activity of soybean isoflavones and their glycosides. Food Chem 90:735-41.
Lee KW, Hur HJ, Lee HJ, Lee CY. 2005b. Antiproliferative effects of dietary phenolic substances and hydrogen peroxide. J Agric Food Chem 53(6):1990-5.
Li H, Ashcraft DK, Freeman BD, Stewart ME, Jank MK, Clark TR. 2008. Non-invasive headspace measurement for characterizing oxygen-scavenging in polymers. Polymer 49(21):4541-5.
Lin Y, Kuo Y, Chen C. 1999. Antioxidative principles from peanut hulls. The Chinese Pharmaceutical Journal 51:397-401.
Liu C-D, Wen Y-Y, Chiou J-M, Wang K-H, Chiou RYY. 2003. Comparative characterization of peanuts grown by aquatic floating cultivation for seed and resveratrol production. J Agric Food Chem 51(6):1582-5.
Llesuy S, Evelson P, Campos AM, Lissi E. 2001. Methodologies for evaluation of total antioxidant activities in complex mixtures. A critical review. Biol Res 34(2).
Lloyd RV, Hanna PM, Mason RP. 1997. The origin of the hydroxyl radical oxygen in the Fenton reaction. Free Radical Biol Med 22(5):885-8.
Lotito S, Frei B. 2006. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free Radic Biol Med 41:1727-46.
Lou H, Yamazaki Y, Sasaki T, Uchida M, Tanaka H, Oka S. 1999. A-type proanthocyanidins from peanut skins. Phytochemistry 51:297-308.
Lou H, Yuan H, Ma B, Ren D, Ji M, Oka S. 2004. Polyphenols from peanut skins and their free radical-scavenging effects. Phytochemistry 65:2391-9.
Lou H, Yuan H, Yamazaki Y, Sasaki T, Oka S. 2001. Alkaloids and flavonoids from peanut skins. Planta Med 67:345-9.
Lu L, Hackett SF, Mincey A, Lai H, Campochiaro PA. 2006. Effects of different types of oxidative stress in RPE cells. J Cell Physiol 206:119-25.
Luc G, Fruchart JC. 1991. Oxidation of lipoproteins and atherosclerosis. AM J Clin Nutr 53(1 Suppl):206S-9S.

Magalhaes Luis M, Segundo Marcela A, Reis S, Lima Jose LFC, Rangel Antonio OSS. 2006. Automatic method for the determination of Folin-Ciocalteu reducing capacity in food products. J Agric Food Chem 54(15):5241-6.

Manna SK, Mukhopadhyay A, Aggarwal BB. 2000. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kB, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. J Immunol 164(12):6509-19.

Mate JI, Saltveit ME, Krochta JM. 1996. Peanut and walnut rancidity: effects of oxygen concentration and relative humidity. J Food Sci 61(2):465-8, 72.

Medina-Bolivar F, Condori J, Rimando A, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan M. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003.

Medina-Remon A, Barrionuevo-Gonzalez A, Zamora-Ros R, Andres-Lacueva C, Estruch R, Martinez-Gonzalez M-A, Diez-Espino J, Lamuela-Raventos RM. 2009. Rapid Folin-Ciocalteu method using microtiter 96-well plate cartridges for solid phase extraction to assess urinary total phenolic compounds, as a biomarker of total polyphenols intake. Anal Chim Acta 634(1):54-60.

Mildner-Szkudlarz S, Jelen HH, Zawirska-Wojtasiak R, Wasowicz E. 2003. Application of headspace-solid phase microextraction and multivariate analysis for plant oils differentiation. Food Chemistry 83(4):515-22.

Mirghani MES, Man YBC, Jinap S, Baharin BS, Bakar J. 2001. A new method for determining aflatoxins in groundnut and groundnut cake using Fourier transform infrared spectroscopy with attenuated total reflectance. J Am Oil Chem Soc 78(10):985-92.

Montecinos V, Guzman P, Barra V, Villagran M, Munoz-Montesino C, Sotomayor K, Escobar E, Godoy A, Mardones L, Sotomayor P, Guzman C, Vasquez O, Gallardo V, van Zundert B, Bono Maria R, Onate Sergio A, Bustamante M, Carcamo Juan G, Rivas Coralia I, Vera Juan C. 2007. Vitamin C is an essential antioxidant that enhances survival of oxidatively stressed human vascular endothelial cells in the presence of a vast molar excess of glutathione. J Biol Chem 282(21):15506-15.

Montoro P, Braca A, Pizza C, De Tommasi N. 2005. Structure-antioxidant activity relationships of flavonoids isolated from different plant species. Food Chem 92(2):349-55.

Nakai VK, Rocha LdO, Goncalez E, Fonseca H, Ortega EMM, Correa B. 2007. Distribution of fungi and aflatoxins in a stored peanut variety. Food Chem 106(1):285-90.

Nawar W. 1996. Lipids. In: Fennema O, editor. Food Chemistry. 3rd ed. New York: Marcel Dekker, Inc.

Nepote V, Grosso NR, Guzman CA. 2000. Antioxidant activity of methanolic extracts from peanut skin. Molecules [Electronic Publication] 5(3):487-8.

Nepote V, Grosso NR, Guzman CA. 2002. Extraction of antioxidant components from peanut skins. Grasas y Aceites 53:391-5.

Nepote V, Grosso NR, Guzman CA. 2005. Optimization of extraction of phenolic antioxidants from peanut skins. J Sci Food Agr 85:33-8.

Nepote V, Mestrallet MG, Grosso NR. 2004. Natural antioxidant effect from peanut skins in honey-roasted peanuts. J Food Sci 69(7):295-300.

Nissen LR, Byrne DV, Bertelsen G, Skibsted LH. 2004. The antioxidative activity of plant extracts in cooked pork patties as evaluated by descriptive sensory profiling and chemical analysis. Meat Sci 68:485-95.

Norata GD, Marchesi P, Passamonti S, Pirillo A, Violi F, Catapano AL. 2007. Anti-inflammatory and anti-atherogenic effects of catechin, caffeic acid and trans-resveratrol in apolipoprotein E deficient mice. Atherosclerosis 191(2):265-71.
O'Brien NM, Woods JA, Aherne SA, O'Callaghan YC. 2000. Cytotoxicity, genotoxicity and oxidative reactions in cell-culture models: modulatory effects of phytochemicals. Biochem Soc T 28(2):22-6.
O'Keefe SF, Wang H. 2006. Effects of peanut skin extract on quality and storage stability of beef products. Meat Sci 73:278-86.
Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. 2002. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. J Agric Food Chem 50:3122-8.
Pace-Asciak CR, Hahn S, Diamandis EP, Soleas G, Goldberg DM. 1995. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implications for protection against coronary heart disease. Clinica Chimica Acta 235(2):207-19.
Pastorelli S, Valzacchi S, Rodriguez A, Simoneau C. 2006. Solid-phase microextraction method for the determination of hexanal in hazelnuts as an indicator of the interaction of active packaging materials with food aroma compounds. Food Addit Contam 23(11):1236-41.
Perez-Jimenez J, Saura-Calixto F. 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Res Int 39:791-800.
Pezet R, Pont V. 1995. Mode of toxic action of vitaceae stilbenes on fungal cells. In: Daniel M, Purkayastha RP, editors. Handbook of Phytoalexin Metabolism and Action. New York: Marcel Dekker, Inc. p 317-31.
Pietta P, Simonetti P, Mauri P. 1998. Antioxidant activity of selected medicinal plants. J Agric Food Chem 46:4487-90.
Pignatelli P, Ghiselli A, Buchetti B, Carnevale R, Natella F, Germano G, Fimognari F, Di Santo S, Lenti L, Violi F. 2006. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. Atherosclerosis 188:77-83.
Prior RL, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290-302.
Przybylski R, Eskin NAM. 1995. Methods to measure volatile compounds and the flavor significance of volatile compounds. In: Warner K, Eskin NAM, editors. Methods to assess quality and stability of oils and fat-containing foods. Champaign, IL: AOCS Press. p 107-33.
Purkayastha RP. 1995. Progress in phytoalexin research during the past 50 years. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 1-39.
Rao PVS, Strange R. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 199-227.
Renaud S, Debord S. 1992. Wine, alcohol, platelets and the french paradox for coronary heart disease. Lancet 339:1523-6.
Richards A, Wijesundera C, Salisbury P. 2005. Evaluation of oxidative stability of canola oils by headspace analysis. J Am Oil Chem Soc 82(12):869-74.

Robb CS, Geldart SE, Seelenbinder JA, Brown PR. 2002. Analysis of green tea constituents by HPLC-FT-IR. J Liq Chromatogr Relat Technol 25(5):787-801.
Romero-Perez AI, Lamuela-Raventos RM, Andres-Lacueva C, de la Torre-Boronat MC. 2001. Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content. J Agric Food Chem 49(1):210-5.
Rowland D, Dorner J, Sorensen R, Beasley JP, Jr., Todd J. 2005. Tomato spotted wilt virus in peanut tissue types and physiological effects related to disease incidence and severity. Plant Pathol 54(4):431-40.
Rudolf JR, Resurreccion AVA. 2005. Elicitation of resveratrol in peanut kernels by application of abiotic stresses. J Agric Food Chem 53(26):10186-92.
Sahin E, Gumuslu S. 2004. Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stress models. Behav Brain Res 155:241-8.
Sanders TH, McMichael RW, Hendrix KW. 2000. Occurrence of resveratrol in edible peanuts. J Agric Food Chem 48:1243-6.
Scheidegger K, Payne GA. 2005. Unlocking the secrets behind secondary metabolism: A review of *Aspergillus flavus* from pathogenicity to functional genomics. In: Abbas H, editor. Aflatoxin and Food Safety. Boca Raton: CRC Press. p 137-66.
Shahidi F. 2000. Antioxidants in food and food antioxidants. Nahrung 44(3):158-63.
Signorelli P, Ghidoni R. 2005. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. J Nutr Biochem 16(8):449-66.
Simonetti P, Ciappellano S, Gardana C, Bramati L, Pietta P. 2002. Procyanidins from *Vitis vinifera* seeds: In vivo effects on oxidative stress. J Agric Food Chem 50:6217-21.
Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 299(Oxidants and Antioxidants, Part A):152-78.
Sobolev V, Cole R. 1999. *trans*-Resveratrol content in commercial peanuts and peanut products. J Agric Food Chem 47:1435-9.
Sobolev V, Horn B, Potter T, Deyrup ST, Gloer JB. 2006a. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. J Agric Food Chem 54:3505-11.
Sobolev V, Potter T, Horn B. 2006b. Prenylated stilbenes from peanut root mucilage. Phytochem Anal 17:312-22.
Sobolev VS. 2008. Localized production of phytoalexins by peanut (Arachis hypogaea) kernels in response to invasion by Aspergillus species. J Agric Food Chem 56(6):1949-54.
Sobolev VS, Deyrup ST, Gloer JB. 2006c. New peanut (Arachis hypogaea) phytoalexin with prenylated benzenoid and but-2-enolide moieties. J Agric Food Chem 54(6):2111-5.
Sobolev VS, Guo BZ, Holbrook CC, Lynch RE. 2007. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. J Agric Food Chem 55(6):2195-200.
Sobolev VS, Neff SA, Gloer JB. 2009. New stilbenoids from peanut (Arachis hypogaea) seeds challenged by an Aspergillus caelatus strain. J Agric Food Chem 57(1):62-8.
Soleas G, Grass L, David Josephy P, Goldberg D, Diamandis E. 2002. A comparison of the anticarcinogenic properties of four red wine polyphenols. Clin Biochem 35:119-24.
Stadtman ER, Berlett BS. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol 10(5):485-94.

Steenson DF, Lee JH, Min DB. 2002. Solid phase microextraction of volatile soybean oil and corn oil compounds. J Food Sci 67(1):71-6.
Suh HJ, Chung MS, Cho YH, Kim JW, Kim DH, Han KW, Kim CJ. 2005. Estimated daily intakes of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) antioxidants in Korea. Food Addit Contam 22(12):1176-88.
Sun C, Zhang F, Ge X, Yan T, Chen X, Shi X, Zhai Q. 2007. SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B. Cell metabolism 6(4):307-19.
Sun T, Ho C-T. 2005. Antioxidant activities of buckwheat extracts. Food Chem 90:743-9.
Tanko JM. 2005. Reaction Mechanisms: Part (i) Radical and radical ion reactions. Annu Rep Prog Chem, Sect B 101:235-63.
Tsai S-H, Lin-Shiau S-Y, Lin J-K. 1999. Suppression of nitric oxide synthase and the down-regulation of the activation of NFkB in macrophages by resveratrol. Brit J Pharmacol 126(3):673-80.
Ungvari Z, Orosz Z, Rivera A, Labinskyy N, Zhao X, Olson S, Podlutsky A, Csiszar A. 2007. Resveratrol increases vascular oxidative stress resistance. Am J Physiol 292(5, Pt. 2):H2417-H24.
Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology 39:44-84.
van Der Heijden CA, Janssen PJCM, Strik JJTWA. 1986. Toxicology of gallates: A review and evaluation. Fd Chem Toxic 24(10/11):1067-70.
van Esch GJ. 1986. Toxicology of *tert*-butylhydroquinone (TBHQ). Fd Chem Toxic 24(10/11):1063-5.
Vanschoonbeek K, Thomassen B, Senden J, Wodzig W, van Loon L. 2006. Cinnamon supplementation does not improve glycemic control in postmenopausal type 2 patients. J Nutr 136(4):977-80.
Vendemiale G, Grattagliano I, Altomare E. 1999. An update on the role of free radicals and antioxidant defense in human disease. Inter J Clin Lab Res 29:49-55.
Vian MA, Tomao V, Gallet S, Coulomb PO, Lacombe JM. 2005. Simple and rapid method for cis- and trans-resveratrol and piceid isomers determination in wine by high-performance liquid chromatography using Chromolith columns. J Chromatogr, A 1085(2):224-9.
Vitaglione P, Sforza S, Galaverna G, Ghidini C, Caporaso N, Vescovi PP, Fogliano V, Marchelli R. 2005. Bioavailability of trans-resveratrol from red wine in humans. Mol Nutr Food Res 49(5):495-504.
Walle T, Hsieh F, DeLegge MH, Oatis JE, Jr., Walle UK. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 32(12):1377-82.
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(1):242-50.
Whitaker TB, Trucksess MW, Weaver CM, Slate A. 2009. Sampling and analytical variability associated with the determination of aflatoxins and ochratoxin A in bulk lots of powdered ginger marketed in 1-lb bags. Anal Bioanal Chem:No pp yet given.
Wood LG, Gibson PG, Garg ML. 2006. A review of the methodology for assessing *in vivo* antioxidant capacity. J Sci Food Agric 86:2057-66.
Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior R. 2004a. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J Agric Food Chem 52:4026-37.

Wu X, Gu L, Holden J, Haytowitz DB, Gebhardt SE, Beecher G, Prior RL. 2004b. Development of a database for total antioxidant capacity in foods: a preliminary study. J Food Compos Anal 17(3-4):407-22.
Xu BJ, Chang SKC. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J Food Sci 72(2):S159-S66.
Yen G, Duh P. 1993. Antioxidative properties of methanolic extracts from peanut hulls. J Am Oil Chem Soc 70(4):383-6.
Yen G, Duh P. 1994. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. J Agric Food Chem 42:629-32.
Yen G, Duh P. 1995. Antioxidant activity of methanolic extracts of peanut hulls from various cultivars. J Am Oil Chem Soc 72(9):1065-7.
Yen G, Duh P, Tsai C. 1993. Relationship between antioxidant activity and maturity of peanut hulls. J Agric Food Chem 41:67-70.
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, Dai J. 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. J Food Compos Anal 19:364-71.
Zhang Z, Pawliszyn J. 1993. Headspace solid-phase microextraction. Anal Chem 65(14):1843-52.

FIGURES

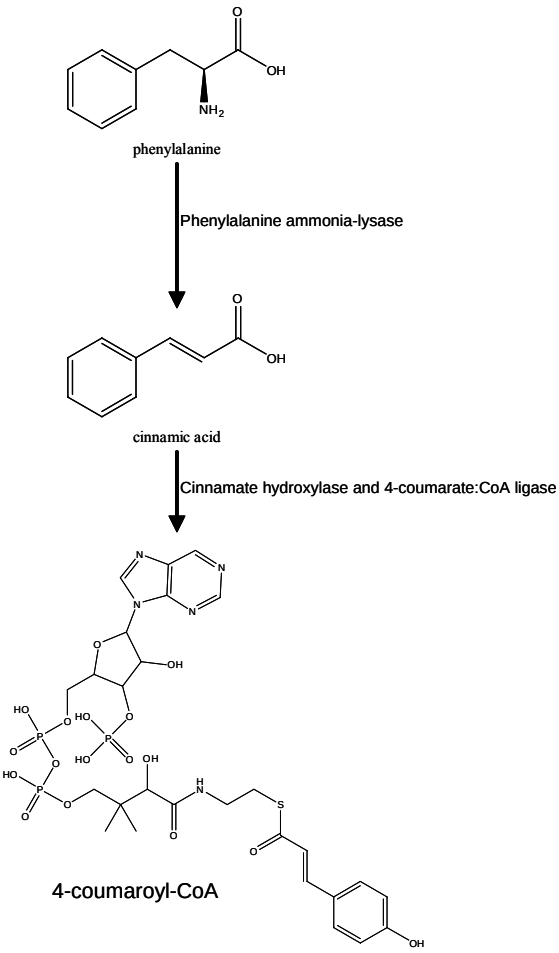

Figure 2.1: Shikimic-polymalonate acid pathway. 4-coumaroyl-CoA then forms an intermediate with three molecules of malonyl-CoA. This intermediate then rearranges to form various phytoalexins.

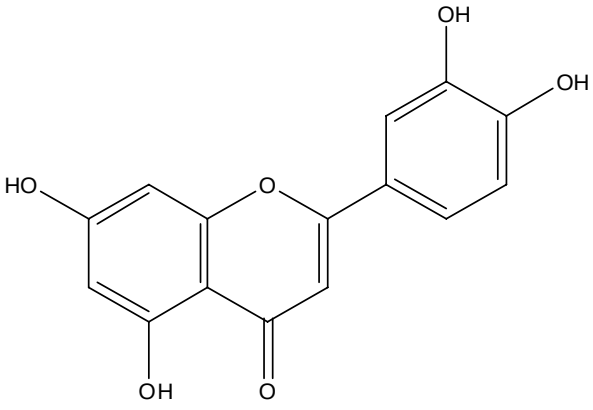

Figure 2.2: Luteolin structure

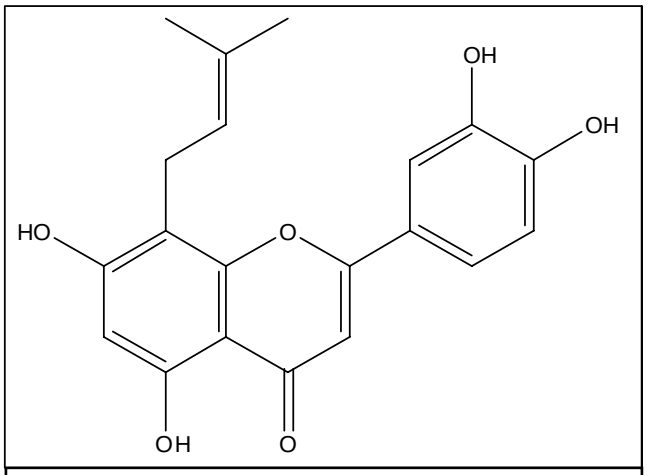

Figure 2.3: 3',4',5,7-tetrahydroxy-8-(3-methylbut-2-enyl)flavone structure

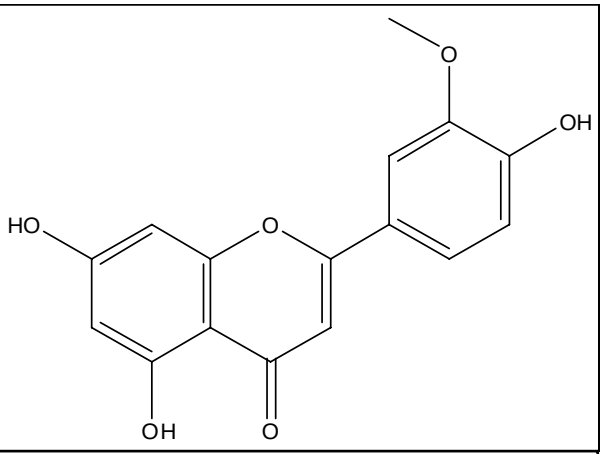

Figure 2.4: Chrysoeriol structure

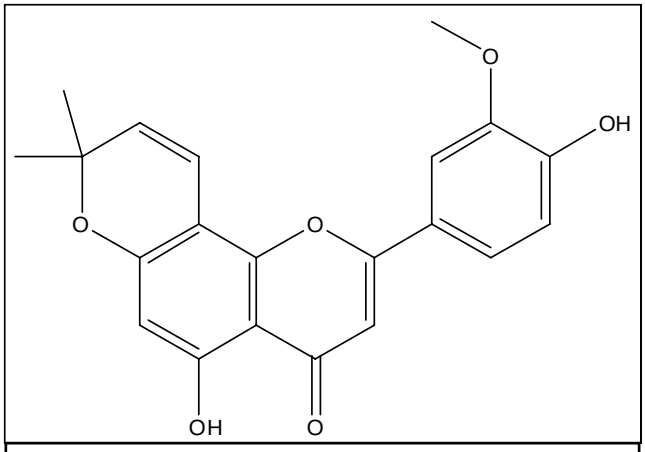

Figure 2.5: 4’,5-dihydroxy-3’-methoxy-2’’,2’’-dimethylpyrano[5’’,6’’:7:8]flavone structure

