ABSTRACT

Title of Document: Promotion of Value-Added Uses of Soybeans

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Soybeans contain a wealth of health-promoting phytochemicals, the amounts of which are known to vary considerably across genotypes and growing conditions. The present work is dedicated to better understand these variations and to improve the methods for investigating soy phytochemicals. To address the first of these concerns, low-linolenic soybeans and colored seed coat soybeans were investigated for their health beneficial components, as well as their chemical and biological properties. A study of low-linolenic soybeans showed their similar antioxidant activity, total phenolics, and isoflavones to normal fatty acid soybeans. The potential for a different trend in tocopherols and lutein was observed between low-linolenic and regular fatty acid soybeans, but the limited study size prevented a definitive conclusion. This work was the basis for further studies on low-linolenic soybeans not included in this report.

Subsequently, a study of eighteen soybeans of brown, green, yellow and black seed coat colors concluded that black seed coat soybeans had significantly higher scavenging activity against hydroxyl, peroxyl, and $ABTS^{+}$ radicals. Black seed coat soybeans also contained higher total phenolic contents and isoflavones than the other colored soybeans, and were the only color to contain the anthocyanin cyanidin-3-glucoside. However, when soybean extracts were tested for their ability to prevent colon cancer cell proliferation, seed coat color and chemical composition were not necessarily predictive of an extracts' bioactivity.

Lastly, a new analytical method was developed and validated for quantification of phytosterols, tocopherols and carotenoids—three separate classes of soy lipophilic phytochemicals—in a single extraction and HPLC run. A ternary, isocratic solvent system of acetonitrile, methanol and water (48:22.5:29.5, v/v/v) was used to achieve separation on a phenyl column. Evaporative light scattering detection (ELSD) was used to quantify β-sitosterol, stigmasterol, campesterol, α-, δ- and γ- tocopherols, while lutein was quantified with visible light absorption at 450 nm. This method offers a more efficient alternative to separate, individual methods for quantifying lutein, tocopherols and sterols in soybeans.

PROMOTION OF VALUE-ADDED USES OF SOYBEANS

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

In love and thanks to my parents, Daniel and Helen Smitka.

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List of Abbreviations

AAPH	2,2'-azinobis (2-amidinopropane) dihydrochloride
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)
ANOVA	Analysis of variance
ATBC	Alpha-tocopherol Beta-Carotene Cancer Prevention Study
ATP	Adenosine triphosphate
AUC	Area under the curve
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BP	Boiling point
CARET	Beta-Carotene and Retinol Efficacy Trial
CHAOS	Cambridge Heart Antioxidant Study
Cy-3-glc	Cyanidin-3-glucoside
DAD	Diode array detector
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescin diacetate
DMPO	5,5-dimethyl-1-pyrroline
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector
ESI	Electron spray ionization
ESR	Electron spin resonance
EtOH	Ethanol
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FC	Folin-Ciocalteu reagent
FDA	(United States) Food and Drug Administration
FID	Flame ionization detector
FL	Fluorescein
FRAP	Ferric-reducing antioxidant power
GAE	Gallic acid equivalents
GC	Gas chromatography
GSH	Glutathione
GSSG	Oxidized (dithiol) glutathione
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HOSC	Hydroxyl radical scavenging capacity
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
IPA	Isopropyl alcohol
LC	Liquid chromatography
LDL	Low density lipoprotein

MeOH	Methanol
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NP-HPLC	Normal phase high performance liquid chromatography
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical
·OH	Hydroxyl radical
ORAC	Oxygen Radical Absorance Capacity
·OOR	Peroxyl radical
PDA	Photodiode Array (detector)
PDCAAS	Protein digestibility corrected amino acid score
PPAR	Peroxisome proliferator-activated receptor
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
RS	Reactive species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
RP-HPLC	Reversed phase high performance liquid chromatography
SE	Seed flour equivalent
TAC	Total Anthocyanin Contents
TBARS	Thiobarbituric acid-reactive substances
TBHQ	Tertiary-butyl hydroquinone
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total Flavonoid Contents
TOC	Total Organic Contents
TPC	Total Phenolic Contents
USDA	United States Department of Agriculture
UV/Vis	Ultraviolet / Visible light

Introduction

A large body of research has been dedicated to studying the health impact of soybean consumption. Broad evidence suggests that soy consumption may have a beneficial effect in preventing the development of many chronic diseases of aging, including cardiovascular diseases, certain cancers, and osteoporosis. Meanwhile, evidence to suggest an effect in other disease states is inconclusive, including obesity, diabetes, and immunological and cognitive diseases. While many factors may impact the results of clinical trials and epidemiological studies, one factor that merits additional attention is the phytochemical composition of the soybeans being consumed.

There are over 20,000 known genotypes of soybeans in the United States Department of Agriculture Soybean Germplasm Collection, and far more around the world. Additionally, phytochemical composition of soybeans is known to vary widely across genotypes and according to different growing, post-harvest, storage and food processing conditions. Therefore, it is prudent to study the phytochemical composition of a variety of soybeans for at least two reasons: 1) to better equip future clinical trials and epidemiological studies to understand the phytochemical composition of soybeans consumed by their subjects; and 2) to select soybeans particularly high in known health beneficial phytochemicals for development of functional foods for the general public.

The overarching goal of this research is to promote the production and consumption of soybeans that are high in health beneficial components. The specific objectives by which this work approaches that goal are to:

- Assess how breeding to lower linolenic acid may affect antioxidants and nutrients.
- Assess colored seed coat soybeans for their potential as health-promoting functional food ingredients.
- Develop an improved HPLC method for studying the composition of soy lipophilic components, to allow for faster, more economical screening of soybeans for phytochemicals in the future.

Chapter 1: Literature Review

1.1 Overview of soybean market

Soybeans [*Glycine max* (L.) Merrill] play an important role in the world's food supply and economy, thus earning the nickname "gold from the soil" from Dr. C. V. Piper of the U.S. Bureau of Plant Industry in the early 1900s (Dies, 1943). Having been grown for millennia in China and its neighboring countries, soybeans made their debut in the United States in the late 1700s, though they only began their climb to current production levels in the early 1920s (Liu, 1997). In 1955, the United States became the world's leading producer of soybeans and has remained so for over half a century, though Brazil is narrowing the gap (Liu, 1997). World production of soybeans in 2007 was 220 million metric tons, with the U. S. responsible for a third of that production at a value of nearly $15 billion, followed by Brazil, Argentina, China and India (FAO, 2009). Soybeans fall third behind rice and wheat in world crop production, and are second to corn in U. S. crop production (FAO, 2009). Approximately 40% of the soybeans grown in the United States are exported (American Soybean Association, 2009).

Soybean use varies by locality. Human consumption in Asian countries tends to include the whole bean, or foods produced from the whole bean, while American consumers are often unaware of their soy consumption, as its components tend to have roles behind the scenes as cooking oils, emulsifiers, bulking agents, and so forth. Just over two-thirds of the edible oil consumed in the United States in 2008 was soybean oil, while the vast majority of the meal left after oil extraction is used for animal feed, primarily chickens and pigs (American Soybean Association, 2009).

In recent years, the price of soybeans has been volatile, as additional factors beyond traditional supply and demand forces have increasingly influenced their value. Increased biofuel production, increased energy prices and depreciation of the U. S. dollar are a few of the factors pinpointed in contributing to the fluctuations (Trostle, 2008).

At the same time, demand for soyfoods has also increased in the U.S.— soyfood sales increased significantly after the 1999 decision by the U. S. Food and Drug Administration allowing soyfood labels to display a health claim connecting soy protein to reduced risk of heart disease. Currently, approximately one-third of Americans are now seeking out and consuming soy food products at least once a month (United Soybean Board, 2009). Additionally, the United Soybean Board reports that consumers' restaurant purchases of soyfoods increased in 2009, with one-quarter of consumers indicating interest but claiming soyfoods are not available in restaurants (United Soybean Board, 2009).

Several research groups have shown that an increase in various types of nutritional knowledge relating to a food increases the person's likelihood to consume it, or vice versa (Wansink et. al, 2005; Ares et. al, 2008; Krystallis et. al, 2008). Though the decision-making process is certainly more complicated than this simple correlation, consumers presumably are seeking soy in greater numbers at least in part because of a growing awareness of its links to health promotion and disease prevention.

Farmers have the potential to benefit from growing cultivars with unique or higher health benefits because they receive monetary incentives for growing specific

or high-demand cultivars. Purveyors were offering premiums of $0.55-0.60 per bushel in the 2009 growing season (soybeanpremiums.org). Identification of soybean genotypes high in health beneficial components, in combination with education of consumers about the health benefits, may serve to create consumer demand for these products and therefore increase premiums to farmers.

1.2 Types of Soybeans

With the numerous uses for soybeans, a plethora of soybean cultivars have been bred or genetically engineered to best suit each use. Soybeans grown for U. S. markets often have higher oil content. Genotypes are chosen to balance the value of the oil with the value of the protein in the meal. Also, soybeans are being bred to add more desirable traits in the meal: low phytate soybeans are being researched to create a more efficient animal feed. Also, low lipoxygenase genotypes are under research for their potential in having less of the undesirable flavor that is typically associated with soy in western culture, in addition to possibly being more stable (Iassonova et. al, 2009; Torres-Penaranda et. al, 1998).

Oil composition has also been manipulated for a variety of reasons. One of particular interest involves reducing the levels of linolenic acid (18:3) to below 3% and increasing oleic (18:1) to 45-60%, thus providing for an oil with greater oxidative stability without the need to hydrogenate and introduce heart disease provoking trans fats to the oil (Wilson, 2004). For optimal health promotion, these low linolenic, high oleic acids would ideally also be low in saturated fats. However, yield becomes a concern when too many fatty acid traits are bred into one line. Low linolenic soybeans will be discussed in more detail in a later section.

For production of tofu, soymilk and more traditional soyfoods, cultivars higher in protein, lower in oil, and higher in seed grade are more appropriate (Liu, 1997). For soybeans eaten as vegetables (or 'edamame') when pods are still immature, the desirable traits include large seed size and tenderness.

Beyond the proximate composition, soybeans also differ in shape from round to a more traditional bean-like shape. However, the morphological features of the seeds are similar across all soybeans: an outer seed coat protects an inner embryo. The embryo is typically ~90% of the seed weight and consists of the radicle (which becomes the primary root upon germination), the hypocotyl (lifts the growing embryo above the surface), epicotyl (the main stem), and the cotyledons (food reserves). The cotyledon, again comprising about 90% of the seed, contains most of the oil and protein. The hard seed coat is composed of mostly cellulosic mass with a small amount of protein.

Finally, soybeans differ by what is called a maturity group. Since soybeans are photoperiod sensitive, they transition from the vegetative stage of growth to the flowering stage of growth in accordance with shortening daylight hours later in summer. However, different cultivars require different numbers of dark hours to begin flowering. Thus, the system of maturity groups was established to correlate the light cycles of certain latitudes with soybeans best acclimated for growth there. Maturity group 00 in Canada represents the soybeans that do well with pronounced changes in the light cycle, whereas maturity group X (Roman numeral 10) spans the Panama region and represents soybeans that do well with less pronounced changes in the light cycle. Maryland falls between the two, primarily in the maturity group IV

territory, with the far edges of the state allotted to groups III and V. Selection of an appropriate maturity group for the growing conditions ultimately allows for better yield, timed appropriately before the frost (Liu, 1997).

1.3 Structure and Nutritional Compostion of Soybeans

Soybeans are well renowned for their nutritional value, particularly their amount and quality of vegetable protein. Though much variation exists, the typical soybean is said to contain approximately 40% protein, 20% oil, 35% carbohydrates and 5% ash, based on dry weight (Snyder and Kwon, 1987). Ranges of protein and oil content of genotypes in the USDA Soybean Germplasm Collection have been reported to be 34 to 57% and 8 to 28%, respectively (Wilson, 2004).

Fatty acid composition is largely controlled by genotype, and again, much variation exists. In general, however, linoleic acid (18:2) is the predominating fatty acid in typical soybean oil, with contents in the neighborhood of 50%, followed by just above 20% oleic (18:1), 10-11% palmitic (16:0), 7-10% linolenic (18:3), and around 4% stearic (18:0) (Liu, 1997; USDA nutrient database # 04044; Wilson, 2004).

Soy carbohydrates are generally less valuable than their oil and protein counterparts. Consequently, significantly less research is devoted to them in the literature. Their composition is usually expressed as soluble and insoluble. The soluble carbohydrates make up about 10% of the total bean weight, half of which is sucrose and the other half the oligosaccharides, which are renowned for their flatulence-producing properties (Liu, 1997). Starch, though present in the immature soybean, declines to near zero amounts in the mature bean (Wilson, 2004).

1.4 Bioactive Phytochemical Compounds in Soy

1.4.1 Antioxidants

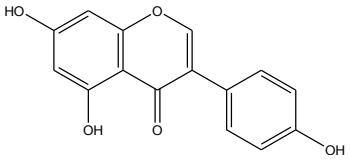
1.4.1.1 Isoflavones

Isoflavones are a class of polyphenolics found almost exclusively in legumes, and most prominently in soybeans. The isoflavone ring structure is the same as that of a flavone, except the B-ring of the isoflavone is attached to C-ring at position 3 instead of position 2. This difference results in isoflavones having structural similarities to that of estradiol, which allows isoflavones to weakly bond to estrogen receptors, eliciting weak estrogenic responses, and earning the title of 'phytoestrogen' (Cederroth and Nef, 2009). Their health effects are discussed below in subsequent sections.

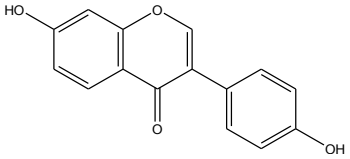
Twelve isoflavone isomers exist in soybeans, with three base molecules in the 'free' aglycone form (genistein, daidzein and glycitein) and three derivatives of each aglycone: β-, malonyl-, and acetyl-glucoside derivatives. The predominating form in soybeans is the malonyl-glucoside derivative, but these are not chemically stable and are largely converted during heat processing to the acetyl- or β-glucosides (Wilson, 2004; Liu, 1997). When fermentation is used as part of soy processing (such as in production of traditional products like tempeh), isoflavones are converted largely to their aglycone form (Liu, 1997).

The isoflavones are found in the hypocotyl and the cotyledon of the soybean seed. The hypocotyl contains concentrations of isoflavones 5-6 times higher than the cotyledon, yet the cotyledon still contains the vast majority of isoflavones in the

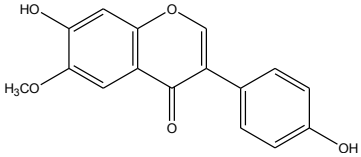
soybean due to its relative size (Liu, 1997). The seed coat contains minimal isoflavones.



Genistein = 4',5,7-trihydroxyisoflavone



Daidzein = 4',7-dihydroxyisoflavone



Glycitein = 4'7-dihydroxy-6-methoxyisoflavone

Figure 1.1 Soy isoflavone structures

The prominent factors known to affect the isoflavone content of soybeans are genotype, environment, and a combination of the two (Eldridge and Kwolek, 1983; Hoeck et al, 2000). Many factors of the growing environment have been identified as potential reasons for variation in isoflavone level, including temperature during seed fill, planting date, and precipitation (Tsukamoto et al, 1995; Rasolohery et al, 2008).

The type of analysis used may also affect the isoflavones detected. The extraction procedure used has the potential to greatly affect isoflavones extracted and therefore detected. Solvents used for the extraction of isoflavones have included

various percentages of acetone, water, methanol, ethanol, acetonitrile, and acids (Xu et al., 2007; Lin and Giusti, 2005). No clear optimum extraction solvent has emerged despite a variety of researchers attempting to answer the question. The differences in polarity of the isoflavone glucoside derivatives prevent any one solvent from being optimal for extracting all forms. Also, the instability of the malonyl-glucoside derivative may lead to structural changes during extraction, particularly when an acidified solvent or heat is used (Lin and Giusti, 2005). Ultimately, these differences highlight the need to 1) select the best solvent and extraction procedure based on available information, 2) treat all samples that are to be compared with the selected protocol, and 3) carefully scrutinize any comparisons of samples extracted with different protocols. Despite differences in procedures, it has become generally accepted that soybeans contain up to 3 mg isoflavone/g soybean dry weight (Liu, 1997).

1.4.1.2 Anthocyanins

The chemical ring structure of anthocyanins is similar to the flavones, except there is no carbonyl group on the C-ring. Varying substitution of hydrogens for hydroxyl and methoxy groups on the 3' and 5' carbons produces the anthocyanidins commonly found in nature: pelargonidin, cyanidin, peonidin, delphinidin, petrunidin, and malvidin (Lee et al, 2005a). The term anthocyanin refers to the glycoside forms, while anthocyanidin refers to the aglycone structures. This family of chemicals is well-known for their pigmentation of deep reds and blues. Not surprisingly then, only soybeans with black seed coat soybeans have anthocyanins, while both brown and black have anthocyanidins (Todd and Vodkin, 1993).

1.4.1.3 Other Phenolics

Though they receive less attention than isoflavones and anthocyanins, other phenolics associated with health benefits have also been detected in soy. Over 10 phenolic acids were identified in defatted soybean flakes by Seo and Morr (1984), with syringic, ferulic, and sinapic acids contributing just over 200 μg/g each, with lesser amounts of isoferulic, salicylic, para-coumaric, ortho-coumaric, vanillic, caffeic, para-hydroxybenzoic, and gentistic acids. The total amount of phenolic acids in defatted soy flakes was 1.1 mg/g.

1.4.1.4 Tocopherols

Tocopherols are members of the Vitamin E family. All four isomers, α, β, δ, and γ, have been detected in soybeans and their oil (Liu, 1997), though only three (α, δ, and γ) are in appreciable amounts. γ-tocopherol represents the largest constituent of the three, at about 50-80% of total tocopherols (Liu, 1997; Wilson, 2004; Britz et al, 2008). Total amounts are in the range of 1-2 g/kg refined oil. As with isoflavones, genotype and environment have been shown to affect the content and composition of tocopherols in soybeans (Britz et al, 2008; Wilson, 2004).

With a hydroxyl group on a chroman ring, tocopherols act as antioxidants by scavenging peroxyl radicals that would cause lipid peroxidation. Thus, their presence adds to the stability of the 18:2 and 18:3 fatty acids present in soybean oils. Cultivars low in 18:3 have been sought as a way to increase the oxidative stability of oils, though low levels of γ-tocopherol may also result in soybeans that have a genetic

predisposition for low 18:3 (Wilson, 2004). However, other results show that some low-18:3 lines did not experience a decrease in total tocopherols, and those that did have less tocopherols had proportionately less of all three isomers (McCord et al, 2004). Additional evidence that tocopherol contents are related to fatty acid composition in soybeans comes from data indicating an inverse relationship between tocopherol content and palmitic acid (Scherder et al, 2006).

1.4.1.5 Carotenoids

Carotenoids are antioxidant, lipid-soluble, pigment molecules found in soybeans. They are tetraterpenoids and exist in two classes: carotenes are purely hydrocarbons and xanthophylls contain one or more oxygen atoms. Their highly conjugated structure leaves them extremely vulnerable to oxidation in the presence of light, heat, and oxygen. Three carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) are considered precursors to Vitamin A. Though detectable, soybeans are generally not considered to be a good source of these provitamins—lutein is considered the major carotenoid component in soybeans (Lee et al, 2009b).

1.4.2 Sterols

Plant sterols are found in significant quantities in soybean oils, in the range of 200-300 mg/100 g commercial oil. Soybean oil sterols exist mostly in the non-esterified form (Phillips et al, 2002), with β-sitosterol predominating (Wilson, 2004). Stigmasterol and campesterol have also been detected in lesser quantities (Warner & Mounts, 1990). Plant sterols have a base structure very similar to that of human cholesterol. Additionally, the 3-hydroxy group can found esterified to a fatty acid or

hydroxycinnamic acid, or it can be glycosylated with a hexose. The esterified sterols have been shown to reduce serum cholesterol levels when consumed in sufficient quantities. More on this topic is available in the cardiovascular disease section below.