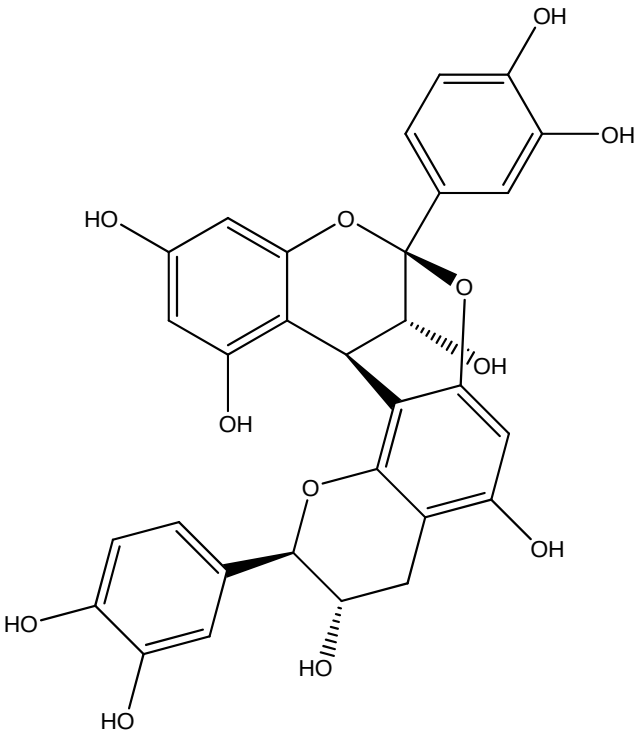

Figure 2.6: Representative A-type proanthocyanidin

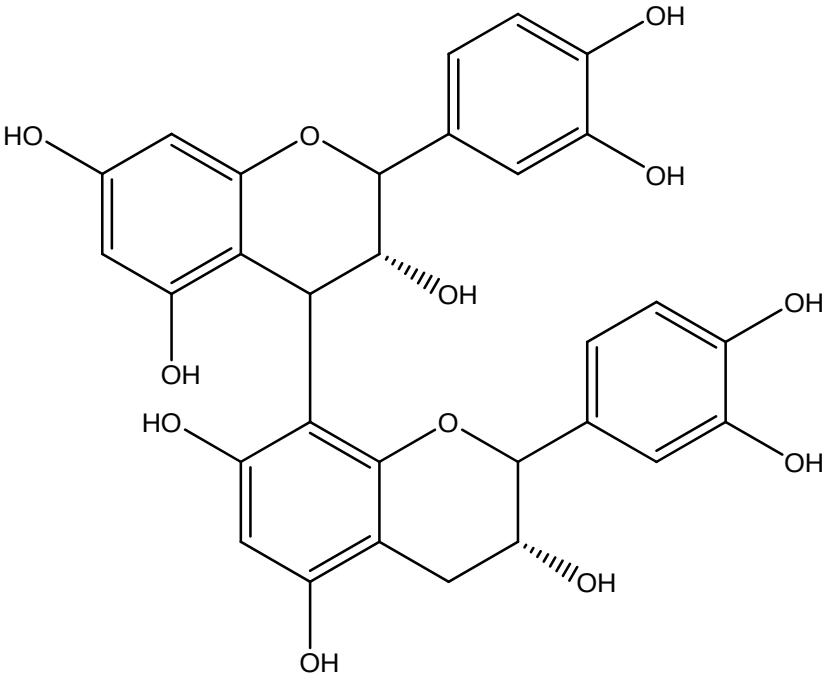

Figure 2.7: Representative B-type proanthocyanidin

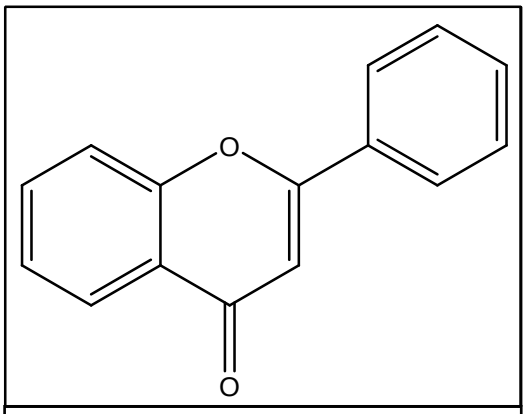

Figure 2.8: Basic flavonoid structure

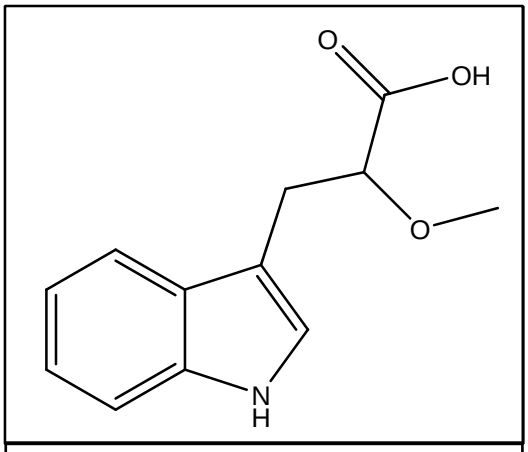

Figure 2.9: Indole alkaloid structure

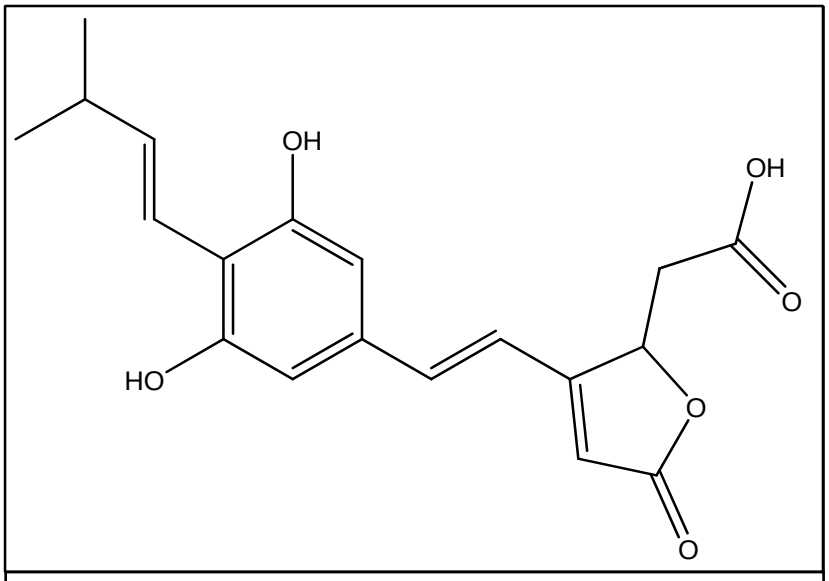

Figure 2.10: Proposed structure of SB-1

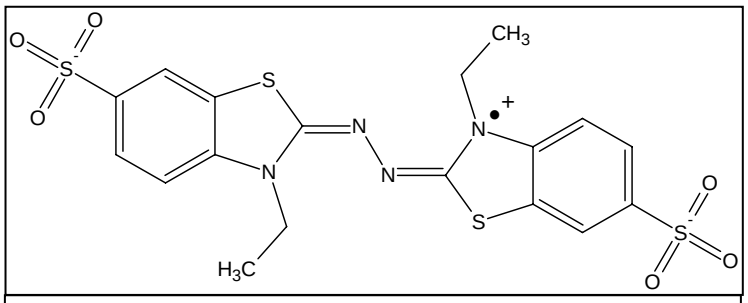

Figure 2.11: ABTS radical

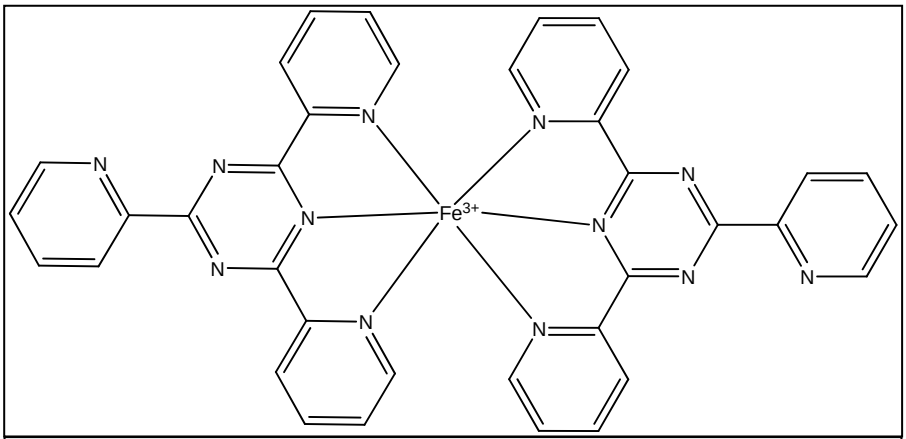

Figure 2.12: FRAP reagent.

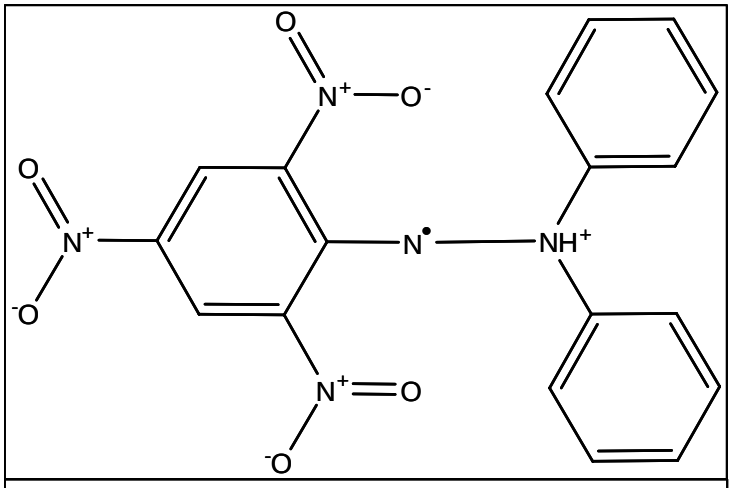

Figure 2.13: DPPH (2,2-diphenyl-1-picrylhydrazyl)

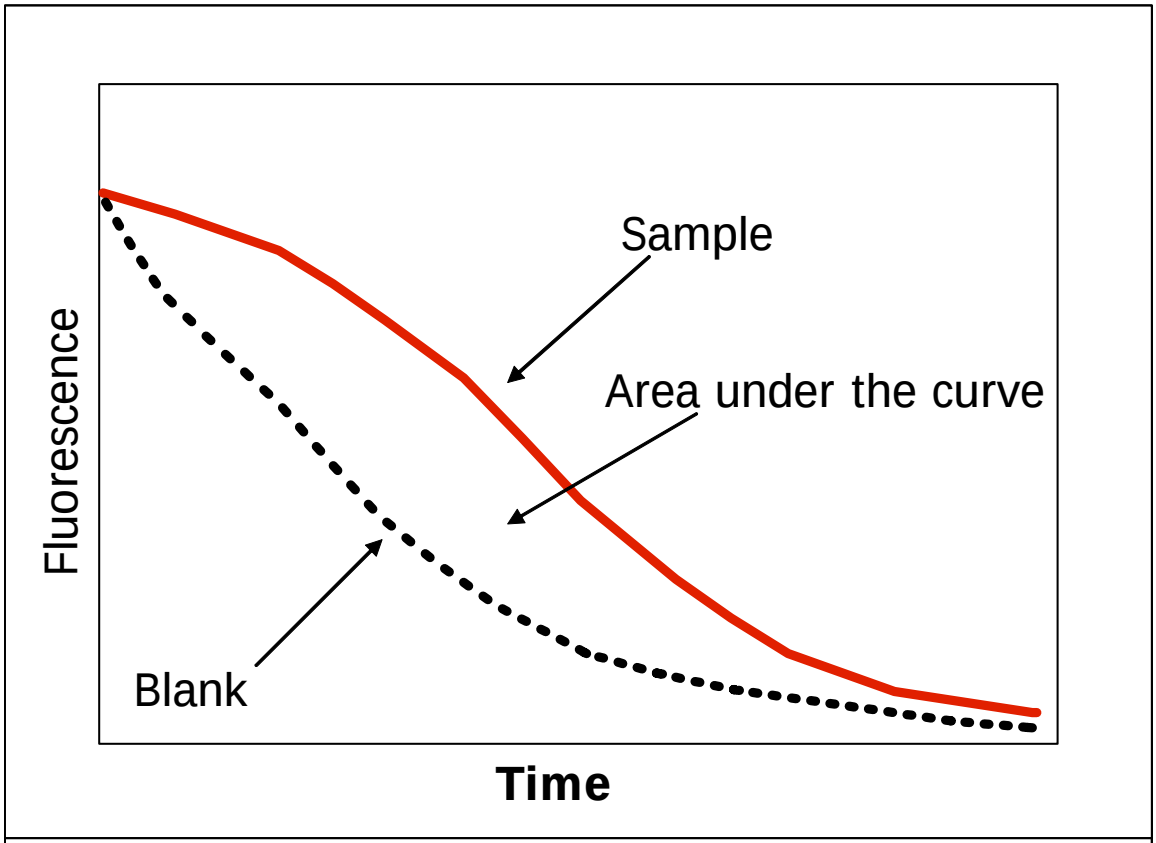

Figure 2.14: Sample ORAC curve. Area under the curve is measured from initial time to a later time specified by the researcher.

CHAPTER 3: LEVELS OF *TRANS*-RESVERATROL IN VIRGINIA-TYPE PEANUT (*ARACHIS HYPOGAEA)* ROOTS

Abstract

Dietary *trans*-resveratrol has been shown to have positive health effects, lowering risk of cancer and heart disease as well as having anti-inflammatory actions. Resveratrol has been reported in peanut products and in peanut roots. The purpose of this work was to measure *trans*-resveratrol levels in US-grown peanut roots from the Tidewater area of Virginia and to investigate the effect of tomato spotted wilt virus (TSWV) infestation on root resveratrol levels.

Virginia-type peanut roots from individual plant samples grown in replicate rows with two levels of TSWV infestation were extracted with 75% aqueous methanol with ultrasonic assistance. Eight cultivars were examined: CHAMPS, Wilson, Brantley, NC 12C, Philips, Perry, NC-V 11, and VA 98R. Two samples of each cultivar were examined. The extracts were analyzed via HPLC using a C-18 column and acidified aqueous methanol gradient elution with diode array detection. Concentrations were determined by using a commercially available *trans*-resveratrol standard.

Resveratrol was identified by retention time match and UV absorbance spectrum similarity with standard. The concentrations of *trans*-resveratrol found in root samples ranged from below detection limits to 46.3 μg/g. Root samples with high levels of disease did not exhibit significantly higher *trans*-resveratrol levels then plants with low disease level. Cultivar and TSWV were not significant factors in predicting resveratrol concentration either.

Key Words: resveratrol, peanut, roots, phytoalexin, *Arachis hypogaea*

Introduction

Plants produce different chemicals in the course of their life for many reasons. One class of these compounds is phytoalexins. Phytoalexins are compounds that are produced when a plant cell is under attack and are used to thwart the attack (Purkayastha, 1995). The elicitation of phytoalexins can be induced by numerous factors such as microbes, fungi, UV light, and heavy metal salts (Rao and Strange, 1995). These stressors activate the shikimic-polymalonate acid pathway that synthesizes flavonoids and stilbenes. Once created, the phytoalexins are secreted into the extracellular space to defend against the threat. Some phytoalexins reportedly can inhibit microbial/fungal invasion (Rao and Strange, 1995). However, the phytoalexins face the threat of being broken down or used in synthesis of polymers by peroxidases (Barz et al., 1990). This may be one explanation for the low concentration of phytoalexins in tissue, unless the cells are producing them at a high level induced by stress, then degrade them at high rates.

The mechanism by which the phytoalexins inhibit fungal growth is not fully understood, but one idea has gained support. It is believed that their main action is on the cell membrane of the invasive cells directly or indirectly. Phytoalexins disrupt the cellular membranes and lower the respiration rate of the fungal cells. Pezet and Pont (1995) found that stilbenes almost immediately slow down fungal cells' respiration. They believe that stilbenes attacked membranes in the order of mitochondria>nucleus>endoplasmic reticulum>cytoplasmic membrane. Pezet and Pont conclude that the stilbenes cause their damage by impeding the action of flavin enzymes, which allows lipid peroxidation to proceed unchecked.

Some of these phytoalexins have been shown in cell culture to have healthful benefits. One of the most well known phytoalexins and stilbene present in foods is *trans*-resveratrol and its associated forms (figure 3.1). *trans*-Resveratrol is the form considered most active and the form synthesized in the greatest abundance in peanut roots (Dixon, 2001; Roupe et al., 2006). *In vitro* experiments with resveratrol have shown it to have vasodilatory effects and to inhibit cancer (Dong, 2003; King et al., 2006). Resveratrol is thought to be a factor involved in the "French Paradox." The "French Paradox" refers to people living around the Mediterranean Sea consuming diets high in fat, but do not experience the high heart disease rates as expected, given their diets. Their high fat diet is thought to be counteracted by their ingestion of red wine with a high concentration of *trans*-resveratrol (Frankel et al., 1993a). Alcohol has been shown to decrease the risk of heart disease, however it does not account for the low risk the French

typically encounter (Renaud and Debord, 1992). Renaud and de Lorgeril (1992) found that platelet aggregation was quite decreased in the French population that they tested. They believe this is due to wine consumption, but did not elaborate on a mechanism. Frankel and fellow researchers (1993b) were the first to link phenolic compounds with the French paradox. They found that red wine, diluted 1000 times, significantly inhibited LDL oxidation *in vitro*, even more than α-tocopherol. The diluted red wine decreased hexanal formation and copper-catalyzed oxidation. Frankel and colleagues noted that the phenolics were not inhibiting oxidation through metal chelation because the antioxidant effect was not diminished when an excess of copper was introduced. They believe that the phenolics present in wine are superb electron donors. The evidence to support this claim is given by the phenolics' ability to reduce the myoglobin oxoferryl radical, produced by mixing equal amounts of hydrogen peroxide and myglobin. Other compounds found in grapes are reported to exhibit vasodilatory effects. Fitzpatrick and colleagues (2000) observed various trimers, tetramers, pentamers, and polymers of proanthocyanidins responsible for vasodilatory effects in a rat aorta model system.

Further evidence of resveratrol's healthy attributes has been published in a recent article where the researchers fed mice a high-calorie diet. Mice were treated with resveratrol supplements (either 5.2 or 22.4 mg/kg/day), which increased their health and survival (Baur et al., 2006). Baur *et al*. also observed an increase in insulin sensitivity among mice treated with resveratrol compared to the control mice or mice fed just the high calorie diet. They found that the most significant effects were seen in the high dose of resveratrol. Peanut plants (*Arachis hypogaea*) contain resveratrol, *trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene and other phytoalexins (Sobolev et al., 2007) (figure 3.2). Resveratrol has been found at high concentrations in the roots (Chen et al., 2002). Peanut roots have the potential to become a dietary supplement and could contain more phytoalexins than currently known.

Resveratrol has been shown to have antioxidant capacity, which in turn reduces low-density lipoprotein (LDL) oxidation *in vitro* (Frankel et al., 1993b). LDL oxidation is believed to be a major factor in the initiation and progression of atherosclerosis (Vinson et al., 1999; Virgili et al., 2003). Resveratrol can also protect against blood clots by preventing platelet aggregation and promoting vasodilation (Olas and Wachowicz, 2005). There is some evidence that suggests resveratrol fights cancer by retarding tumor initiation and promotion (Cao et al.,

2005; Dong, 2003; Jang et al., 1997; King et al., 2006). Resveratrol suppresses the NF-κβ pathway, discouraging cancer growth and spread. It also participates in the AP-1 pathway, which is also important in its anticancer properties. How resveratrol partakes in these pathways is still unknown, but is being investigated (Aggarwal and Shishodia, 2006).

The evidence regarding the bioavailability of resveratrol in humans has shown it to be absorbed in high amounts, but plasma levels are typically low. One team of researchers gave human subjects 25 mg of radiolabeled resveratrol. They found that most of it was absorbed (70%), but very little could be found in the plasma. They concluded that some benefit may still be gained in cells lining the digestive tract (Walle et al., 2004). Further research will need to be done exploring this hurdle to resveratrol utilization. However, resveratrol may induce health effects indirectly. Lotito and Frei (2006) argue that the antioxidant effects seen after ingestion of foods high in phytoalexins is due to these compounds increasing the uric acid concentration found in the plasma. Uric acid is an endogenous antioxidant. The bioavailability of resveratrol may be small, but it may generate its many benefits via secondary pathways such as increasing uric acid levels.

Resveratrol was first identified in peanut roots in 1976 (Ingham, 1976). Levels of *trans*-resveratrol have been reported as high as 1330 μg/g in field grown peanut roots (Chen et al., 2002). This is much higher then the level found in other peanut products. The highest concentration reported in peanut seeds is 19.98 μg/g (Chukwumah et al., 2007b). Peanut skins have also been reported to contain resveratrol at levels up to 0.7 μg/g (Sanders et al., 2000). Red wine has received the most press in regards to its *trans*-resveratrol levels. Burns *et al*. (Burns et al., 2002) found concentrations in four different red wines ranging from 48 μg/100 mL to 1057 μg/100 mL. The variation in *trans*-resveratrol levels in red wine can be attributed to the grape variety and the various winemaking techniques employed. Cocoa products have also garnered attention in relation to resveratrol concentrations. Hurst *et al*. (2008) measured resveratrol concentrations in 19 different cocoa products. They found levels ranging from 0.09 μg/g in chocolate syrups to 1.85 μg/g in cocoa powders. This work was carried out to explore the level of resveratrol found in the roots of Virginia-type peanut cultivars.

Materials and Methods

Peanut Root Samples

Peanut roots were collected from 8 different cultivars with two levels of TSWV infestation (low or high) in Virginia. Roots from 4 plants were combined for pooled sampling. The roots were stored at -18 C until extraction.

Peanut Root Extraction

Preliminary data (not shown) of different concentrations of methanol versus *trans*-resveratrol recovery from peanut skins indicates that 75% aqueous methanol obtains the best extraction. The preliminary study consisted of extracting 5 g of peanut skins with five levels of aqueous methanol (0%, 25%, 50%, 75%, and 100%). The peanut skins were extracted with 100 mL of aqueous methanol. The samples were shaken at 240 rpm for 20 hours, and then centrifuged 300 x g at room temperature. Resveratrol levels were determined by HPLC (procedure described below).

The roots were extracted by adding 100 mL 75% aqueous methanol to 5 g of peanut root. The peanut root pieces used were the smallest rootlets available. This mixture was then blended for 30 seconds in a Waring Commercial Blendor (New Hartford, Conneticut) set on high powered by a Tenma rheostat (Centerville, Ohio) set at 70 volts, allowed to sit for five minutes, and then blended again for one minute at 70 volts. The mixture was then placed in a Fisher FS20 ultrasonic bath (Pittsburgh, PA) for 10 minutes. Following the ultrasonic bath, the suspension was placed in 50 mL plastic centrifuge vials and centrifuged for 30 minutes at 300 x g at room temperature (Damon IEC HN-S, Pittsburgh, PA). The supernatant was then transferred to 10 mL glass centrifuge tubes and centrifuged again for 30 minutes at 300 x g. The finished extracts were then transferred to HPLC vials for further analysis.

HPLC Analysis

The HPLC work was carried out with an Agilent (Santa Clara, CA) 1200 Series HPLC consisting of degasser, quaternary solvent pump, autosampler with refrigeration, column oven, and a diode array detector and a Phenomenex (Torrance, CA) Luna 5µ C18 (250 x 4.6mm) column with a Phenomenex Security Guard column. An acidified methanol gradient consisting of two solvents: solvent A (0.5% aqueous acetic acid) and solvent B (0.5% acetic acid in

methanol) was utilized (Table 3.1). Flow rate was 0.8 mL/min. The sample injection was 100 μL. UV absorbance was recorded at 305 nanometers and UV absorption spectra obtained from 190-400 nanometers. Concentrations were determined by using a commercially available *trans*-resveratrol standard (Sigma-Aldrich, St. Louis, MO) dissolved in 75% aqueous methanol. Resveratrol peaks were identified by retention time and UV absorbance spectra. The detection limit for *trans*-resveratrol was determined to be 0.025 μg/mL, which correlated to about 0.5 μg *trans*-resveratrol per gram of peanut roots. Statistical analysis was performed with JMP 7.0 (SAS, Cary, NC). Means were considered significantly different at $P<0.05$.

Results and Discussion

Concentrations of *trans*-resveratrol are shown in Table 3.2. The cultivars and their resistance to TSWV are listed in Table 3.3 (Huber, 2007; Isleib et al., 2006). Resveratrol concentration was not significantly different between high and low disease levels ($P=0.09$). Concentrations of resveratrol was not significantly different among cultivars ($P=0.7$) or susceptibility to TSWV ($P=0.22$). Sobolev *et al*. (2007) looked at five different cultivars of peanuts grown in Georgia and found that the cultivars that had a high disease resistance reported in the literature produced the most phytoalexins in the pods when they were damaged by the lesser cornstalk borer. *trans*-Resveratrol was one of the phytoalexins found in substantial concentrations. They also measured *trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, and *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene, but only in the pods. Our results suggest that disease resistance may not predict phytoalexin production in roots. This contrasting data could be explained by the accumulation of resveratrol in the segment of the plant that is experiencing the stressor, different plant responses for different stresses, or the particular cultivars we used did not react that same as those chosen by Sobolev *et al*.

Sobolev (2008) investigated which phytoalexins were produced in peanut seeds under duress from different fungal strains. Sobolev infected seeds with different strains of fungi and then sliced the seeds to determine where the phytoalexins were most concentrated. Resveratrol was the dominant phytoalexin after 24 hours of incubation, but after 48 hours arachidin-3 and SB-1 were the major phytoalexins. Sobolev discovered that the seeds reacted similarly with different fungal strains and that the highest concentration of phytoalexins were areas of the seed nearest to the infection.

Our results do agree with levels previously reported in peanut roots. However, our levels are not as high as the highest reported 1330 μg/g by Chen et al.(Chen et al., 2002). Chen *et al*. investigated 3 different cultivars during two growing seasons (Tainan 9, Tainan 11, and Tainan 12). They found average resveratrol contents ranging from 27 μg/g to 905 μg/g. The values reported for this experiment are within the range reported by Chen *et al*. They reported little variation among cultivars, but the largest concentration difference was between growing seasons. This variation in resveratrol levels may be due to variations in analytical procedures, different plant cultivars, growing conditions including disease or other stressors or yearly variations (Sobolev et al., 2007). Each plant experiences different levels of stress depending on location, disease prevalence, insect prevalence, etc.

Resveratrol has been found in peanut seeds at concentrations of 0.02 to 19.98 μg/g (Chukwumah et al., 2007b; Sanders et al., 2000). Burns *et al*. (2002) measured resveratrol concentrations in wine, grapes, itadori root, peanut seeds, peanut butter and itadori tea. They discovered *trans*-resveratrol in peanut butter at 0.3 μg/g and at 5.1 μg/g in boiled peanut seeds. Chukwumah *et al*. (2007a) reported 0.9 μg/g of *trans*-resveratrol in boiled peanut seeds, but did not detect it in raw or roasted peanuts. Ibern-Gomez (2000) also measured levels of resveratrol in peanut butters. They found levels of resveratrol ranging from 0.265 μg/g to 0.753 μg/g in various peanut butters found in the market.

Chukwumah *et al*. (2007b) used various extraction methods to measure the level of *trans*-resveratrol and other phytoalexins in the peanut seed. They measured levels of resveratrol ranging from 0.9 to 19.98 μg/g. Chukwumah and fellow colleagues utilized stirring, sonication, Soxtec, and microwave-assisted sonication as methods to extract phytoalexins. They also performed different treatments on the peanut seeds prior to extraction to determine the effect of fat on phytoalexin extraction. Chukwumah *et al*. extracted nondefatted peanut seeds, mechanically defatted peanut seeds, and hexane-defatted peanut seeds. They found that microwave-assisted sonication yielded the highest measured levels of resveratrol followed by stirring. Chukwumah *et al*. also discovered that they were able to extract significantly higher concentrations of phytoalexins from defatted peanuts versus nondefatted peanuts. These results suggest that many of the published levels of resveratrol may be underestimating the true concentration found in foodstuffs and plant materials. The concentration of resveratrol found in peanut roots is reportedly much higher then the level found in the peanut seed.

Peanut hairy root cultures have been investigated as a possible commercial source of resveratrol (Medina-Bolivar et al., 2007). Hairy root culture isolates cells that are responsible for the secretion of resveratrol and allow tight control of the conditions to elicit resveratrol production. These cultures lend themselves to commercial production of resveratrol on a large scale. Medina-Bolivar and colleagues (Medina-Bolivar et al., 2007) tried 5 different stressors to induce resveratrol production. They used cellulose, chitosan, laminarin, sodium acetate, and copper sulfate. These resveratrol elicitors were added at levels proven to produce phytoalexins. They chose sodium acetate after preliminary work showed it to be the best elicitor of resveratrol and is relatively inexpensive. This group was able to entice the hairy root cultures to produce *trans*-resveratrol upwards of 98 μg/mg extract dry weight. This research shows that peanut roots are not only high producers of resveratrol in the field, but are also significant producers of resveratrol in culture. Peanut roots may become an important and easily available source of resveratrol.

Chang and colleagues (2006) employed a different technique to produce resveratrol. Chang *et al*. only found trace amounts of resveratrol prior to incubation in peanut seeds. They sliced Spanish peanut seeds and incubated (23 C to 25 C) them for 3 days with aeration in darkness. They were able to produce 147.3 μg/g of *trans*-resveratrol after 20 hours of incubation. This level is substantially lower then what Medina-Bolivar *et al*. (Medina-Bolivar et al., 2007) achieved with peanut hairy root cultures, but is still an improvement upon the initial levels measured. In another study, researchers measured a maximum of 0.06 μg/g of resveratrol in 5 day old peanut seedling roots (Sobolev et al., 2006). They found the largest concentration of resveratrol in the testa of the seedling at 1.27 μg/g. Another group investigating peanut seedlings as a possible functional food, measured the levels of resveratrol in the sprouts (Wang et al., 2005). Wang *et al*. found that resveratrol concentrations increased from 2.3-4.5 μg/g to 11.7-25.7 μg/g during sprouting with levels dependent upon the peanut cultivar. They also found that antioxidant activity, as measured by DPPH, increased with sprouting. This research supports the commercialization of peanut sprouts as a functional food with an increased resveratrol concentration over peanut seeds.

Rudolf and Resurreccion (2005) investigated abiotic resveratrol elicitors in peanut seeds. They specifically looked at postharvest techniques such as UV light, size reduction, and ultrasound. The peanut seeds were reduced in size by various methods and then subjected to one

of the abiotic stresses. Rudolf and Resurreccion found that these postharvest abiotic stresses caused an increase in *trans*-resveratrol concentrations compared to untreated peanut seeds. By optimizing the peanut seed treatment, they were able to elicit resveratrol concentrations of 3.96 μg/g. This concentration was achieved by slicing the kernels, submitting them to ultrasonic waves for 4 minutes, and then incubating for 36 hours. However, these treatments did not increase the overall phenolic content or antioxidant activity.