1.4.3 Lean Protein

Soy protein is considered to be the only plant-based nutritionally complete protein with soy protein concentrate having a protein digestibility corrected amino acid score (PDCAAS) near 1.0, the highest rating possible (Liu, 1997). Soy protein isolate values are lower (around 0.9), which is still considered a high quality protein. The primary limiting amino acid is methionine followed by cysteine, both sulfur-containing (Liu, 1997).

Its high biological value, in combination with it being lower in saturated fat and cholesterol, highlights soy protein as a healthy alternative to meat. Interestingly, studies have suggested there may be additional benefits to soy protein beyond these basic nutritional qualities. In 1999, the U.S. Food and Drug Administration allowed for a health claim connecting soy protein intake to reduced risk for coronary heart disease (Federal Register 64 FR 57699). More on this topic will be discussed in the cardiovascular disease section below.

1.5 Health Benefits of Soy Consumption

Consumption of soy foods has been recognized by various cultures as health beneficial, if not medicinal, long before scientific evidence was available to support or refute these claims. Recently, scientific attention has turned to associate soy with

reduced risk for diseases of aging (and lifestyle) such as cardiovascular disease, cancer, osteoporosis and others.

1.5.1 Cardiovascular Disease

The meta-analysis of 29 controlled clinical trials by Anderson et al. (1995) played a pivotal role in identifying the benefits of soy consumption on serum lipid levels. Results showed that an average consumption of 47 g soy protein/day resulted in significant decreases of total cholesterol, LDL cholesterol and triglycerides by 9.3, 12.9, and 10.5%, respectively. Though previous studies had pointed to the relationship between soy and lipid levels, Anderson's paper was the first serious review to highlight these results. The work later became an important piece of evidence in the U.S. FDA's decision to approve a statement linking soy protein to reduced risk for coronary heart disease as a health claim meeting significant scientific agreement (Federal Register 64 FR 57699).

A more recent review by the American Heart Association (AHA) Nutrition Committee found only a 3% decrease in LDL with soy protein consumption from 8 randomized trials providing approximately 50 g soy protein/day, and no apparent benefit in 14 other trials (Sacks et al, 2006). They deemed the LDL decrease to be a small benefit in relation to the sizeable consumption of soy necessary to achieve it. The differing results between the Anderson meta-analysis and the AHA review may be explained by the extent of hyperlipidemia in patients. The meta-analysis of Anderson et al. (1995) included earlier studies with strongly hyperlipidemic patients (total cholesterol > 250 mg/dL). These subjects with more extreme hyperlipidemia benefited from greater percentage reductions in total and LDL cholesterol than those

with moderate or minor hyperlipidemia. As research progressed, it became compulsory to pharmaceutically reduce cholesterol levels in these strongly hyperlipidemic patients. Therefore, later studies may not see as strong of cholesterol-lowering benefits, because the population it most helped no longer exists.

Beyond cholesterol levels, epidemiological studies have long linked soy consumption to a reduced risk for coronary heart disease and cardiovascular disease (Sacks et al, 2006—discussing Seven Countries Study by Ansel Keys). The early research in this area compared soy consumption between Asians and Westerners, but review articles back up the findings with multiple articles associating soy protein, vegetable protein, and/or legume intake with reduced risk of cardiovascular disease in varied populations (Sirtori et al, 2009; Flight and Clifton, 2006).

The association between soy intake and reduced cardiovascular risk may be purely related to our understanding of 'classical' nutrition: soy is low in saturated fats, high in PUFAs, cholesterol-free, and high in vitamins, minerals and fiber. For an individual reducing consumption of animal protein (typically higher in saturated fat and cholesterol, and lower in PUFAs), it follows classical nutrition that his/her serum cholesterol levels would decrease, and overall health would be promoted. Also, intake of soy protein may be indicative of an overall healthier diet and/or lifestyle. Still, 11 studies controlled for saturated fat and cholesterol while studying the effects of soy, and cholesterol and saturated fat were found to not modulate the effect of soy on LDL overall (Sacks et al, 2006).

Attempts have been made to understand the mechanism behind reduction in lipid levels and in the overall occurrence of cardiovascular disease in soy eaters. One

proposed mechanism of risk reduction involves the inhibition of low density lipoprotein (LDL) oxidation, which theoretically would reduce the formation of plaque in the arteries. Soybean extracts (Takahashi et al. 2005; Astadi et al., 2009) and peptides (Rho et al., 2007) have both been shown to reduce the oxidation of LDL cholesterol *in vitro* (Astadi et al, 2009; Takahashi et al, 2005) or in rats (Rho et al, 2007), but black soybeans have been shown to have greater inhibitory effect against lipid peroxidation in human LDL than yellow ones (Takahashi et al., 2005). Along these lines, anthocyanins found in black seed coat soybeans have been shown to independently inhibit LDL oxidation and display anti-inflammatory properties (Kong et al, 2003; Valachovicova et al., 2004).

A more recent suggestion aimed at elucidating the mechanism behind the reduction of lipid levels in soy eaters involves the liver's LDL receptors, which may be affected upon soy consumption to enhance their uptake of LDL from the serum and consequently lower serum LDL levels (van Horn et al., 2008). A very recent review by Sirtori et al. (2009) implicates soy proteins, in particular the 7S globulin, as at least part of the active component responsible for soy's lipid-lowering effects. It has also been suggested that isoflavones may act on vascular tissue to improve bloodflow (Ghosh and Scheepens, 2009).

Finally, plant sterols found in soy have been linked to the ability to reduce serum cholesterol levels associated with increased risk of cardiovascular disease. The dose established for maximum efficacy is approximately 2 g of esterified plant sterols per day (Lichtenstein and Deckelbaum, 2001). It is inconceivable that a person could consume enough soybean oil to achieve this level of consumption, even

if the sterols in oil were all in the esterified form. Thus, sterols isolated from soybeans may serve as a nutraceutical ingredient, but unmodified soybean oil is not itself a functional food with regard to sterol content. Interestingly, soybeans with lowered 18:3 contents had differences in the individual sterols (increased stigmasterol and decreased β-sitosterol), though the total levels did not seem to change (Wilson, 2004).

1.5.2 Cancer

Health beneficial factors in soy have also shown potential in reducing the risk of certain types of cancer (MacDonald et al., 2005; Nagata et al., 2007; Messina et al., 2006). Breast and prostate cancers are the most heavily researched, primarily because of their potential for sensitivity to estrogen.

The relationship between consumption of soyfoods (and/or isoflavones) and breast cancer remains controversial despite intensive research in this area. While limited animal and cell studies have suggested a potential for isoflavones to increase estrogen-sensitive breast cancer risk, some reviews allay these concerns by citing a vast number of human clinical and epidemiological studies that have not yet found a danger (Messina and Wood, 2008; Messina and Wu, 2009). Others contend that though the anticarcinogenic potential is promising, the matter of safety still needs to be resolved (Mense et al., 2008; Velentzis et al., 2008). Factors that currently prevent a more thorough understanding of soy's effects on breast cancer include the typical obstacles in clinical studies, but unique difficulties inherent to soy include ethnicities of the populations studied, the amount and type of isoflavones in the particular soy

food/supplement administered, the equol-producing status of the individual subjects, and incompletely understood age/gender differences in metabolism and use of isoflavones. (Equol production is further discussed in Section 1.10.1.) Though details remain to be elucidated, some research suggests that isoflavone consumption during adolescence may be protective against breast cancer in adulthood (Messina et al., 2009b).

There is more agreement that soy may not only reduce the risk of prostate cancer (Messina, 2003), it is also being studied in clinical trials for the treatment of prostate cancer (Banerjee et al., 2008). More limited studies point to possible benefits in colon.

Mechanisms of cancer prevention by isoflavones have also been the focus of intense research, and results suggest that multiple methods of action are responsible for their bioactivity. Isoflavones have been shown to affect the cell cycle, apoptosis, differentiation, proliferation, growth, along with a variety of effects on cell signaling (Mense et al., 2008; Banerjee et al., 2008). Of particular interest to this research is the mechanisms by which they act as antioxidants. They are well-known as scavengers of reactive oxygen species, but recent research is suggestive of additional antioxidant activity beyond direct scavenging of radicals. Genistein in particular has been shown to activate transcription factors and stimulate gene expression (Banerjee et al, 2008), indicating that it may have the ability to impact overall cellular oxidative status through a variety of mechanisms.

Genistein has also been found to decrease the growth of various types of cancer, colorectal cancer included (Kim et al., 2005). In one study, genistein dose-

dependently slowed the proliferation of HT-29 human colon cancer cells and induced cell death through apoptosis and inhibition of DNA synthesis (Kim et al., 2005). Genistein has also been found to exhibit anti-proliferative effects on Caco-2 human colon cancer cells and pancreatic cancer cells (Kim et al., 2005; Chen and Donovan, 2004). Additionally, anthocyanins found in black seed coats of soybeans have also been shown to inhibit cancer cell growth *in vitro* (Kim et al., 2008).

1.5.3 Symptoms of Menopause

The structure of the isoflavone aglycones is similar to that of 17β-estradiol, giving them the ability to weakly invoke both estrogenic and anti-estrogenic responses *in vivo*. Epidemiological studies have indicated that Asian postmenopausal women experience less bone loss and report fewer hot flashes than their Western counterparts. The high dietary intake of isoflavone-containing soyfoods in Asian cultures is suggested as a possible explanation for this difference. Thus, isoflavones have been researched as an alternative to hormone replacement therapy (HRT) in menopausal and post-menopausal women to alleviate hot flashes and reduce menopausal bone loss.

Evidence of an effect on bone density remains controversial since the initial studies were performed in the mid-1990's, with the same problems associated with soy-cancer studies plaguing soy-menopause research: inadequate attention to equol producer status and difficulty comparing studies due to considerable differences in isoflavone form and dose (Vatanparast and Chilibeck, 2007). However, a recent 3-year clinical intervention providing 54 mg/d isolated genistein increased bone mineral

densities by 8-9%, whereas control densities decreased by about 10% (Messina et al., 2009b)—this is by far the most successful intervention in this field of study and is being investigated further. Also, two prospective studies—the Singapore Chinese Health Study and the Shanghai Women's Health Study—both showed a dose-dependent relationship connecting higher soy/isoflavone intake to lower risk of bone fractures, at least in women (Messina et al., 2009b). Review articles have yet to include these data in their meta-analyses.

Hot flashes seem to also moderately benefit from soy/isoflavone intake. Results are mixed, as usual, though a critical review of the literature in 2009 suggested that there may be a dose relationship, in that all studies using 15 mg genistein or greater resulted in a modest decrease in hot flash frequency (Messina et al, 2009b). A 2006 review by Howes and colleagues came to a similar conclusion.

Further research is necessary to assess the safety of isoflavone supplementation. Isoflavones are antioxidants and are therefore prone to exude pro-oxidant effects in an excessive dose. However, additional caution needs to be exercised relative to their estrogenic effects, particularly in regard to their unquantified risk of developing breast cancer and other estrogen-sensitive cancers. However, a major dietary supplement brand in the United States is already marketing dietary supplements with genistein to promote bone health.

1.5.4 Obesity

A newer and less mainstream area of soy research involves its potential to mitigate obesity and/or the complications that arise because of it (Orgaard and Jensen, 2008; Azadbakht and Esmaillzadeh, 2008). Cellular mechanism studies have

suggested the estrogenic-like activity of isoflavones may play a role in regulating adipogenesis by binding to estrogen receptors, thus decreasing lipoprotein lipase activity, though there also appears to be some relationship with peroxisome proliferator-activated receptors (PPAR) (Orgaard and Jensen, 2008). Levels of genistein in the 0.1 to 1.0 μM range have been shown to inhibit adipogenesis, while higher concentrations (25-50 μM) enhanced adipogenesis, showing a biphasic effect (Orgaard and Jensen, 2008), which is notably similar to their antioxidant/proxidant activities. Because of its biphasic nature, caution has been suggested in drawing conclusions about genistein's effect on obesity, particularly when different organs and species may react to differing degrees (Dang, 2009). Results with rodent studies are generally in agreement with *in vitro* results, whereas human studies are less conclusive. Many human clinical experiments do not show a decrease in body weight as would be expected from animal trials, but improvements in blood lipids seem to be mitigated (Orgaard and Jensen, 2008) or improvements in insulin resistance may be observed (Bhathena and Velasquez, 2002). As with soy's potential effects on breast cancer prevention, recent research suggests that soy consumption early in life may impact levels of the adipose-produced hormone leptin later in life (Maskarinec et al, 2009; Dang, 2009).

1.5.5 Other Chronic Diseases

Soy's connection to a variety of other diseases has been studied, though not to the same depth as those discussed above. Diabetes metrics, cognitive function and immune function are among the list of conditions said to potentially improve with

consumption of soy or its components (Cederroth and Nef, 2009; Bhathena and Velasquez, 2002; Lee et al., 2005b; Ryan-Borchers et al., 2006). As with the other diseases discussed above, it remains unclear whether it is isoflavones, proteins, fiber, or some other component causing the supposed beneficial effects. Finally, because of their generally low levels in soy, carotenoids have not been directly implicated as contributing to its health promoting qualities. However, it should be noted that some carotenoids (including lutein, the primary carotenoid in soy) potentially possess beneficial properties for ocular health (Krinsky and Johnson, 2005) and antioxidant activity (Krinsky, 1989).

1.6 Reactive Species, Oxidative Stress, and Health

Reactive species (RS) include both reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS include peroxyl ($\cdot OOR$), hydroxyl ($\cdot OH$), and superoxide ($O_2^{\cdot -}$) radicals, hypochlorite (^-OCl), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), while RNS include peroxynitrite (^-ONOO), nitric oxide ($\cdot NO$) and nitrogen dioxide ($\cdot NO_2$). The term free radical is often used, meaning a chemical species with an unpaired electron; however, reactive species has a broader context and encompasses species like peroxides and singlet oxygen, which are also considerably reactive and potentially detrimental to biological molecules, though not technically free radicals.

RS are generated *in vivo* through both intentional and unintentional mechanisms. An example of intentional generation of RS can be seen in the phagocyte, in which non-self stimuli elicit the respiratory burst—a blast of superoxide

radicals capable of killing invading organisms (Valko et al, 2007). Peroxisomes represent another example where RS are used to an organism's advantage—they produce hydrogen peroxide and use it as an oxidizing agent in a variety of metabolic actions (Valko et al, 2007). Intentional mechanisms like these are normally tightly controlled, but have the potential to release RS acutely or chronically when control mechanisms fail.

Superoxide is considered the primary unintentionally-generated RS, and arises mainly as a result of the electron leakage onto molecular oxygen from the electron transport chain in mitochondria (Valko et al, 2007). Secondary RS are then generated from superoxide. For example, superoxide causes the release of free iron under stress conditions, allowing for the generation of hydroxyl radicals from hydrogen peroxide (Valko et al 2007). The metabolism of certain drugs along with ionizing and ultraviolet radiation also produce RS (Winterbourn, 2008; Valko et al, 2007).

The human body possesses an endogenous antioxidant system to keep RS species in check and maintain the body's redox balance (Fridovich, 1998). The enzymes superoxide dismutase, catalase, and glutathione peroxidase play a key role in detoxifying superoxide, hydrogen peroxide, and other lipid hydroperoxides. Co-factors for these enzymes, including selenium, Coenzyme $Q_{10,}$ and glutathione, help in maintaining the cellular redox environment. Glutathione, a thiol-containing tripeptide, also participates by directly scavenging $\cdot OH$ and $^1O_2$ and participating in the regeneration of Vitamins C and E from their oxidized forms (Valko et al, 2007). Finally, some exogenous compounds have fairly-well-understood antioxidant effects

on the redox environment *in vivo,* including Vitamins C and E (ascorbic acid and α-tocopherol).

Simplistically, oxidative stress occurs when one of two things happens: an overproduction of RS overwhelms the endogenous antioxidant system or the endogenous antioxidant system becomes deficient and is unable to maintain 'normal' levels (Halliwell and Whiteman, 2004). When the redox balance tips in favor of oxidative stress, the excess RS cause oxidative damage to DNA, RNA, proteins, lipids and other tissue components. However, some amount of oxidative damage is inevitable regardless of redox status. Hydroxyl radicals are one of the most reactive species known and will react with almost any molecule they contact, making a protection system against them unfeasible and a repair system essential (Winterbourn, 2008).

It is hypothesized that the immune-promoting response of RS was enhanced through natural selection and evolution (Halliwell, 2009). Only since health care has lengthened our life spans well beyond the reproductive years do we recognize the negative consequences of this evolutionary trait—the constant low-level excess of RS damages biomolecules, which at least participates in the pathologies we view as chronic diseases of aging, including cancer, cardiovascular disease, neurodegenerative disease, arthritis, and others. While oxidative stress has been described as 'serious imbalance between RS production and antioxidant defenses,' which may lead to potential damage (Halliwell and Whiteman, 2004), this gives no clear rubric to define a 'serious imbalance' or what level of damage may accompany it. Another definition describes oxidative stress as "the harmful effect of free radicals

causing potential biological damage" (Valko et al, 2007). This perspective gives credence to the idea that damage is the key issue at hand. Finally, a last opinion suggests that oxidative stress is "a disruption of redox signaling and control," this definition focusing on the controlling mechanisms (Jones, 2006).

1.7 Antioxidants and Health

Chemically speaking, an antioxidant would be a chemical species capable of preventing oxidation (and therefore damage) of some molecule. Biologically speaking, however, an antioxidant of this definition is only *relevant* if it functions as such in the intended biological system *and* prevents damage to the intended molecules.

Antioxidants can come in a great many different forms and still fit this definition. As discussed above, the body utilizes an 'antioxidant defense system' (Halliwell et al, 1995) to protect itself from the damaging effects of RS, consisting of enzymes, their co-factors and direct radical scavengers. The antioxidants of most interest to aid in disease prevention are those from exogenous sources, because their amounts and administration can be easily modified. Chemically-produced antioxidants (butylated hydroxytoluene [BHT] and butylated hydroxyanisole [BHA]) used to extend shelf-life of foods and other products are intentionally left out of this discussion due to lack of consumer acceptance. Consumers view antioxidants from natural sources to be safer than their chemically-produced counterparts, though this may not actually be true. The structure of many antioxidant compounds (natural or synthetic) allows them to act as both anti- and pro-oxidant (Halliwell, 2000). This

dual nature of so-called antioxidants has raised many questions in the scientific community, including the safety of consumption of antioxidants, of natural or synthetic origin. Results have been seen in *in vitro* testing as well as in several large clinical trials: the British CHAOS study, the Finnish ATBC study, and the American Physicians' Health Study, Women's Health Study and CARET study have all published either negative effects or neutral results with consumption of β-carotene or vitamin E supplements (Heinonen and Albanes, 1994; Stephens et al, 1996; Omenn et al, 1996; Hennekens et al, 1996; Lee et al, 1999). The pro-oxidant effects of β-carotene and α-tocopherol may be responsible for these results. Also, it has been suggested that the oxidative status of the organism may play a role in how antioxidant molecules will act *in vivo*, which may explain why some trials showed no effect (Halliwell, 2000).

Flavonoids are a class of polyphenolics found in plants that has received much attention because of their demonstrated antioxidant activity in *in vitro* testing. They are found in a wide variety of forms in a wide variety of edible plants, with high concentrations in certain fruits (grapes, berries, apples), soy, cocoa, and tea. Several thousand forms of flavonoids have been identified (Fraga, 2007), but the basic 3-ring structure remains. Antioxidant capacity of many flavonoid sub-classes has been observed in chemical assays, and this activity is dependent on the hydroxyl groups for donation of a hydrogen to directly scavenge radicals, while the substitution pattern of these and other groups on the rings also appears to have an effect on scavenging efficiency (Fraga, 2007).