Resveratrol was detected in all but one variety of Virginia-type peanut roots. Resveratrol concentrations ranged from below detection limits to 46.3 μg/g. Disease level of TSWV was not a significant factor in resveratrol concentration, nor was any significance found between cultivars or published disease susceptibility. Resveratrol concentration should be sampled in a larger population to test for other trends in phytoalexin production.

Literature Cited

Aggarwal, B., and S. Shishodia. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem. Pharmacol. 71:1397-1421.

(ed.) 1990. Ciba Foundation Symposium.

Baur, J.A., K.J. Pearson, N.L. Price, H.A. Jamieson, C. Lerin, A. Kalra, V.V. Prabhu, J.S. Allard, G. Lopez-Lluch, K. Lewis, P.J. Pistell, S. Poosala, K.G. Becker, O. Boss, D. Gwinn, M. Wang, S. Ramaswamy, K.W. Fishbein, R.G. Spencer, E.G. Lakatta, D. Le Couteur, R.J. Shaw, P. Navas, P. Puigserver, D.K. Ingram, R. de Cabo, and D.A. Sinclair. 2006. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337-342.

Burns, J., T. Yokota, H. Ashihara, M.E.J. Lean, and A. Crozier. 2002. Plant foods and herbal sources of resveratrol. J. Agric. Food Chem. 50:3337-3340.

Cao, Y., Z.-D. Fu, F. Wang, H.-Y. Liu, and R. Han. 2005. Anti-angiogenic activity of resveratrol, a natural compound from medicinal plants. J. Asian Nat. Prod. Res. 7:205-213.

Chang, J.-C., Y.-H. Lai, B. Djoko, P.-L. Wu, C.-D. Liu, Y.-W. Liu, and R.Y.Y. Chiou. 2006. Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (Arachis hypogaea L.) arachidin-1, arachidin-3, and isopentadienylresveratrol. J. Agric. Food Chem. 54:10281-10287.

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

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

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

Dixon, R.A. 2001. Natural products and plant disease resistance. Nature 411:843-847.

Dong, Z. 2003. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat. Res., Fundam. Mol. Mech. Mutagen. 523-524:145-150.

Fitzpatrick, D.F., R.C. Fleming, B. Bing, D.A. Maggi, and R.M. O'Malley. 2000. Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. J. Agric. Food Chem. 48:6384-90.

Frankel, E.N., A.L. Waterhouse, and J.E. Kinsella. 1993a. Inhibition of human LDL oxidation by resveratrol. Lancet 341:1103-4.

Frankel, E.N., J. Kanner, J.B. German, E. Parks, and J.E. Kinsella. 1993b. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341:454-7.

Huber, A. 2007. 2007 Variety guide [Online]. Available by Peanut Grower http://www.peanutgrower.com/home/2007_FebVarietyGuide.html (verified 17 Oct).

Hurst, W.J., J.A. Glinski, K.B. Miller, J. Apgar, M.H. Davey, and D.A. Stuart. 2008. Survey of the trans-resveratrol and trans-piceid content of cocoa-containing and chocolate products. J. Agric. Food Chem. 56:8374-8378.

Ibern-Gomez, M., S. Roig-Perez, R.M. Lamuela-Raventos, and M.C. de la Torre-Boronat. 2000. Resveratrol and piceid levels in natural and blended peanut butters. J Agric Food Chem 48:6352-6354.

Ingham, J.L. 1976. 3,5,4'-Trihydroxystilbene as a phytoalexin from groundnuts (Arachis hypogaea). Phytochemistry 15:1791-3.
Isleib, T.G., P.W. Rice, R.W. Mozingo II, S.C. Copeland, J.B. Graeber, W.P. Novitzky, H.E. Pattee, T.H. Sanders, R.W. Mozingo, and D.L. Coker. 2006. Registration of 'Brantley' peanut. Crop Science 46:2309-2310.
Jang, M., L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W.W. Beecher, H.H.S. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, and J.M. Pezzuto. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218-220.
King, R., J. Bomser, and D. Min. 2006. Bioactivity of resveratrol. Compr. Rev. Food Sci. Food Saf. 5:65-70.
Lotito, S., and B. Frei. 2006. Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: cause, consequence, or epiphenomenon? Free Radic Biol Med 41:1727-1746.
Medina-Bolivar, F., J. Condori, A. Rimando, J. Hubstenberger, K. Shelton, S.F. O'Keefe, S. Bennett, and M. Dolan. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003.
Olas, B., and B. Wachowicz. 2005. Resveratrol, a phenolic antioxidant with effects on blood platelet functions. Platelets 16:251-260.
Pezet, R., and V. Pont. 1995. Mode of toxic action of vitaceae stilbenes on fungal cells, p. 317-331, *In* M. Daniel and R. P. Purkayastha, eds. Handbook of Phytoalexin Metabolism and Action. Marcel Dekker, Inc, New York.
Purkayastha, R.P. 1995. Progress in phytoalexin research during the past 50 years, p. 1-39, *In* M. Daniel and R. P. Purkayastha, eds. Handbook of phytoalexin metabolism and action. Marcel Dekker, Inc, New York.
Rao, P.V.S., and R. Strange. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack, p. 199-227, *In* M. Daniel and R. P. Purkayastha, eds. Handbook of phytoalexin metabolism and action. Marcel Dekker, Inc, New York.
Renaud, S., and S. Debord. 1992. Wine, alcohol, platelets and the french paradox for coronary heart disease. Lancet 339:1523-1526.
Roupe, K.A., C.M. Remsberg, J.A. Yanez, and N.M. Davies. 2006. Pharmacometrics of stilbenes: seguing towards the clinic. Curr. Clin. Pharmacol. 1:81-101.
Rudolf, J.R., and A.V.A. Resurreccion. 2005. Elicitation of resveratrol in peanut kernels by application of abiotic stresses. J. Agric. Food Chem. 53:10186-10192.
Sanders, T.H., R.W. McMichael, and K.W. Hendrix. 2000. Occurrence of resveratrol in edible peanuts. J. Agric. Food Chem. 48:1243-1246.
Sobolev, V., B. Horn, T. Potter, S.T. Deyrup, and J.B. Gloer. 2006. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. J Agric Food Chem 54:3505-3511.
Sobolev Victor, S., Z. Guo Baozhu, C.C. Holbrook, and E. Lynch Robert. 2007. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. J Agric Food Chem FIELD Full Journal Title:Journal of agricultural and food chemistry 55:2195-200.
Sobolev, V.S. 2008. Localized production of phytoalexins by peanut (Arachis hypogaea) kernels in response to invasion by Aspergillus species. J Agric Food Chem 56:1949-1954.

Sobolev, V.S., B.Z. Guo, C.C. Holbrook, and R.E. Lynch. 2007. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. J. Agric. Food Chem. 55:2195-2200.

Vinson, J.A., J. Jang, J. Yang, Y. Dabbagh, X. Liang, M. Serry, J. Proch, and S. Cai. 1999. Vitamins and especially flavonoids in common beverages are powerful *in vitro* antioxidants which enrich lower density lipoproteins and increase their oxidative resistance after *ex vivo* spiking in human plasma. J. Agric. Food Chem. 47:2502-2504.

Virgili, F., C. Scaccini, L. Packer, and G. Rimbach. 2003. Nutritional phenolics and cardiovascular disease. Phytochem. Functional Foods:5-17.

Walle, T., F. Hsieh, M.H. DeLegge, J.E. Oatis, Jr., and U.K. Walle. 2004. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab. Dispos. 32:1377-1382.

Wang, K.-H., Y.-H. Lai, J.-C. Chang, T.-F. Ko, S.-L. Shyu, and R.Y.-Y. Chiou. 2005. Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. J. Agric. Food Chem. 53:242-246.

FIGURES

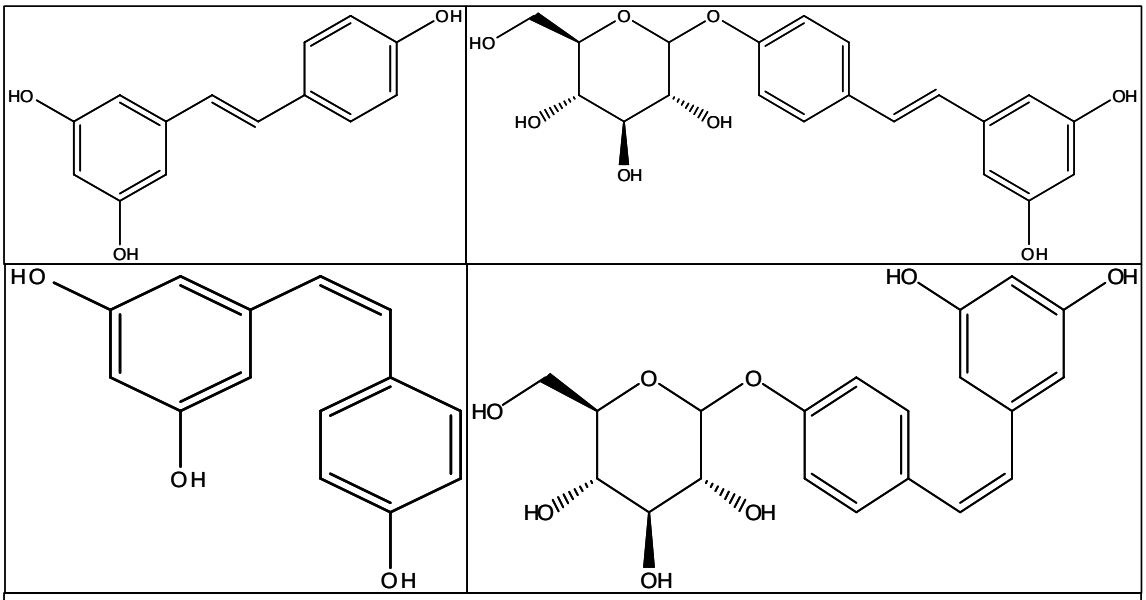

Figure 3.1: Structures of *trans*-resveratrol (top left), *trans*-resveratrol glucoside (top right), *cis*-resveratrol (bottom left), and *cis*-resveratrol glucoside (bottom right).

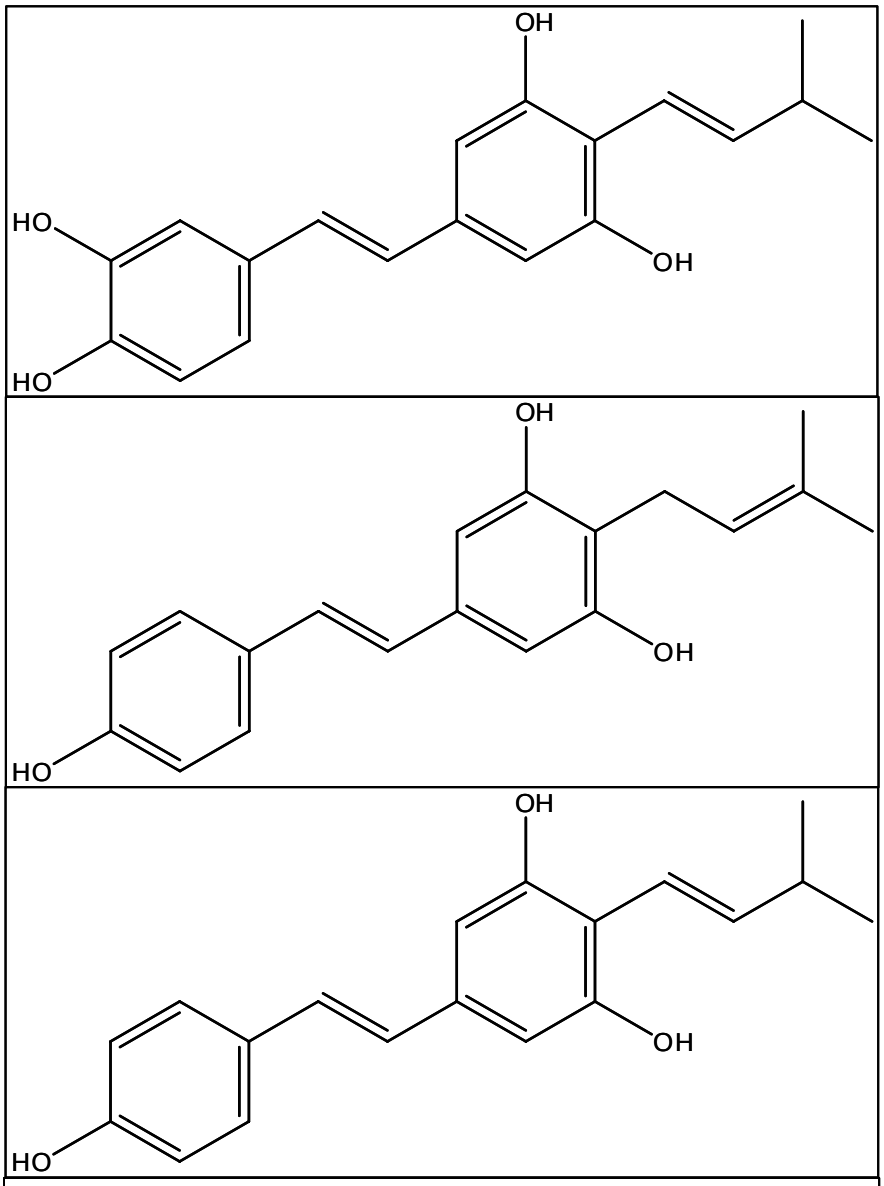

Figure 3.2: Structures of *trans*-arachidin-1 (top), *trans*-arachidin-2 (middle), and *trans*-arachidin-3 (bottom).

TABLES

Table 3.1: HPLC gradient schedule used for identification of *trans*-resveratrol in peanut roots matched with a commercial standard. Solvent A is 0.5% aqueous acetic acid and solvent B is 0.5% acetic acid in methanol.

Time (minutes)	% A	% B
0	100	0
2	100	0
4	95.3	4.7
15	69.1	30.9
42	25.3	74.7
54.5	5	95
55	100	0
65	100	0

Table 3.2: Concentration of *trans*-resveratrol present in peanut roots of Virginia-type peanut cultivars as measured by HPLC.
* below minimum detectable concentration 0.5 µg/g

Sample	***trans*-Resveratrol concentration (µg/g)**	
	Low disease	High disease
CHAMPS	3.2	3.1
Wilson	6.9	13.1
Brantley	2.3	46.3
NC 12C	1.8	40.3
Philips	7.0	1.2
Perry	Not detected*	Not detected*
NC-V 11	1.6	3.2
VA 98R	1.4	9.4

Table 3.3: Susceptibility of Virginia-type peanut cultivars to tomato spotted wilt virus (TSWV) on a scale of 1-4, with 1 being the least susceptible and 4 the most. Compiled from Huber (2007) and Isleib *et al*. (2006).

Cultivar	Susceptibility to TSWV
CHAMPS	2
Wilson	1
Brantley	4
NC 12C	4
Philips	3
Perry	4
NC-V 11	2
VA 98R	2

CHAPTER 4: ORACHROMATOGRAPHY AND TOTAL PHENOLICS CONTENT OF PEANUT ROOT EXTRACTS

Abstract

Compounds found in peanut plants include *cis*-resveratrol, *trans*-resveratrol, 4-isopentenylresveratrol, arachidins, medicarpin, *p*-coumaric acid, caffeic acid, ferulic acid, demethylmedicarpin, daidzein, formononetin, isoflavonones, nonyl phenol, and dienols. Many of these compounds are phytoalexins, which are produced by plants experiencing environmental stress and often exhibit antioxidant activity. The objectives of this research were to examine the ORAC activity and total phenolic contents of peanut root extracts and peanut root extract fractions collected via HPLC.

Peanut roots were extracted from four different cultivars (Brantley, NC-12, Phillips, and Wilson) with 70% aqueous ethanol with ultrasonic assistance. Each cultivar was sampled in duplicate. The extracts were fractionated into 18 3-minute fractions by HPLC using a C-18 column. Fractions and crude extracts were freeze dried and reconstituted with a known volume. ORAC and total phenolic content was then determined for all fractions and crude extracts.

ORAC activity and total phenolic content were found to be not significant by cultivar. Fractions were found to be significantly different by ORAC activity and total phenolic content. The ORAC activity and total phenolic content were then used to determine μM TE/mg GAE for the fractions. Both cultivar and fractions were found to be significantly different for μM TE/mg GAE.

Keywords: ORAC, total phenolics, peanut roots, antioxidant, phytoalexin

Introduction

The peanut plant (*Arachis hypogaea*) has been extensively studied in recent years as the search for nutraceuticals has intensified. Peanut skins, hulls, and roots have all received much attention. Peanut skins have been studied for their antioxidant activity and possible applications. The first published study regarding peanut skin extracts performed a methanol extraction and then utilized the extract in sunflower oil (Nepote and others 2000). The oil containing the peanut skin extract had significantly less oxidation then the control, but was outperformed by BHT on a given weight basis. Yu and colleagues (2006) identified peanut skin compounds by LC-MS and employed DPPH to determine the antioxidant activity of the skin extracts. In this study, they used 80% aqueous ethanol for extraction. They homogenized 30 mL of solvent with 1 g of skin powder for 2 minutes and then centrifuged for 15 minutes at 3,000 x *g*. Yu *et al*. identified catechins, A-type procyanidins, and B-type procyanidins. They also observed peaks that they believe to be caffeic acid, chlorogenic acid, resveratrol, and resveratrol glycoside piceid. According to the DPPH analysis, the peanut skin extracts from roasted and raw peanuts had a higher antioxidant activity then Trolox and vitamin C. Compounds found in peanut plants include *cis*-resveratrol, *trans*-resveratrol, 4-isopentenylresveratrol, arachidins, medicarpin, *p*-coumaric acid, caffeic acid, ferulic acid, demethylmedicarpin, daidzein, formononetin, isoflavonones, nonyl phenol, and dienols (Keen and Ingham 1976; Aguamah and others 1981; Arora and Strange 1991; Rao and Strange 1995; Sobolev and others 2006a). Many of these compounds are phytoalexins, which are produced by plants experiencing environmental stress. Stress can be elicited by an actual fungal or microbial infection or in the lab with compounds derived from fungi/microbes such as proteins and polysaccharides (Chung and others 2003; Rowland and others 2005; Medina-Bolivar and others 2007). Phytoalexins can also be induced by UV light, heavy metal salts, physical damage, and other environmental stressors (Barz and others 1990; Arora and Strange 1991; Chung and others 2003).

The roots of the peanut plant are of great interest because they tend to show a higher infection rate than the rest of the plant. Rowland and colleagues (2005) infected three different cultivars (NC-V11, ANorden, and Georgia Green) with tomato spotted wilt virus (TSWV). They found the roots had a higher rate of infection then the leaves. This higher rate of root infection may cause higher concentrations of phytoalexins in the roots then other parts of the peanut plant. Rowland *et al*. also noticed that root infection coincided with a lower photosynthesis rate (up to a

42% decrease), but root infection had a mixed effect on transpiration of different cultivars. ANorden showed an increase in transpiration, but Georgia Green and NC-V11 showed a decrease in transpiration.

Extracts of peanut plant parts have been investigated as a food ingredient to prevent oxidation and subsequent spoilage of food. Chen and associates (2002) added peanut root powders (0.6 g) to pork patties (60 g) and then cooked. After the patties cooled, the oil was stored at 60°C and checked for conjugated dienes periodically. The oil from the patties cooked with peanut root powders was less prone to oxidation then the control oil. A similar study investigated peanut skin extract effects in ground beef (O'Keefe and Wang 2006). O'Keefe and Wang found that peanut skin extract significantly reduced oxidation in ground meat (as measured by TBARS) with no ill effects to microbial growth, texture, aroma, and other sensory attributes. They found that 200 and 400 ppm of peanut skin extracts were effective with little additional effects seen at higher levels. Nepote, Mestrallet, and Grosso (2004) investigated the antioxidant effects of peanut skin extracts in honey-roasted peanuts. They measured peroxide and thiobarbituric acid reactive substance (TBARS) values to determine the level of oxidation and utilized a sensory panel. Peanut skin extracts inhibited lipid oxidation, but not as well as BHT. The sensory panel determined the extracts did not change the acceptability of the peanuts.

Extract solvent has a significant effect on the antioxidant effectiveness of plant extracts. Xu and Chang (2007) examined the effects of solvents on total phenolic content, total flavonoids content, condensed tannin content, DPPH activity, FRAP activity, and ORAC activity of legume extracts. They extracted the legumes with 50% aqueous acetone, 80% aqueous acetone, 70% acidified aqueous acetone, 70% aqueous ethanol, 70% aqueous methanol, and 100% ethanol. The highest total phenolics content was given by 50% acetone, followed by 70% ethanol. The highest total flavonoid content and condensed tannin content were both given by 80% acetone, followed by 70% acidic acetone. However, the results of the antioxidant assays lean towards other solvents. The highest DPPH activity was 70% methanol, followed by 70% ethanol and 70% ethanol also gave the highest FRAP and ORAC value. Nepote and colleagues (2002) compared the antioxidant extraction of methanol, ethanol, acetone, and aqueous extracts of peanut skins. They found that the methanol, ethanol, and acetone extracts extracted the highest levels of phenolics and antioxidants as measured by Folin-Ciocalteu total phenolics method and DPPH respectively. Nepote and fellow researchers also measured the effectiveness of these

extracts to combat oxidation with the DPPH assay and a model food system of sunflower oil. The methanol and ethanol extracts had the highest DPPH scavenging activity of 93% at a concentration of 10 μg/mL. There was not a significant difference between these extracts and their defatted counterparts. The sunflower oil portion of this experiment revealed that the peanut skin extracts did not reduce oxidation as much as BHT, but they did reduce oxidation more then the negative control. Nepote and colleagues (2005) performed a follow-up study that looked at optimizing the extraction process by manipulating the concentration of ethanol in an aqueous solution, size of the peanut skin particles, time, ratio of skins to solvent, and number of extractions. They measured the total phenolics content with the Folin-Ciocalteu method and utilized DPPH to assess the antioxidant activity. They found that the optimal extraction occurred with a 70% ethanol aqueous solvent, with uncrushed skins, with 10 minutes of shaking, 20 mL of solvent for every gram of skins, and three extraction stages. This combination resulted in a yield of 0.118 g of phenolic compounds per gram of skins. Ballard and fellow researchers (2009) reported that 30% aqueous methanol gave the highest ORAC values from peanut skins when extracted at 52.9°C for 30 minutes. This extraction protocol was estimated by using response surface methodology and gave an estimated ORAC activity of 2149 μM TE/g of dry skins. They tried using methanol, ethanol, and water in 4 different concentrations (0, 30, 60, 90%). Aqueous ethanol was the second best extraction solvent for high ORAC activities. In regards to total phenolics though, 30.8% ethanol is estimated to give the highest total phenolic content with an estimated total phenolics content of 118 mg GAE/g of dry skins.

There is no agreed upon standard method to determine antioxidant capacity of a sample, and this causes difficulty when trying to compare one substance to another. Currently there are numerous antioxidant capacity assays available to scientists. Each assay measures the antioxidant capacity differently, which can lead to different results for the same antioxidant. Some of these assays use radicals that are foreign to reactions in the body and in food systems, such as ABTS$\bullet^{+}$ and DPPH•, and this leads to difficulties in interpretation. The oxygen-radical absorbing capacity (ORAC) has become a popular method to measure an antioxidant's effectiveness at quenching free radicals. First described in 1993, ORAC uses a fluorescent molecular probe that undergoes attack by a free radical thus diminishing its fluorescence (Cao and others 1993). The fluorescent molecule (often fluoroscein) is added to the sample and then the radical generator is added. Peroxyl radicals are typically generated by 2,2'-azobis(2-

amidinopropane) dihydrochloride (AAPH) in this assay. Fluorescence is measured over time and the area under the curve is compared to a standard, usually Trolox, and then reported as equivalent units. ORAC has found widespread use with some manufacturers actually putting the ORAC value on their labels (Prior and others 2005). ORAC has also been used extensively by the USDA (Wu and others 2004).

The Folin-Ciocalteu assay has been used for many years and has become a standard for measuring total phenolic content. Investigators will often measure total phenolic content and optimize their experimental conditions to extract the highest phenolic content (Nepote and others 2005). The Folin-Ciocalteu assay measures ability of the sample compound to reduce the Folin-Ciocalteu reagent (a heteropoly phophotungstate molybdate compound) forming a blue color that is typically measured at 765 nm. This method is preferred over measuring individual concentrations of phenolic compounds and summing them because it gives a more accurate picture of the phenolic content. It is also simple to carry out and due to its popularity investigators can compare results among many different samples (Singleton and others 1999). One disadvantage in the past was the assay required a large amount of solvents and generated much waste. This has been overcome in recent years as researchers have scaled down the procedure with waste per sample being less then a milliliter (Magalhaes Luis and others 2006; Ainsworth and Gillespie 2007). The objectives of this research were to examine the ORAC activity and total phenolic contents of peanut root extracts and peanut root extract fractions collected via HPLC.

Materials and Methods

Peanut Root Extraction

Peanut roots were collected from four plants from each of 4 different cultivars (Brantley, NC-12, Phillips, and Wilson) in Virginia and separated into two replications. Roots from 2 plants were combined for pooled sampling. The roots were rinsed with water to remove dirt, dried at 40°C, and stored at -18°C until extraction.

The roots were extracted by adding 100 mL 70% aqueous ethanol to 5 g of peanut root. The peanut root pieces used were the smallest rootlets available. This mixture was then blended for 30 seconds in a Waring Commercial Blender (New Hartford, Conneticut) set on high powered by a Tenma rheostat (Centerville, Ohio) set at 70 volts, allowed to sit for five minutes,

and then blended again for one minute at 70 volts. The mixture was then placed in a Fisher FS20 ultrasonic bath (Pittsburgh, PA) for 10 minutes. Following the ultrasonic bath, the suspension was placed in 50 mL plastic centrifuge vials and centrifuged for 30 minutes at 300 x g at room temperature. The supernatant was then transferred to 10 mL glass centrifuge tubes and centrifuged again for 30 minutes at 300 x g. The supernatant was then stored at -18°C until analysis.

HPLC Analysis and Fraction Collection

The HPLC work was carried out with an Agilent (Santa Clara, CA) 1200 Series HPLC consisting of degasser, quaternary solvent pump, autosampler with refrigeration, column oven, and a diode array detector and a Phenomenex (Torrance, CA) Luna 5µ C18 (250 x 4.6mm) column with a Phenomenex Security Guard column. An acidified methanol gradient consisting of two solvents: solvent A (0.5% aqueous acetic acid) and solvent B (0.5% acetic acid in methanol) was utilized (table 4.1). Flow rate was 0.8 mL/min. The sample injection was 100 µL. UV absorbance was recorded at 305 nanometers and UV absorption spectra obtained from 190-400 nanometers.

Fractions were manually collected twice into 10 mL glass centrifuge tubes after exiting the diode array detector. Eighteen 3-minute fractions were collected from each sample. The fractions were dried under nitrogen to remove methanol, water was added, and samples frozen at –18°C for lyophilization. The crude extracts were freeze-dried in the same manner. Both fractions and crude extracts were lyophilized in a VirTis FreezeMobile 12SL (Gardiner, New York).

ORAC

A black 96-well plate was utilized for ORAC. Samples (including blanks and Trolox standards) were run in duplicate. Both crude extracts and fractions were analyzed. Fractions were reconstituted with 2 mL 70% aqueous ethanol and crude extracts were analyzed at 50 µg/mL in 70% aqueous ethanol. Each well contained 40 µL of sample and 200 µL of 100 mM fluoroscein. The plate was then incubated at 37°C for 20 minutes. After this time, 35 µL of 0.36 M AAPH in warmed 75 mM PBS (pH 7.4) was added and the plate was read with a Victor mulitlabel plate reader (Perkin-Elmer, Turku, Finland) with an excitation wavelength of 485 nm

and an emission wavelength of 535 nm. Results were reported in μM Trolox equivalents (TE) for the fractions and as μM TE/mg dry extract for crude extracts.

Total Phenolics Content

A clear 96-well plate was utilized for total phenolics content. Samples (including blanks and gallic acid standards) were run in duplicate. Both crude extracts and fractions were analyzed. Fractions were reconstituted with 2 mL 70% aqueous ethanol and crude extracts were analyzed at 50 μg/mL in 70% aqueous ethanol. Each well contained 18 μL of sample and 36 μL of 10% aqueous Folin-Ciocalteu (v/v) reagent. After the Folin-Ciocalteu reagent was added, 146 μL 100 mM Na_2CO_3 was added to each well and then the plate was incubated at room temperature in darkness for 2 hours. The absorbance of each well was recorded at 765 nm with a Victor mulitlabel plate reader (Perkin-Elmer, Turku, Finland). Results were reported in mg gallic acid equivalents (GAE)/L for fractions and mg GAE/g dry extract.

Statistical analysis

All statistical analysis was carried out using JMP 7.0 (SAS Institute, Cary, NC). Oneway ANOVA was used for determining significance among factors (Cultivar, fraction) for ORAC activities and total phenolic contents. Tukey-Kramer Honestly Significant Difference (HSD) was used to determine which treatments in the factors were significant. Means were considered significantly different at $P<0.05$.

Results and Discussion

Peanut root extracts contain numerous UV-absorbing compounds as illustrated by a sample chromatogram in figure 4.1. One problem facing researchers investigating peanut root extracts is determining which area of the chromatogram is of interest. One method is to search for known antioxidants. Chakraborty and Mandal (2008) searched for known phenolic acids in peanut root nodules extracted with 80% aqueous methanol. They identified *p*-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, caffeic acid, and protocatechuic acid by matching known standards with HPLC-DAD. Chakraborty and Mandal also ran *t*-cinnamic acid and benzoic acid standards, but were unable to identify these peaks in their samples. However, this method has limitations. It limits the search to known compounds that are available in pure

forms. It is also limited by the researcher choosing which compounds may be found in the sample and running those standards exclusively. The chosen standards may or may not be in the sample and may not contribute substantially to the bioactive effects of these extracts. One advantage to this method is that it is available to groups with an instrument lacking a mass spectrometer. It can also be coupled with mass spectrometry to ensure positive identification. Sobolev and his group (2006b) searched peanut root mucilage extracted with 90% aqueous acetonitrile for compounds found in other peanut plant parts using HPLC-MS. They were particularly interested in *t*-arachidin-1, *t*-arachdidin-2, *t*-arachidin-3, which they previously measured in peanut pods subjected to damage by the lesser cornstalk borer (Sobolev and others 2007). Another method to select peaks of interest is to choose the HPLC peaks with the largest area. Sobolev and his group (2006b) also utilized this approach when dealing with peanut root mucilage compounds. While looking for known compounds in peanut root mucilage, they also analyzed a large peak based on UV absorbance, which turned out to be a previously unknown compound. Through mass spectroscopy analysis, they determined the structure to be 4-(3-methyl-but-1-enyl)-3,5-dimethoxy-4'-hydroxy-*trans*-stilbene and named it mucilagin A (figure 4.2). A disadvantage of the two previously discussed methods is that neither allows the researcher to determine which compounds are responsible for the activity expressed by an extract. A procedure that addresses this disadvantage would determine the antioxidant activity of portions of the extract and allow the researcher to focus his efforts to identify peaks that have high antioxidant activity. Antioxidant activity determined using this approach would of course combine intrinsic antioxidant activity with compound concentration. Nevertheless, the compounds that have the highest antioxidant effect can be determined this way.

Duenas and colleagues (2003) chose to collect all major peaks from a methanol mocan seed extract by HPLC several times and dried them under vacuum. They subjected all of these peaks to antioxidant activity analysis by DPPH. They then obtained mass spectroscopy data for all peaks via HPLC-MS. Duenas and colleagues were able to positively identify several compounds, including (+) catechin and several procyanidins dimers and trimers. Duh and fellow researchers (1992) utilized thin-layer chromatography (TLC) to fractionate methanolic peanut hull extract into 18 different fractions. The antioxidant activities of these fractions were measured using the thiocyanate method. This method measures the ability of the test compounds to inhibit the oxidation of linoleic acid at 40 C as measured by peroxide values. After

determining the antioxidant activity of the peanut hull fractions, Duh and fellow researchers chose to pursue several fractions with higher antioxidant activity levels and further purified them with TLC. These subfractions were then subjected to the antioxidant activity assay. From this data, the authors narrowed down an entire peanut hull extract to one compound of interest. Using primarily ^{1}H NMR, combined with UV and melting point, this compound was determined to be luteolin. This research followed a similar path, but utilized different methods and source materials.

The ORAC activity was measured in the crude extracts (table 4.2). ORAC activity was found to be not significant by cultivar (P=0.73) analyzed by oneway ANOVA. This finding can easily be explained by plant to plant variation is greater then cultivar variation. Each plant experiences different levels of stress depending on location, disease prevalence, insect prevalence, drought stress, etc. Stress leads to phytoalexin production and many of these phytoalexins are antioxidants. This effect can be illustrated by resveratrol levels previously measured in peanut roots. Chen *et al*. investigated 3 different peanut cultivars during two growing seasons (Tainan 9, Tainan 11, and Tainan 12) and measured *trans*-resveratrol levels in roots. They found average resveratrol contents ranging from 27 μg/g to 905 μg/g. They reported little variation among cultivars, but the largest concentration difference was between growing seasons. This variation in resveratrol levels may be due to variations in analytical procedures, different plant cultivars, growing conditions including disease or other stressors or yearly variations (Sobolev and others 2007).

The crude peanut root extract ORAC activity is quite high compared to foods that have been typically regarded as high in antioxidants. According to the USDA, raw blueberries have an ORAC value of 65.2 μM TE/g and raw cranberries have an ORAC value of 93.82 μM TE/g (2007). The lowest obtained ORAC value for peanut root extract is 600.3 μM TE/g with up to a 10-fold increase in this value depending on the sample. Peanut root extract is a rich source of natural antioxidants that has potential to become an antioxidant food ingredient or natural supplement as shown by this ORAC data. It has been tested as a natural antioxidant in pork to inhibit oxidation. Chen and fellow associates (2002) added peanut root powders (0.6 g) to pork patties (60 g) and then cooked them. After the patties cooled, the extracted oil was stored at 60°C and checked for conjugated dienes periodically. The oil from the patties cooked with peanut root powders was less prone to oxidation then the control oil. A similar study

investigated methanolic peanut skin extract effects in ground beef (O'Keefe and Wang 2006). O'Keefe and Wang found that peanut skin extract significantly reduced oxidation in ground meat (as measured by TBARS) with no ill effects to microbial growth, texture, aroma, and other sensory attributes. They found that 200 and 400 ppm of peanut skin extracts were effective with little additional effects seen in higher levels. These studies both lend credence to turning peanut industry waste into value added products.

After collecting 18 3-minute fractions, the fractions were lyophilized and the ORAC activities were determined for the fractions. The ORAC activities can be seen in figure 4.3 for all fractions. The fractions were found to be significantly different with oneway ANOVA ($P<0.0001$) (figure 4.4). Fractions 27-30, 30-33, and 3-6 were found to have the highest ORAC values over all cultivars. These findings are not surprising since one would assume that different fractions would have different antioxidant activities based on the compounds eluting during the collection window. An interesting find is that the ORAC activity of the fractions is significantly affected by cultivar ($P<0.0001$) as determined by oneway ANOVA. The cultivars are split into two significantly different groups ($\alpha=0.05$) by applying Tukey-Kramer HSD. NC-12 and Wilson form a group having fractions with higher ORAC activity, on average 58.5 and 60.9 μM TE/g respectively, and Brantley and Phillips' fractions form a group having lower ORAC activity, on average 26.6 and 29.4 μM TE/g respectively. This finding is most likely caused by differing concentrations of individual antioxidant compounds among cultivars with some possible synergistic effects occurring in the crude extracts. Sobolev and associates (2007) measured several different phytoalexins in various peanut cultivars and found support for different levels of phytoalexin production among cultivars. Sobolev et al. looked at five different cultivars of peanuts grown in Georgia and found that the cultivars that had a high disease resistance to tomato spotted wilt virus and late leaf spot produced the most phytoalexins in the pods when they were damaged by the lesser cornstalk borer. *trans*-Resveratrol was one of the phytoalexins found in substantial concentrations. They also measured *trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, and *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene in peanut pods.

Total phenolic content was measured in the lyophilized crude extracts (table 4.3). Oneway ANOVA revealed that cultivar was not a significant factor for total phenolic content of the crude extracts ($P=0.73$). This result is most likely caused by plant stress variation having a greater effect then genetics producing phytoalexins as explained above regarding non-

significance of cultivar on ORAC activity in crude extracts. Total phenolic content was also measured for the collected fractions (figure 4.5). Peanut root fractions are significantly different in regards total phenolic content ($P<0.0001$) analyzed by oneway ANOVA. Tukey-Kramer HSD only singles out the 3-6 minute fraction as significantly different ($\alpha=0.05$) then all the other fractions. This result is not surprising given that many phenolic acids share a similar structure and will have a tendency to elute near the same time frame. In this instance, the phenolic compounds have limited interactions with the C18 column and elute rather quickly when the solvent is mostly water (table 4.1). The goal of measuring the total phenolic content of the fractions was to be able to normalize the ORAC data from the fractions. It would be rather difficult, if not impossible, to determine the weight of each fraction and subsequently determine μM TE/g fraction. Combining the ORAC and total phenolic content data allows the calculation of μM Trolox (TE) equivalents/mg gallic acid equivalent (GAE) and reveals the antioxidant "strength" of a fraction (figure 4.6). Statistical analysis of this data by oneway ANOVA reveals both cultivar ($P<0.0001$) and fraction ($P<0.0001$) to be significantly different for μM TE/mg GAE (table 4.4 and figure 4.7). Statistical significance of the fractions is again not surprising given that different compounds elute at different compounds with varying degrees of antioxidant activity. However it appears that some cultivars are higher in antioxidant activity with lower total phenolic profiles.

Literature Cited

2007. Oxygen radical absorbance capacity (ORAC) of selected foods-2007. USDA.

Aguamah G, Langcake P, Leworthy D, Page J, Pryce R, Strange R. 1981. Two novel stilbene phytoalexins from *Arachis hypogaea*. Phytochemistry 20(6):1381-3.

Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc 2(4):875-7.

Arora MK, Strange RN. 1991. Phytoalexin accumulation in groundnuts in response to wounding. Plant Sci 78(2):157-63.

Ballard TS, Mallikarjunan P, Zhou K, O'Keefe SF. 2009. Optimizing the extraction of phenolic antioxidants from peanut skins using response surface methodology. J Agr Food Chem 57:3064-72.

Barz W, Bless W, Boerger-Papendorf G, Gunia W, Mackenbrock U, Meier D, Otto C, Sueper E. Phytoalexins as part of induced defense reactions in plants: their elicitation, function and metabolism; 1990. p 140-56.

Cao G, Alessio HM, Cutler RG. 1993. Oxygen-radical absorbance capacity assay for antioxidants. Free Radical Biol Med 14(3):303-11.

Chakraborty D, Mandal SM. 2008. Fractional changes in phenolic acids composition in root nodules of Arachis hypogaea L. Plant Growth Regul 55(3):159-63.

Chen R, Wu P, Chiou R. 2002. Peanut roots as a source of resveratrol. J Agric Food Chem 50:1665-7.

Chung I-M, Park MR, Chun JC, Yun SJ. 2003. Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. Plant Sci 164(1):103-9.

Duenas M, Hernandez T, Estrella I, Rabanal R. 2003. Phenolic composition and antioxidant activity of mocan seeds (Visnea mocanera L.f). Food Chem 82(3):373-9.

Duh P, Yeh D, Yen G. 1992. Extraction and identification of an antioxidative component from peanut hulls. J Am Oil Chem Soc 69(8):814-8.

Keen NT, Ingham JL. 1976. New stilbene phytoalexins from American cultivars of Arachis hypogaea. Phytochemistry 15(11):1794-5.

Magalhaes Luis M, Segundo Marcela A, Reis S, Lima Jose LFC, Rangel Antonio OSS. 2006. Automatic method for the determination of Folin-Ciocalteu reducing capacity in food products. J Agric Food Chem 54(15):5241-6.

Medina-Bolivar F, Condori J, Rimando A, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan M. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003.

Nepote V, Grosso NR, Guzman CA. 2000. Antioxidant activity of methanolic extracts from peanut skin. Molecules [Electronic Publication] 5(3):487-8.

Nepote V, Grosso NR, Guzman CA. 2002. Extraction of antioxidant components from peanut skins. Grasas y Aceites 53:391-5.

Nepote V, Grosso NR, Guzman CA. 2005. Optimization of extraction of phenolic antioxidants from peanut skins. J Sci Food Agr 85:33-8.

Nepote V, Mestrallet MG, Grosso NR. 2004. Natural antioxidant effect from peanut skins in honey-roasted peanuts. J Food Sci 69(7):295-300.

O'Keefe SF, Wang H. 2006. Effects of peanut skin extract on quality and storage stability of beef products. Meat Sci 73:278-86.

Prior RL, Wu X, Schaich K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290-302.
Rao PVS, Strange R. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 199-227.
Rowland D, Dorner J, Sorensen R, Beasley JP, Jr., Todd J. 2005. Tomato spotted wilt virus in peanut tissue types and physiological effects related to disease incidence and severity. Plant Pathol 54(4):431-40.
Singleton VL, Orthofer R, Lamuela-Raventos RM. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 299(Oxidants and Antioxidants, Part A):152-78.
Sobolev V, Horn B, Potter T, Deyrup ST, Gloer JB. 2006a. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. J Agric Food Chem 54:3505-11.
Sobolev V, Potter T, Horn B. 2006b. Prenylated stilbenes from peanut root mucilage. Phytochem Anal 17:312-22.
Sobolev VS, Guo BZ, Holbrook CC, Lynch RE. 2007. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. J Agric Food Chem 55(6):2195-200.
Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior R. 2004. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. J Agric Food Chem 52:4026-37.
Xu BJ, Chang SKC. 2007. A comparative study on phenolic profiles and antioxidant activities of legumes as affected by extraction solvents. J Food Sci 72(2):S159-S66.
Yu J, Ahmedna M, Goktepe I, Dai J. 2006. Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. J Food Compos Anal 19:364-71.

FIGURES

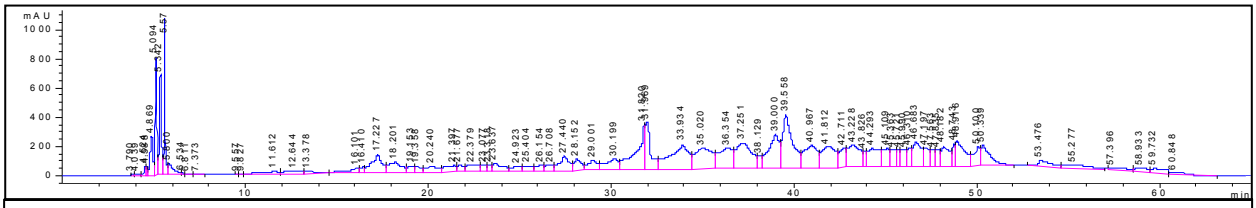

Figure 4.1: HPLC-DAD (280 nm shown) chromatogram of NC-12 70% aqueous ethanol extract.

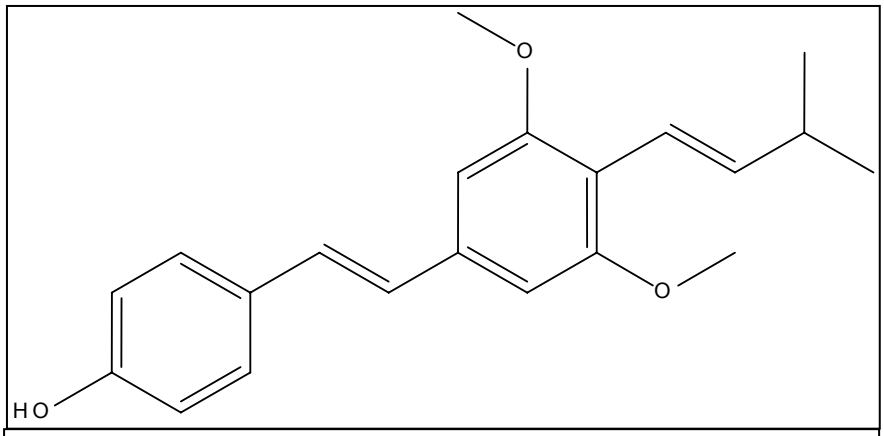

Figure 4.2: Mucilagin A structure as proposed by Sobolev *et al*. (2006b).

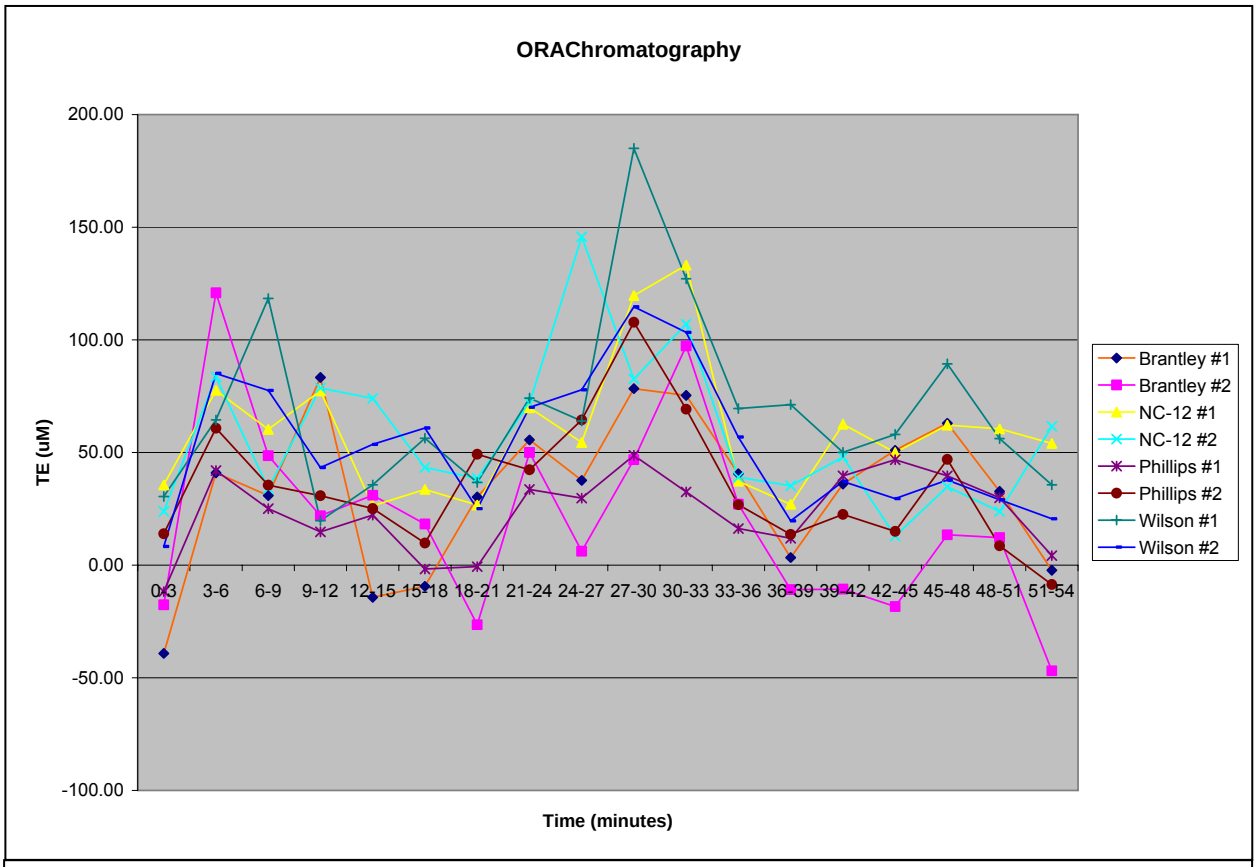

Figure 4.3: Antioxidant activity of collected 70% aqueous ethanol peanut root extract fractions as measured by ORAC. Fractions were separated by HPLC using a C18.

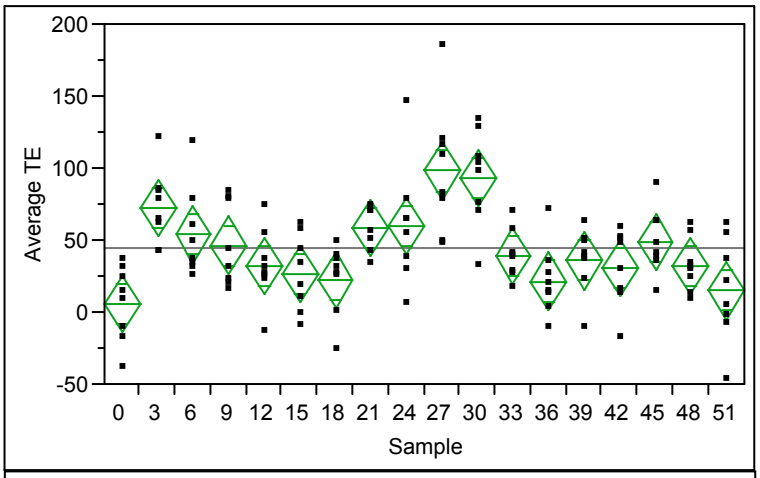

Figure 4.4: Oneway analysis of average antioxidant activity measured by ORAC of collected 70% aqueous ethanol peanut root extract fractions as measured by ORAC (α=0.05). Values reported as μM Trolox equivalents (TE). Fractions were separated via HPLC using a C18.

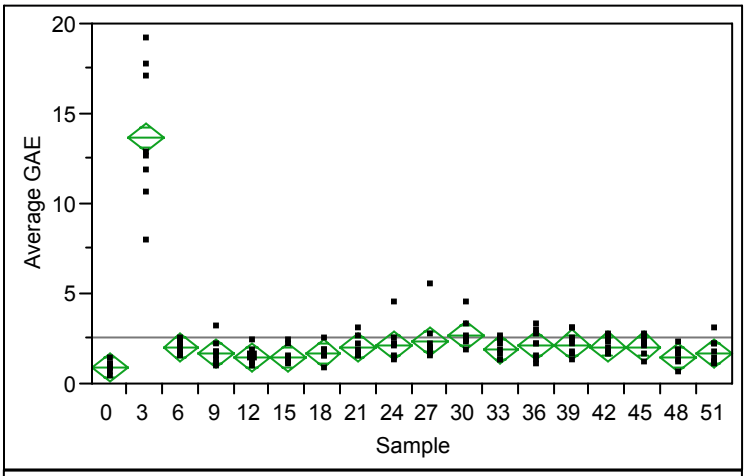

Figure 4.5: Oneway ANOVA of total phenolic content (mg gallic acid equivalents (GAE)/L) of collected 70% aqueous ethanol peanut root extract fractions (α=0.05).

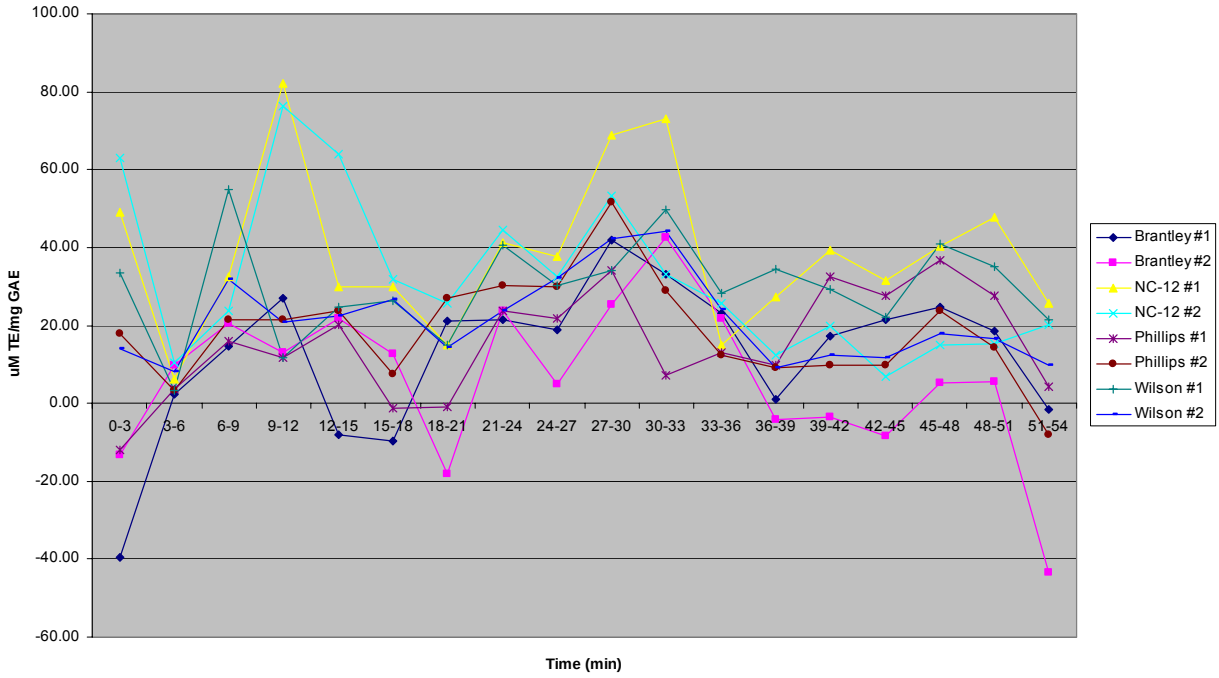

Figure 4.6: Combined antioxidant activity measured by ORAC and total phenolic content data for 70% aqueous ethanol peanut root extract fractions fractionated by HPLC using a C18 column.