It was originally believed that the direct RS scavenging and/or chelating activities of flavonoids and other polyphenols observed in chemical assays was also responsible for beneficial effects in the body. More recent research, however, indicates that low levels of these compounds in the blood (with maximum values in the low micromolar range) are often below what is necessary to function as effective RS scavengers, and suggests their activity arises instead from action as signaling molecules (Fraga, 2007; Stevenson and Hurst, 2007). Polyphenols may act to promote health by up-regulating the endogenous antioxidant enzyme system, modulating inflammatory mechanisms, and participating in signaling pathways for cell differentiation, proliferation and apoptosis (Stevenson and Hurst, 2007).

Despite all the controversy surrounding the action of the individual components, diets high in the antioxidants β-carotene, α-tocopherol, and polyphenols have been associated with lower risk of age-related diseases (Halliwell, 2008; Steinmetz & Potter, 1996). Also, animal and tissue culture studies have shown promising results with supplementation of antioxidants, despite mixed results in human trials (Vivekananthan et al, 2003). Research has not yet fully explained the discrepancy in results between the whole foods and their isolated components, or between animal results and human trials.

In summary, consumption of foods high in antioxidants promotes health, though the underlying mechanisms are not completely understood. Recent research has guided expert opinions to suspect antioxidants work via complex, signaling pathways rather than (or in addition to) direct scavenging.

1.8 Low-Linolenic Soybeans

Low linolenic soybeans have been grown commercially since 1994, and breeding to that end began in 1952 (Fehr, 2007). But only in 2004 did commercial markets for the oil see significant sales increases, because of a confluence of consumer and government concerns over the detrimental health effects associated with *trans* fats in partially hydrogenated vegetable oils. Hydrogenation reduces the number of double bonds in the fats and increases their oxidative stability. Low-linolenic soybean oils, already low in double bonds, inherently have increased oxidative stability, which reduces the need for hydrogenation. A recent human study reinforced the connection between heart health and the altered fat soybeans, showing that consumption of low-linolenic, high-oleic and low-saturated fatty acid soybean oils instead of hydrogenated soybean oil resulted in an improved plasma lipid profile (Lichtenstein et al, 2006). Many food purveyors have switched from *trans* fat laden hydrogenated oils to low-linolenic soybean oils, and still others continue to search for viable alternatives, leaving a wide gap in the market that the low-linolenic and low-saturated fat soybean oils may fill.

This market demand has stimulated the breeding effort and production of soybeans with low-linolenic acid and other improved fatty acid profiles, including low saturated fat. Because of the demand for the healthy edible oils and the higher premiums that accompany them, the production of the altered lipid soybeans is expected to continue to increase (Fehr, 2007).

Little research has been published with regards to the effects of low-linolenic breeding efforts on other properties in the soybean, particularly in regard to their

antioxidants. McCord and others (2004) indicated that though previous research had found low linolenate soybean lines to be lower in tocopherols than conventional soybeans, their research had identified some reduced-linolenate lines with tocopherols not significantly different from conventional lines. Also, high temperature during growth of conventional soybeans has been shown to decrease the ratio of linoleic plus linolenic to total fatty acids (Tsukamoto et al, 1995). A similar environmental effect has also been shown to yield decreased levels of isoflavones.

1.9 Colored Seed Coat Soybeans

Color of the seed coat varies across genotypes. In the typical yellow soybean, chlorophyll disappears as seeds mature and dry in the field, allowing the remaining flavonoid pigments' color to show. Some soybeans do not lose their chlorophyll as they dry and remain green at maturation (Snyder and Kwon, 1987). Still others have additional pigments, including anthocyanins and proanthocyanins, that impart deep black and brown seed coat colors (Xu and Chang, 2008a; Todd and Vodkin, 1993).

A number of studies have shown that soybeans with black, brown, green, and yellow seed coats might differ in their antioxidant properties, total phenolic contents, and proanthocyanidins (Takahashi et al., 2005; Xu Yuan and Chang., 2007; Takahata et al., 2001), indicating that this may alter their ability to affect health.

In vitro studies have shown that the anthocyanins, in addition to the isoflavone genistein, are capable of inhibiting the growth of cancer cells through various mechanisms (Valachovicova et al., 2004; Kim et al., 2008). Black soybeans are the only color reported to contain anthocyanins (Xu and Chang, 2008a; Todd and Vodkin, 1993), and only brown and black contain proanthocyanins. Since these

pigments are phenolic compounds, they have also been shown to have antioxidant activity, and may therefore confer additional health benefits beyond the traditional yellow soybeans.

1.10 Chemical and Bioactive Analysis of Soy

As discussed previously with mention of isoflavone extractions, the choice of extraction solvent and the method of extraction have the potential to greatly affect the phytochemicals extracted from soybeans and other biological samples. This is understood and taken into account throughout the following discussions on measuring the presence and activity of antioxidant compounds.

1.10.1 Bioavailability, Absorption and Metabolism of Isoflavones

Isoflavone bioavailability, absorption and metabolism are well-studied areas in soy research, and it has become well-accepted that isoflavones of all forms are absorbed through the gastrointestinal tract (though glycosides are only absorbed after enzymatic hydrolysis to the aglycone form), metabolized by the intestinal cells and/or the liver, after which the metabolites are distributed systemically throughout the body, and removed through enteric recycling and urinary and biliary excretions (Larkin et al, 2008). Thus, given their presence throughout the body, it is well justified to study the bioactivity of isoflavones. An understanding of the nuances of isoflavone bioavailability then becomes tantamount before exploring their bioactivity, on both the level of the whole organism and the level of the individual cell.

Much is known about the transit and metabolism in the body, but only the information relative to tissue culture work is discussed here due to the nature of this

work. A recent review by Larkin and others (2008) well covers data involving the whole organism.

Absorption of aglycone isoflavones occurs by passive diffusion across the intestinal epithelium (Larkin et al, 2008), with genistein and daidzein being absorbed 10 times more efficiently than their glucosides (Murota et al, 2002). Prior to absorption, most sugar side chains of the β-, acetyl-, and malonyl-glucoside isoflavones are hydrolyzed in the intestinal lumen by glucosidases from the intestinal mucosal cells or microbes present in the intestine, leaving the aglycone form (Larkin et al, 2008). The aglycones are more hydrophobic and can thus diffuse more readily through the lipophilic cell membranes. Despite intense research in the area, disagreement remains whether genistein or daidzein is more bioavailable, though the argument seems to favor genistein due to higher post-prandial levels typically seen in the blood. (Glycitein's bioavailability has yet to be studied adequately in human consumption trials to enter this discussion.)

Many factors affect the rate of absorption of isoflavones after ingestion, including the food matrix, the dose, the form of isoflavone (aglycone vs. glucoside), intestinal transit speed, and fiber content of the diet, among others (Nielsen and Williamson, 2007). Furthermore, some studies have indicated that isoflavone absorption is more efficient in soy foods as compared to isoflavone supplements (Walsh et al, 2003; Gardner et al, 2009). Thus, *in vitro* studies are inherently limited by their lack of ability to accurately mimic the real intestinal conditions that affect absorption rate.

A common criticism of tissue culture treatments is that they expose cells to the compound of interest but not the metabolites that would circulate in the blood. In this case, intestinal cells have been shown to convert isoflavones to their glucuronide and sulfate derivatives, both in *in vitro* cultures and on the whole organism level (Murota et al, 2002; Larkin et al, 2008). The glucuronides predominate the isoflavone metabolites in the plasma after absorption, followed by the sulfated metabolites (Larkin et al, 2008).

Finally, one factor of digestion and metabolism that has yet to be completely understood involves the conversion of daidzein to equol, an isoflavandiol. Equol has both stronger antioxidant and estrogenic activities, but only 30-50% of the population is able to produce it (Cassidy, 2006). The conversion is performed by intestinal bacteria, but it is not understood why some individuals with the bacteria are not equol producers. Equol is similarly glucuronated or sulfated in the liver and is then circulated in the blood, but its kinetics and clearance from the body are slower (Larkin et al, 2008). Studying *in vitro* exposure of intestinal cells to daidzein will therefore not give a complete picture of soy exposure for equol producers.

1.10.2 Identification and Quantification of Antioxidant Components

1.10.2.1 Total Phenolic Content

One of the most commonly used assays in assessing phenolic antioxidants, the total phenolic content assay has a misleading name because it actually measures reducing power. The assay is designed where samples in a basic solution reduce the molybdenum-based Folin-Ciocalteu (FC) reagent. Though the mechanism of the

reaction remains to be completely elucidated (Huang et al, 2005), it is believed that the reduction of Mo(VI) complexes to Mo(V) by an electron transfer from the antioxidant molecule is associated with a color change from yellow to blue, which is measurable at 765 nm. The reducing power of sample solutions are thus compared against a standard phenolic acid, typically gallic acid, and results are expressed as mg gallic acid equivalents per unit of sample.

There are a number of interfering factors that may skew the results of the TPC assay, including various nonphenolic organic compounds, certain inorganic salts, and aromatic amino acids, among others (Prior et al, 2005). Also, some groups have expressed results as equivalents of a phenolic standard other than gallic acid, limiting the ability to compare results across labs. As for the TPC method's positive outlook, it is relatively quick (2 h), requires easily accessible equipment, and is reproducible. Finally, there is often agreement between this method and the ORAC method (Prior et al, 2005).

1.10.2.1 Total Flavonoid Content

The total flavonoid content (TFC) assay is used much less frequently than other 'total content' assays, but has been used in the assessment of soybeans and thus deserves mention. The method is colorimetric. It involves the addition of solvent, $AlCl_3$ and the sample. An acid-stable complex forms between aluminum chloride and the C-4 keto group and one of the hydroxyl groups at C-3 or C-5 of the flavones or flavonols. The complexation is monitored by spectrophotometer. However, different flavones and flavonols have different λ_{max} values, necessitating different absorption wavelengths with different predominating flavonoids (Chang et al, 2002; Zhishen et

al, 1999). Similarly, the choice of flavonoid standard becomes important, and predominance of flavonoids in the actual sample should be considered when picking a flavonoid for a standard curve. The method is easy to perform and quick enough for routine analysis. However, it is definitely limited in the flavonoids it can detect, not recognizing flavonones and flavanonols (Chang et al, 2002), though these are not found in significant quantities in soy.

1.10.2.3 Total Anthocyanin Content

The commonly used total anthocyanin content (TAC) assay by Lee et al (2005a) takes advantage of the reversible structural change that occurs in the anthocyanin between pH 1.0 and 4.5. At the lower pH, the oxonium form predominates, the characteristic pink color is seen, and absorbance at 520 nm is high. At the higher pH, the hemiketal form dominates; consequently, the color disappears, the absorbance at 520 nm drops, and the differential between absorbances at the two pH values is proportional to the amount of anthocyanin present. The method is relatively quick, easy, inexpensive, and the necessary equipment and reagents are readily available in any moderately equipped lab. Potential limiting factors for the assay include its ability to only measure monomeric anthocyanins and not polymerized, the ability of food colorants to interfere with its readings, and the method of calculation relying on the molar extinction coefficient of the main anthocyanin (most typically cyanidin-3-glucoside), though this may not adequately account for the variety of anthocyanin forms present. Finally, the assay is not sensitive enough to accurately measure samples below 20 mg cyanidin-3-glucoside equivalents/L.

1.10.2.4 High Performance Liquid Chromatography

High performance liquid chromatography is the gold standard for identification and quantification of individual as well as total flavonoids, phenolics, anthocyanins and the lipid-soluble carotenoids and tocopherols. Methods for analyzing each are well established in the literature—specifics regarding carotenoid, tocopherol and sterol analysis will be discussed in a later section. Barriers to using HPLC as compared to the previous colorimetric methods include the high cost of the system and high-purity solvents, the need for more skilled personnel to run the system, the extended time needed to prepare/purify samples, a much lengthier analysis time per sample, and the potential to lose desired compound during additional sample purification. However, no other method is capable of providing the same level of detail, particularly with the more advanced methods of detection, including mass spectrometry.

1.10.3 Antioxidant Activity Assays

Various methods have been developed to measure the activity of antioxidants through chemical assays, which offer the benefit of measuring very specific activity toward a particular radical but also the downside of not necessarily reflecting the true physiological or food system conditions under which antioxidants and radicals interact naturally. Studying each component individually can lead to a greater understanding of which component is responsible for which activity. Test tube assays also offer cost and time savings compared to *in vivo* tests and lesser so to *in vitro* cell models. Due to the differences between assays and the low barrier to perform them,

it is considered essential in antioxidant activity research to use multiple activity assays to evaluate a sample, hopefully including a method to study both aqueous- and lipid-soluble radicals, reactions that occur via radical quenching and reducing mechanisms, and finally via metal complexing (Prior et al, 2005).

1.10.3.1 Electron Spin Resonance

Electron spin resonance (ESR) remains the only method that allows for the 'direct' observation of free radicals. Thus, by monitoring the levels of radicals present in a test solution compared to a control reaction, antioxidant activity of samples can be observed. However, because many physiological free radicals are too short-lived to be detectable, molecules such as 5,5-dimethyl-1-pyrroline (DMPO) are used to trap them, creating a more stable radical that can then be detected (Yu and Cheng, 2008). While an essential tool for validating antioxidant methods and affirming critical results, ESR is beyond reach for most antioxidant screening work, requiring highly unique and expensive equipment, trained personnel, and a significant amount of time to perform.

1.10.3.2 Oxygen Radical Absorbance Capacity

Perhaps the most commonly used antioxidant activity assay, the oxygen radical absorbance capacity (ORAC) assay provides an indicator of a sample's ability to scavenge peroxyl radicals (·OOR). To accomplish this, the assay monitors the degradation of a fluorescent probe (most typically fluorescein), which loses its fluorescence upon oxidation by a peroxyl radical. Antioxidants interfere in the oxidation via a classic radical chain breaking mechanism involving a hydrogen atom

transfer (Ou et al, 2001). The ability of an antioxidant to protect the probe from oxidation is measured by comparison to trolox, a known antioxidant. Calculations of the area under the fluorescence decay curve (AUC) provide a route to standardization—values are typically reported as trolox equivalents, after comparison to a standard curve of trolox AUC vs. concentration. The choice of solvents and radical source can be altered to study both hydrophilic and lipophilic antioxidants, and the method has been adapted for high-throughput use with a 96-well plate reader (Huang et al, 2001). Potential caveats include the strict temperature sensitivity of the assay and reactions with solvent.

1.10.3.3 Hydroxyl Radical Scavenging Capacity

The hydroxyl radical scavenging capacity (HOSC) assay is a more recent addition to the antioxidant arsenal (Moore et al, 2006b). It estimates the ability of a compound to scavenge hydroxyl radicals (·OH). The method is similar to ORAC in that it uses competitive kinetics, in which the fluorescein probe and antioxidants compete to react with radicals. However, it differs in the radical generation system: whereas ORAC uses AAPH to generate peroxyl radicals, the HOSC assay uses a Fenton-like Fe^{3+}/H_2O_2 reaction to generate hydroxyl radicals (Moore et al, 2006). HOSC does not have the same temperature restrictions as ORAC, but certain solvents (ethanol, methanol and DMSO) cannot be used due to interference with probe degradation. The assay is currently limited to hydrophilic antioxidant analysis; however, a method using ESR spin trapping detection and acetonitrile as the solvent has been developed for analysis of hydroxyl scavenging abilities on lipophilic antioxidants (Cheng et al, 2007). Like ORAC, the method is adapted for high-

throughput use, is performed on commonly available equipment, and uses inexpensive chemicals. An additional benefit is that HOSC is performed at a physiological pH.

The study of hydroxyl radical scavenging activity (in general, not any particular assay) has been criticized due to the highly reactive nature of the radical (Magalhaes et al, 2008)—hydroxyl radicals are one of the most reactive naturally occurring chemical species and react with almost every molecule they contact. Thus, the only way for such highly reactive radicals to be scavenged would be to have a higher concentration of the scavenging molecules.

1.10.3.4 DPPH Radical Scavenging

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical is very stable, non-physiological, nitrogen-centered radical with a deep purple color and absorbance at 515 nm (Prior et al, 2005). Antioxidants react directly with the radical via an electron transfer reaction (Magalhaes et al, 2008), reducing it and leaving it a pale yellow. Thus, absorbance at 515 nm is monitored as an indicator of reaction progress. Results are often expressed as the EC_{50}, or the dose at which 50% of the radicals are consumed, but a standardized method has been developed where results are expressed relative to trolox (Cheng et al, 2006).

The assay is easy to perform, partly because the radical is commercially available. It is also adapted for use on a 96-well plate reader for high-throughput data accumulation. However, the use of $DPPH^{\cdot}$ scavenging assays has been criticized because not only is it already not physiological, its long-life bears little similarity to short-lived physiological radicals (Huang et al, 2005). Also, its large steric bulk

prevents larger antioxidants from gaining access to reduce it. Because of these limitations, its relevance to biological activity of antioxidants is questioned, though it is still a commonly used assay.

1.10.3.5 ABTS Radical Cation Scavenging

The ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)] radical cation scavenging assay has also been called the TEAC (Trolox equivalent antioxidant capacity) assay, though it is prudent to refer to it as $ABTS^{\bullet+}$ due to the advent of other methods comparing to a trolox standard. The assay involves the generation of ABTS radical cation from a commercially available chemical and manganese dioxide, AAPH, or enzymes (Magalhaes et al, 2008). The radical cation is an intense blue-green color, which disappears when the radical is reduced. Absorbance is monitored at 734 nm at a pre-determined time point, and results are often expressed as trolox equivalents per unit of food. The method is commonly used for hydrophilic antioxidants but can be adapted to study lipophilic antioxidants. It can also be performed at a variety of pH's and in a microplate for high-throughput analysis (Prior et al, 2005). However, it too is a non-physiological radical, and its biological relevance is questioned. Finally, different standards have shown different kinetic behaviors (Magalhaes et al, 2008; Prior et al, 2005); therefore, measuring activity at only one time point may not provide a complete understanding of a sample's activity.

1.10.3.6 Superoxide Scavenging Capacity

Superoxide radicals are generated through normal metabolic processes, most notably the electron transport chain, where an extra electron is donated to molecular oxygen. The enzyme superoxide dismutase converts it to oxygen and hydrogen peroxide, and another enzyme catalase converts the hydrogen peroxide to water and oxygen. An enzyme capable of performing the reverse reactions, xanthine oxidase, is used in this assay to generate the superoxide radical (MacDonald-Wicks et al, 2006). The assay involves a competition between antioxidants and a probe to react with the superoxide radical. The probe is nitro blue tetrazolium, which turns from yellow to blue when reduced by superoxide, and can be followed at an absorption of 560 nm spectrophotometrically. Other variations exist, involving non-enzymatic radical generating systems and cytochrome c or chemiluminescent probes (Magalhaes et al, 2008). The method benefits from a generally simple protocol and use of easily available equipment. Like other direct reduction methods, it is potentially limited if the antioxidant compounds are capable of directly reducing the probe. Additionally, an excess of substrate in the reaction mixture would allow a two-electron transfer mechanism to occur, a hydroperoxide to form, and results to be inaccurate (MacDonald-Wicks et al, 2006).

1.10.3.7 Ferric-reducing Antioxidant Power

The ferric-reducing antioxidant power (FRAP) assay directly measures a sample's ability to reduce a ferric (Fe^{3+}) salt to a ferrous (Fe^{2+}) salt. A blue color develops as the reaction proceeds and is monitored at a wavelength of 593 nm. The

reaction occurs wholly from electron transfer, making it an ideal method for identifying the dominant mechanism of particular antioxidants (Prior et al, 2005). However, the method and mechanism behind it are very similar to the $ABTS^{\cdot+}$ scavenging method discussed above, particularly because the two have very similar redox potentials (Prior et al, 2005; Huang et al, 2005; MacDonald-Wicks et al, 2006). The two methods differ in the pH of the reaction solution: $ABTS^{\cdot+}$ scavenging is performed at neutral pH and the FRAP assay requires acidic conditions (pH 3.6) to maintain solubility of the iron ions. The method is easy to perform, can be automated for high-throughput but is nonetheless quick when performed manually, inexpensive and requires no specialized equipment. It is limited, particularly with polyphenols, in that some molecules react at different rates—the single time point analysis that is traditionally performed does not easily allow for appropriate analysis of this effect.

1.10.3.8 Inhibition of LDL oxidation

The oxidation of low density lipoproteins (LDL) has been associated with enhanced atherosclerosis *in vivo*. Because of this, *in vitro* tests have been designed to test phytochemicals for their ability to inhibit LDL oxidation. The method most commonly used currently involves the measurement of thiobarbituric acid-reactive substances (TBARS), secondary lipid oxidation products. For the test, isolated LDL is introduced to an oxidizing agent—either Cu(II) or AAPH—and suspected antioxidant compound. The reaction proceeds for a specified time period, and is then reacted with thiobarbituric acid. The extent of TBARS formation is estimated by comparing absorbance at 532 nm with standards. The method is valuable because of

its physiological relevance, but dispute remains about the most appropriate oxidizing agent to mimic physiological conditions (Frankel and Meyer 2000; Huang et al, 2005; Prior et al, 2005). Additionally, the mechanism of LDL protection by phenolics is not completely understood and may vary depending on the oxidizing agent. Protection may occur via direct radical scavenging, chelation, and binding to proteins and/or amino acids (Frankel and Meyer, 2000).

1.10.3.9 Lipid Peroxidation Measures

Inhibition of lipid peroxidation assays use a similar protocol as the LDL method utilizing TBARS described above. The study of lipid peroxidation (often linoleic acid), however, benefits from greater consistency than LDL-oxidation assays, where some variability is inevitable lot-to-lot. Nonetheless, it is still criticized for bearing little similarity to lipids in an actual biological system *in vivo* (Huang et al, 2005).

1.10.4 Cell-based Assays

The use of cell-based assays to measure oxidative stress is an exciting prospect—the assays provide a relatively quick assessment of bioactivity and are certainly less expensive than *in vivo* trials. The measurement of transient RS in relatively low levels in an ever-changing biological system poses a considerable challenge to researchers (Dikalov et al., 2007). One particular demerit of cell assays worthy of mention here is that cell culture is inherently a condition of increased oxidative stress for cells (Halliwell, 2003). High levels of dissolved oxygen and lack of the complete *in vivo* endogenous antioxidant system (culture media are typically

absent of Vitamin C) are some of the components that add to this stress. Additionally, cell culture assays are also limited by the lack of digestion, absorption and metabolism of chemicals before delivery to the cells. Thereby, it is recognized that a cell-based assay can never provide a complete understanding of the effects of exogenous antioxidants on oxidative stress *in vivo*. Nonetheless, these types of assays are necessary to gain initial insight of the biological interactions that do occur.

1.10.4.1 Growth Inhibition / Antiproliferation Assays

Growth inhibition and antiproliferation assays are among the most commonly used *in vitro* tests for screening the bioactivity of foods and their components. They are relatively simple to perform (expose a cell culture to a treatment and compare cell growth to a control), though they require several days of readings to construct a growth curve and are limited in the number of samples that can be screened. Also, the mechanism of antiproliferative activity is likely to vary from one active compound to the next, but this assay will only say which is active, not *how*. They have received substantial use in the literature, yet their use has yet to be reviewed in comparison to *in vivo* activity. Numerous methods have developed to speed the process of counting viable cells, including ATP-luminescence and dyes (methylene blue and MTT) (Wang et al, 2007; Wolfe & Liu, 2007).

1.10.4.2 Intracellular Radical Probes

A variety of intracellular radical probes have been reported in the literature—the most popular in the food science realm has been dichlorofluorescin diacetate (DCFH-DA). Originally thought to be a probe that could uniquely detect hydrogen

peroxide (LeBel et al, 1992), it has been shown to detect a variety of radicals and for this reason has been suggested to be a better indicator of overall oxidative stress. This property is very enticing for use in screening foods and/or their components for health-promoting qualities.

A number of protocols exist for measuring oxidative stress with DCFH-DA (LeBel et al, 1992; Wang & Joseph, 1999; Wolfe & Liu, 2007; Girard-Lalancette et al, 2009), and the assay is heavily used in radiation therapy research in addition to food science. All assays rely on the membrane permeability of DCFH-DA. The dissolved probe passively enters cells, upon which the two acetates are cleaved off by esterases, trapping the resulting polar DCFH inside. Oxidation of DCFH by RS produces dichlorofluorescein (DCF), which fluoresces strongly. Fluorescence is monitored to determine the presence of RS in cells (λ_{ex}= 485 nm, λ_{em}=530 nm). Protocols vary with respect to order of addition of oxidizing agent, protective agents and the probe.

Unfortunately, there are a number of interfering factors that limit the usefulness of the DCFH-DA assay. It does not directly react with $O_2^{\bullet -}$, H_2O_2 and other peroxides, without a catalyst, meaning it cannot directly detect the two most common RS (Wardman letter, 2008; Wardman, 2007). However, peroxyl, alkoxyl, peroxynitrite, $NO_2^{\cdot}$, $CO_3^{\cdot -}$, and $OH^{\cdot}$ are all capable of oxidizing DCFH to DCF (Halliwell and Whiteman, 2004). Other biological molecules are also capable of oxidizing DCFH—hemes, cytochrome c, and peroxidase—which has the potential to provide faulty readings (Wardman letter, 2008; LeBel et al, 1992). Also, light may induce photooxidation, again interfering with results through increased background

fluorescence (Wang and Joseph, 1999; Soh, 2006). Finally, the assay results are subject to variations in probe or antioxidant concentrations, giving varying results independent of RS levels (Wardman, 2008). Another potential interfering factor is the potential for extracellular reactions between probe and components of the cell culture media or extracellular phenolics, but the problem is not easily solved due to a loss in sensitivity when cells are washed with fresh media after probe loading (Hafer and Schiestl letter, 2008). Perhaps of most concern is the ability of the probe to generate superoxide radicals (Wardman, 2007).

Ultimately, the many limitations of the DCFH-DA assay make it difficult to draw definitive conclusions about its results, which are compounded by previous researchers' lack of attention to these faults, resulting in a largely unusable set of references for comparison.

Other less popular fluorescent probes include dihydrorhodamine 123, dihydrocalcein and Amplex Red. These have similar drawbacks as the DCFH-DA assay, including predisposition to photooxidation and a lack of selectivity for radicals (Soh, 2006). Chemiluminescent probes are also of interest, including lucigenin, luminol, luciferin, and their analogs. Similar to the fluorescent probes, these probes are oxidizable by multiple (though not all) RS. Also, they too participate in the generation of superoxide radicals in the presence of molecular oxygen. Luminol in particular has been chided as having too many interferences to be of use in live cells (Wardman, 2007). Wardman reviews the use of fluorescent and luminescent probes in cells (Wardman, 2007).

A number of probes have been designed to detect specific RS, a trait which may serve to provide an understanding of mechanisms. They have yet to gain ground in food science and nutraceutical research, and perhaps will only do so when a specific mechanism tied to a disease state is identified that can be targeted by food components. A review by Soh (2006) discusses novel fluorescent probes (though not necessarily for cell use). Until specific mechanisms (preferably associated with disease states) are identified, probes that assess general oxidative stress are arguably better for food bioactive component screening.

1.10.4.3 Damage assays

As oxidative damage is considered to be the link between oxidative stress and disease, it is not a surprise that a multitude of assays have been developed to detect it. DNA appears to be the most commonly measured biomolecule, due to the connection between DNA damage and cancer.

DNA damage can be measured in a number of ways, including (from most to least specificity) LC-MS/MS, GC-MS, HPLC, ELISA and the comet assay (Loft et al, 2008). The chromatographic assays require specific extraction and preparation steps, adding time and cost to the analysis in addition to extraneous oxidation during the additional steps (Loft et al, 2008). The widely used comet assay does not require extra preparation steps but is limited because it is less specific.

The comet assay can measure DNA damage and repair—it involves encapsulation of individual cells (already treated with an oxidant and/or antioxidant) in agarose gel, after which the cells are lysed in place, and electrophoresis is run to separate the intact DNA (forming the 'head' of the comet) from damaged DNA

(forming the 'tail' of the comet). The use of lesion-specific enzymes lends increased specificity to the assay (Wasson et al, 2008).

Measures of protein and lipid oxidation are also used. Lipid oxidation can be measured in tissue culture or on biopsy samples using the TBARS assay or malondialdehyde detection through HPLC. Thiobarbituric acid-reactive substances (TBARS) provides less specificity but a faster, simpler method, whereas HPLC offers specific detection but is more complex. Both require extraction and preparation of the biological samples.

Studying the oxidative damage of proteins is complicated by the large number of oxidized products that can result from this attack (Halliwell &Whiteman, 2004). The most commonly used method involves measuring the amount of protein carbonyl groups present, the formation of which has been linked to oxidative stress (Kadiiska et al, 2005; Dalle-Donne et al, 2006), thus measurements of these species have also been used as a rubric for determining oxidative stress under various diseases and treatments.

1.10.4.4 Physiological ESR

Electron spin resonance (ESR) techniques as described in section 2.10.3.1 can also be applied to cells in culture using spin traps (Halliwell and Whiteman, 2004). The limitations described previously persist here. Also, the detectable radical-spin trap adducts are rapidly metabolized to a species that does not generate an ESR signal, further complicating evaluation of results (Halliwell and Whiteman, 2004). The current recommended probe for biological samples is cyclic hydroxylamines, while new nitrone probes are under development to resist to degradation (Dikalov et

al, 2007). Also of interest, measurements may be specific to either the intracellular or extracellular compartments by varying the spin probe used (Dikalov et al, 2007). Nonetheless, ESR remains the only direct method of observing free radicals and is used despite the cost and faults. Applications for ESR evaluation of tissue samples and whole (animal) organisms are also being refined.

1.10.4.5 Antioxidant enzyme assays

Enzymes of interest in the endogenous antioxidant system include superoxide dismutase, catalase, and peroxidases. Superoxide dismutase aids in the conversion of $O_2^{\bullet-}$ to H_2O_2; catalase further dismutes H_2O_2 to H_2O and O_2; glutathione peroxidase also dismutes peroxides including H_2O_2 to H_2O and ROH but oxidizes glutathione in the process, requiring glutathione reductase and NADPH to reduce it back.

Assessing the activity of these antioxidant enzymes (and/or the up-regulation of their encoding genes) gives another view of the activity of the endogenous antioxidant system *in vitro*. A number of kits have been developed by various purveyors to be medium-to-high throughput, typically in 96-well formats using spectroscopic detection (Cayman Chemical, Ann Arbor, MI; Luminos Assays, Ann Arbor, Michigan). Most often, cells need to be lysed and centrifuged prior to the assay.

1.10.4.6 Cellular Redox Measurement (GSH:GSSG)

As the oxidative stress paradigm has shifted from direct phenolic scavenging of radicals to a more complex signaling system, so too have new techniques been added to the antioxidant assay arsenal. One that has gained considerable attention

involves the measurement of the redox pair of glutathione (GSH) and its oxidized dithiol form (GSSG).

As mentioned above in section 2.6, glutathione plays a role in a number of pathways for managing oxidative stress. The ratio of GSH:GSSG has also been reported as a biomarker for cancer, cardiovascular disease, rheumatoid arthritis, Alzheimer's and Parkinson's diseases, and diabetes mellitus (Valko et al, 2007). GSH is the major thiol in the cytosol and the GSH/GSSG pair is considered the major "cellular redox buffer" (Valko et al, 2007). Methods have been adapted to measure this ratio *in vitro*. Traditional measurements are cumbersome, involving protein precipitation, centrifugation, and finally detection through spectrometry, fluorometry, bioluminescence, or HPLC (Pastore et al, 2003), which significantly limits the number of samples that can be tested. Also, the rather involved preparation steps cause oxidation, potentially leading to incorrectly high GSSG levels. Commercial kits have been developed to detect GSH in cell cultures. More recently, an assay capable of detecting both GSH and GSSG in the same well has been introduced (Luminos Assays, Ann Arbor, Michigan). It uses a proprietary fluorescent probe, and takes two GSH readings—one of free GSH, and then another after the GSSG has been reduced back to GSH.

Other redox pairs that have been studied or suggested to have potential for use as oxidative stress indicators include reduced ascorbate, cysteine, and thioredoxin with their oxidized forms (Jones, 2006; Valko et al, 2007).

1.10.4.7 Unique Cell Lines

Several unique cell lines have been developed to be highly resistant to a particular RS, which when paired with a similar normally-susceptible cell line provides a unique opportunity to study the effects of that radical *in vitro*.

One such pairing of cell lines is the human leukemia HL-60 cells and its H_2O_2-resistant 'clone', HP100. The HP100 line has been shown to have 18 times more catalase activity and twice the amount of superoxide dismutase activity as HL-60 cells, giving them a measured 340 times greater resistance to H_2O_2 than their parent line (Kasugai & Yamada, 1992). Studying the pairing of cells is useful in combination with the other cell assays discussed here. It has primarily been used to study the mechanism of DNA lesion formation in response to various treatments: green tea catechins (Oikawa et al, 2003; Furukawa et al, 2003) and the synthetic antioxidant propyl gallate (Kobayashi et al, 2004).

1.11 Methods of Analysis for Quantifying Lipophilic Soy Health Beneficial Factors

A table summarizing the pertinent extraction and analytical HPLC methods for carotenoids, tocopherols and sterols are found in **Appendices A** and **B**.

1.11.1 Carotenoids

Carotenoids can fall into one of two classifications: carotenes are purely conjugated hydrocarbons, while xanthophylls are conjugated hydrocarbons with one or more oxygen atoms. Reversed phase high performance liquid chromatography

(RP-HPLC) has become the method of choice for identifying and quantifying carotenoids, particularly after the advent of the C_{30} column in the 1990s. Because of the inability of C_{18} columns to produce adequate separation of xanthophylls, the C_{30} stationary phase was designed specifically to improve their separation (Sander et al, 1994).

Due to their high degree of conjugation, carotenoids are highly susceptible to oxidation—care must be taken during sample preparation to avoid unnecessary light, heat, oxygen and prooxidant metals (de Quiros & Costa, 2006). The more classical process of analyzing food carotenoids involves extraction first, followed by saponification (if it is performed), then HPLC analysis. Newer methods save time by directly saponifying foods prior to extraction (**Appendix A**), though these methods have not yet been applied to the analysis of soy carotenoids.

For protective purposes, antioxidants are used in the saponification solutions, extraction solvents and/or mobile phases to prevent carotenoid oxidation during sample preparation. BHT and BHA are among the antioxidants commonly used, generally in the range commonly used to protect lipids—from 0.01 to 0.1% (w/v). Ascorbic acid and pyrogallol are also used in the literature, particularly during the saponification step where a more hydrophilic antioxidant may be necessary due to the reaction conditions. (**Appendix A**.)

Extraction solvents are highly variable among food studies of carotenoids. Hexane, ethyl acetate, acetone, petroleum ether, 2-propanol, ethanol, chloroform, isopropanol and others have all been used in different proportions. Hexane and combinations containing it have predominated the literature in recent years.

Chloroform and other chlorinated solvents are generally not recommended due to loss of carotenoids, though ammonium acetate in the mobile phase may mitigate the loss (Huck et al, 2000).

Saponification is sometimes used to remove unwanted chlorophylls and lipids from samples, while also hydrolyzing carotenol esters. However, the harsh conditions of saponification are associated with a loss of carotenoids, particularly xanthophylls, in many food samples, though modifying the temperature and strength of base appear to provide some controlling power over these losses (de Quiros & Costa, 2006; Muzhingi et al, 2008).

Column temperature has been shown to play a crucial role in the separation of carotenoids in RP-HPLC. There is relative agreement in the literature that lower column temperatures allow for the preservation of carotenoids while providing adequate separation—recommended temperatures fall between 20-23 ºC (Böhm, 2001; Huck et al, 2000; de Quiros & Costa, 2006; Bell et al, 1997). Böhm's results (2001) cautioned that temperatures too low interfered with separation of a commonly used internal standard.

A variety of detection methods are available for carotenoid HPLC analysis, including UV/Vis absorbance, photodiode array detection, electrochemical, and mass spectrometry utilizing either electron spray ionization (ESI) or atmospheric pressure ionization interfaces (APCI) (Huck et al, 2000; Also see **Appendix B**).

1.11.2 Tocopherols

Tocopherol analysis is conducted very similarly to that of carotenoids. Tocopherols are not as susceptible to oxidative loss, though their high degree of

unsaturation requires precautions still be taken in these areas. Extraction, saponification, and HPLC detection methods are largely the same as for carotenoids—the use of antioxidant protectants during these steps is also seen for tocopherol analysis.

Capillary gas chromatography has been used in tocopherol analysis in the past, prior to the advent of HPLC technology (Abidi, 2000). Subsequently, normal phase HPLC (NP-HPLC) was used because it required less sample clean-up than did GC. More recently, favor has shifted to the use of RP-HPLC because NP-HPLC requires the use of toxic and environmentally-harmful mobile phases. However, NP-HPLC is easily able to separate the constitutional isomers beta- and gamma-tocopherol, where RP-HPLC has had much difficulty. Fortunately, beta-tocopherol constitutes a fairly insignificant amount of total tocopherols in most foods, thus making the lack of separation seemingly inconsequential. C_{18} columns have been employed most frequently with RP-HPLC, though C_{30} columns have been used, particularly when analyzing carotenoids and tocopherols together (Abidi, 2000; **Appendix B**.) Though C_{18} columns are capable of separating beta-tocopherol from gamma-tocopherol, the analysis requires a very slow flow rate and increases the analysis time by a factor of at least 5 (Abidi, 2000).

1.11.3 Sterols

The analysis of sterols in foods follows a similar pattern as seen with carotenoids and tocopherols: extraction, saponification, and chromatographic analysis (Lagarda et al, 2006). In the case of sterols, an acid hydrolysis step may also be added after saponificiation to cleave sterol glycosides since they are resistant to

basic saponification. Sterols, though inherently more stable than carotenoids and tocopherols, also benefit from protection against oxidative damage throughout the various extraction steps.

Capillary GC has been used most frequently in the analysis of plant sterols, offering great selectivity (Lagarda et al, 2006; Abidi, 2001). However, HPLC operates at milder temperatures, does not require derivitization, and offers the choice of non-destructive detection methods. Thus, HPLC use for sterol analysis has been increasing, RP-HPLC in particular. See **Appendices A and B** for representative methods of extraction and HPLC analysis of sterols. Also, Abidi presents a thorough review of chromatographic analysis of sterols (2001), followed by a separate review by Lagarda et al (2006), which continued where Abidi left off.

Solvents used for extraction of sterols are slightly different than those for carotenoids and tocopherols—methanol, hexane and acetone remain as potential players, but chloroform and methylene chloride are also used with sterols (Lagarda et al, 2006; Abidi, 2001). Detection methods coupled with HPLC are again similar to carotenoids and tocopherols: UV absorbance, photodiode array detectors, HPLC-MS, along with evaporative light scattering detectors and refractive index.

Chapter 2: Antioxidant Properties and Phenolic, Isoflavone, Tocopherol and Carotenoid Composition of Maryland-grown Soybean Lines with Altered Fatty Acid Profiles

Slavin, M., Cheng, Z., Luther, M., Kenworthy, W., and Yu, L. (2009). *Food Chemistry*, *114*, 20-27.