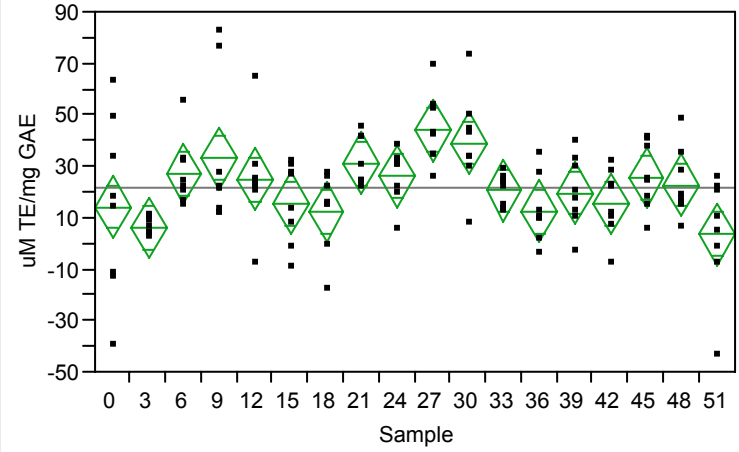

Figure 4.7: Oneway ANOVA of combined antioxidant activity measured by ORAC and total phenolic content data for 70% aqueous ethanol peanut root extract fractions fractionated by HPLC using a C18 column. Values reported as µM Trolox equivalents (TE)/mg gallic acid equivalents (GAE) (α=0.05).

TABLES

Table 4.1: HPLC gradient schedule used to separate 70% aqueous ethanol peanut root extracts using a C18. Solvent A is 0.5% aqueous acetic acid and solvent B is 0.5% acetic acid in methanol.

Time (minutes)	**% A**	**% B**
0	100	0
2	100	0
4	95.3	4.7
15	69.1	30.9
42	25.3	74.7
54.5	5	95
55	100	0
65	100	0

Table 4.2: Antioxidant activity of 70% aqueous ethanol peanut root extracts as measured by ORAC.	
Cultivar	**Trolox equivalents (µM/g dry extract)**
Brantley	2261.2
	4515.6
NC-12	600.3
	6564.4
Phillips	1166.0
	1471.4
Wilson	1335.8
	2658.8

Table 4.3: Total phenolic content of 70% aqueous ethanol peanut root extracts as measured by the Folin-Ciocalteu method.	
Cultivar	**Gallic acid equivalents (mg GAE/g dry extract)**
Brantley	40.2
	49.1
NC-12	24.2
	79.6
Phillips	23.1
	36.2
Wilson	45.5
	50.5

Table 4.4: Combined antioxidant activity measured by ORAC and total phenolic content data for 70% aqueous ethanol peanut root extract fractions by cultivar fractionated by HPLC using a C18 column. Values reported as μM Trolox equivalents (TE)/mg gallic acid equivalents (GAE). Means not sharing a letter are significantly different by Tukey-Kramer HSD (α=0.05).

Cultivar	**Average μM TE/mg GAE**
Brantley	9.5[c]
NC-12	35.2[a]
Phillips	16.9[b,c]
Wilson	25.5[a,b]

CHAPTER 5: EFFECT OF PEANUT ROOT EXTRACT ON OXIDATIVELY CHALLENGED HUMAN BRAIN MICROVASCULAR ENDOTHELIAL CELLS

Abstract

Many of the compounds found in peanut plants have been shown to have positive health effects. These effects range from insulin-like effects to anticancer properties. The objectives of this research was to identify the toxic levels of peanut root extracts, the level of hydrogen peroxide necessary to see a significant decrease in cell viability, and the ability of peanut root extract, quercetin, and Trolox to protect endothelial cells against oxidative stress induced by hydrogen peroxide as measured by cell viability and protein oxidation.

Peanut root extracts were incubated with human brain microvascular endothelial cells (HBMEC) at several concentrations (0.5, 5, 50, 250, and 500 μg/mL) for 20 hours and then hydrogen peroxide was added at 1500 μM for 4 hours. Trolox and quercetin were also used as antioxidant treatments due to their known effects. Cell viability and protein carbonyl concentration were then determined.

Peanut root extract concentration significantly affected cell viability ($P<0.0001$) with concentrations above 250 μg/mL found to be toxic. Peanut root extracts, quercetin, and Trolox did not exhibit a protective antioxidant effect for HBMEC challenged by hydrogen peroxide.

Keywords: HBMEC, human brain microvascular endothelial cells, hydrogen peroxide, MTT, peanut root, protein oxidation, protein carbonyls

Introduction

Plants produce different chemicals in the course of their life for many different reasons. One class of these compounds is the phytoalexins. Phytoalexins are compounds that are produced when a plant cell is under attack and are used to thwart the attack (Purkayastha 1995). The elicitation of phytoalexins can be induced by numerous factors such as microbes, fungi, UV light, and heavy metal salts. These stressors activate the shikimic-polymalonate acid pathway to create flavonoids and stilbenes (Rao and Strange 1995). The phytoalexins produced then proceed to defend against the threat by numerous mechanisms.

Many of our popular drugs were first extracted from plants (Heinrich and others 2004) and the search for medicine from plants continues to this day. Many of the compounds found in peanut plants have been shown to have positive health effects. These effects range from insulin-like effects to anticancer properties (Jang and others 1997; Soleas and others 2002; Delmas and others 2003; Signorelli and Ghidoni 2005; Baur and others 2006; Ungvari and others 2007). It is believed that many of these healthy influences are due to the compounds' antioxidant effects. It is thought that some of these compounds may also participate in cell signaling pathways that lead to health benefits (Dong 2003; Kundu and Surh 2004; Signorelli and Ghidoni 2005).

Oxidation has been implicated as the causative agent in many diseases. Oxidation occurs when a molecule is robbed of an electron, hydrogen atom or gains an oxygen atom. This often occurs when a free radical gains an electron to become a more stable moiety. Oxidation is a self-propagating reaction as free radicals lead to more free radicals in a chain reaction (Ingold 1961). Protein oxidation is thought to be a cause of cataracts and aging. Oxidation is a destructive agent in arteriosclerosis and injuries caused by reperfusion (Vendemiale and others 1999). The free radical theory of aging was first proposed by Denham Harman (1956). The free radical theory of aging proposes that aging is the result of additive damage to cells caused by oxidative species that is not repaired due to repair enzyme failure. This damage can lead to cancer, aging, and disease. Antioxidants would lower the oxidative stress experienced by the body and would subsequently lessen the effect of aging and oxidation related diseases.

Natural sources of antioxidants have shown potential in reducing the oxidative stress experienced by the body. Simonetti and colleagues (2002) investigated the effects of procyanidins from grape seeds in ten volunteers. The volunteers took 110 mg of the supplement for 30 days. The researchers found that α-tocopherol levels rose in the subjects' red blood cells

and DNA oxidation was reduced. The authors concluded that the procyanidins exhibited a protective effect for α-tocopherol while at the same time reducing DNA oxidation. In another study, Pignatelli and colleagues (2006) explored the "French paradox." They used 30 volunteers. They had ten of them drink 300 mL of white wine per day, ten drink 300 mL of red wine per day, and ten abstain from alcohol. The researchers measured urinary PGF-2α-III as a marker of oxidative stress. The level of PGF-2α-III is directly correlated with oxidative stress levels (Pignatelli and others 2006). They found PGF-2α-III levels decreased in both wine drinking groups and the decrease was highest in the red wine group. Red wine has been shown to have a higher polyphenol content then white wine. Pignatelli and fellow authors conclude that the effect they saw was due to the polyphenolic content found in wine. Frankel and colleagues (1993a) separated ethanol from the wine phenolics and examined the effects they had on retarding oxidation of LDL. They found that wine phenolics were effective at inhibiting LDL oxidation and were more effective then α-tocopherol. LDL oxidation has been shown to be responsible for atherosclerosis (Luc and Fruchart 1991; Alexander 1995).

Much experimentation has been performed with cell culture and testing antioxidants' effect against oxidation. Reports of the ability of antioxidants and phytoalexins to inhibit oxidative damage to cells have been mixed. The results vary among cell types and concentrations of the tested compounds. This is best demonstrated by a study carried out by Ram and Hiebert (2004) where they investigated the protective effects of α-tocopherol on both porcine and bovine aortic endothelial cells. The investigator oxidatively-challenged the endothelial cells with hydrogen peroxide and discovered that α-tocopherol was able to protect the porcine cells, but not the bovine cells. Human endothelial cells are used frequently when studying the effects of oxidation and the ability of substances to lessen the damage caused by induced oxidation. Montecinos and colleagues (2007) found that ascorbic acid was an effective inhibitor of oxidation in human vascular endothelial cells when oxidatively stressed by hydrogen peroxide. Ek, Strom, and Cotgreave (1995) experimented with human umbilical vein endothelial cells and measured the ability of ascorbic acid to protect them against hydrogen peroxide from xanthine oxidase. They also measured the uptake of ascorbic acid. This group found that endothelial cells actively take up ascorbic acid, but it weakly protects the cells from hydrogen peroxide. Phenolics have also been investigated. Quercetin has been shown to protect human umbilical vein vascular endothelial cells from oxidative attack from homocysteine at

concentrations ranging from 12.5-50 μM (Lin and others 2007). A disadvantage of adding phenolic compounds to cell culture experiments is the production of hydrogen peroxide. This may lead to misleading results when studying the ability of phenolic compounds to inhibit cancer cells. Lee and fellow researchers (2005) investigated the ability of gallic acid and quercetin to produce hydrogen peroxide in cell media. Gallic acid produced 269 μM of hydrogen peroxide at 100 μg/mL (587.8 μM) and quercetin produced 59 μM of hydrogen peroxide at the same concentration in 1 hour. Both phenolics were shown to exhibit antiproliferative effects in colon cancer cells and rat liver epithelial cells, but gallic acid was shown to have a stronger effect then quercetin. Another research group examined the protective effects of myricetin, quercetin, and rutin against hydrogen peroxide in HepG2 and Caco-2 cells (O'Brien and others 2000). They found that these antioxidants were protective against hydrogen peroxide (50 μM) as measured by DNA damage by the comet assay up to 200 μM. Kaneko and Baba (1999) investigated the ability of flavonoids to protect human umbilical vein endothelial cells against oxidative damage caused by linoleic acid hydroperoxide. They determined that flavonols were able to increase cell viability as measured by a colormetric cell-counting kit. The flavanones had no measurable effect in this model system.

A widely used assay to determine the amount of protein oxidative damage that has occurred to the cell is 2, 4-dinitrophenylhydrazine (DNPH), which measures the concentration of protein carbonyls present in cell culture (Lee and Choi 2008; Matos and others 2008). Protein carbonyls are most often formed by hydroxyl radicals (HO•) (Headlam and Davies 2004). Hydroxyl radicals are frequently the result of hydrogen peroxide produced by cellular metabolism. This hydrogen peroxide, if not degraded to harmless products, can go on to form hydroxyl radicals via the Fenton reaction (Ciolino and Levine 1997). The Fenton reaction produces hydroxyl radicals when hydrogen peroxide oxidizes ferrous iron (II) to ferric iron (III). Iron is commonly found in biological systems. An example protein carbonyl formation reaction is shown in figure 5.1 (Headlam and Davies 2004). DNPH is used to derivitize the protein to a colored hydrazone as shown in the following two reactions (Levine and others 1990):

Protein–C=O + H_2N–NH–2,4–DNP→ Protein=N–NH–2,4–DNP + H_2O

Protein–C=N–Protein + H_2N–NH–2,4–DNP→ Protein=N–NH–2,4–DNP + H_2O

The MTT assay is a commonly used assay to measure cell viability (Schiller and others 1992). The MTT reagent is metabolized by cellular enzymes resulting in a formazan compound with a purple color (figure 5.2) that is not water soluble. The mechanism behind the MTT reaction dictates that the magnitude of metabolism of the cells will determine the level of formazan produced and this can lead to variability between experiments.

The objectives of this research was to identify the toxic levels of peanut root extracts, the level of hydrogen peroxide necessary to see a significant decrease in cell viability, and the ability of peanut root extract, quercetin, and Trolox to protect endothelial cells against oxidative stress induced by hydrogen peroxide as measured by cell viability and protein oxidation.

Materials and Methods

Peanut Root Extraction

Peanut roots were collected from 4 different cultivars (Brantley, NC-12, Phillips, and Wilson) in Virginia and separated into two replications. Roots from 2 plants were combined for pooled sampling. The roots were rinsed with water to remove dirt, dried at 40°C, and stored at -18°C until extraction.

The roots were extracted by adding 100 mL 70% aqueous ethanol to 5 g of peanut root. The peanut root pieces used were the smallest rootlets available. This mixture was then blended for 30 seconds in a Waring Commercial Blender (New Hartford, Conneticut) set on high powered by a Tenma rheostat (Centerville, Ohio) set at 70 volts, allowed to sit for five minutes, and then blended again for one minute at 70 volts. The mixture was then placed in a Fisher FS20 ultrasonic bath (Pittsburgh, PA) for 10 minutes. Following the ultrasonic bath, the suspension was placed in 50 mL plastic centrifuge vials and centrifuged for 30 minutes at 300 x g at room temperature. The supernatant was then transferred to 10 mL glass centrifuge tubes and centrifuged again for 30 minutes at 300 x g. The supernatant was then frozen and lyophilized.

ORAC

A black 96-well plate was utilized for ORAC. Samples (including blanks and Trolox standards) were run in duplicate. Crude extracts were analyzed at 50 μg/mL in 70% aqueous ethanol. Each well contained 40 μL of sample and 200 μL of 100 mM fluoroscein. The plate

was then incubated at 37°C for 20 minutes. After this time, 35 μL of 0.36 M AAPH in warmed 75 mM PBS (pH 7.4) was added and the plate was read with a Victor mulitlabel plate reader (Perkin-Elmer, Turku, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Results were reported in μM TE/mg dry extract for crude extracts.

Cell Culture

The human brain microvascular endothelial cells (HBMEC) were cultured as previously described (Layman and others 2009). Briefly, cells were cultivated in RPMI 1640-based medium containing 10% fetal bovine serum (Mediatech), 10% Nuserum (Becton Dickinson), 30 μg/mL of endothelial cell growth supplement (Becton Dickinson), 15 U/mL heparin (Sigma), 2 mM L-glutamine, 2 mM sodium pyruvate, nonessential amino acids, vitamins, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Mediatech). Cells were maintained at 37°C in a 5% CO_2 humid environment.

MTT Assay

HBMEC were plated onto clear, collagen coated 96-well plates at an approximate concentration of 5000 cells per well 24 hours prior to experimentation. Quercetin (Sigma), Trolox (Sigma), and peanut root extract were all dissolved in RPMI 1640 and syringe filtered at 0.45 μm to remove any potential bacteria and molds. 100 μL of quercetin, Trolox, peanut root extract, or RPMI 1640 (control) was then added to each well and incubated for 20 hours at 37°C in a 5% CO_2 humid environment. After 20 hours, the cells were rinsed with 100 μL of HBSS and hydrogen peroxide diluted in RPMI 1640 was then added for 4 hours. Following treatment, the cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT, Sigma) conversion assay. MTT reagent was added at a concentration of 0.5 mg/mL in RPMI 1640 with 100 μL per well. The plates were then incubated for 4 hours at 37°C. The solution was then aspirated and the formazan product was solubilized with DMSO. Absorbance of the plate was read at 570 nm with a Molecular Devices Corp. SPECTRAmax M2 microplate reader.

Protein Carbonyl Analysis

HBMEC were plated onto 6-well plates at an approximate concentration of 100,000 cells per well and allowed to grow to confluence. Quercetin, Trolox, and peanut root extract were dissolved in RPMI 1640 and syringe filtered at 0.45 μm to remove any potential bacteria and molds. 2 mL of quercetin, Trolox, peanut root extract, or RPMI 1640 (control) was then added to each well and incubated for 20 hours at 37°C in a 5% CO_2 humid environment. After incubation, cells were rinsed with 2 mL of HBSS and hydrogen peroxide diluted in RPMI 1640 was then added for 4 hours. Following treatment, protein carbonyl concentration was determined using a protein carbonyl assay kit (Cayman Chemical). Cell lysate was collected by removing cells with trypsin, adding RPMI 1640, and physically agitating the culture vessel, thus releasing the cells into the RPMI 1640. The cells were then collected by centrifugation for 10 minutes at 4°C and 1,000 x g. The supernatant was removed and 1 mL of 50 mM PBS, pH 6.7 with 1 mM EDTA was added. The cells were frozen at -20°C to aid in collection of the lysate. The cells were sonicated twice for 1 minute, then centrifuged for 10 minutes at 10,000 x g at 4°C. The supernatant was removed and utilized for the protein carbonyl analysis. The supernatant absorbance was checked at 280 nm and 260 nm to ensure no nucleic acid contamination. 200 μL of supernatant was transferred to two tubes. One tube was for the protein carbonyl determination and the other tube served as the control for the sample. 800 μL of 10mM DNPH solution was added to the sample tube and 800 μL of 2.5 M HCl was added to the control tube. The samples were then incubated at room temperature in darkness and vortexed every 15 minutes. Then, 1 mL of 20% TCA was added to each tube, vortexed, incubated for 5 minutes on ice, and centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed, incubated for 5 minutes on ice, and then centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was discarded and the protein pellet was resuspended in 1 mL of ethanol/ethyl acetate (1:1) solvent. The tubes were then centrifuged for 10 minutes at 10,000 x g at 4°C. The supernatant was removed and the protein pellet was solubilized in 500 μL guanidine hydrochloride. The samples were centrifuged at 10,000 x g for 10 minutes at 4°C. The samples (220 μL) were then transferred to two wells on a clear, 96-well plate and absorbance was read at 370 nm with a Molecular Devices Corp. SPECTRAmax M2 microplate reader. The control absorbance was then subtracted from the sample absorbance giving the corrected absorbance. Protein carbonyls were determined using the equation:

Protein carbonyl (nmol/mL)= [corrected absorbance/0.011 μM^{-1}]*(500 μL/200 μL).

Statistical Analysis

All statistical analysis was carried out using JMP 7.0 (SAS Institute, Cary, NC). Mean separation by one-way ANOVA was carried out for the MTT assays as well as the protein carbonyl assay to determine any significant effects by treatments (peanut root extract, quercetin, Trolox, control). Tukey-Kramer Honestly Significant Difference (HSD) was used to determine which treatments in the factors were significant. Means were considered significantly different at $P<0.05$.

Results and Discussion

ORAC activities were measured in four different cultivars of peanut root extracts in duplicate (table 5.1). From this data it was decided to use the peanut root extract with the highest ORAC value for further study in cell culture, which was an NC-12 root extract with an ORAC value of 6564.4 μM Trolox equivalent/g dry extract.

Peanut root extracts were tested for toxic effects at levels ranging from 0.5 μg/mL to 500 μg/mL in endothelial cells by measuring cell viability via MTT assay (figure 5.3). Peanut root extract concentration significantly affected cell viability ($P<0.0001$). Mean separation by one-way ANOVA showed that treatment levels of 250 μg/mL and 500 μg/mL were significantly different ($P<0.0001$) and had a negative effect on cell viability. The relationship between peanut root extract concentration and cell viability is quadratic as illustrated by figure 5.3 ($P<0.0001$) with an r-squared of 0.88. Figure 5.4 further illustrates this point. Martin and fellow researchers (2008) tested for protective effects of cocoa extract against oxidative damage from *tert*-butylhydroperoxide in HepG2 cells. Martin *et al*. used cocoa extract levels ranging from 0.05 to 50 μg/mL and did not observe a toxic effect from the extracts in unchallenged cells. Cell viability was measured by the lactate dehydrogenase leakage assay. The researchers chose cocoa extract concentrations from levels of antioxidants previously found to induce health benefits. It is almost certain that had they tested higher levels, they would have found a concentration that is toxic. The toxicity of plant extracts probably varies in different extracts and different cell lines/types. The toxicity of peanut root extract may be due to the phenolic compounds present producing hydrogen peroxide. This has been shown to be the case in apple extracts (Lapidot and

others 2002). Apple extracts had previously been shown to be inhibitory to tumor cells, but Lapidot *et al*. further investigated this by measuring the hydrogen peroxide present when apple extract is added to cell culture. Apple extract was tested from 0-225 μM of phenolics and was found to inhibit proliferation of tumor cells. It was also shown to produce hydrogen peroxide when added to the cell media. Metmyoglobin was added to reduce hydrogen peroxide concentrations and the apple extracts no longer had inhibiting effects on the tumor cells. Labieniec and Gabryelak (2005) found that concentrations of ellagic acid, tannic acid, and gallic acid above 1 μM produced protein carbonyls in B14 Chinese hamster cells. This study illustrates the importance of testing different concentrations and measuring different markers of cell viability and oxidative damage. Antioxidants can have pro- or antioxidant activity depending on concentration.

After establishing the toxic levels of peanut root extract, the dose of hydrogen peroxide needed to be determined. The concentration of hydrogen peroxide added to cell culture and how long to incubate the cells with hydrogen peroxide treatments varies in literature reports. Youdim and fellow researchers (2002) investigated the ability of flavonoids to protect human microvascular endothelial cells against oxidative damage from hydrogen peroxide. They measured the formation of lipid peroxides when the cells were exposed to 100 μM hydrogen peroxide for 2 hours. Riso and colleagues (2002) used hydrogen peroxide at concentrations ranging from 10 to 200 μM for 15 minutes while investigating the ability of green tea extract to protect DNA in Jurkat T cells. Rah and fellow investigators (2005) also looked at the ability of green tea polyphenols, but the measured cell viability in human microvascular endothelial cells exposed to 10 mM of hydrogen peroxide for 24 hours. They found that this level of hydrogen peroxide reduced cell viability by 32% as measured by flow cytometry. Jarret, Albon, and Boulton (2006) studied the ability of different cells to resist oxidative stress induced by hydrogen peroxide. They studied retinal pigment epithelial cells, corneal fibroblasts, alveolar type II epithelial cells, and skin epidermal cells. The cells were exposed to 1500 μM hydrogen peroxide for 1 hour and then the cell viability was assessed using the MTT assay. This level of hydrogen peroxide produced cell viability reductions of around 25% with some variation being seen among cell types. Retinal pigment endothelial cells had the least reduction, which was expected due to their high innate tolerance to oxidative stress.

Concentrations ranging from 1000-15,000 μM of hydrogen peroxide were tested for toxicity and the targeted loss of cell viability occurred between 1000 and 2000 μM (figure 5.5). From this data, the decision to use 1500 μM hydrogen peroxide for the remainder of the experimentation was made. Hydrogen peroxide concentration rapidly decreases in cell culture due to the presence of catalase, which is dependent upon the cell density (Gille and Joenje 1992). Hydrogen peroxide passes into the cells and is then broken down by catalase. The more cells present, the more quickly the hydrogen peroxide concentration diminishes. Hydrogen peroxide administers most of its damage by being converted into hydroxyl radicals by transition metals. If transition metals are present in low concentrations, then hydrogen peroxide will have less of a devastative effect then when transition metals are prevalent. This is an important consideration in cell culture since the cells are reliant on the cell culture media for their nutrients. If the transition metals are not restored, then cell lines will become deficient in these compounds (Varani and others 1992). Ciolino and Levine (1997) took this concept into consideration when they investigated oxidative damage of proteins in bovine aortic endothelial cells. Ciolino and Levine added 3 μM iron (III) chloride with a lipophilic metal chelator to the cells and measured the iron uptake by the ferrozine assay. The iron (III) chloride was added with a lipophilic metal chelator because iron chloride alone will not pass through the cellular membrane. The authors found that the bovine aortic endothelial cells did not experience oxidative damage by 75 μM hydrogen peroxide prior to iron restoration.