2.1 Abstract

One cultivar and five experimental lines of Maryland-grown soybeans with improved fatty acid profiles were investigated for their phytochemical and antioxidant properties. Soybean oils were extracted and analyzed for their fatty acid profiles, tocopherols, and carotenoids. Two of the experimental lines were low in α-linolenic acid and another one was low in both α-linolenic and total saturated fatty acids. The defatted flours were extracted in 50% acetone and 70% ethanol, and estimated for isoflavone compositions, total phenolic contents (TPC), and scavenging capacities against peroxyl (ORAC), hydroxyl (HOSC), and 2,2-diphenyl-1-picryhydrazyl (DPPH) radicals. No difference was observed in tocopherols, carotenoids, TPC, and antioxidant properties between soybeans with reduced α-linolenic and/or saturated fats and the ones with 'normal' fatty acid profiles. This study indicates the possibility to produce soybeans with reduced linolenic and/or saturated fat and with desirable levels of health beneficial phytochemicals and antioxidant properties.

2.2 Introduction

Soybean is one of the primary agricultural commodities in the United States and had the second highest farm value in 2005 (Ash, Livezey, & Dohlman, 2006). Soybean oils account for about two-thirds of the total vegetable oils consumed in the

U.S. for food production, with and without hydrogenation (Ash et al., 2006; United States Census Bureau, 2008). New soybean cultivars were introduced to the market in 2004, largely because of the health concerns associated with trans fats from partially hydrogenated vegetable oils (Ash et al., 2006). These new soybeans have been bred to have lower α-linolenic acid (18:3n-3), thus increasing their oxidative stability and reducing the need for hydrogenation. Soybean oils low in saturated fat and/or high in oleic acid (18:1n-9) are also available and desirable for human health. A recent human study reinforced the connection between heart health and the altered fat soybeans, showing that consumption of low-linolenic, high-oleic and low-saturated fatty acid soybean oils instead of hydrogenated soybean oil resulted in an improved plasma lipid profile (Lichtenstein et al., 2006).

Starting in January of 2006, the U.S. Food and Drug Administration (FDA) required trans fats to be listed on the nutrition facts panel of packaged foods. More recently, several major cities have announced initiatives that may prohibit or restrict the use of trans fats in restaurants (Department of Health & Mental Hygiene, 2006; Philadelphia City Council, 2007). Food purveyors are searching for viable alternatives, leaving a wide gap in the market that the low linolenic and low saturated fat soybean oils may fill. This market demand has stimulated the breeding effort and production of soybeans with improved fatty acid profiles (Ash et al., 2006; Monsanto Company, 2007).

Soybean seed meal is the major by-product from soybean oil production and is highly valuable because it typically contains many health beneficial components including soy protein, fiber, isoflavones and other phytochemicals. In 1999, the FDA

permitted for the use of a health claim on food labels linking intake of soy protein to a reduction of cholesterol, and thus a risk for heart disease. Health beneficial factors in soy have also shown potential in reducing the risk of certain types of cancer (MacDonald et al., 2005; Messina, Kucuk, & Lampe, 2006; Nagata et al., 2007), improving cognitive and immune functions (Lee, Lee, & Sohn, 2005a; Ryan-Borchers et al., 2006), and providing relief of menopause symptoms in women, including the prevention of osteoporosis (Omoni & Aluko, 2005).

Tocopherols, carotenoids, isoflavones, and other phenolic compounds have also been detected in soybeans and may contribute to the overall health benefits of soy food intake (Malencic, Popovic, & Miladinovic, 2007; Riedl et al., 2007). The major isoflavone compounds in soy are genistein, daidzein, and glycitein plus their glycosidic forms. Genistein and daidzein, as well as their glycosides, showed scavenging capacity against 2,2-diphenyl-1picrylhydrazyl (DPPH) radicals, reducing power and suppression of low density lipoprotein oxidation (Lee et al., 2005b). Recently, soybean extracts have shown ability to directly react with and quench DPPH radicals (Malencic et al., 2007; Riedl et al., 2007). Changes in the levels of these components in soybeans may cause a loss in their overall beneficial activities. Consumers are responding to the link to decreased risk of heart disease and the montage of other reported health benefits. Recent surveys indicate the percentage of Americans seeking out soy products for health reasons continues to grow— up 6% in one year to 37% in 2007 (United Soybean Board, 2006; 2007). Similarly, sales of soy foods and supplements in 2004 totaled $4 billion, a 5% increase from the previous year (Ash et al., 2006). These data indicate the huge potential of developing value-

added nutraceutical products from soybean seed meals for supplemental and functional food products.

It has been noted that the amount of isoflavones in soybeans may be altered considerably by the growing, processing and storage conditions besides the genotype (Lee, Yan, Ahn, & Chung, 2003; Riedl et al., 2007). Several studies also have shown that breeding of soybeans to reduce linolenic and palmitic acids has altered the tocopherol contents of the oils (McCord et al., 2004; Scherder, Fehr, Welke, & Wang, 2006). Furthermore, studies have suggested that the antioxidant contents of other crops, including wheat, may also vary with growing conditions (Moore, Liu, Zhou, & Yu, 2006a). To date, no information is available whether these breeding efforts may alter antioxidant properties and isoflavone compositions of soybean seeds.

The present research was conducted to take an initial step toward studying the possible effects of breeding to improve soybean fatty acid compositions on the value-adding factors, such as antioxidant properties and phytochemical profiles. The ultimate goal of this research is to identify soybeans with healthy fatty acid profiles and high concentrations of health beneficial components, thereby enhancing their production and utilization in order to improve the health of the human population, while enhancing market value of the seed for growers.

2.3 Materials and Methods

2.3.1 Materials and Chemicals

Whole soybeans from the 2005 growing season grown at a single location were obtained from Dr. William Kenworthy of the Department of Plant Science and

Landscape Architecture, University of Maryland (College Park, MD). The soybeans result from a traditional cross-breeding program designed to achieve low linolenic and/or low saturated fatty acid contents in soybean lines that are adapted for growth in the Maryland climate. Manokin, a cultivar commonly grown in Maryland, is used as a standard for comparison, while the five experimental lines analyzed include MD 03-5517, MD 03- 5527, MD 04-5493, MD 04-5550 and MD 0304 WN-35.

Iron (III) chloride, fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) were purchased from Sigma–Aldrich (St. Louis, MO). 2,2'-Azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals, USA (Richmond, VA). Thirty percent ACS-grade H_2O_2 was purchased from Fisher Scientific (Fair Lawn, NJ), while α, γ, δ-tocopherols were purchased from EMD chemicals, Inc., (San Diego, CA). Lutein, β-carotene, β-cryptoxanthin, and zeaxanthin were obtained from Indofine Chemical Company (Hillsborough, NJ). Ultrapure water was prepared by an ELGA Purelab ultra Genetic polishing system with <5 ppb total organic carbon and resistivity of 18.2 mX (Lowell, MA) and was used for all experiments. All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.3.2 Oil extraction

Whole soybeans were ground in a standard household coffee grinder to pass through a 20-mesh sieve. Five grams of ground soybeans were extracted in approximately 70 mL hexane for 3 h via a Soxhlet apparatus. The hexane was evaporated using a Buchi Rotovapor R-200 (Flawil, Switzerland) at a reduced

pressure, and the remaining oils were weighed and percent oil was calculated. The oils were stored at ambient temperature under nitrogen in the dark until testing.

2.3.3 Antioxidant extraction

The soy flour remaining after oil extraction was allowed to dry overnight at ambient temperature. Approximately 1 g of the flour was extracted with 10 mL of either 70% ethanol or 50% acetone at ambient temperature. Solvents were selected according to a solvent comparison study by Xu and Chang (2007), which found 50% acetone extracts exhibited the highest TPC values and 70% ethanol the highest ORAC values. The soy flour-solvent mixtures were then sonicated for 20 min, and the extracts were filtered through Whatman No. 41 filter paper. The extracts were held under nitrogen in the dark until testing.

2.3.4 Fatty acid composition

Fatty acid methyl esters (FAME) were prepared from the oils by saponification followed by methylation according to a previously described laboratory protocol and subjected to gas chromatography (GC) analysis (Yu, Adams, & Watkins, 2003). A Shimadzu GC- 2010 with a FID and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD) was used for fatty acid analysis. Helium was used as the carrier gas at a flow rate of 2.2 mL/min through a fused silica capillary column SPTM-2380 (30 m x 0.25 mm with a 0.25 lm film thickness) from Supelco (Bellefonte, PA). Injection volume was 1 µL at a split ratio of 10/1. Oven temperature was initially 136 °C, increased by 6 °C/min until 184 °C where it was held for 3 min, then increased again by 6 °C/min to a final temperature of 226 °C. Individual fatty

acids were identified through comparison of GC retention time with those of fatty acid methyl ester standards. Quantification was based on the area under each fatty acid peak as compared to the total area of all fatty acid peaks.

2.3.5 Tocopherol contents

Oil samples and standard compounds were dissolved in methanol and were analyzed using the liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MSMS) method. Briefly, LC analysis was performed using a TSQ Quantum tandem mass spectrometry (Thermo-Electron Company, San Jose, CA, USA) equipped with an ESI interface and Agilent 1100 Micro- LC system (Agilent Technologies, Palo Alto, CA, USA). The LC separation was achieved by using a Zorbax SB-C18 column (Agilent Technologies, Palo Alto, CA, USA), 0.3 mm i.d. x 150 mm, 3.5 μm particle size, at 40 °C. The tocopherols were eluted using a mobile phase of 4% water and 96% methanol. The flow rate was 12 μL/min and the injection volume was 1 μL. The TSQ Quantum was operated in the positive-ion mode using nitrogen (>99.7%) as the sheath gas and auxiliary gas at pressure of 35 psi and 5 units, respectively. ESI spray voltage was 4.5 kV at the ESI interface. The capillary temperature was maintained at 300 °C. A collision induced dissociation (CID) was achieved using argon as the collision gas at the pressure adjusted to more than 0.8 mTorr above the normal, and the applied collision offset energy was set from -35 eV to -45 eV for individual analyte. The optimal energy level was adjusted against analyte standards each time before analyzing the sample. Identification of tocopherols was accomplished by comparing the LC retention time and the selected reactant monitoring (SRM) analysis of the sample peaks with that of the certified pure

commercial tocopherol standards. The quantitative m/z: from 430.4 (molecular ion) to 165.2 (major fragment) was set for α-tocopherol, 416.4 (molecular ion) to 151.0 (major fragment) was set for γ-tocopherol, and m/z: 402.3→137.0 was set for δ-tocopherol. Data were acquired with Xcalibur software system (Thermo-Electron Company, San Jose, CA, USA). The quantification for tocopherol compounds was conducted using the total ion counts with the responding external standard. β-tocopherol was not analyzed because its contents are assumed to be negligible in soybeans and its very similar molecular structure to γ-tocopherol makes it difficult to separate from the latter.

2.3.6 Carotenoid contents

Six oil samples were dissolved in 1 mL $CHCl_3$–Methanol (3:7, v/ v) for LC–MS analysis. Briefly, LC analysis was performed using a TSQ Quantum tandem mass spectrometry (Thermo-Electron Company, San Jose, CA, USA) equipped with an ESI interface and Agilent 1100 Micro-LC system (Agilent Technologies, Palo Alto, CA, USA). The LC separation was achieved by using a DeveloSil column (Nomura Chemical Co., Ltd. Aichi, Japan), 150 mm x 1.0 mm i.d. and 5 μm particle size, at 5 °C. The four carotenoids were eluted using a mobile phase of 4% water and 96% methanol. The flow rate was 10 μL/min and the injected volume was 1 μL. The TSQ Quantum was operated in the positive-ion mode under following conditions: nitrogen (>99.7%) was used for sheath gas and auxiliary gas at pressure of 35 psi and 5 units, respectively. ESI spray voltage was 4.5 kV at the ESI interface. The temperature of the heated capillary was 300 °C. A collision induced dissociation was achieved using

argon as the collision gas at the pressure adjusted to more than 0.8 mTorr above the normal, and the applied collision offset energy was adjusted against analyte standards before analyzing each sample in the range from -35 eV to -45 eV. The four carotenoids were identified by comparing the LC retention time and the selected reactant monitoring analysis of the sample peaks with that of the carotenoid standards. The quantitative m/z: 536.5 (molecular ion) to 119.2 (major fragment) was set for β-carotene, and m/z 552.5→119.2, 550.5→119.2 and 568.5→119.2 were set for β-cryptoxanthin, lutein and zeaxanthin, respectively. Data were acquired with Xcalibur software system (Thermo-Electron Company, San Jose, CA, USA). The concentration of each carotenoid compound was estimated using the total ion counts with the responding external standard.

2.3.7 Total Phenolic Contents (TPC)

The TPC of each 50% acetone and 70% ethanol soybean flour extract was measured according to a laboratory procedure described previously (Yu, Haley, Perret, & Harris, 2002). In short, 100 μL of soybean flour extract, 500 μL of the Folin–Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL ultrapure water comprised the reaction mixture. Gallic acid was used as the standard. Absorbance was read at 765 nm on a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) after 2 h reaction at ambient temperature in order to calculate TPC. Reactions were conducted in triplicate and results were reported as gallic acid equivalents (GAE) per gram of soybean.

2.3.8 Isoflavone composition

Approximately 3 mL of each 50% acetone and 70% ethanol extract was mixed with 0.75 mL 12 M HCl and heated in a water bath for 2 h at 55 °C. The reaction mixtures were cooled to room temperature, and the ethanol and acetone were evaporated using a nitrogen evaporator. The remaining aqueous suspension containing the isoflavones was extracted three times with 4 mL of ethyl ether/ethyl acetate each time (1:1, v/v). The combined organic phases were washed with 3 mL of water, and dried using anhydrous sodium sulfate. After removing the organic solvents in a nitrogen evaporator, residues were re-dissolved in 0.5 mL methanol, and the resulting solutions were filtered through a 0.45 μm membrane, and subjected to HPLC analysis.

HPLC analysis was performed on a Waters 600 system (Milford, MA) with a Photodiode Array Detector 996, a 717 autosampler and an in-line degasser with a Phenomenex C18 column (250 mm × 4.6 mm) according to the protocol by Lee et al (Lee, Kim, Zheng, & Row, 2007) with modifications. A binary solvent system was employed with solvent A consisting of water–acetic acid at a ratio of 99.9:0.1 (v/v) and solvent B consisting of acetonitrile–acetic acid (99.9:0.1, v/v). The gradient changed linearly from 85:15 (A:B, v/v) to 65:35 (A:B, v/v) from 0 to 50 min, was held steady at 65:35 (A:B, v/v) for 10 min before returning to the initial condition for an additional 10 min to re-equilibrate the system. The flow rate was 1 mL/min, injection volume was 10 μL, and the detection wavelength was set at 254 nm. The isoflavones were identified via comparison to standards for retention time and UV

spectra, and amounts of isoflavones were estimated via the area under the curve using external standards.

2.3.9 Antioxidant activity assays

2.3.9.1 DPPH radical scavenging capacity estimation

A laboratory protocol was used for the DPPH radical scavenging capacity estimation that was performed using a Victor3 multilabel plate reader (PerkinElmer, Turku, Finland) (Cheng, Moore, & Yu, 2006). Briefly, each reaction mixture contained 100 μL of soybean flour 50% acetone or 70% ethanol extracts at different concentrations and 100 μL of 0.2 mM $DPPH^{\bullet}$ solution. Six different concentrations were used for each soybean extract in the study for estimating the EC_{50} value against $DPPH^{\bullet}$. The $DPPH^{\bullet}$ solution was added into each well to initiate the antioxidant–radical reactions. The absorption at 515 nm was determined immediately after initiation of reactions. A blank with only 200 μL of solvent, and a control with the mixture of 100 μL of solvent and 100 μL of 0.2 mM $DPPH^{\bullet}$ were also determined for absorbance at 515 nm. The percent radical remaining at 40 min is determined according to the following equation: % $DPPH^{\bullet}$ remaining = $[(A_{sample} - A_{blank})/(A_{control} - A_{blank})] \times 100\%$, where A_{sample}, A_{blank}, and $A_{control}$ stand forthe absorbance of sample, blank, and control reactions at 40 min, respectively. Based on the values of % $DPPH^{\bullet}$ remaining at 40 min, the EC_{50} of each sample was obtained by plotting the % $DPPH^{\bullet}$ remaining against the antioxidant concentrations. The EC_{50} value is the concentration of an antioxidant required to quench 50% radicals in the reaction mixture under the assay condition.

2.3.9.2 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was conducted according to a previously reported laboratory protocol (Moore et al., 2005) with modifications. Fluorescein (FL) was used as the fluorescent probe and a Victor3 multilabel plate reader (Perkin-Elmer, Turku, Finland) was used to measure fluorescence. Trolox, a water-soluble antioxidant, was used as the standard. Trolox standards were prepared in 50% acetone and 70% ethanol, while all other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). In the initial reaction mixtures, 225 µL of freshly made 8.16 x 10^{-8} M FL and 30 µL 50% acetone or 70% ethanol extract of the soybean flour, standard or blank were combined in the wells of a 96-well plate and were pre-heated in the plate reader for 20 min at 37 °C. Then, 25 µL of freshly made 0.36M AAPH was added to each well. The fluorescence of the assay mixture was recorded once every two minutes for three hours at 37 °C, with excitation and emission wavelengths of 485 nm and 535 nm, respectively. Trolox equivalents (TE) were calculated for samples based on area-under-the-curve (AUC) calculations used by Ou and others (Ou, Hampsch-Woodill, & Prior, 2001). Results are expressed as micromoles of TE per gram of defatted soybean flour.

2.3.9.3 Hydroxyl radical scavenging (HOSC) estimation

The HOSC assay was conducted using FL as the fluorescent probe and a Victor3 multilabel plate reader (Perkin-Elmer, Turku, Finland) according to a previously reported laboratory protocol (Moore, Yin, & Yu, 2006b). The reaction mixture contained 170 µL of 9.28 x 10^{-8} M FL, 30 µL sample, 40 µL of 0.1990 M

H_2O_2 and 60 µL of 3.43 M $FeCl_3$. The fluorescence of the reaction mixture was recorded approximately once every four minutes for seven hours with no temperature control. Excitation and emission wavelengths were 485 nm and 535 nm, respectively. Due to the incompatibility of ethanol with the assay, 1 mL of the 70% ethanol extract was evaporated and re-dissolved in 2 mL 70% acetone. Standards were prepared in 50% and 70% acetone. The 3.43 M $FeCl_3$ solution was prepared daily and 0.1990 M H_2O_2 solution was prepared fresh for each assay, each in ultrapure water. The 9.28×10^{-8} M FL was prepared fresh for each assay from stock FL solution and 75 mM sodium phosphate buffer (pH 7.4). Trolox equivalents (TE) were calculated for samples using the same AUC calculations (Moore et al., 2006b). Results are expressed as micromoles of TE per gram of defatted soybean flour.

2.3.10 Statistical analysis

Tests were conducted in triplicate with data reported as mean ± standard deviation. Differences in means were detected using one-way ANOVA and Tukey's test. Correlation among means was determined using a two-tailed Pearson correlation test. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.05$.

2.4 Results and Discussion

2.4.1 Fatty acid composition

As shown in **Table 2.1**, the fatty acid composition of the oils from Manokin, MD 03-5517, and MD 03-5527 had fatty acid compositions similar to that observed

in conventional soybean oils. MD 04-5493 and MD 04-5550 soybeans contained about 3.1 g and 3.3 g α-linolenic acid (18:3n-3) in 100 g of total fatty acids, respectively, which were significantly lower than that of 6.7–7.4 g/100 g fatty acids observed in the Manokin, MD 03-5517 and MD 03-5527 soybean oils (**Table 2.1).** The MD 0304-WN-35 soybean line had the lowest palmitic acid (16:0) concentration of 3.6% among all tested soybean lines, along with a low α-linolenic acid level of 3.6% (**Table 2.1**). This soybean line also had lowest stearic acid concentration and significantly lower total saturated fatty acid content.

The oleic acid concentration was significantly higher in soybean lines with reduced a-linolenic acid and palmitic acid levels (**Table 2.1**). The greatest oleic acid concentration was observed in MD0304-WN-35 line at a level of 40.5 g/100 g of total fatty acids. The other two low α-linolenic acid soybean lines had 36.5% and 29.7% oleic acid in their total fatty acids, respectively. Also noted was that these low α-linolenic acid and saturated fat soybean lines also had significantly lower concentrations of linoleic acid (18:2n-6) ranging from 42.1% to 48.7% of total fatty acids as compared to 50.4% to 52.6% total fatty acids in the 'normal' lipid soybeans. These data indicate it is possible to obtain soybean lines with high monounsaturated fatty acids (MUFA) and low in saturated fatty acids. It has been suggested that a diet with a higher level of MUFA could serve as an alternative for the low-fat diet and may reduce the plasma cholesterol concentrations (Hargrove, Etherton, Pearson, Harrison, & Kris-Etherton, 2001). A previous study also showed that diets rich in MUFA reduced the aortic accumulation of oxidized LDL and was more effective in suppressing atherosclerosis development in hypercholesterolemic hamsters (Nicolosi

et al., 2002). A recent study investigated the selectively bred and genetically modified soybean oils with low α-linoleate or saturated fatty acids, high in oleic acid, and regular and partially hydrogenated soybean oils for their effects on risk factors of cardiovascular diseases using moderately hyperlipidemic human subjects (Lichtenstein et al., 2006). All the soybean oils with altered fatty acid compositions and the regular soybean oil resulted in more favorable lipoprotein and lipid profiles than the partially hydrogenated oil, leading to the conclusion that these specially bred soybean oils are viable for food utilizations to reduce the trans fat content.

Table 2.1. Fatty Acid Composition of Soybeans (g/100 g total fatty acids)*

	16:0	18:0	18:1	18:2	18:3	PUFA	TSA
Manokin	10.31c ± 0.21	4.30bc ± 0.04	22.25a ± 0.61	52.58e ± 0.36	7.37e ± 0.07	59.95e ± 0.41	14.62b ± 0.18
MD 03-5517	11.82d ± 0.31	4.18b ± 0.11	21.79a ± 0.47	50.37d ±0.36	7.44e ± 0.16	57.92d ± 0.99	16.00c ± 0.41
MD 03-5527	12.01d ± 0.02	4.63d ± 0.02	22.85a ± 0.87	51.11d ± 0.16	6.70d ± 0.03	57.81d ± 0.16	16.64d ± 0.03
MD 04-5493	9.02b ± 0.04	5.32e ± 0.01	36.53c ± 0.23	42.17a ± 0.10	3.05a ± 0.04	45.22a ± 0.06	14.33b ± 0.05
MD 04-5550	10.31c ± 0.03	4.40c ± 0.01	29.74b ± 0.04	48.71c ± 0.16	3.33b ± 0.01	52.03c ± 0.16	14.71b ± 0.04
MD 0304 WN-35	3.62a ± 0.01	3.04a ± 0.02	40.52d ± 0.05	46.30d ± 0.05	3.60c ± 0.00	49.90b ± 0.05	6.66a ± 0.01

* Data expressed as mean ± standard deviation (n = 3). Within each column, means marked with the same letter are not significantly different ($P \leq 0.05$). PUFA = total polyunsaturated fatty acids. TSA = total saturated fatty acids.

2.4.2 Tocopherol contents

The tocopherol contents of soybeans with altered lipid contents were of particular interest because previous studies showed that breeding to lower linolenic acid decreased levels of α, δ, and γ-tocopherols, though not all examined lines differed significantly from the soybean lines with normal linoleate level (McCord et al., 2004). It was also observed that genetically modified soybeans with lower palmitic and stearic acids contained less total tocopherol, although β-tocopherol concentration was increased in their oils (Mounts, Abidi, & Rennick, 1996). In 2006, another study investigated the relationship between palmitate content and tocopherol concentration in soybean lines with similar genetic background, and concluded that a decrease in palmitate correlated to an average increase in total tocopherols by 15% (Scherder et al., 2006). These previous studies suggested the potential changes of tocopherol contents in soybeans with altered fatty acid profiles.

The present study examined and compared α, δ, and γ-tocopherol concentrations in Maryland-grown soybeans with different fatty acid profiles. As shown in **Table 2.2**, Manokin and MD 0304-WN-35 lines had same total tocopherols, and the MD 04-5550 line contained the lowest total tocopherols. Furthermore, MD 04-5493 contained low δ and γ-tocopherols, but not α-tocopherol. These data suggest that reduction of α-linolenic acid in soybean oil through breeding efforts does not necessarily decrease its tocopherol contents, although it may alter the tocopherol composition. This points to the possibility of finding a soybean that is low in linolenic and palmitic acids and still reasonably high in total tocopherols. A Pearson correlation analysis failed to show a significant relationship between fatty acid

contents and tocopherol contents. It needs to be pointed out that this preliminary study involved a small number of soybean lines. Comprehensive research involving larger a number of soybean samples under different growing conditions is required to draw a clear conclusion on how breeding to optimize the fatty acid profile may influence the composition of other beneficial factors in soybeans.

Table 2.2. Tocopherol Contents of Soybean Oils*

	Oil Type	α-tocopherol (μg/g)	δ-tocopherol (μg/g)	γ-tocopherol (μg/g)	Total tocopherols (μmol/g)
Manokin	Normal	430a ± 9	199c ± 8	767b ± 95	3.34b ± 0.15
MD 03-5517	Normal	1847c ± 107	335e ± 18	1065c ± 100	7.68d ± 0.34
MD 03-5527	Normal	545a ± 28	202c ± 9	2157d ± 193	6.94d ± 0.54
MD 04-5493	Low 18:3	1327b ± 66	134b ± 3	504ab ± 13	4.62c ± 0.17
MD 04-5550	Low 18:3	468a ± 35	41a ± 4	267a ± 4a	1.83a ± 0.08
MD 0304-WN-35	Low 18:3, low saturated	437a ± 7	267d ± 6	704b ± 70	3.37b ± 0.15

* Data expressed as mean ± standard deviation (n = 3). Within each column, means marked with the same letter are not significantly different.

2.4.3 Carotenoid contents

The carotenoid contents are of interest because, like tocopherols, they are a lipid-soluble, health beneficial component found in soybean oil. Carotenoid contents with respect to a changing fatty acid profile have not been reported in the literature.

This study found a large variation in the amount of carotenoids present in the tested six hexane-extracted soybean oils (**Table 2.3**). Manokin and MD 03-5517, two of the 'normal' lipid soybeans, had the highest amounts of lutein, cryptoxanthin, and total carotenoids. MD 03-5527, the third 'normal' lipid soybean, contained the lowest amount of lutein, cryptoxanthin and total carotenoids. Meanwhile, MD 0304-WN-35, the soybean low in both 18:3n-3 and SFA had a content of zeaxanthin that was not significantly different than MD 03-5517, which had the highest zeaxanthin measurement. Correlation analysis did not show any significant relationship between fatty acid profile and carotenoid contents, individually or total, which may be partially due to the small sample size. These data do not show a clear effect of altering fatty acid profile on carotenoid contents in soybeans, but highlight the importance of screening soybeans for carotenoid contents, because a soybean otherwise normal in its lipid profile and tocopherol content may have a low content of carotenoids.

Table 2.3. Carotenoid Contents of Soybean Oils*

	Oil Type	Cryptoxanthin (μg/g)	Lutein (μg/g)	Zeaxanthin (μg/g)	Total carotenoids (μmol/g)
Manokin	Normal	13.5c ± 1.2	5142e ± 48	34.6c ± 1.9	9.13d ± 0.09
MD 03-5517	Normal	24.1d ± 2.2	4887d ± 153	95.6d ± 7.2	8.80d ± 0.25
MD 03-5527	Normal	0.1a ± 0.1	1256a ± 7	13.7ab ± 0.3	2.23a ± 0.01
MD 04-5493	Low 18:3	0.3b ± 0.02	1665b ± 37	22.6b ± 1.1	2.97b ± 0.07
MD 04-5550	Low 18:3	0.1a ± 0.02	1504b ± 44	4.6a ± 0.4	2.65b ± 0.08
MD 0304-WN-35	Low 18:3, low saturated	8.8c ± 1.0	2694c ± 106	104.8e ± 2.8	4.94c ± 0.19

* Data expressed as mean ± standard deviation (n = 3). Within each column, means marked with the same letter are not significantly different.

2.4.4 Total phenolic content (TPC)

The major antioxidants found in soybeans are isoflavones, a group of polyphenolic compounds. TPC was measured as an overall indicator of contents of these molecules with antioxidant properties. The TPC values for 50% acetone and 70% ethanol extractions of soybean flour are shown (**Fig. 2.1**). Higher TPC value was observed in the 50% acetone extract for all soybean lines, suggesting 50% acetone is a better solvent than 70% ethanol for extraction of phenolics from soybeans. This result is in agreement with the results of Xu and Chang (2007). The three 'normal' lipid soybeans had a range from 2.0 to 2.5 mg gallic acid equivalents (GAE)/g soybean, while the lipid-altered soybeans had a similar range from 2.1 to 2.6 mg

GAE/g soybean. No clear trend was found between fatty acid contents and TPC. Pearson correlation analysis showed that TPC using 50% acetone as the extraction solvent was correlated to the ORAC and HOSC values, as well as genistein, daidzein, and total isoflavone contents. TPC in the 70% ethanol extracts was correlated only to ORAC values and total isoflavones.

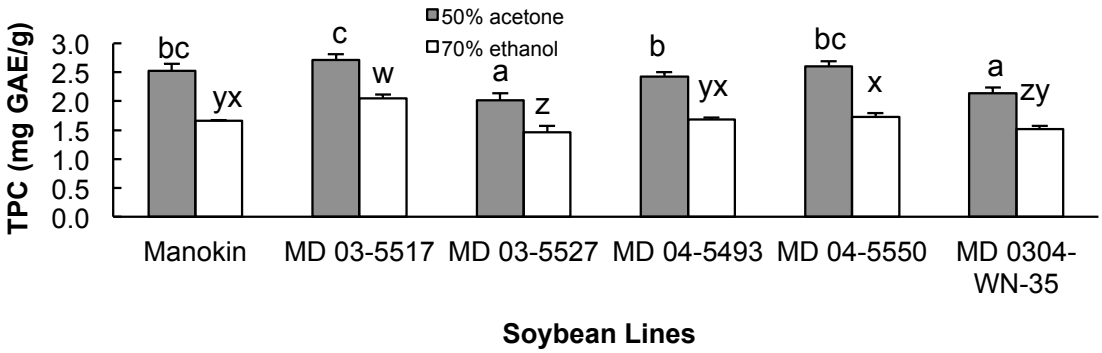


Figure 2.1. Total phenolic contents of defatted soybean flours.
TPC were quantified using the Folin-Ciocalteu reagent with gallic acid as the standard. Absorbance was read at 765 nm at 2 h reaction time. Results expressed as mg gallic acid equivalents (GAE)/g defatted soybean flour. Analyses were conducted in triplicate, with mean values shown and standard deviation depicted by the vertical bars. Columns of the same extraction solvent marked by the same letter did not have significantly different values ($P \leq 0.05$).

2.4.5 Isoflavone composition

The extracts were treated with acid to cleave the glycoside forms of isoflavones into aglycones prior to HPLC analysis, a process which may also occur in the gut prior to absorption (Liggins, Bluck, Coward, & Bingham, 1998; Murphy, Song, Buseman, & Barua, 1997). The isoflavones including daidzein, genistein and glycitein were identified in every extract. In both 50% acetone and 70% ethanol extracts, genistein and glycitein alternated as the most prominent aglycone (**Fig. 2.2**). Also, MD 03-5517 had the highest total isoflavones and MD 03-5527 had the lowest

total isoflavones in both extraction solvents. Both lines contain normal values of linolenate and saturated fatty acids. These data again indicate that reducing the level of α-linolenic acid in soybeans through breeding might not decrease their isoflavonoid content, suggesting that soybeans with desirable fatty acid profile and isoflavone composition may be obtained through breeding.

The USDA-Iowa State University Database on the isoflavone content of foods lists the mean isoflavone content of raw, food-quality United States soybeans to be 1280 μg/g edible portion (Agricultural Research Service, 2002), whereas the total isoflavones in the soybeans reported in this paper ranged from 251 to 624 μg/g whole soybean. The USDA database compiled data from published articles using various extraction solvents, which undoubtedly have variations in their extraction efficiency. Using 50% acetone and 70% ethanol in this publication would not have extracted insoluble-bound isoflavones and may account in part for the difference between the presented results and the USDA database amount.

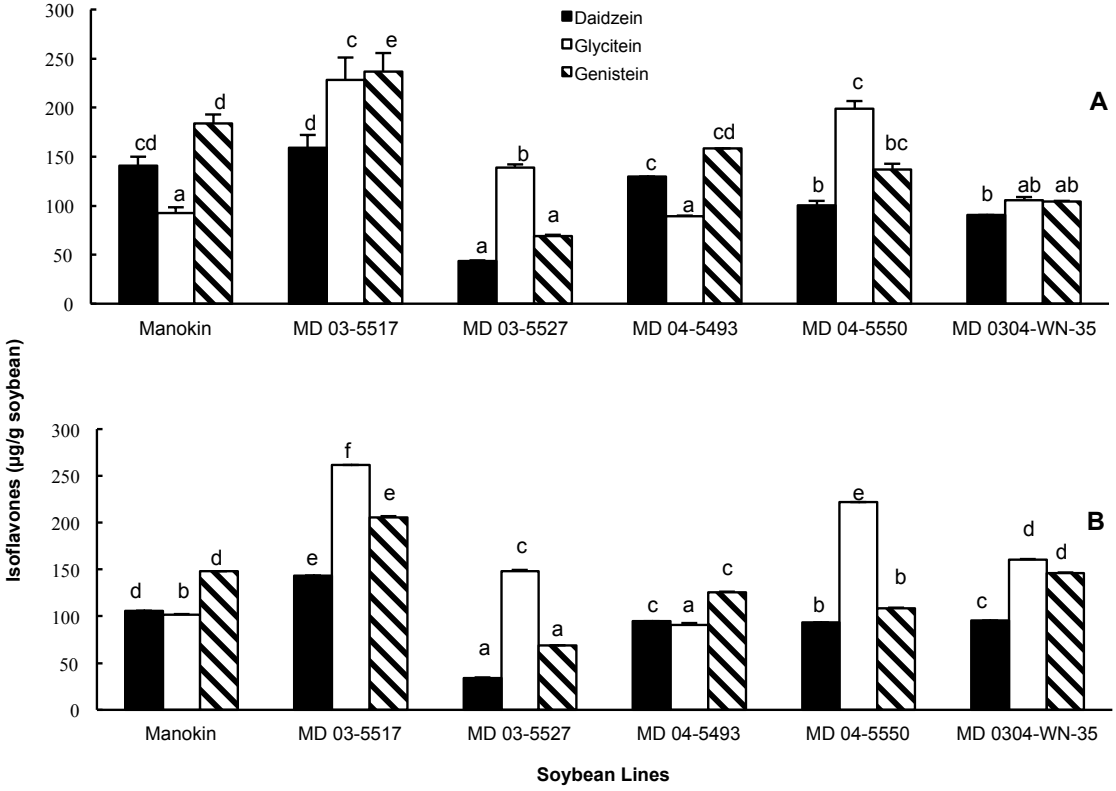


Figure 2.2. Isoflavone content of defatted soybean flours.
A) 50% acetone and B) 70% ethanol extracts of defatted soy flours were hydrolyzed with acid to release isoflavones. RP-HPLC analysis was conducted on a C18 column, using the area under the curve of external standards to quantify the genistein, daidzein, and glycitein content of samples. Results are expressed as µg isoflavone/g soybean. Analyses were conducted in duplicate, with mean values shown and standard deviation depicted by the vertical bars. Columns in the same series marked by the same letter are not significantly different ($P \leq 0.05$).

2.4.6 Antioxidant activity

Antioxidant activity was measured with a variety of tests in order to assess their ability to counteract the effects of various radicals. ORAC measures the absorbance capacity of peroxyl radicals, HOSC measures the scavenging capacity against hydroxyl radicals, and the DPPH tests measure against the 2,2-diphenyl-1-picryhydrazyl radical, a non-biological radical.

2.4.6.1 DPPH radical scavenging capacity

The EC_{50} values were used to report the $DPPH^{\bullet}$ scavenging capacity of the soybean cultivar and lines. The EC_{50} is the required initial concentration of a selected antioxidant sample to quench 50% of the free radicals initially in the reaction system; therefore, a higher EC_{50} value corresponds to a lower antioxidant activity in the sample. All 50% acetone and 70% ethanol extracts of defatted soybean flours directly reacted and quenched $DPPH^{\bullet}$ in the testing system under the experimental conditions (**Fig. 2.3**). For the 50% acetone extracts, five of the samples – Manokin, MD 03-5517, MD 03-5527, MD 04-5550, and MD 0304-WN-35 – had values around 11 mg seed flour equivalents (SE)/mL that were not significantly different from each other. The only sample with a different value was MD 04-5493, with a value of 16.1 mg SE/mL, indicating it has lesser ability to quench DPPH radicals. The 70% ethanol extracts had an EC_{50} value ranging from 7 to 10 mg SE/mL, with the lipid-altered soybeans having the highest values, or lowest ability to scavenge the DPPH radicals. Taken together, these data indicate that reduction of α-linolenic and saturated fatty acids may not significantly ($P \leq 0.05$) alter the antioxidant properties of soybeans.

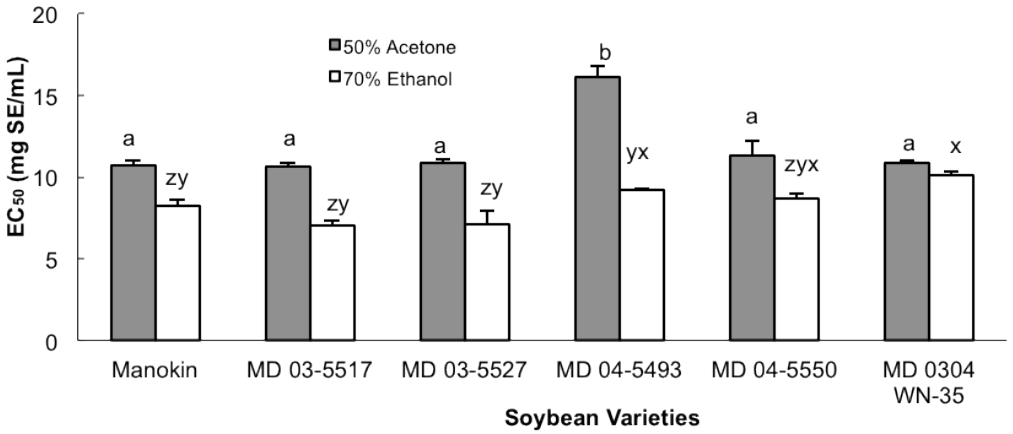


Figure 2.3. EC_{50} values against $DPPH^{\bullet}$ for defatted soybean flours.
EC_{50}, the required initial concentration of a selected antioxidant sample to quench 50% of the free radicals in the reaction system, was measured at 40 min reaction time. The initial concentration of $DPPH^{\bullet}$ in the reaction mixture was 0.1 mM. Results expressed as mg soybean flour equivalent/mL testing solution. Tests were conducted in duplicate, with mean values shown and standard deviations depicted by the vertical bars. Columns of the same extraction solvent marked by the same letter did not have significantly different values ($P \leq 0.05$).

2.4.6.2 Oxygen radical absorbance capacity (ORAC)

ORAC values of both 50% acetone and 70% ethanol extracts were examined. Similar to the observation of TPC, the ORAC values did not appear to be significantly ($P \leq 0.05$) affected by the change of fatty acid profile regardless of the extraction solvent (**Fig. 2.4**). The range of ORAC values for the lipid-altered soybeans was similar to that of the 'normal' lipid soybeans. The values for the lipid-altered soybeans for 50% acetone extracts ranged from 80.5 to 95.7 trolox equivalents/g soybean, while the values for the 'normal' lipid soybeans ranged from 71.2 to 106.0 trolox equivalents/g soybean. The 70% ethanol extract values were substantially lower than their 50% acetone counterparts, by up to a 2.5-fold difference, suggesting that 50% acetone is a preferred solvent for soybean antioxidant extraction and thus contradicting previous work indicating 70% ethanol soybean extracts to produce higher ORAC values (Xu and Chang, 2007). A difference in extraction protocol (a single extraction with the aid of sonication) may account for the differing results seen in this study. Still, there was no observed correlation between ORAC values and fatty acid profile for either 50% acetone or 70% ethanol extraction. ORAC values for 50% acetone extracts were significantly correlated at the 5% level

to values for TPC, genistein, daidzein, and total isoflavones, while ORAC in the 70% ethanol extracts was correlated only to TPC and total isoflavones.