Peanut root extract was tested for efficacy against hydrogen peroxide induced oxidation measured by two assays. Cell viability was measured by the MTT assay to observe whether peanut root extract was able to increase cell viability when endothelial cells were assaulted by 1500 μM hydrogen peroxide (figure 5.6). There were no significant differences among treatment levels for cell viability by one-way ANOVA (P=0.26). Previous research regarding the ability of plant extracts to protect cells from oxidative stress has been more successful at inhibiting oxidative damage. Vitor and fellow researchers (2004) examined the extract of *Pterospartum tridentatum* to inhibit oxidative damage in human umbilical vein endothelial cells caused by 3 μM hydrogen peroxide. Cell viability was measured by the MTT assay. The authors tested concentrations of 0.001 to 1 mg/mL of *Pterospartum tridentatum* extract. Increased cell viability against hydrogen peroxide was not seen until plant extract concentrations reached 300 μg/mL. Peanut root extract was found to be toxic at these concentrations. No toxic effect was seen at

any level of *Pterospartum tridentatum* extract, but cell viability was not measured in cells exposed solely to the plant extracts without the addition of hydrogen peroxide. There have been several studies investigating green tea in preventing oxidation in cell culture. Rah and colleagues (2005) used human microvascular endothelial cells and tested green tea polyphenols at either 1 or 10 μg/mL concentrations. After incubating the cells with the green tea polyphenol mixture for 1 hour, 10 mM hydrogen peroxide was added and the cells were incubated for 24 hours. Cell viability was measured via flow cytometry following treatment. Rah *et al*. found that 10 μg/mL of green tea polyphenol mixture was able to maintain cell viability when exposed to hydrogen peroxide and 1 μg/mL exhibited some positive effect, but not to the same degree as the 10 μg/mL concentration. Riso and fellow researchers (2002) tried 10 μg/mL of green tea extract to reduce DNA damage caused by hydrogen peroxide in Jurkat T cells. DNA damage was measured by the Comet assay, which is an electrophoresis technique. If DNA is undamaged, then it will exhibit as a fluorescent circle in the gel. If the DNA is damaged, then the fluorescent circle will breakdown into a comet-like shape (Riso and others 1999). Hydrogen peroxide levels of 10 to 200 μM were incubated with the cells for 15 minutes. The authors found no detectable decrease in DNA damage in cells treated with green tea extract. Riso *et al*. (2002) concluded that other compounds may be interfering with the antioxidant acivity when using a crude extract. Martin and fellow investigators (2008) examined the ability of cocoa extract to protect HepG2 cells from *tert*-butylhydroperoxide-induced oxidative damage measured by cell viability. Cell viability was measured by the lactate dehydrogenase leakage assay. Cocoa extracts were added at concentrations ranging from 0.05 to 50 μg/mL to cell culture for either 2 or 20 hours. Following treatment with the cocoa extract, the cells were oxidatively challenged with the addition of 200 μM *tert*-butylhydroperoxide for 3 hours. The authors found that the levels of cocoa extract tested were not toxic and did increase cell viability when compared to non-treated cells exposed to the oxidative stress. Hseu and colleagues (2008) investigated a plant associated with traditional Chinese medicine, *Antrodia camphorata*, for antioxidant activity against peroxyl radicals from AAPH. Human umbilical vein endothelial cells were used as the cell of choice in this study and Trolox was used as a positive control. Cell viability was recorded as was DNA damage. *Antrodia camphorata* extracts were found to increase cell viability in cells treated with AAPH without any toxic effects at the levels tested (up to 200 μg/mL). The conclusions drawn regarding the ability of a substance to inhibit oxidation will depend upon

which cell lines were chosen, which oxidative treatment was chosen, and the assays used to measure the degree of oxidative damage.

Another avenue that investigators have taken when testing natural antioxidants in cell culture is to subject the cells to pure compounds as opposed to crude extracts as mentioned previously. The researchers will identify potential antioxidants that are found in crude plant extracts either through a literature search or through preliminary data and then test them directly. Youdim and fellow colleagues (2002) took this approach when measuring the ability of anthocyanins found in blueberries and cranberries and hydroxycinnamic acids to protect human microvascular endothelial cells against hydrogen peroxide oxidative damage as measured by lipid peroxidation, inflammatory markers, cell viability via MTT assay. Anthocyanins and hydroxycinnamic acids both showed an ability to protect the cells against oxidative damage in this model system. Lee and Choi (2008) took this approach a step further when they singled in on one compound, myricetin, and judged its ability to protect MC3T3-E1 cells against oxidative reactions from 2-deoxy-D-ribose. MC3T3-E1 cells are osteoblastic cells or cells that are responsible for bone formation. Lee and Choi measured lipid oxidation products (malondialdehyde) and protein carbonyls. Myricetin was able to reduce these oxidative markers in MC3T3-E1 cells significantly. If the research elucidates a positive effect from a single compound or a purified extract, then researchers can begin to look for more effective means of synthesizing it for possible health supplementation products. This has been the case with resveratrol. Resveratrol is thought to be a factor involved in the "French Paradox." The "French Paradox" refers to people living around the Mediterranean Sea consuming diets high in fat, but do not experience the high heart disease rates as expected, given their diets. Their high fat diet is thought to be counteracted by their ingestion of red wine with a high concentration of *trans*-resveratrol (Frankel and others 1993b). After resveratrol was identified as a possible factor in regards to the French paradox, much research was carried out to discover any effects resveratrol may have. Ou and fellow researchers (2006) tested resveratrol's ability to protect human umbilical vein endothelial cells from oxidative damage from oxidized LDL. The researchers concluded that resveratrol does have positive effects in cell culture and that this research supports the theory that resveratrol is a factor in the French paradox.

Interestingly, neither Trolox nor quercetin showed an ability to increase cell viability at the levels tested. Trolox has been shown to increase cell viability and decrease PGI_2 (an

oxidative marker) at 30 μM in endothelial cells (Hseu and others 2008). It is possible that the level used was too high, but Trolox was tested down to 10 μM with no effect on cell viability in the preliminary work. Quercetin has been extensively studied in cell culture with a proven ability to protect cells from oxidative damage. It has been shown to protect human umbilical vein endothelial cells from oxidative damage from homocysteine at concentrations of 6.25 to 100 μM (Lin and others 2007). In this same study, quercetin reduced malondialdehyde concentrations in the cells at 12.5 to 50 μM levels. It has also been shown to protect HepG2 cells from *tert*-butylhydroperoxide-induced oxidative damage at 0.1 to 10 μM concentrations. Quercetin reduced malondialdehyde production in HepG2 cells as well (Alia and others 2006). Neurons also experienced a protective effect from quercetin when confronted with oxidative damage induced by amyloid beta-peptides (Ansari and others 2009). This research concluded that quercetin has a protective effect at lower levels, but found a toxic effect at concentrations of 40 μM and higher. It is possible that the concentration used in the present experiment was too high and led to cell death. Both ascorbic acid and gallic acid were also tested with no hydrogen peroxide present. Ascorbic acid and gallic acid did not have a significant effect ($P=0.054$) on cell viability at 10 and 100 μM (figure 5.7), but did appear to have some negative effect on cell viability at the tested levels. This was not surprising given the evidence in the literature, but with the variation seen among cell lines, confirmation was solicited. Gallic acid has been shown to produce hydrogen peroxide in DMEM cell media (Lee and others 2005). In this study, 269 μM of hydrogen peroxide were produced when gallic acid was solubilized in DMEM at 100 μg/mL (587.8 μM) for 1 hour. The hydrogen peroxide produced will have a negative effect on cell viability. Ascorbic acid has also been shown to produce hydrogen peroxide in cell media (Duarte and others 2007). Duarte and colleagues found that ascorbic acid has no effect on cell viability in human diploid fibroblasts at 100 μM as measured by the MTT assay, but did cause DNA damage. Other studies have shown ascorbic acid to protect cells. Jarrett, Cuenco, and Boulton (2006) subjected retinal pigment epithelial cells to oxidative stress via 1.5 mM hydrogen peroxide and observed reduced oxidative damage with addition of antioxidants. Ascorbic acid was shown to increase cell survival at both 1 and 10 μM, but reduced cell survival at 100 μM. This is most likely due to its pro-oxidant activity at this level.

Protein carbonyl concentration was measured in cells pretreated with 0.5 μg/mL peanut root extract, 5 μg/mL peanut root extract, 1000 μM Trolox, 500 μM quercetin and solely RPMI

1640 (figure 5.8). There were no significant differences among treatments by one-way ANOVA (P=0.21). Protein carbonyl analysis is a proven technique as a means to evaluate the extent of oxidative damage experienced in cell culture and tissue samples (Ciolino and Levine 1997; Headlam and Davies 2004; Grune and others 2005; Lee and Choi 2008). Although the differences were not significant, both quercetin and 5 μg/mL peanut root extract had higher average protein carbonyl contents. It is quite possible that at these levels a pro-oxidant effect is beginning to be seen. Labieniec and Gabryelak (2005) measured pro-oxidant effects in polyphenolic compounds (tannic and gallic acid) added to B14 Chinese hamster cells. Oxidative damage to protein and DNA was observed in concentrations of polyphenols of 1 μM and higher. This study illustrates the ability of an antioxidant to become a pro-oxidant at relatively small concentrations, which can have devastating effects.

Literature Cited

Alexander RW. 1995. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. Hypertension 25(2):155-61.

Alia M, Ramos S, Mateos R, Granado-Serrano AB, Bravo L, Goya L. 2006. Quercetin protects human hepatoma HepG2 against oxidative stress induced by *tert*-butyl hydroperoxide. Toxicol Appl Pharm 212:110-8.

Ansari MA, Abdul HM, Joshi G, Opii WO, Butterfield DA. 2009. Protective effect of quercetin in primary neurons against amyloid beta (1-42): relevance to Alzheimer's disease. J Nutr Biochem 20(4):269-75.

Ciolino HP, Levine RL. 1997. Modification of proteins in endothelial cell death during oxidative stress. Free Radical Bio Med 22(7):1277-82.

Dong Z. 2003. Molecular mechanism of the chemopreventive effect of resveratrol. Mutat Res, Fundam Mol Mech Mutagen 523-524:145-50.

Duarte TL, Almeida GM, Jones GDD. 2007. Investigation of the role of extracellular H2O2 and transition metal ions in the genotoxic action of ascorbic acid in cell culture models. Toxicol Lett 170:57-65.

Ek A, Stroem K, Cotgreave IA. 1995. The uptake of ascorbic acid into human umbilical vein endothelial cells and its effect on oxidant insult. Biochem Pharmacol 50(9):1339-46.

Frankel EN, Kanner J, German JB, Parks E, Kinsella JE. 1993a. Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341(8843):454-7.

Frankel EN, Waterhouse AL, Kinsella JE. 1993b. Inhibition of human LDL oxidation by resveratrol. Lancet 341(8852):1103-4.

Gille JJP, Joenje H. 1992. Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. Mut Res 275:405-14.

Grune T, Merker K, Jung T, Sitte N, Davies KJA. 2005. Protein oxidation and degradation during postmitotic senescence. Free Radic Biol Med 39:1208-15.

Harman D. 1956. Aging: A theory based on free radical and radiation chemistry. J Gerontol 11:298-300.

Headlam HA, Davies MJ. 2004. Markers of protein oxidation: different oxidants give rise to variable yields of bound and released carbonyl products. Free Radical Biol Med 36(9):1175-84.

Heinrich M, Barnes J, Gibbons S, Williamson E. 2004. Fundamentals of pharmacognosy and phytotherapy. Edinburgh: Churchill Livingstone.

Hseu Y-C, Chen S-C, Yech Y-J, Wang L, Yang H-L. 2008. Antioxidant activity of Antrodia camphorata on free radical-induced endothelial cell damage. J Ethnopharmacol 118(2):237-45.

Ingold KU. 1961. Inhibition of the autoxidation of organic substances in the liquid phase. Chem Rev 61:563-89.

Jarrett SG, Albon J, Boulton M. 2006. The contribution of DNA repair and antioxidants in determining cell type-specific resistance to oxidative stress. Free Radic Res 40(11):1155-65.

Jarrett Stuart G, Cuenco J, Boulton Mike E. 2006. Dietary antioxidants provide differential subcellular protection in epithelial cells. Redox Report 11:144-52.

Kaneko T, Baba N. 1999. Protective effect of flavonoids on endothelial cells against linoleic acid hydroperoxide-induced toxicity. Biosci Biotechnol Biochem 63(2):323-8.

Kundu JK, Surh Y-J. 2004. Molecular basis of chemoprevention by resveratrol: NF-kB and AP-1 as potential targets. Mutat Res 555(1-2):65-80.
Labieniec M, Gabryelak T. 2005. Measurement of DNA damage and protein oxidation after the incubation of B14 Chinese hamster cells with chosen polyphenols. Toxicol Lett 155:15-25.
Lapidot T, Walker MD, Kanner J. 2002. Can apple antioxidants inhibit tumor cell proliferation? Generation of H2O2 during interaction of phenolic compounds with cell culture media. J Agric Food Chem 50(11):3156-60.
Layman JM, Ramirez SM, Green MD, Long TE. 2009. Influence of polycation molecular weight on poly(2-dimethylaminoethyl methacrylate)-mediated DNA delivery in vitro. Biomacromolecules 10(5):1244-52.
Lee K-H, Choi E-M. 2008. Myricetin, a naturally occurring flavonoid, prevents 2-deoxy-D-ribose induced dysfunction and oxidative damage in osteoblastic MC3T3-E1 cells. Eur J Pharmacol 591(1-3):1-6.
Lee KW, Hur HJ, Lee HJ, Lee CY. 2005. Antiproliferative effects of dietary phenolic substances and hydrogen peroxide. J Agric Food Chem 53(6):1990-5.
Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186(Oxygen Radicals Biol. Syst., Pt. B):464-78.
Lin R, Liu J, Gan W, Ding C. 2007. Protective effect of quercetin on the homocysteine-injured human umbilical vein vascular endothelial cell line (ECV304). Basic Clin Pharmacol Toxicol 101(3):197-202.
Luc G, Fruchart JC. 1991. Oxidation of lipoproteins and atherosclerosis. AM J Clin Nutr 53(1 Suppl):206S-9S.
Martin MA, Ramos S, Mateos R, Granado Serrano AB, Izquierdo-Pulido M, Bravo L, Goya L. 2008. Protection of human HepG2 cells against oxidative stress by cocoa phenolic extract. J Agric Food Chem 56(17):7765-72.
Matos M, Augusto E, Oliveira CR, Agostinho P. 2008. Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: Involvement of oxidative stress and mitogen-activated protein kinase cascades. Neuroscience 156(4):898-910.
Montecinos V, Guzman P, Barra V, Villagran M, Munoz-Montesino C, Sotomayor K, Escobar E, Godoy A, Mardones L, Sotomayor P, Guzman C, Vasquez O, Gallardo V, van Zundert B, Bono Maria R, Onate Sergio A, Bustamante M, Carcamo Juan G, Rivas Coralia I, Vera Juan C. 2007. Vitamin C is an essential antioxidant that enhances survival of oxidatively stressed human vascular endothelial cells in the presence of a vast molar excess of glutathione. J Biol Chem 282(21):15506-15.
O'Brien NM, Woods JA, Aherne SA, O'Callaghan YC. 2000. Cytotoxicity, genotoxicity and oxidative reactions in cell-culture models: modulatory effects of phytochemicals. Biochem Soc T 28(2):22-6.
Ou H-C, Chou F-P, Sheen H-M, Lin T-M, Yang C-H, Sheu WH-H. 2006. Resveratrol, a polyphenolic compound in red wine, protects against oxidized LDL-induced cytotoxicity in endothelial cells. Clinica Chimica Acta 364(1-2):196-204.
Pignatelli P, Ghiselli A, Buchetti B, Carnevale R, Natella F, Germano G, Fimognari F, Di Santo S, Lenti L, Violi F. 2006. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. Atherosclerosis 188:77-83.

Purkayastha RP. 1995. Progress in phytoalexin research during the past 50 years. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 1-39.
Rah DK, Han D-W, Baek HS, Hyon S-H, Park J-C. 2005. Prevention of reactive oxygen species-induced oxidative stress in human microvascular endothelial cells by green tea polyphenol. Toxicol Lett 155:269-75.
Ram JI, Hiebert LM. 2004. Vitamin E protects porcine but not bovine cultured aortic endothelial cells from oxygen-derived free radical injury due to hydrogen peroxide. Cell Biol Toxicol 20(1):55-67.
Rao PVS, Strange R. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 199-227.
Riso P, Erba D, Criscuoli F, Testolin G. 2002. Effect of greeen tea extract on DNA repar and oxidative damage due to H2O2 in Jurkat T cells. Nutr Res 22:1143-50.
Riso P, Santangelo A, Porrini M. 1999. The comet assay for the evaluation of cell resistance to oxidative stress. Nutr Res 19:325-33.
Schiller CD, Kainz A, Mynett K, Gescher A. 1992. Assessment of viability of hepatocytes in suspension using the MTT assay. Toxicol in Vitro 6(6):575-8.
Signorelli P, Ghidoni R. 2005. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. J Nutr Biochem 16(8):449-66.
Simonetti P, Ciappellano S, Gardana C, Bramati L, Pietta P. 2002. Procyanidins from *Vitis vinifera* seeds: In vivo effects on oxidative stress. J Agric Food Chem 50:6217-21.
Varani J, Dame MK, Gibbs DF, Taylor CG, Weinberg JM, Shayevitz J, Ward PA. 1992. Human umbilical vein endothelial cell killing by activated neutrophils. Loss of sensitivity to injury is accompanied by decreased iron content during in vitro culture and is restored with exogenous iron. Lab Invest 66:708-14.
Vendemiale G, Grattagliano I, Altomare E. 1999. An update on the role of free radicals and antioxidant defense in human disease. Inter J Clin Lab Res 29:49-55.
Vitor RF, Mota-Filipe H, Teixeira G, Borges C, Rodrigues AI, Teixeira A, Paulo A. 2004. Flavonoids of an extract of Pterospartum tridentatum showing endothelial protection against oxidative injury. J Ethnopharmacol 93(2-3):363-70.
Youdim KA, McDonald J, Kalt W, Joseph JA. 2002. Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults. J Nutr Biochem 13(5):282-8.

FIGURES

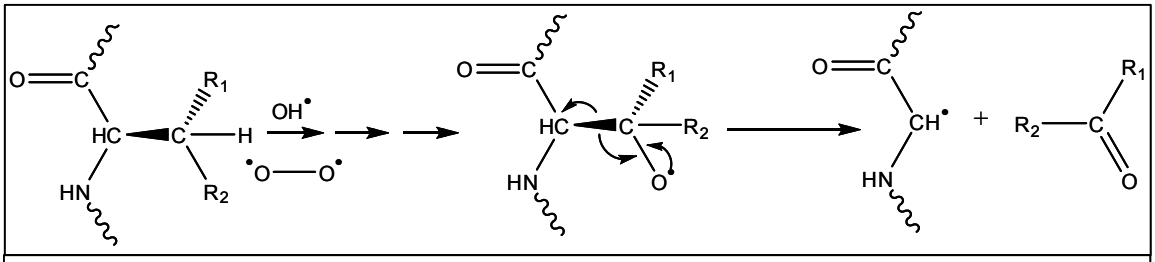

Figure 5.1: Protein carbonyl formation by β-scission

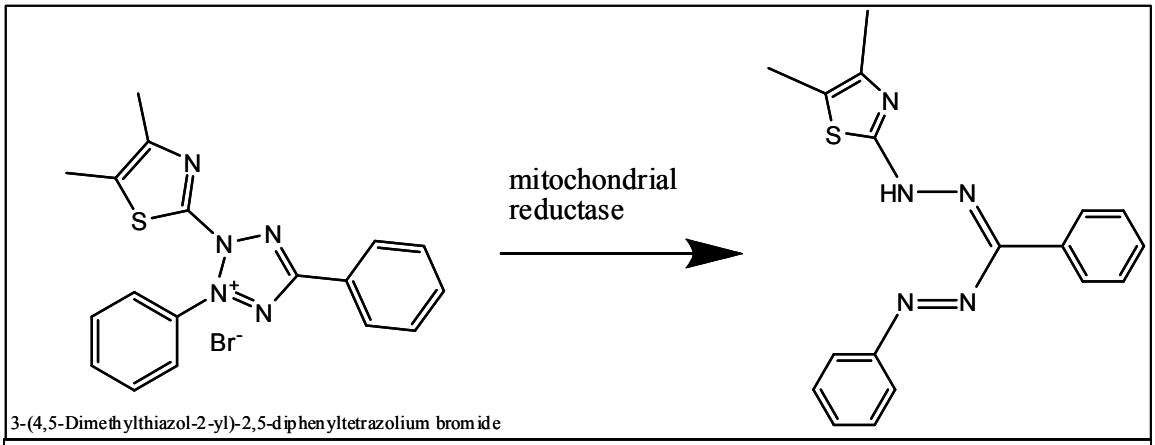

Figure 5.2: MTT reaction schematic.

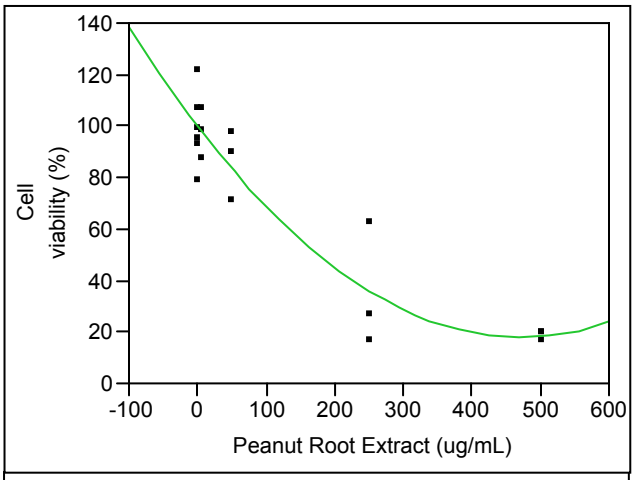

Figure 5.3: Peanut root extract effect on cell viability as measured by the MTT assay (r^2=0.88). HBMEC were incubated with several concentration of peanut root extracts (0.5, 5, 50, 250, and 500 µg/mL) for 20 hours and then the cell viability was measured.

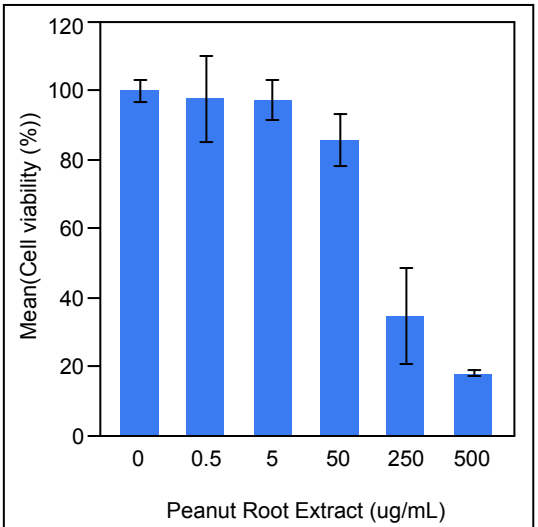

Figure 5.4: Cell viability means for various peanut root extract concentrations. HBMEC were incubated with several concentration of peanut root extracts (0.5, 5, 50, 250, and 500 µg/mL) for 20 hours and then the cell viability was measured by the MTT assay.

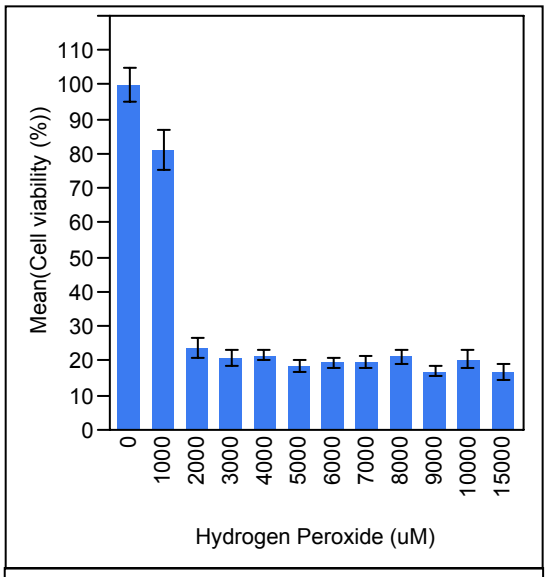

Figure 5.5: Hydrogen peroxide toxicity in HBMEC. Cells were incubated with hydrogen peroxide for 4 hours and the cell viability was measured via the MTT assay

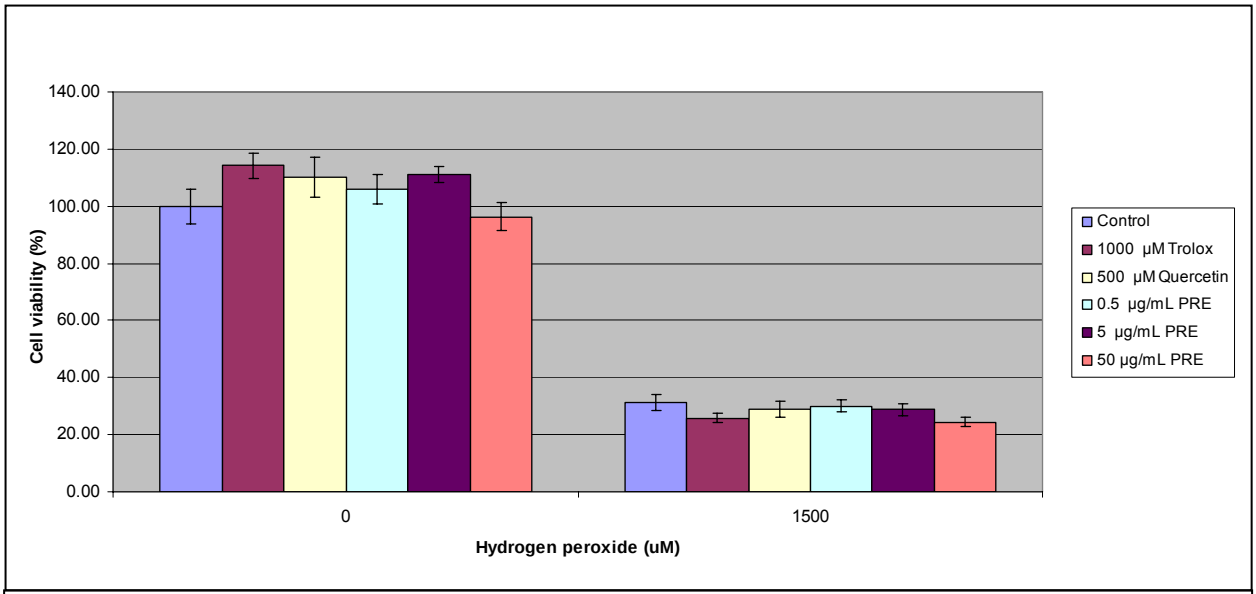

Figure 5.6: Peanut root extract effect on oxidatively challenged endothelial cells compared to Trolox and quercetin. HBMEC were incubated with antioxidants (peanut root extract (PRE), Trolox, or quercetin) for 20 hours. The antioxidants were then removed and the cells subjected to 1500 µM hydrogen peroxide for 4 hours. The cell viability was measured via the MTT assay.