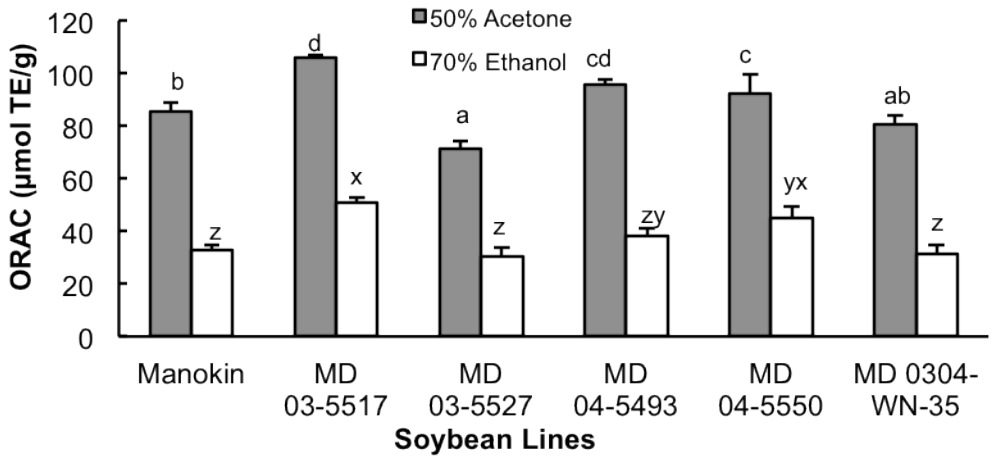


Figure. 2.4. Oxygen radical absorbing capacity (ORAC) of defatted soybean flours.
ORAC values were obtained using fluorescein as the fluorescent probe and a plate reader, with trolox as the standard. Results expressed as µmol trolox equivalents (TE)/g of defatted soybean flour. Tests were conducted in triplicate, with mean values shown and standard deviations depicted by the vertical bars. Columns of the same extraction solvent marked by the same letter did not have significantly different values ($P \leq 0.05$).

2.4.6.3 Hydroxyl radical scavenging capacity

Both 50% acetone and 70% ethanol extracts showed hydroxyl radical scavenging capacities under the experimental conditions (**Fig. 2.5**). The range of HOSC values for the 50% acetone extracts of the lipid altered soybeans, 68.3–103.5 µmoles of trolox equivalents/g soybean, is similar to the range for the 'normal' lipid soybeans from 75.2 to 109.5 µmoles of trolox equivalents/g soybean. The 70% ethanol extracts, re-dissolved in 70% acetone for assay compatibility, had lower values than the 50% acetone extracts, similar to ORAC results.

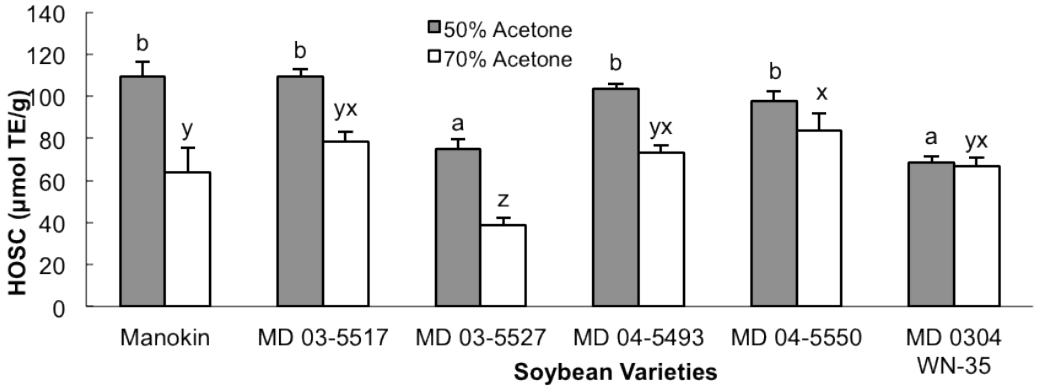


Figure 2.5. HOSC values were obtained through an assay utilizing fluorescein as the fluorescent probe and a plate reader.
The 70% ethanol extract was evaporated and re-dissolved in 70% acetone due to ethanol's incompatibility with the HOSC assay. Results expressed as micromoles trolox equivalents (TE) per gram of defatted soybean flour. Tests were conducted in triplicate, with mean values shown and standard deviations depicted by the vertical bars. Columns of the same extraction solvent marked by the same letter did not have significantly different values ($P \leq 0.05$).

In summary, results from this preliminary study indicate the possibility of obtaining soybean lines with a desirable fatty acid profile for improved oxidative stability and food application without reducing the levels of other beneficial components such as natural antioxidants, tocopherols, carotenoids, and isoflavones. It still remains unclear whether a definite relationship exists between fatty acid composition and the level of one or a group of beneficial phytochemicals of soybean. Additional studies with larger sample sizes and detailed growing conditions are required to fully elucidate these relationship(s).

2.5 Acknowledgements

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Chapter 3: Antioxidant Properties, Phytochemical Composition and Antiproliferative Activity of Maryland-grown Soybeans with Colored Seed Coats

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3.1 Abstract

This study characterized and compared eighteen colored-seed-coat soybeans for the isoflavone, total phenolic and cyanidin-3-glucoside (Cy-3-glc) contents of their flour extracts, and the fatty acid composition and carotenoid and α-tocopherol contents of their oils. Antioxidant assays also assessed activity of the flour extracts against peroxyl, hydroxyl and $ABTS^+$ radicals. Black seed coat soybeans had the highest TPC, ORAC, HOSC, and $ABTS^+$ radical scavenging values, in addition to the highest isoflavone content, and were the only color to contain Cy-3-glc. Then, five soybeans (two black and one each brown, yellow and green) were selected to test their effects on HT-29 human colorectal cancer cell growth. The effects of the hydrolyzed and unhydrolyzed extracts were compared to an aglycone isoflavone standard mixture of the same total molar concentration as the highest soybean concentration of 15 mg seed flour equivalents/mL treatment media. All high doses of hydrolyzed soybean treatments except the green genotype significantly ($P < 0.05$) reduced cell number compared to control at 3 h treatment time, whereas the high dose of isoflavone standard treatment took 72 h to show a significant reduction ($P < 0.05$).

3.2 Introduction

Consumption of soyfoods has been recognized to lower risk of aging associated diseases, including cardiovascular disease and cancer, among others (McCue & Shetty, 2004; Valachovicova et al, 2004; Dixon & Ferreira, 2002). These health benefits have often been studied in relation to a particular soy component. Isoflavones, a class of flavonoids found almost exclusively in legumes and most prominently in soy, have been studied heavily in this regard. There are three aglycone isoflavones—genistein, daidzein, and glycitein. Each aglycone has three derivatives based on the placement of sugar constituents—β-, malonyl-, and acetyl-glucoside. The aglycone forms are absorbed faster by intestinal cells due to their less polar structure. Additionally, enzymes present in the intestine are capable of cleaving the glucosides to their aglycones, thus providing for better absorption. Isoflavones' antioxidant abilities are well known, and their biological activity has been demonstrated in a great number of studies—they inhibit cancer cell growth *in vitro*, prevent tumor development in animal models, inhibit LDL oxidation, and are capable of binding to estrogen receptors and inhibition of bone resorption by osteoclasts, among a list of other activities (Valachovicova et al, 2004; Dixon & Ferreira, 2002). These results suggest that isoflavones may be at least in part responsible for the health benefits associated with soyfood consumption mentioned above.

Interestingly, a few studies indicated that soybeans with black, brown, green, and yellow seed coats might differ in their antioxidant properties, flavonoid levels, total phenolic contents, and proanthocyanidins (Takahashi et al, 2005; Xu et al, 2007; Takahata et al, 2001), indicating that this might alter their ability to affect health.

Soybean extracts (Takahashi et al, 2005; Astadi et al, 2009) and peptides (Rho et al, 2007) have both been shown to reduce the oxidation of LDL cholesterol *in vitro* (Takahashi et al, 2005; Astadi et al, 2009) or in rats (Rho et al, 2007), but black soybeans have been shown to have greater inhibitory effect against lipid peroxidation in human LDL than yellow ones (Takahashi et al, 2005). Additionally, in vitro studies have shown that the isoflavone genistein and anthocyanins are independently capable of inhibiting the growth of cancer cells through various mechanisms (Valachovicova et al, 2004; Kim et al, 2008). Since black soybeans are the only color reported to contain anthocyanins (Xu et al, 2008a; Todd & Vodkin, 1993), and only brown and black contain proanthocyanins, this may result in a differing level or type of bioactivity amongst soybeans with different seed coat colors. Also, these data suggest the possibility to develop novel soybean lines with a selected seed coat color to be used as bioactive ingredients in functional foods targeting different health problems.

The farm value for soybeans has varied greatly in recent years and is highly dependent on factors well beyond the control of farmers, including international demand, weather conditions, and the market supply size (Trostle, 2008). If a high-demand, consistent retail outlet can be established for a novel soybean line because of its special health properties, small farms growing it may be better able to withstand the uncertain market and retain more consistent profitability. As part of our continuous effort to enhance the quality and value of Maryland-grown soybeans, this study aimed to characterize the antioxidant activity and phytochemical composition of Maryland-grown soybeans with various seed coat colors. Furthermore, this study

sought to compare the phytochemical composition of extracts of these soybeans with their bioactivity in HT-29 human colorectal cancer cells, as compared to the activity of treatments of pure isoflavones.

3.3 Materials and Methods

3.3.1 Materials and Chemicals

Whole soybeans of brown, green, yellow and black seed coat colors from the 2007 growing season grown at a single location in Maryland were obtained from Dr. William Kenworthy of the Department of Plant Science and Landscape Architecture, University of Maryland (College Park, MD). In Maryland, 2007 was a drought season, which should be considered in interpreting results due to the well-accepted link between growing conditions and levels of various phytochemicals, including isoflavones and tocopherols.

2,2-azobis (3-ethylbenzothizide-6-sulfonic acid) diammonium salt was manufactured by Calbiochem (San Diego, CA). Iron (III) chloride, fluorescein (FL), biotech grade DMSO, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\cdot}$), genistein, daidzein, cyanidin, and α, δ, and γ-tocopherols were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Thirty percent ACS-grade H_2O_2 was purchased from Fisher Scientific (Fair Lawn, NJ). Lutein, β-carotene, β-

cryptoxanthin, zeaxanthin, and glycitein were obtained from Indofine Chemical Company (Hillsborough, NJ, USA). Cyanidin-3-glucoside was purchased from Polyphenols Laboratory AS (Norway). Ultrapure water was manufactured by Cayman Chemical Company (Ann Arbor, MI, USA) and was used for all experiments. ATP-Lite 1step Luminescence Assay System was obtained from Perkin Elmer (Waltham, MA, USA). All cell culture media components were purchased from Invitrogen (Carlsbad, CA, USA). All other chemicals and solvents were of the highest commercial grade available and used without further purification.

3.3.2 Seed Coat Color

A monolayer of whole, undamaged soybeans was placed in a clean glass sample container and analyzed for Hunter color values L*, a* and b* using a HunterLab ColorFlex (Reston, VA, USA) according to manufacturer directions. Color value was obtained using a D65/10° (daylight 65 illuminant/10° observer) setting. Triplicate measurements were taken, with seeds gently shaken in between each reading.

3.3.3 Oil Extraction

Whole soybeans were ground in a standard household coffee grinder to fit through a 20-mesh sieve. Five grams of ground soybeans were extracted at room temperature in 10 mL of petroleum ether (BP 35-60 °C) a total of four times. The petroleum ether was evaporated in a nitrogen evaporator to a constant weight. The oils were stored at ambient temperature under nitrogen in the dark until testing.

3.3.4 Antioxidant Extraction

The soy flour remaining after oil extraction was then extracted with 50% acetone at a ratio of 1 g:10 mL. The solvent was chosen according to the results of Xu and Chang (Xu & Chang, 2007) and to coincide with our previous work on soybeans (Slavin et al, 2009). The tubes were stirred to disturb the unwetted portion at the bottom, blown off with nitrogen, vortexed three times for 15 seconds each, and allowed to extract overnight in the dark. The soy flour-solvent mixtures were then sonicated for 15 minutes, and the extracts were filtered through 0.45 μm syringe filters. The extracts were held under nitrogen in the dark until testing.

3.3.5 Fatty Acid Composition

Fatty acid methyl esters (FAME) were prepared from the oils by saponification followed by methylation according to a previously described laboratory protocol and subjected to gas chromatography (GC) analysis (Yu et al, 2003). A Shimadzu GC-2010 with an FID, an AOC-20i injector and an AOC-20S autosampler (Shimadzu, Columbia, MD) was used for fatty acid analysis. The carrier gas was helium at a flow rate of 2.2 mL/min through a fused silica capillary column SP^{TM}-2380 (30 m × 0.25 mm with a 0.25 μm film thickness) from Supelco (Bellefonte, PA). Injection volume was 1 μL at a split ratio of 10/1. Oven temperature was initially 136 ºC, increased by 6 ºC/min until 184 ºC where it was held for 3 min, then increased again by 6 ºC/min to a final temperature of 226 ºC. Individual fatty acids were identified through comparison of GC retention time with

those of fatty acid methyl ester external standards. Quantification was based on the area under each fatty acid peak as compared to the total area of all fatty acid peaks.

3.3.6 Carotenoid and α-tocopherol Contents

Oil samples and standards were dissolved in hexane and analyzed via normal phase liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (NP-LC-APCI-MS/MS) according to Hao et al. (Hao et al, 2005). Briefly, a Zorbax RX-SIL column, 2.1 mm i.d. × 150 mm, 5 μm particle size (Agilent Technologies, Palo Alto, CA, USA) was used at ambient temperature. Separation was achieved through gradient elution with a flow of 0.5 mL/min, with hexane for solvent A and 1% isopropanol in ethyl acetate for solvent B. A 5 min linear gradient from 1 to 10% solvent B was followed by a 15 min linear gradient from 10 to 50% B. The column was allowed to re-equilibrate at initial conditions for 10 minutes prior to injection of the next sample. Injection volume was 5 μL. Quantification was done using total ion counts compared to that of external standards.

3.3.7 Total Phenolic Contents (TPC)

The TPC of each soybean extract was measured according to a laboratory procedure described previously (Yu et al, 2002). The reaction mixture was comprised of 100 μL of 50% acetone soybean extract, 500 μL of Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL ultrapure water. Absorbance was read at 765 nm on a Thermo Spectronic (Waltham, MA, USA) Genesys spectrophotometer after 2 hours of reaction at ambient temperature. Different concentrations of gallic

acid were used to create the standard curve. Reactions were conducted in triplicate and results were reported as mg gallic acid equivalents (GAE) per gram of soybean flour.

3.3.8 Isoflavone Composition

50% acetone soybean extracts were hydrolyzed with acid, re-dissolved in methanol, and filtered through a 0.45 μm syringe filter prior to being subjected to HPLC analysis. The protocol by Lee et al. (2009) was followed with modifications. The column used was a Phenomenex (Torrance, CA, USA) Gemini C18 column (150 mm × 4.6 mm × 5.0 μm) and was housed in an oven set to 40 °C. A binary solvent system was employed with solvent A consisting of water-acetic acid at a ratio of 99.9:0.1 (v/v) and solvent B consisting of acetonitrile-acetic acid (99.9:0.1, v/v). The gradient changed linearly from 75:25 (A:B, v/v) to 67:33 (A:B, v/v) from 0 to 20 minutes, then was returned to initial conditions for 5 minutes to re-equilibrate the column prior to the next run. The flow rate was 1 mL/min, injection volume was 10 μL, and the detection wavelength was set to 254 nm. The isoflavones were identified and quantified via comparison to external standards.

3.3.9 Cyanidin-3-glucoside Contents

Cyanidin-3-glucoside (Cy-3-glc) was determined since it is the predominant anthocyanin present in black soybean seed coats (Xu & Chang, 2008a; Lee et al, 2009; Choung et al, 2001). An HPLC protocol by Lee and colleagues (Lee et al, 2009) was adapted for use on a Shimadzu Prominence UFLC system (Columbia, MD,

USA) with autosampler, in-line degasser, CTO-20AC oven and SPD-20A UV/Vis detector. A gradient flow of 1.0 mL/min was employed on a Phenomenex (Torrance, CA, USA) Gemini C18 column (150 mm × 4.6 mm × 5.0 μm) in an oven set to 25 °C. Solvent A consisted of 0.1% acetic acid in water, while solvent B consisted of 0.1% acetic acid in acetonitrile. The gradient changed linearly from 100:0 (A:B, v/v) to 85:15 (A:B, v/v) over 10 minutes, then to 75:25 (A:B, v/v) over another 10 minutes. Then, a 5 minute wash at 10:90 (A:B, v/v) was performed, after which the column was re-equilibrated at 100:0 (A:B, v/v) for 5 minutes. Absorbance was read at 530 nm and injection volume was 20 μL of unhydrolyzed extract. Cy-3-glc was identified and quantified via comparison to peak area of an external standard.

3.3.10 Antioxidant Activity Assays

3.3.10.1 $ABTS^{+\bullet}$ Scavenging Ability

The scavenging capacity against $ABTS^{+\bullet}$ was measured according to a previously reported method (Moore et al, 2009). Briefly, $ABTS^{+\bullet}$ working solution was prepared by reacting 2,2-azobis (3-ethylbenzothizide-6-sulfonic acid) diammonium salt with manganese oxide (MnO_2) in solution, which was then filtered and diluted to an absorbance of 0.700 ± 0.005 at 734 nm on a Thermo Spectronic (Waltham, MA, USA) Genesys spectrophotometer. 80 μL sample or standard was added to 1 mL of the working $ABTS^{+\cdot}$ solution, vortexed for 30 sec and absorbance was read at 734 nm after 90 sec of reaction. Trolox standards in 50% acetone were used to create a standard curve and samples were diluted as necessary to fit on the curve.

3.3.10.2 Oxygen Radical Absorbance Capacity (ORAC) Assay

The ORAC assay was conducted according to a previously reported laboratory protocol (Moore et al, 2005) with minor modifications. Trolox standards were prepared in 50% acetone, while all other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). The initial reaction mixture contained 225 μL freshly made 8.16×10^{-8} M fluorescein and 30 μL sample, standard or blank. Initial reaction mixtures were pipetted into a 96-well plate and pre-heated at 37 °C for 20 minutes. Then, 25 μL of freshly made 0.36 M AAPH was added to each well. Fluorescence of the assay mixture was recorded on a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) once every two minutes for three hours at 37 °C, with λ_{Ex} = 485 nm and λ_{Em} = 535 nm. Trolox equivalents (TE) were calculated for samples based on area-under-the-curve (AUC) calculations used by Ou and others (Ou et al, 2001). Results are expressed as micromoles of TE per gram of defatted soybean flour.