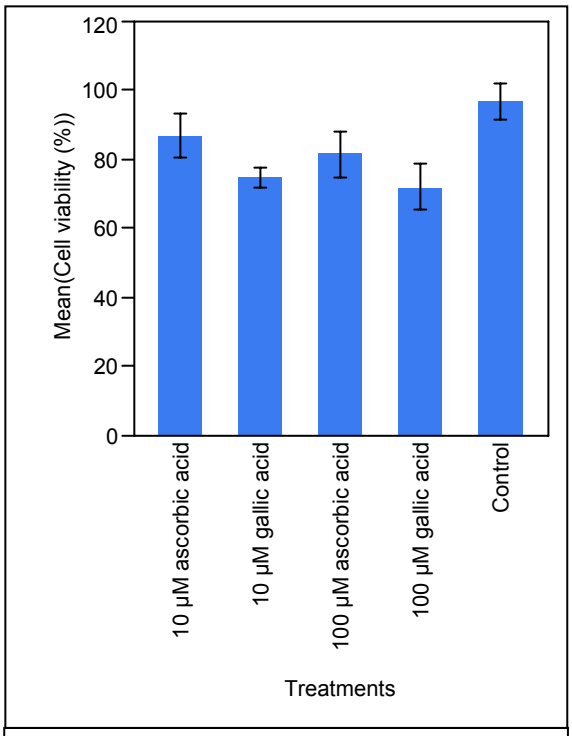

Figure 5.7: Effect of ascorbic acid and gallic acid on HBMEC viability. Cells were incubated with antioxidants for 4 hours and viability measured by MTT assay.

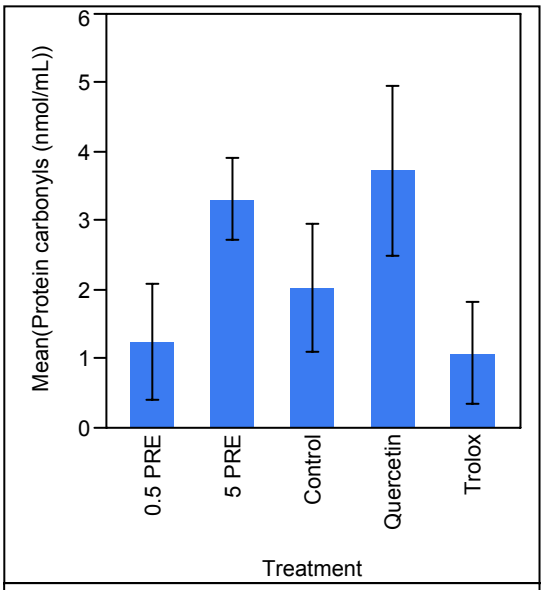

Figure 5.8: Protein carbonyl concentrations in HBMEC exposed to 1500 μM hydrogen peroxide for 4 hours. Cells were incubated with antioxidants for 20 hours prior to hydrogen peroxide treatment. Protein carbonyls were quantified using DNPH.

TABLES

Table 5.1: Antioxidant activity of 70% aqueous ethanol peanut root extracts as measured by ORAC.

Cultivar	**Trolox equivalents (µM/g dry extract)**
Brantley	2261.2
	4515.6
NC-12	600.3
	6564.4
Phillips	1166.0
	1471.4
Wilson	1335.8
	2658.8

CHAPTER 6: EFFECT OF PEANUT ROOT EXTRACT ON OXIDATION OF PEANUT PASTE

Abstract

The criticism of synthetic antioxidants has spawned research in natural antioxidants as replacements. This research investigated peanut root extracts as a possible antioxidant food ingredient to reduce lipid oxidation. Aflatoxin analysis was carried out to ensure consumer safety prior to commercialization.

Peanut root extracts were tested for aflatoxins using an ELISA kit. Both 70% aqueous ethanol and 70% aqueous methanol were tested as possible extraction solvents for aflatoxins. Peanut root extract was added to peanut paste at 200 ppm of GAE and TBHQ was added at 200 ppm. The peanut paste was stored for 14 days at 45°C. Headspace oxygen, nonanal, and hexanal were quantified.

The peanut root extracts were found to have aflatoxin contamination ranging from 30.35 ng/g dry extract to 123.84 ng/g. Ethanol and methanol were both found to be suitable for extracting aflatoxins for analysis. Headspace oxygen, nonanal, and hexanal were not significant by treatment (TBHQ, peanut root extract, control). Headspace oxygen and nonanal were both significant by time.

Keywords: peanut paste, peanut root, antioxidants, oxidation, SPME, headspace oxygen, hexanal, nonanal, aflatoxins

Introduction

The peanut plant (*Arachis hypogaea*) has been extensively studied in recent years as the search for nutraceuticals has intensified. Peanut skins, hulls, and roots have all received much attention. Compounds found in peanut plants include *cis*-resveratrol, *trans*-resveratrol, 4-isopentenylresveratrol, arachidins, medicarpin, *p*-coumaric acid, caffeic acid, ferulic acid, demethylmedicarpin, daidzein, formononetin, isoflavonones, nonyl phenol, and dienols (Keen and Ingham 1976; Aguamah and others 1981; Arora and Strange 1991; Rao and Strange 1995; Sobolev and others 2006). Many of these compounds are phytoalexins, which are produced when the plant experiences environmental stressors. This can be elicited by an actual fungal or microbial infection or in the lab with compounds derived from fungi/microbes such as proteins and polysaccharides (Chung and others 2003; Rowland and others 2005; Medina-Bolivar and others 2007). Phytoalexins can also be induced by UV light, heavy metal salts, physical damage, and other environmental stressors (Barz and others 1990; Arora and Strange 1991; Chung and others 2003).

The roots of the peanut plant are of great interest because they tend to show a higher infection rate than the rest of the plant. Rowland and fellow colleagues (2005) infected three different cultivars (NC-V11, ANorden, and Georgia Green) with tomato spotted wilt virus (TSWV). They found the roots had a higher rate of infection then the leaves. This higher rate of root infection may cause higher concentrations of phytoalexins in the roots then other parts of the peanut plant. Rowland *et al*. also noticed that root infection coincided with a lower photosynthesis rate (up to a 42% decrease), but root infection had a mixed effect on transpiration of different cultivars. ANorden showed an increase in transpiration, but Georgia Green and NC-V11 showed a decrease in transpiration.

The antioxidants currently in widespread food use include 3,5-di(tertiary-butyl)4-hydroxytoluene (BHT), 2-tert-butyl-4-hydroxyanisole (BHA), propyl gallate (PG), *tert*-butylhydroquinone (TBHQ), and α-tocopherol. However, BHT, BHA, TBHQ and PG carry the negative connotations associated with synthetic additives and some evidence of detrimental health effects (Ito and others 1986; Barlow 1990).

Synthetic antioxidants can be added directly to food at a concentration of 200 ppm (in the United States) as specified under Title 21 of the Code of Federal Regulations. The United States allows higher levels of synthetic antioxidants to be added then other countries. Japan does not

allow BHA or TBHQ in its food and Canada and much of Europe do not allow TBHQ (Shahidi 2000). It is estimated that Americans ingest 80% (0.4 mg kg^{-1} body weight) of the acceptable daily intake (ADI) of BHA and 130% (0.39 mg kg^{-1} body weight) of the ADI of BHT (Suh and others 2005).

BHT and BHA have been found to be carcinogenic in some organs and inhibit carcinogenesis in other organs. Ito and colleagues (1986) found BHA induced stomach lesions in rats at 109.6 mg kg^{-1} body weight and up. The rats were followed for up to 104 weeks. The researchers also found that BHA concentration was indirectly correlated with body weight. Some of the higher concentrations of BHA caused cancer in the stomach of the rats. Ito and fellow researchers induced colon cancer in some rats and found that BHT decreased the amount of tumors present. BHA and BHT were found to increase the development of bladder cancer. The 3-*tert* isomer of BHA seemed to be more carcinogenic then 2-*tert*-BHA, this is unfortunate because 3-*tert*-BHA is the more potent antioxidant (Ingold 1961). These results illustrate that BHA and BHT have different effects on different organs and that a high dosage is required to induce these effects over the short term. In monkeys, which are genetically closer to humans than rats, it has been shown that 500 mg kg^{-1} body weight of BHT caused a significant increase in liver size, but BHA at the same level had no ill effects (Branen 1975).

Propyl gallate is used in baked goods and a few other foods. Van Der Heijdan and colleagues (1986) conducted a metastudy and found that propyl gallate has no carcinogenic effects. They propose a 0.2 mg kg^{-1} body weight ADI for propyl gallates and other gallates. Propyl gallate has been found to cause dermatitis to those who have close contact with it. In rats it was found that no effects occurred at or below 1,000 mg kg^{-1} body weight. This dose is quite high when compared to the ADI, but one must be careful when comparing rats and humans. Humans may metabolize these antioxidants differently then rats or other small mammals.

Rosemary extract is being used in the food industry with positive results. Rosemary extract contains phenolics such as carnosol, rosmanol, and carnosic acid (Georgantelis and others 2007). It is typically used at levels up to 1000 ppm, although it is a GRAS flavor extract. Several studies have investigated rosemary extract's effectiveness at retarding oxidation in various products. Estevez and Cava (2006) examined frankfurters and found that rosemary extract was effective at reducing levels of protein and lipid oxidation at 150 ppm, as measured by carbonyl content and TBARS respectively. However at 300 and 600 ppm, rosemary extract

showed pro-oxidant qualities in protein oxidation and no inhibition of lipid oxidation. Another study compared rosemary extract to coffee, green tea, and grape skin extract in cooked pork patties store at 4°C in normal atmosphere for 10 days (Nissen and others 2004). Rosemary was found to be the best natural extract of these at inhibiting lipid oxidation as measured by TBARS. Georgantelis and colleagues (2006) compared rosemary extract to chitosan and α-tocopherol in inhibiting oxidation of beef burgers. Chitosan was the most effective antioxidant and was best when used with rosemary extract. All antioxidant treatments significantly reduced lipid oxidation when compared to the control. α-Tocopherol has found widespread use in foods with high levels of lipids. Many commercially available oils are protected by α-tocopherol. The availability of natural antioxidants is still quite limited with α-tocopherol, ascorbate, and rosemary extract being the most widely used. Both of these antioxidants have their limitations and the search for a better choice continues.

Many foods contain unsaturated fatty acids, which are prone to lipid oxidation. Lipid oxidation leads to off-flavors, loss of nutrition, and loss of shelf-life. It is one of the leading causes of food spoilage. One of the initial products of lipid oxidation is lipid hydroperoxides. Hydroperoxides are odorless and tasteless. These hydroperoxides are unstable and breakdown into a multitude of compounds of various odor characters and thresholds (Nawar 1996). Different fatty acids have a tendency to produce certain oxidation products. Nonanal, octanal, 2-decenal, and heptane are the most common products formed from free radical-induced oxidation of oleic acid. Linoleic will form hexanal, 2,4-decadienal, pentane, and 2-nonenal from free radical-induced oxidation. There are more compounds formed, but those listed are the main byproducts listed from highest to lowest concentrations observed (Przybylski and Eskin 1995).

A GC method that has been gaining in popularity for measuring such compounds in foods is solid phase microextraction (SPME). SPME allows researchers to sample headspace volatiles without the use of solvents with sensitivity (down to parts-per-trillion) and the ability to target classes of compounds (Arthur and Pawliszyn 1990; Zhang and Pawliszyn 1993). Numerous fibers coated with liquid compounds with varying polarities are available for SPME. The fiber used is determined by the targeted compound(s). Jelen and fellow colleagues (2000) investigated which fiber would be ideal for measuring headspace above oxidized rapeseed oil and looked specifically at 14 different byproducts of lipid oxidation. The compounds targeted were *n*-pentane, 2-butanone, *n*-butanol, pentanal, pentanol, hexanal, 2-heptanone, heptanal, 2-heptanal,

1-octen-3-ol, octanal, 2-nonanone, nonanal, and *trans*, *trans*-2,4-decadienal. The following four fibers were tested: 85 μm polyacrylate, 100 μm poly (dimethylsiloxane) (PDMS), carbowax/divinylbenzene, and divinylbenzene/carboxene/PDMS. They concluded that a divinylbenzene/carboxene/PDMS fiber was the optimal fiber for this situation due to the linearity and sensitivity. SPME has been used to measure oxidation products in beef bouillon, infant formula, numerous oils, hazelnuts, peanuts, and walnuts (Mate and others 1996; Steenson and others 2002; Mildner-Szkudlarz and others 2003; Giuffrida and others 2005; Richards and others 2005; Garcia-Llatas and others 2006; Pastorelli and others 2006).

Earth's atmosphere contains approximately 21% oxygen. If the atmosphere of a packaged food is not modified, then the initial headspace oxygen is 21%. However, as oxidative reactions take place, this oxygen is consumed. Measurement of this oxygen can be used as an indicator of degree of oxidation provided the packaging does not allow the transfer of oxygen. Consumption of headspace oxygen can be used to calculate theoretical peroxide values and has been shown to have strong correlations with measured peroxide values and conjugated dienes (Chung and others 2004). In the past, headspace oxygen was measured by puncturing the package and using a gas analyzer or GC, but new instruments allow headspace oxygen to be measured without puncturing the packaging. One such instrument is made by Oxysense®. It measures the time of fluorescence of oxygen-sensitive films placed inside the packaging (Li and others 2008). Jensen and fellow researchers (2004) found that headspace oxygen was a good indicator of susceptibility to oxidation. This research investigated the oxidation of pork scratchings, peanuts, oatmeal, and muesli. Pork scratchings consumed little oxygen and muesli consumed approximately 6% over 23 weeks, but peanuts and oatmeal consumed all headspace oxygen by week 12 of the study. All samples were stored at 27 °C under fluorescent lights during the entire storage period.

Aflatoxins are natural toxins that are most commonly produced by *Aspergillus flavus*, *A. parasiticus*, *A. nomius*. Four different aflatoxins are produced B_1, B_2, G_1, and G_2, so named due to the color of fluorescence given off (B=blue and G=green). B_1 is the most toxic, followed by G_1. Aflatoxins are toxic to liver cells and are highly carcinogenic (Dvorackova 1990). In the United States, the level of aflatoxins must be at 20 ng/g or less in food. Aflatoxins cost the peanut industry $25 million annually in the southeast United States (Lamb and Sternitzke 2001). Aflatoxin contamination is most likely to occur 30 °C due to stress experienced by the plant

making it more vulnerable to attack by fungi (Scheidegger and Payne 2005). Aflatoxins can be found in numerous foodstuffs including medicinal herbs, peanuts, ginger roots, and corn (Gomez-Catalan and others 2005; D'Ovidio and others 2006; Nakai and others 2007; Fu and others 2008).

One of the most important steps in detecting aflatoxins in foodstuffs is ensuring proper sampling techniques. Aflatoxin contamination is often not uniform throughout the product; so many samples should be taken to ensure that an accurate picture of aflatoxin contamination. Aflatoxins are extracted from samples with 70% aqueous methanol with little variation. In one study, the variation in aflatoxin contamination due to sampling in powdered ginger was found to be 87% (Whitaker and others 2009). Due to the fluorescent nature of aflatoxins, TLC and HPLC with a fluorescence detector are often employed. Enzyme-linked immunosorbent assay (ELISA) kits are also quite common and typically easy to use (Gilbert and Vargas 2005; Zheng and others 2005). Some research has looked into using FTIR to detect aflatoxins, but it has not gained widespread use (Mirghani and others 2001). This research investigated peanut root extracts as a possible antioxidant food ingredient to reduce lipid oxidation. Aflatoxin analysis was carried out to ensure consumer safety prior to commercialization.

Materials and Methods

Peanut Root Extraction

Peanut roots were collected from 4 different cultivars (Brantley, NC-12, Phillips, and Wilson) in Virginia and separated into two replications. Roots from 2 plants were combined for pooled sampling. The roots were rinsed with water to remove dirt, dried at 40°C, and stored at -18°C until extraction.

The roots were extracted by adding 100 mL 70% aqueous ethanol to 5 g of peanut root. The peanut root pieces used were the smallest rootlets available. This mixture was then blended for 30 seconds in a Waring Commercial Blender (New Hartford, Conneticut) set on high powered by a Tenma rheostat (Centerville, Ohio) set at 70 volts, allowed to sit for five minutes, and then blended again for one minute at 70 volts. The mixture was then placed in a Fisher FS20 ultrasonic bath (Pittsburgh, PA) for 10 minutes. Following the ultrasonic bath, the suspension was placed in 50 mL plastic centrifuge vials and centrifuged for 30 minutes at 300 x g at room

temperature. The supernatant was then transferred to 10 mL glass centrifuge tubes and centrifuged again for 30 minutes at 300 x g. The supernatant was then frozen and lyophilized.

ORAC

A black 96-well plate was utilized for ORAC. Samples (including blanks and Trolox standards) were run in duplicate. Crude extracts were analyzed at 50 μg/mL in 70% aqueous ethanol. Each well contained 40 μL of sample and 200 μL of 100 mM fluoroscein. The plate was then incubated at 37°C for 20 minutes. After this time, 35 μL of 0.36 M AAPH in warmed 75 mM PBS (pH 7.4) was added and the plate was read with a Victor mulitlabel plate reader (Perkin-Elmer, Turku, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Results were reported in μM TE/mg dry extract for crude extracts.

Total Phenolics Content

A clear 96-well plate was utilized for total phenolics content. Samples (including blanks and gallic acid standards) were run in duplicate. Crude extracts were analyzed at 50 μg/mL in 70% aqueous ethanol. Each well contained 18 μL of sample and 36 μL of 10% aqueous Folin-Ciocalteu (v/v) reagent. After the Folin-Ciocalteu reagent was added, 146 μL 100 mM Na_2CO_3 was added to each well and then the plate was incubated at room temperature in darkness for 2 hours. The absorbance of each well was recorded at 765 nm with a Victor mulitlabel plate reader (Perkin-Elmer, Turku, Finland). Results were reported in mg GAE/g dry extract.

Aflatoxin Analysis

An Agraquant 4-40 ppb total aflatoxin ELISA kit (Romer Labs, Union, MO) was used for aflatoxin analysis. Ground pooled peanut roots (2 g) spiked with 10 ng of mixed aflatoxin were extracted with either 40 mL 70% aqueous ethanol or 40 mL 70% aqueous methanol to determine if ethanol was able to extract aflatoxins. This mixture was vortexed for 2 minutes, filtered with Whatman No. 1 paper, and then used with the ELISA kit. Aflatoxin conjugate (200 μL) was mixed with 100 μL of sample. Then 100 μL of this solution was transferred to an antibody coated well and incubated for 15 minutes. The well was then washed five times with distilled water. Enzyme substrate (100 μL) was then added and incubated for 5 minutes. Then 100 μL of stop solution was added to the well and the absorbance read at 450 nm (Organon Teknika Reader

520, Oss, Netherlands). Aflatoxin standards (Romer Labs, Union, MO) were analyzed from 0.0 to 8 ng/mL. Log concentration was plotted on the x-axis and the logit of the sample absorbance divided by the absorbance of 0 ng/mL aflatoxin standard on the y-axis. Sample aflatoxin contamination was determined by comparing the sample absorbance to the standards. Following confirmation that ethanol was able to extract aflatoxins, 50 mg of lyophilized crude extract was extracted with 250 μL 70% aqueous methanol and centrifuged at 300 x g for 10 minutes at room temperature. The supernatant was then analyzed for aflatoxin contamination.

Peanut Paste

Peanuts were obtained from the Tidewater Area Peanut Research and Education Center. Peanuts were dry roasted in an oven on a metal pan at 177°C until an L-value of 50 was reached. A Minolta CHROMA METER CR-200 (Osaka, Japan) was used to record color. The peanuts were cooled to room temperature and made into peanut paste. The peanuts were ground in a Black & Decker Quick 'N Easy food processor (Towson, MD) until a creamy texture was observed. The peanut paste was then processed through a Speco Model 2.5 colloid mill (Beverly, MA) with a stainless steel grinding wheel twice to ensure a homogenous mixture. TBHQ was added at 200 ppm and peanut root extract was added at 200 ppm of GAE. Both of the treatments were dissolved in 3 mL of absolute ethanol and the same amount of ethanol was added to the control peanut paste. The treatments were mixed well into the peanut paste. Peanut paste was then aliquoted into 10 mL glass headspace vials at 5 g per vial and caps with PTFE/silicone septums were crimped on. Samples were stored at 45°C in darkness. Samples were randomly taken in triplicate at time 0 and at 14 days for each treatment and stored at -18°C until analysis with GC-MS SPME. Headspace oxygen was analyzed at time of sampling.

Headspace Oxygen

An OxySense 4000B (Dallas, TX) was used to measure headspace oxygen. Fluorescent O_2xyDots® were affixed to the interior of headspace vials using translucent adhesive (RTV 108, Momentive Performance Materials, Albany, NY) as suggested by the manufacturer. The headspace oxygen was measured by placing the O_2xyDot® in front of the reader pen, adjusting the gain, and capturing the fluorescence decay. The fluorescent dot was excited by a blue pulse of 470 nm and the fluorescent decay was measured at 600 nm. The instrument was calibrated

using a 0% oxygen standard consisting of a headspace vial of 1% sodium sulfite and a 21% oxygen standard consisting of a headspace vial with 1 mL of distilled water. The instrument measures sample temperature as well and adjusts the headspace oxygen accordingly. Headspace oxygen was used to calculate theoretical peroxide values similarly to the method employed by Chung, Colakoglu, and Min (2004).The headspace oxygen was then used to calculate moles of oxygen using the ideal gas equation and an approximate headspace volume of 7.7 mL. It is assumed that for every mole of oxygen consumed, one mole of peroxide is formed. Theoretical peroxide values are then reported as milliequivalents/kg of oil.

Fatty Acid Analysis

Peanuts were analyzed for fatty acid content prior to the shelf life study. Approximately 12 g of peanuts were blended (Waring Commercial Blendor, New Hartford, Conneticut) with 40 mL methylene chloride for 2 minutes on low. The mixture was then filtered with Whatman No. 4 paper, 3 g sodium sulfate was added, held for 15 minutes, and then filtered again. The solvent was evaporated in a rotary evaporator (Buchi, Rotavapor R-3000, Switzerland) at 40°C. Then 20 mg of this lipid was dissolved in 2 mL iso-octane in a 10 mL glass centrifuge tube with a Teflon-lined cap. To this, 200 μL of 2N KOH in methanol was added, vortexed for 1 minute, and centrifuged at 300 x g at room temperature for 5 minutes. The bottom layer was discarded, and 0.5 mL saturated ammonium acetate (aqueous) was added, vortexed, and centrifuged. The bottom layer was discarded, and aqueous ammonium acetate was added again, vortexed, and centrifuged as before. The bottom layer was discarded, 0.5 g sodium sulfate was added for 5 minutes, and then centrifuged. The supernatant was then removed and 1 μL was direct injected into a Shimadzu GC-17A gas chromatograph (Kyoto, Japan) coupled to a QCMS-QP5050A mass spectrometer with a split ratio of 1:20. A Supelco SP2560 (100 m x 0.25 mm i.d. x 0.25 μm film thickness) column (Bellefonte, PA) was used. The injection temperature was 275 C, interface temperature was 230°C, and the oven was ramped from 200°C to 240°C at 2°C per minute and held for 5 minutes. Helium was used as the carrier gas at 1.4 mL/min. The mass spectrometer was set in scan mode for 50 m/z to 650 m/z and recorded data from 7.72 minutes to 25 minutes at a scan speed of 2000.

SPME GC-MS

A Hewlett Packard 5890A gas chromatograph (Valley Forge, PA) was utilized coupled to a Hewlett Packard 5972 Mass Selective mass spectrometer. A CTC Analytics CombiPal autosampler (Zwingen, Switzerland) was used in conjunction with the GC-MS. An HP-5MS (crosslinked 5% PH ME siloxane) (30 m x 0.25 i.d. x 0.25 μm film thickness) column was used. The injector and interface temperature were 250°C. The oven was heated from 35°C to 120°C at 5°C per minute then heated to 220°C at 20°C per minute and held for 5 minutes. Helium was the carrier gas at 1 mL/min. A Supelco DVB/CAR/PDMS (grey) fiber (Bellefonte, PA) was used for all headspace analysis. The fiber was heated with the sample for 30 minutes at 40°C. The fiber was then desorbed for 12 minutes. The mass spectrometer was operated in scan mode from 50 m/z to 550 m/z. Hexanal (Sigma, St Louis, MO) and nonanal (Acros Organics, New Jersey) standards were analyzed at a volume of 2 mL following the same method as noted above. Sample peaks were matched to standards by retention times and mass chromatograms and quantitated by linear regression.

Statistical Analysis

All statistical analysis was carried out using JMP 7.0 (SAS Institute, Cary, NC). Mean separation by one-way ANOVA was carried out for aflatoxin contamination by cultivar. Standard least squares modeling was used to determine significance of time, treatment, and time*interaction on nonanal and hexanal concentrations. Tukey-Kramer Honestly Significant Difference (HSD) was used to determine which treatments in the factors were significant. Means were considered significantly different at $P<0.05$.

Results and Discussion

Aflatoxin analysis was carried out on spiked root samples to determine whether ethanol was able to extract aflatoxins. Each root sample was spiked with 10 ng of mixed aflatoxin standard. Aqueous methanol extracted 32.89 ng of aflatoxin and aqueous ethanol extracted 36.60 ng of aflatoxin. This finding dictated that all extracts be tested due to the ability of aqueous ethanol to extract aflatoxin at similar levels as aqueous methanol. This result also indicated that the roots had aflatoxin contamination. If the roots that were extracted were contaminated with aflatoxins, then the extracts should also be contaminated with aflatoxins.

Aflatoxin contamination has not been reported in peanut roots before, probably due to the roots not entering the food stream. Aflatoxins have been reported in peanuts. Nakai and fellow researchers (2007) analyzed B_1 and B_2 aflatoxin concentration in peanut kernels and peanut hulls. Aflatoxin contamination was found in 6.7% of hulls in Tupa, Brazil. Kernels had a higher rate and concentration of contamination. Approximately one-third of tested kernels were positive for aflatoxins with a highest reported concentration of 116 ppb of B_1. Although aflatoxin levels have not been reported in peanut roots, they have been reported for other roots that humans have historically ingested. Ginger roots have been examined from US grocery stores and found to be free of aflatoxins (D'Ovidio and others 2006). The authors did find one moldy ginger root in a grocery store and found that it contained 16 ng/g, which is below the 20 ppb legal limit. The easiest way to eliminate aflatoxin contamination is by avoiding mold growth conditions.

Aqueous methanol and aqueous acetonitrile have both been reported as extraction solvents for aflatoxins (Gomez-Catalan and others 2005; Fu and others 2008). A review of the literature reveals a preference for aqueous methanol when extracting samples to test for aflatoxin contamination (Ribeiro and others 2006; Essono and others 2009; Whitaker and others 2009). There is a lack of information regarding the ability of aqueous ethanol to extract ethanol. Aqueous ethanol has been examined as a possible detoxification method in peanut and cottonseed meal (Rayner and others 1970). The authors used 85°C ethanol and found that it removed between 96% and 98% of the aflatoxin contamination in peanut meal. This study supports the finding that ethanol extracts most of the aflatoxins in a sample. Further confirmation is needed, but it seems likely that aqueous ethanol offers a less toxic option when sampling for aflatoxin contamination.

Aflatoxin contamination was found in the peanut root extracts (table 6.1) at relatively high levels, far above the limit of 20 ppb. Aflatoxin contamination was not significant for cultivar ($P=0.89$). These high levels of aflatoxin contamination may be due to storage of the whole peanut plant prior to washing and drying the roots, but the storage history of this set of roots is unknown.. In barley rootlets, *Aspergillus flavus* produced the most aflatoxins at a water activity of 0.95 (Ribeiro and others 2006). Prior to washing and drying, the roots would have had a high water activity and would have been prone to fungal growth. If peanut root extracts

are used in a commercial application, then lots will have to be screened for aflatoxins and measures put in place to reduce potential of aflatoxin contamination.

The ORAC activity was measured in the crude extracts (table 6.2). Two cultivars' extracts (Brantley and Wilson) were mixed together in order to have enough dried extract for the shelf life study. These extracts were chosen based on their higher ORAC activity. The Folin-Ciocalteu assay was used to analyze the total phenolic content of this mixed extract. The mixed extract contained approximately 49.3 mg gallic acid equivalents/g dry extract of phenolics. This was important when determining the amount of extract to add by gallic acid equivalents.

Extracts of peanut plant parts have been investigated as a food ingredient to prevent oxidation and subsequent spoilage of food. Chen and associates (2002) added peanut root powders (0.6 g) to pork patties (60 g) and then cooked. After the patties cooled, the oil was stored at 60°C and checked for conjugated dienes periodically. The oil from the patties cooked with peanut root powders was less prone to oxidation then the control oil. Another similar study investigated peanut skin extract effects in ground beef (O'Keefe and Wang 2006). O'Keefe and Wang found that peanut skin extract significantly reduced oxidation in ground meat (as measured by TBARS) with no ill effects to microbial growth, texture, aroma, and other sensory attributes. They found that 200 and 400 ppm of peanut skin extracts were effective with little additional effects seen in higher levels. Nepote, Mestrallet, and Grosso (2004) investigated the antioxidant effects of peanut skin extracts in honey-roasted peanuts. They measured peroxide and thiobarbituric acid reactive substance (TBARS) values to gauge the level of oxidation and utilized a sensory panel. Peanut skin extracts inhibited lipid oxidation, but not as well as BHT. The sensory panel determined the extracts did not change the acceptance level of the peanuts. The results from this experiment did not show an antioxidant effect from peanut root extract.

The fatty acid revealed an oleic fatty acid content of 55% and a linoleic content of 28%. This fatty acid profile indicates normal oleic peanuts and also suggests which lipid oxidation products are expected (Frankel 1998; Isleib and others 2006). Hexanal concentrations increased from 0.014 ppm at day 0 to 0.015 ppm at day 15. Hexanal concentration was not significant by time ($P=0.36$), by treatment ($P=0.1$), or by the interaction of time by treatment ($P=0.1$) by standard least squares modeling. If there was significant oxidation, then hexanal would be an expected byproduct as linoleic acid would oxidize more readily then oleic acid producing hexanal as the main compound. This was not the case. Lee and fellow investigators (2002)

analyzed the ability of whey protein to reduce peanut oxidation as a coating. The area under the hexanal increased significantly over time for control peanuts in this experiment. The peanuts were stored at 60°C and the hexanal peak area increased many times over. It is possible that the peanut paste was not stored at a high enough temperature to induce oxidation in the short period of time used in this study. Another possibility is that hexanal is not released at the same rate from peanut paste as peanuts during exposure of the SPME fiber due to lower surface area and differing partition coefficients. Another scenario is the peanut paste requires mixing while the SPME fiber is exposed to the headspace to facilitate the release of hexanal. Abegaz, Kerr, and Koehler (2004) found hexanal concentrations of over 200 ppm for peanut pastes samples without TBHQ stored at 21°C at time 4 weeks. Some of the peanut pastes samples treated with TBHQ were over 200 ppm hexanal as well at this time point. Without agitation, very little of the hexanal may be released from the peanut paste surface and thus leading to little change in the hexanal concentration over time. Grosso and colleagues (2008) investigated hexanal changes in cracker-coated peanuts and roasted peanuts. Normal oleic peanuts were stored at several different temperatures (-19°C, 23°C, 30°C, and 40°C) and some were also flushed with nitrogen. There was very little change in hexanal concentration until after 50 days of storage. Grosso *et al*. found that sensory data was a much better predictor of shelf life then headspace hexanal concentration.

Nonanal increased from a mean of 0.016 ppm at day 0 to 0.027 ppm at day 14. Nonanal was significant by time ($P<0.0001$) and by treatment ($P=0.04$), but was not significant for the interaction of time and treatment ($p=0.08$) by standard least squares modeling. Tukey-Kramer HSD separated the TBHQ treatment as significantly different from peanut root extract at day 14 with a mean of 0.023 ppm nonanal. This increase is not practically significant. Nonanal is a major product of the oxidation of oleic acid (Przybylski and Eskin 1995). Warner and fellow investigators (1996) analyzed the headspace volatiles of normal oleic peanuts stored for 68 days at 65°C. They found both hexanal and nonanal to increase significantly over time. Hexanal increased from 32.1 ng/mL of headspace to 91.3 ng/mL of headspace. In the same amount of time, nonanal increased from 1.4 ng/mL to 5.8 ng/mL. This result is expected because linoleic acid is much more prone to oxidation and would produce hexanal at a quicker rate then oleic acid breaking down and forming nonanal. The same forces at work are seen in an experiment that measured headspace volatiles in peanut oil heated to several different temperatures (50 to 200°C)

(Chung and others 1994). The peak area for hexanal increased with increasing temperatures as did nonanal. Once again though the hexanal peak area increased at a larger rate then the nonanal; this is to be expected from normal oleic peanut products.

Chung, Colakoglu, and Min (2004) found that theoretical peroxide values calculated from headspace oxygen allowed the calculation of decomposed peroxides if actual peroxide values are measured. This leads to a better estimation of the extent of oxidation experienced by a product. Jensen and fellow researchers (2004) also found this to be the case when they investigated pork scratchings, peanuts, oatmeal, and muesli. Headspace oxygen over time was used to determine which products were more oxidized. Peanuts had consumed all of the headspace oxygen in 11 weeks, while oatmeal took only 8 weeks at 27°C. Headspace oxygen decreased from 21% at day 0 to a mean of 14% at day 14. Headspace oxygen was significantly different by time ($P<0.0001$), but was not significantly different by treatment ($P=0.81$) or by the interaction of treatment and time ($P=0.39$) as determined by standard least squares modeling. Theoretical peroxide values were not significant by treatments ($P=0.89$) by one-way ANOVA (figure 6.1). These theoretical peroxide values fall near expected values. Mugendi and fellow researchers (1998) stored normal and high oleic peanuts at 40°C and found peroxide values for normal oleic peanuts averaging near 7 milliequivalents/kg after two weeks of storage. High oleic peanuts had very little accumulation of peroxides after two weeks. Abegaz, Kerr, and Koehler (2004) found similar results when they tested the effect of moisture, sugar, and TBHQ on peroxide values of peanut paste. By four weeks, the first time point, the peanut pastes had reached a peroxide value of greater then 10 meq/kg except for the TBHQ samples and the samples with 5 g of water per 100 g peanut paste, which had barely perceptible peroxide values. All of the samples were stored at 21°C. These findings contrast with the level of theoretical peroxide values calculated for the TBHQ treatment assuming a linear growth of peroxide values from the initial measurement to the 4 week measurement. The addition of ethanol may have affected the rate of oxidation of peanut butter or the ability of TBHQ to inhibit oxidation. Reed and colleagues (2002) studied the effect water activity had on storage of normal and high oleic peanuts. They measured peroxide values after 3 weeks of storage at 25°C. The normal oleic peanuts had peroxide values of 5 meq/kg and 10 meq/kg while both cultivars of high oleic peanuts exhibited lower concentrations of peroxide values. The normal oleic peanuts with a lower water activity

(0.19) experienced more lipid oxidation then normal oleic peanuts with higher water activity (0.6).

Peanut root extract will be contaminated with aflatoxin if the peanut roots used for extraction are contaminated. Peanut root extract and TBHQ did not significantly affect the concentration of hexanal or nonanal present in the headspace of peanut paste stored for 14 days at 45°C. Headspace oxygen did significantly decrease over time. Peanut paste may be a relevant model system for lipid oxidation, but may need increased storage time in order to see measurable lipid oxidation.

Literature Cited

Abegaz EG, Kerr WL, Koehler PE. 2004. The role of moisture in flavor changes of model peanut confections during storage. Lebensmittel-Wissenschaft und -Technologie 37(2):215-25.

Aguamah G, Langcake P, Leworthy D, Page J, Pryce R, Strange R. 1981. Two novel stilbene phytoalexins from *Arachis hypogaea*. Phytochemistry 20(6):1381-3.

Arora MK, Strange RN. 1991. Phytoalexin accumulation in groundnuts in response to wounding. Plant Sci 78(2):157-63.

Arthur CL, Pawliszyn J. 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal Chem 62(19):2145-8.

Barlow SM. 1990. Toxicological aspects of antioxidants used as food additives. In: Hudson BJF, editor. Food Antioxidants. Essex, England: Elsevier Science Publishing. p 253-307.

Barz W, Bless W, Boerger-Papendorf G, Gunia W, Mackenbrock U, Meier D, Otto C, Sueper E. Phytoalexins as part of induced defense reactions in plants: their elicitation, function and metabolism; 1990. p 140-56.

Branen AL. 1975. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. J Am Oil Chem Soc 52:59-63.

Chen R, Wu P, Chiou R. 2002. Peanut roots as a source of resveratrol. J Agric Food Chem 50:1665-7.

Chung HJ, Colakoglu AS, Min DB. 2004. Relationships among headspace oxygen, peroxide value, and conjugated diene content of soybean oil oxidation. J Food Sci 69(2):FCT83-FCT8.

Chung I-M, Park MR, Chun JC, Yun SJ. 2003. Resveratrol accumulation and resveratrol synthase gene expression in response to abiotic stresses and hormones in peanut plants. Plant Sci 164(1):103-9.

Chung TY, Eiserich JP, Shibamoto T. 1994. Volatile compounds produced from peanut oil heated with different amounts of cysteine. J Agric Food Chem 42(8):1743-6.

D'Ovidio K, Trucksess M, Weaver C, Horn E, McIntosh M, Bean G. 2006. Aflatoxins in ginseng roots. Food Addit Contam 23(2):174-80.

Dvorackova I. 1990. Aflatoxins and Human Health. Boca Raton, FL: CRC Press.

Essono G, Ayodele M, Akoa A, Foko J, Filtenborg O, Olembo S. 2009. Aflatoxin-producing Aspergillus spp. and aflatoxin levels in stored cassava chips as affected by processing practices. Food Control 20(7):648-54.

Estevez M, Cava R. 2006. Effectiveness of rosemary essential oil as an inhibitor of lipid and protein oxidation: Contradictory effects in different types of frankfurters. Meat Sci 72:348-55.

Frankel EN. 1998. Lipid Oxidation. Davis, CA: The Oily Press.

Fu Z, Huang X, Min S. 2008. Rapid determination of aflatoxins in corn and peanuts. J Chromatogr, A 1209(1-2):271-4.

Garcia-Llatas G, Lagarda MJ, Romero F, Abellan P, Farre R. 2006. A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: Application to liquid infant foods and powdered infant formulas. Food Chem 101(3):1078-86.

Georgantelis D, Ambrosiadis I, Katikou P, Blekas G, Georgakis SA. 2007. Effect of rosemary extract, chitosan and a-tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4 DegC. Meat Sci 76(1):172-81.

Georgantelis D, Blekas G, Katikou P, Ambrosiadis I, Fletouris DJ. 2006. Effect of rosemary extract, chitosan, alpha -tocopherol on lipid oxidation and colour stability during frozen storage of beef burgers. Meat Sci 75(2):256-64.
Gilbert J, Vargas E. 2005. Advances in sampling and analysis for aflatoxins in food and animal feed. In: Abbas H, editor. Aflatoxins and Food Safety. Boca Raton: CRC Press. p 237-68.
Giuffrida F, Golay P-A, Destaillats F, Hug B, Dionisi F. 2005. Accurate determination of hexanal in beef bouillons by headspace solid-phase microextraction gas-chromatography mass-spectrometry. Eur J Lipid Sci Technol 107(11):792-8.
Gomez-Catalan J, Pique E, Falco G, Borrego N, Rodamilans M, Llobet JM. 2005. Determination of aflatoxins in medicinal herbs by HPLC. An efficient method for routine analysis. Phytochem Anal 16(3):196-204.
Grosso NR, Resurreccion Anna VA, Walker GM, Chinnan MS. 2008. Sensory profiles and hexanal content of cracker-coated and roasted peanuts stored under different temperatures. J Food Process Preserv 32:1-23.
Ingold KU. 1961. Inhibition of the autoxidation of organic substances in the liquid phase. Chem Rev 61:563-89.
Isleib TG, Pattee HE, Sanders TH, Hendrix KW, Dean LO. 2006. Compositional and sensory comparisons between normal- and high-oleic peanuts. J Agric Food Chem 54(5):1759-63.
Ito N, Hirose M, Fukushima S, Tsuda H, Shirai T, Tatematsu M. 1986. Studies on antioxidants: Their carcinogenic and modifying effects on chemical carcinogenesis. Fd Chem Toxic 24(10/11):1071-82.
Jelen HH, Obuchowska M, Zawirska-Wojtasiak R, Wasowicz E. 2000. Headspace Solid-Phase Microextraction Use for the Characterization of Volatile Compounds in Vegetable Oils of Different Sensory Quality. Journal of Agricultural and Food Chemistry 48(6):2360-7.
Jensen PN, Danielsen B, Bertelsen G, Skibsted LH, Andersen ML. 2004. Storage stabilities of pork scratchings, peanuts, oatmeal and muesli: Comparison of ESR spectroscopy, headspace-GC and sensory evaluation for detection of oxidation in dry foods. Food Chem 91(1):25-38.
Keen NT, Ingham JL. 1976. New stilbene phytoalexins from American cultivars of Arachis hypogaea. Phytochemistry 15(11):1794-5.
Lamb MC, Sternitzke DA. 2001. Cost of aflatoxin to the farmer, buying point, and sheller segments of the southeast United States peanut industry. Peanut Sci 28:59-63.
Lee SY, Trezza TA, Guinard JX, Krochta JM. 2002. Whey-protein-coated peanuts assessed by sensory evaluation and static headspace gas chromatography. J Food Sci 67(3):1212-8.
Li H, Ashcraft DK, Freeman BD, Stewart ME, Jank MK, Clark TR. 2008. Non-invasive headspace measurement for characterizing oxygen-scavenging in polymers. Polymer 49(21):4541-5.
Mate JI, Saltveit ME, Krochta JM. 1996. Peanut and walnut rancidity: effects of oxygen concentration and relative humidity. J Food Sci 61(2):465-8, 72.
Medina-Bolivar F, Condori J, Rimando A, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan M. 2007. Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992-2003.
Mildner-Szkudlarz S, Jelen HH, Zawirska-Wojtasiak R, Wasowicz E. 2003. Application of headspace-solid phase microextraction and multivariate analysis for plant oils differentiation. Food Chemistry 83(4):515-22.

Mirghani MES, Man YBC, Jinap S, Baharin BS, Bakar J. 2001. A new method for determining aflatoxins in groundnut and groundnut cake using Fourier transform infrared spectroscopy with attenuated total reflectance. J Am Oil Chem Soc 78(10):985-92.
Mugendi JB, Sims CA, Gorbet DW, O'Keefe SF. 1998. Flavor stability of high-oleic peanuts stored at low humidity. J Am Oil Chem Soc 75(1):21-5.
Nakai VK, Rocha LdO, Goncalez E, Fonseca H, Ortega EMM, Correa B. 2007. Distribution of fungi and aflatoxins in a stored peanut variety. Food Chem 106(1):285-90.
Nawar W. 1996. Lipids. In: Fennema O, editor. Food Chemistry. 3rd ed. New York: Marcel Dekker, Inc.
Nepote V, Mestrallet MG, Grosso NR. 2004. Natural antioxidant effect from peanut skins in honey-roasted peanuts. J Food Sci 69(7):295-300.
Nissen LR, Byrne DV, Bertelsen G, Skibsted LH. 2004. The antioxidative activity of plant extracts in cooked pork patties as evaluated by descriptive sensory profiling and chemical analysis. Meat Sci 68:485-95.
O'Keefe SF, Wang H. 2006. Effects of peanut skin extract on quality and storage stability of beef products. Meat Sci 73:278-86.
Pastorelli S, Valzacchi S, Rodriguez A, Simoneau C. 2006. Solid-phase microextraction method for the determination of hexanal in hazelnuts as an indicator of the interaction of active packaging materials with food aroma compounds. Food Addit Contam 23(11):1236-41.
Przybylski R, Eskin NAM. 1995. Methods to measure volatile compounds and the flavor significance of volatile compounds. In: Warner K, Eskin NAM, editors. Methods to assess quality and stability of oils and fat-containing foods. Champaign, IL: AOCS Press. p 107-33.
Rao PVS, Strange R. 1995. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In: Daniel M, Purkayastha RP, editors. Handbook of phytoalexin metabolism and action. New York: Marcel Dekker, Inc. p 199-227.
Rayner ET, Dollear FG, Codifer LP. 1970. Extraction of aflatoxins from cottonseed and peanut meals with ethanol. J Am Oil Chem Soc 47:26.
Reed KA, Sims CA, Gorbet DW, O'Keefe SF. 2002. Storage water activity affects flavor fade in high and normal oleic peanuts. Food Res Int 35(8):769-74.
Ribeiro JMM, Cavaglieri LR, Fraga ME, Direito GM, Dalcero AM, Rosa CAR. 2006. Influence of water activity, temperature and time on mycotoxins production on barley rootlets. Lett Appl Microbiol 42(2):179-84.
Richards A, Wijesundera C, Salisbury P. 2005. Evaluation of oxidative stability of canola oils by headspace analysis. J Am Oil Chem Soc 82(12):869-74.
Rowland D, Dorner J, Sorensen R, Beasley JP, Jr., Todd J. 2005. Tomato spotted wilt virus in peanut tissue types and physiological effects related to disease incidence and severity. Plant Pathol 54(4):431-40.
Scheidegger K, Payne GA. 2005. Unlocking the secrets behind secondary metabolism: A review of *Aspergillus flavus* from pathogenicity to functional genomics. In: Abbas H, editor. Aflatoxin and Food Safety. Boca Raton: CRC Press. p 137-66.
Shahidi F. 2000. Antioxidants in food and food antioxidants. Nahrung 44(3):158-63.
Sobolev V, Horn B, Potter T, Deyrup ST, Gloer JB. 2006. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. J Agric Food Chem 54:3505-11.
Steenson DF, Lee JH, Min DB. 2002. Solid phase microextraction of volatile soybean oil and corn oil compounds. J Food Sci 67(1):71-6.

Suh HJ, Chung MS, Cho YH, Kim JW, Kim DH, Han KW, Kim CJ. 2005. Estimated daily intakes of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butyl hydroquinone (TBHQ) antioxidants in Korea. Food Addit Contam 22(12):1176-88.

van Der Heijden CA, Janssen PJCM, Strik JJTWA. 1986. Toxicology of gallates: A review and evaluation. Fd Chem Toxic 24(10/11):1067-70.

Warner KJH, Dimick PS, Ziegler GR, Mumma RO, Hollender R. 1996. 'Flavor-fade' and off-flavors in ground roasted peanuts as related to selected pyrazines and aldehydes. J Food Sci 61(2):469-72.

Whitaker TB, Trucksess MW, Weaver CM, Slate A. 2009. Sampling and analytical variability associated with the determination of aflatoxins and ochratoxin A in bulk lots of powdered ginger marketed in 1-lb bags. Anal Bioanal Chem:No pp yet given.

Zhang Z, Pawliszyn J. 1993. Headspace solid-phase microextraction. Anal Chem 65(14):1843-52.

Zheng Z, Humphrey CW, King RS, Richard JL. 2005. Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. Mycopathologia 159:255-63.

FIGURES

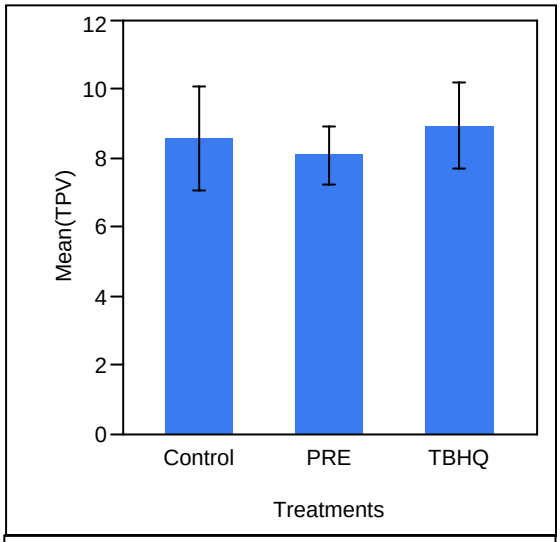

Figure 6.1: Theoretical peroxide values reported milliequivalents of peroxide per kilogram oil. Theoretical peroxide values were determined using headspace oxygen data and calculating moles of oxygen consumed. Error bars are standard error.

TABLES

Table 6.1: Aflatoxin contamination in 70% aqueous ethanol peanut root extracts by cultivar. Aflatoxin contamination was quantified by ELISA and compared to a commercial standard.

Cultivar	Aflatoxin (ng/g dry extract)
Brantley	30.35
	101.56
NC-12	31.31
	123.84
Phillips	44.93
	40.13
Wilson	38.97
	92.22

Table 6.2: Antioxidant activity of 70% aqueous ethanol peanut root extracts as measured by ORAC. Values followed by * were mixed together for a mixed extract for shelf-life testing.

Cultivar	**Trolox equivalents (µM/g dry extract)**
Brantley	2261.2*
	4515.6*
NC-12	600.3
	6564.4
Phillips	1166.0
	1471.4
Wilson	1335.8*
	2658.8*

CHAPTER 7: CONCLUSION

Peanut roots have not been investigated in this scope before this research. Peanut roots were extracted with 70% aqueous ethanol and tested in two model systems, peanut paste and HBMEC. Peanut root extracts were fractionated with HPLC with a C18 column. These fractions were then analyzed for ORAC activity and total phenolic content. The effect of cultivar was not significant for the ORAC activities of the crude extracts. This finding can easily be explained by plant to plant variation being greater then cultivar variation. Each plant experiences different levels of stress depending on location, disease prevalence, insect prevalence, drought stress, etc. Stress leads to phytoalexin production and many of these phytoalexins are antioxidants. However, cultivar was a significant factor for ORAC activity of the fractions. This finding is most likely caused by differing concentrations of individual antioxidant compounds among cultivars with some possible synergistic effects occurring in the crude extracts. The fractions had significantly different ORAC activities and total phenolic contents based on time. These findings are not surprising since one would assume that different fractions would have different antioxidant activities and phenolic contents based on the compounds eluting during the collection window.

The extract was tested in two model systems: peanut paste and HBMEC. The peanut paste treatments were peanut root extract (200 ppm of GAE) and TBHQ (200 ppm). The peanut paste was stored for 14 days at 45°C. Headspace oxygen, nonanal, and hexanal were not significant by treatment (TBHQ, peanut root extract, control). Headspace oxygen and nonanal were both significant by time. Nonanal increased from 0.02 ppm to 0.03 ppm. Although this result was statistically significant, it is not of practical importance. Headspace oxygen did experience a significant drop from 21% oxygen to 14% oxygen. Based on the hexanal and nonanal data, it does not appear that a measurable amount of oxidation has occurred in the short time frame used in this experiment. However, the headspace oxygen data would disagree with this conclusion. If the sample did oxidize, then the SPME procedure needs to be investigated further. The peanut paste sample was not stirred during exposure of the SPME fiber. This may lead to low concentrations of volatiles in the headspace and would account for the low levels that were seen. It is possible that the sample did not oxidize in the short period of time. The question then becomes: where did the headspace oxygen go? One possibility is the sample absorbed some of the headspace oxygen. The sample was warm when it was put into the headspace vial and as

it cooled, it could have absorbed oxygen. This model system needs to be further investigated, but does have potential as an effective model system based on the ease of use.

The aflatoxin contamination of peanut root extract is of great concern. This contamination may have been due to the time period between harvest and drying the roots. Much of this contamination can be prevented through proper handling techniques and removing infected roots from the extract material.

Peanut root extracts were incubated with human brain microvascular endothelial cells (HBMEC) at several concentrations (0.5, 5, 50, 250, and 500 μg/mL) for 20 hours and then hydrogen peroxide was added at 1500 μM for 4 hours. Trolox and quercetin were also used as antioxidant treatments due to their antioxidant effects as noted in the cell culture literature. Cell viability and protein carbonyl concentration were determined. Peanut root extract concentration significantly affected cell viability ($P<0.0001$), and concentrations above 250 μg/mL were found to be toxic. Peanut root extracts, quercetin, and Trolox did not exhibit a protective antioxidant effect for HBMEC challenged by hydrogen peroxide. Trolox and quercetin did not exhibit any antioxidant effects during the cell culture portion of the research. This model system can be improved by determining if there is a concentration of quercetin and Trolox that exhibit protective effects against oxidation. This will be advantageous when comparing natural antioxidants to known antioxidants.

Peanut root extracts do have high antioxidant activities as elucidated by ORAC. However they fail to exhibit any antioxidant activity in the model systems tested. Unfortunately, the known antioxidants tested in the model systems failed to inhibit oxidation as well. Further research is needed to optimize the extraction and identify more compounds. Peanut roots may be a viable option for production of certain phytoxalexins. Peanut root extracts are not a viable commercial food ingredient or health supplement at this time, but further research is necessary to confirm if peanut root extract will remain a nonviable option.