3.3.10.3 Hydroxyl Radical Scavenging Capacity (HOSC) Estimation

The HOSC assay was conducted also using fluorescein as the fluorescent probe and a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) according to a previously reported laboratory protocol (Moore et al, 2006). The reaction mixture contained 170 μL of 9.28×10^{-8} M fluorescein, 30 μL sample, 40 μL of 0.1990 M H_2O_2 and 60 μL of 3.43 M $FeCl_3$. The fluorescence of the reaction mixture was recorded approximately once every four minutes for seven hours at ambient temperature, with λ_{Ex}= 485nm and λ_{Em} = 535 nm. Standards were prepared in 50% acetone. The 9.28×10^{-8} M fluorescein was prepared fresh for each assay from stock

solution and 75 mM sodium phosphate buffer (pH 7.4). Trolox equivalents (TE) were calculated for samples using the same AUC calculations as in ORAC (Moore et al, 2006). Results are expressed as micromoles of TE per gram of defatted soybean flour.

3.3.11 Anti-proliferative Activity Against HT-29 Cells

The anti-proliferation test was adopted from Wang et al. (Wang et al, 2007). HT-29 human colorectal adenocarcinoma cells were cultured in a humidified incubator at 37 °C and 5% CO_2. Cell culture media consisted of McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution.

Cells were grown to 95% confluence, then plated at 2,500 cells/well in a black 96-well viewplate treated for tissue culture. After 24 hours, the media was replaced with 100 μL control or treatment media. Proliferation of cells was assessed using the ATPlite 1step Luminescence Assay System from Perkin Elmer (Waltham, MA, USA) prior to treatment and at 3, 24, 48, 72, and 96 h after initial treatment. To take a reading, plates were allowed to equilibrate at room temperature for 30 min, then 100 μL of reconstituted ATPlite solution was added to each well immediately prior to taking luminescence readings on a Victor^3 multi-well plate reader (Perkin Elmer, Turku, Finland). A separate plate was used for each reading. Treatment and control media were replaced every 24 hours until a reading was taken on that plate.

All treatment media contained 1.2% DMSO, including the control, and were filtered through a 0.2 μm retrograde cellulose syringe filter prior to treatment of cells.

For the study of the antiproliferative activity of soybean extracts, cells were treated with hydrolyzed or unhydrolyzed soybean extracts in three doses: 1.5, 5 and 15 mg seed flour equivalents/mL media. All treatment media, including the control, contained a final concentration of 1.2% DMSO (v/v). Isoflavone compositions of the hydrolyzed extracts were calculated for genistein, daidzein and glycitein based on HPLC data and are shown in **Table 3.1**. Since the unhydrolyzed extracts had not had their glucoside isoflavones hydrolyzed to their aglycone forms, it is likely their isoflavones were mainly in the glucoside form. However, all 12 isoflavone forms were not quantified in unhydrolyzed extracts. For the unhydrolyzed extracts, the values in **Table 3.1** therefore represent isoflavone "aglycone equivalents". For comparison purposes, a standard isoflavone treatment was tested on the cells. It included a mixture of genistein, daidzein and glycitein (each ≥99% pure and purchased from Sigma-Aldrich or Indofine), and the concentration of isoflavones in the mixture was equivalent to that of Pi 88788, which had the highest isoflavone contents of soybean extracts tested on cells.

For the study of the antiproliferative activity of isoflavones, cells were treated with the aglycone isoflavones (genistein, daidzein and glycitein) individually and in all possible combinations at three doses each. Again, the final concentration of DMSO in the treatment media was 1.2%. Doses were chosen to be concentrations equivalent to the high dose of soybean Pi 88788. Actual doses and combinations can be seen in **Table 3.2**.

Another study was conducted to test the antiproliferative effect of an isoflavone and cyanidin mixture which mimic the concentration of both isoflavones

and cyanidin in Peking and Pi88788 extracts. The isoflavone and cyanidin concentrations of these two soybeans were re-created with standard compounds, with final treatment media again containing 1.2% DMSO and anti-proliferative effects were again compared to a control with 1.2% DMSO. Actual concentrations are shown in **Table 3.2**.

3.3.12 Statistical Analysis

Tests were conducted in triplicate with data reported as mean ± standard deviation. Differences in means were detected using one-way ANOVA and Tukey's test. For those instances where average values were calculated for a seed coat color group, differences between color groups were determined using one-way ANOVA with the Tukey-b post hoc option, allowing for the comparison of unequal sample sizes. Correlation among means were determined using a two-tailed Pearson correlation test. Statistics were analyzed using SPSS for Windows (version rel. 10.0.5, 1999, SPSS Inc., Chicago, IL). Statistical significance was defined at $P \leq 0.05$.

Table 3.1 Isoflavone composition of soybean extract treatments at three concentrations (μM)*

	Low Dose			Medium Dose			High Dose		
	Daid	**Gen**	**Gly**	**Daid**	**Gen**	**Gly**	**Daid**	**Gen**	**Gly**
Emerald	0.5	0.7	0.9	1.6	2.3	3.0	4.8	6.9	8.9
MD 0304 WN-46	0.4	0.4	1.2	1.2	1.5	4.0	3.6	4.5	12.0
MD 0304 WN-46-1	0.2	0.3	0.7	0.6	1.0	2.2	1.7	2.9	6.7
Peking	1.8	1.2	2.1	5.9	4.0	7.0	17.7	12.1	20.9
Pi 88788	1.2	1.4	2.5	4.1	4.5	8.5	12.2	13.6	25.4
Isoflavone mixture	1.2	1.4	2.5	4.0	4.5	8.5	12.0	13.6	25.4

Daid = daidzein; Gen = genistein; Gly = glycitein.
Low, medium and high doses represent concentrations of 1.5, 5 and 15 mg seed flour equivalents/mL media. Concentrations of isoflavones in treatment media were calculated by HPLC analysis of the original 50% acetone extracts.

Table 3.2. Isoflavone composition of isoflavone treatment media concentrations.*

	Daid	**Gen**	**Gly**	**Cy**
		(μM)		
Control	0	0	0	0
Daid only	12.0	0	0	0
Gen only	0	13.6	0	0
Gly only	0	0	25.4	0
Daid + Gen	12.0	13.6	0	0
Daid + Gly	12.0	0	25.4	0
Gen + Gly	0	13.6	25.4	0
Daid + Gen + Gly	12.0	13.6	25.4	0
Pi88788 mimic	12.0	13.6	25.4	7.0
Peking mimic	17.7	12.1	20.9	17.7

* Daid = daidzein; Gen = genistein; Gly = glycitein; Cy = cyanidin.

3.4 Results and Discussion

3.4.1 Seed Coat Color

In the typical yellow soybean, chlorophyll disappears as seeds mature and dry in the field, allowing the remaining flavonoid pigments' color to show. Some soybeans do not lose their chlorophyll as they dry and remain green at maturation, while others have additional pigments, including anthocyanins and proanthocyanins, that impart deep black and brown seed coat colors. It is these pigments that are believed to contribute at least in part to the antioxidant capacity differences discussed later.

Seed coat color values are shown in **Table 3.3**. The L* reading is an indicator of blackness of the sample, with closer to zero being more black. Appropriately, the black seed coat L* values are in the vicinity of 20, whereas the others are higher: brown are around 33, green are around 50 and yellow are around 60. Negative a* readings indicate a green color, whereas positive a* readings indicate a red color. As expected, the green seed coats have the most negative a* value, and the brown have the highest positive a* value. Lastly, b* represents the yellow to blue continuum, with positive values indicating yellow and negative values indicating blue. The yellow seed coats have the highest positive b*, while the black seed coats have the lowest value.

Table 3.3. HunterLab Color Values of Soybeans

	L*	a*	b*
		Black	
MD 06-5437-1	19.6 ± 0.3	0.2 ± 0.2	1.0 ± 0.2
MD 06-5440-2	16.5 ± 0.44	0.1 ± 0.2	-0.5 ± 0.1
Peking	22.7 ± 0.3	0.0 ± 0.0	0.3 ± 0.0
Pi 88788	22.3 ± 0.2	0.0 ± 0.1	0.0 ± 0.1
Pi 90763	19.3 ± 0.6	0.0 ± 0.1	-0.6 ± 0.1
		Brown	
MD 0304 WN46-1	33.9 ± 0.2	11.6 ± 0.2	14.5 ± 0.1
MD 0304 WN46-2	33.3 ± 0.4	11.4 ± 0.3	14.4 ± 0.6
MD 0304 WN46-3	33.6 ± 0.5	11.4 ± 0.3	13.9 ± 0.6
MD 0304 WN46-4	32.7 ± 1.0	10.9 ± 0.6	13.5 ± 0.7
		Green	
Emerald	49.6 ± 1.5	-3.4 ± 0.2	24.6 ± 0.6
Verde	51.4 ± 1.1	-3.4 ± 0.5	24.9 ± 0.3
		Yellow	
MD 0304 WN-46	61.1 ± 0.7	7.9 ± 0.4	30.8 ± 0.5
MD 05-6073	63.7 ± 0.5	6.7 ± 0.1	29.3 ± 0.2
MD 05-6077	62.4 ± 0.4	6.9 ± 0.3	30.6 ± 1.1
MD 05-6079	62.4 ± 0.5	6.5 ± 0.2	29.7 ± 0.4
MD 06-5433-1	60.7 ± 1.5	7.2 ± 0.2	34.6 ± 1.8
MD 06-5433-3	60.7 ± 1.5	6.6 ± 0.1	30.7 ± 0.5
MD 06-5445-5	58.3 ± 1.3	6.0 ± 0.1	30.9 ± 1.0

Values based on triplicate readings. Mean ± standard deviation shown. (n = 3).

3.4.2 Fatty Acid Composition

As shown in **Table 3.4,** oils from soybeans with different seed coat color might differ significantly in their fatty acid compositions ($P \leq 0.05$). This is relevant due to the considerable efforts dedicated to altering fatty acid composition of soybeans oils for specific uses: low-linolenic cultivars have been sought due to their improved oxidative stability, while other cultivars (including low-saturated or high-linolenic) have been sought for their health-promoting attributes.

The black genotypes had the lowest 18:1 contents and the highest average 16:0 level, along with relatively high levels of total saturated fatty acids (SFA) around 15% of total fatty acids and on average the highest 18:2 and 18:3 contents (**Table 3.4**). The brown genotypes had the highest 18:1 content ranging 41.0-41.5% of total fatty acids in their oils, along with around 10% SFA and low 18:3 contents at 3%, with higher 18:1 and 18:2 to compensate. The two green genotypes had SFA at 13 and 14.5% and an average 18:3 content of 8%. This 18:3 level is comparable to that of 8.0-10.5% detected in the soybeans with black coats (**Table 3.4**). Finally, the most variation in fatty acid composition was seen among the yellow genotypes, with SFA ranging from 7 to 23%, 18:1 from 22 to 35%, 18:2 from 39 to 54% and 18:3 from 3 to 9%. In summary, black coated soybeans may serve as dietary source for n-3 fatty acid, whereas soybeans with brown coats may be a candidate for future breeding effort to develop high oleic and low linolenic and saturated fatty acids lines.

Table 3.4. Fatty Acid Composition of Soybean Oil by Seed Coat Color (g/100 g oil).

	16:0	18:0	Total SFA	18:1	18:2	18:3	Total PUFA
				Black			
MD 06-5437-1	11.27i ± 0.05	3.91d ± 0.03	15.18k ± 0.03	21.26c ± 0.07	52.35ij ± 0.12	8.48k ± 0.06	60.94j ± 0.17
MD 06-5440-2	11.44j ± 0.05	4.05e ± 0.01	15.49m ± 0.05	22.99d ± 0.04	50.63g ± 0.05	7.99i ± 0.01	58.66h ± 0.04
Peking	11.21i ± 0.08	3.46b ± 0.05	14.67i ± 0.04	17.13a ± 0.04	55.14p ± 0.12	10.78p ± 0.07	66.03o ± 0.18
Pi 88788	11.41j ± 0.02	3.48b ± 0.03	14.89j ± 0.02	18.72b ± 0.05	53.57m ± 0.06	10.27n ± 0.03	63.89m ± 0.05
Pi 90763	11.64k ± 0.02	3.03a ± 0.03	14.67i ± 0.01	18.16b ± 0.03	53.93no ± 0.11	10.45o ± 0.02	64.49n ± 0.11
Black average	11.39z ± 0.16	3.59yz ± 0.38	14.98z ± 0.33	19.65w ± 2.23	53.12z ± 1.59	9.59z ± 1.17	62.71z ± 2.73
				Brown			
MD 0304 WN46-1	5.86b ± 0.02	4.24gh ± 0.05	10.10c ± 0.05	41.48j ± 0.04	42.84bc ± 0.08	3.10a ± 0.05	46.02b ± 0.11
MD 0304 WN46-2	6.19d ± 0.02	4.11ef ± 0.03	10.30d ± 0.03	41.02j ± 0.04	43.13d ± 0.03	3.31bc ± 0.07	46.47c ± 0.05
MD 0304 WN46-3	6.05c ± 0.01	4.19fg ± 0.03	10.24cd ± 0.04	41.01j ± 0.08	42.92cd ± 0.09	3.27bc ± 0.05	46.28bc ± 0.13
MD 0304 WN46-4	6.14cd ± 0.06	4.36h ± 0.03	10.49e ± 0.08	41.07j ± 0.10	42.63b ± 0.07	3.23b ± 0.01	45.93b ± 0.07
Brown average	6.06x ± 0.13	4.22z ± 0.10	10.28y ± 0.15	41.15z ± 0.21	42.88y ± 0.20	3.23w ± 0.09	46.11x ± 0.25
				Green			
Emerald	9.58e ± 0.07	3.45b ± 0.03	13.03f ± 0.05	23.51de ± 0.11	52.53jk ± 0.09	8.33j ± 0.03	60.96j ± 0.12
Verde	10.97h ± 0.08	3.51bc ± 0.03	14.48h ± 0.08	26.08f ± 0.13	48.88f ± 0.19	7.76h ± 0.04	56.83f ± 0.16
Green average	10.28y ± 0.76	3.48y ± 0.04	13.76yz ± 0.73	24.80x ± 1.41	50.71z ± 2.01	8.05y ± 0.31	58.75y ± 2.31
				Yellow			
MD 0304 WN-46	6.04c ± 0.04	3.86d ± 0.01	9.90b ± 0.04	36.03i ± 0.06	48.31e ± 0.18	3.37c ± 0.01	51.87d ± 0.18
MD 05-6073	3.54a ± 0.04	3.61c ± 0.02	7.15a ± 0.05	33.26g ± 0.02	53.68mn ± 0.05	3.76e ± 0.92	57.50g ± 0.07
MD 05-6077	3.55a ± 0.2	3.61c ± 0.02	7.16a ± 0.02	33.11g ± 0.07	53.99o ± 0.05	3.52d ± 0.01	57.56g ± 0.05
MD 05-6079	3.59a ± 0.03	3.49bc ± 0.01	7.08a ± 0.02	34.79h ± 1.11	51.36h ± 0.03	3.88f ± 0.01	55.27e ± 0.02
MD 06-5433-1	16.60m ± 0.04	6.08i ± 0.02	22.68n ± 0.04	23.86e ± 0.04	38.98a ± 0.05	5.92g ± 0.01	44.95a ± 0.04
MD 06-5433-3	10.61g ± 0.03	3.88d ±0.11	14.50h ± 0.10	22.71d ± 0.04	52.23i ± 0.07	8.04i ± 0.03	60.34i ± 0.07
MD 06-5445-5	10.36f ± 0.02	3.48b ± 0.07	13.84g ± 0.05	21.71c ± 0.02	52.73k ± 0.04	9.07m ± 0.05	61.84k ± 0.08
Yellow average	7.76xy ± 4.72	4.00yz ± 0.88	11.76yz ± 5.46	29.35y ± 5.96	50.18z ± 5.02	5.37x ± 2.24	55.55y ± 5.44

* Values are based on triplicate readings. Mean ± SD shown. Individual sample values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). Average values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). SFA = saturated fatty acids; PUFA = polyunsaturated fatty acids.

3.4.3 Carotenoid and α-Tocopherol Contents

Lutein, β-carotene, cryptoxanthin and zeaxanthin contents of the soybean oils are summarized in **Table 3.5.** Lutein was the primary carotenoid compound present in the soybeans regardless of seed coat color, and zeaxanthin was also detected in most of the tested soybean oils. The greatest lutein content was 907 μg/g in the oil of MD 05-6079 yellow soybean, and MD 05-6077 yellow soybean oil had no detectable lutein or zeaxanthin. The highest zeaxanthin content was 217 μg/g in the oil of Pi 90763 black soybean oil, and black soybean oils contained, on average, higher zeaxanthin than soybeans with other color coats (**Table 3.5**). The oil of Pi 90763 and MD 06-5437-1 black soybean lines had all four carotenoid compounds, but the oil of MD 05-6079 yellow soybean line had the highest total carotenoid content of 1.62 μmol/g oil, and followed by that of 1.38 μmol/g oil detected in the oil of Pi 90763 soybeans with black seed coats (**Table 3.5**). The average total carotenoid content was about 0.76, 0.48, 0.45, and 0.09 μmol/g oil for soybeans with black, yellow, brown, and green coats, with significant standard deviations existing (0.35, 0.48, 0.35, and 0.09 μmol/g oil, respectively). The yellow genotypes contained the widest variation in carotenoid content from 0.005 to 1.621 μmol/g oil. It was also noted that the oil of Emerald green soybean contained no detectable carotenoids, and the other green coated soybean cultivar (Verde) contained less than 0.2 μmol total carotenoids/g in its oil. However, two samples are not enough to determine whether a significant trend exists.

Table 3.5. Carotenoid and α-tocopherol Content of Soybeans by Seed Coat Color.*

	α-tocopherol	Lutein	β-carotene	Cryptoxanthin	Zeaxanthin	Total Carotenoids
			(μg/g oil)			(μmol/g oil)
			Black			
MD 06-5437-1	526.6hi ± 39.6	171.8b ± 3.7	30.4a ± 3.3	6.9f ± 0.6	95.3e ± 2.1	0.539fg ± 0.011
MD 06-5440-2	1083.8m ± 97.0	191.8b ± 4.4	ND	ND	60.0d ± 3.2	0.443ef ± 0.013
Peking	144.1ab ± 5.3	270.7d ± 16.4	ND	ND	62.5d ± 3.6	0.586g ± 0.034
Pi 88788	744.1jk ± 75.7	375.9e ± 8.2	ND	1.9cd ± 0.4	119.0f ± 1.4	0.874h ± 0.013
Pi 90763	360.8fg ± 17.3	510.3f ± 17.7	49.1b ± 1.8	6.4f ± 0.4	217.4g ± 10.4	1.382i ± 0.037
			Brown			
MD 0304 WN46-1	282.7def ± 6.5	201.4bc ± 8.6	ND	ND	45.0c ± 1.7	0.433e ± 0.013
MD 0304 WN46-2	262.1cdef ± 6.5	391.9e ± 12.8	ND	3.1e ± 0.4	89.7e ± 2.5	0.852h ± 0.024
MD 0304 WN46-3	308.9ef ± 1.7	64.8a ± 2.7	ND	ND	10.6a ± 0.9	0.133b ± 0.005
MD 0304 WN46-4	240.7bcde ± 10.5	178.4b ± 9.2	ND	ND	37.9c ± 1.4	0.380de ± 0.016
			Green			
Emerald	250.0cde ± 6.7	ND	ND	ND	ND	0.000a ± 0.000
Verde	431.3gh ± 26.7	82.4a ± 6.7	ND	ND	14.4a ± 0.9	0.170b ± 0.013
			Yellow			
MD 0304 WN-46	332.7efg ± 15.8	226.9bcd ± 17.0	ND	0.5ab ± 0.3	27.0b ± 1.0	0.447ef ± 0.032
MD 05-6073	161.8abc ± 6.3	80.4a ± 4.7	ND	ND	ND	0.141b ± 0.008
MD 05-6077	109.9a ± 7.5	ND	ND	2.9de ± 0.4	ND	0.005a ± 0.001
MD 05-6079	201.3abcd ± 14.4	906.5g ± 64.0	ND	1.3bc ± 0.2	14.4a ± 1.1	1.621j ± 0.111
MD 06-5433-1	618.7ij ± 23.2	66.9a ± 5.9	36.4a ± 3.4	ND	19.3ab ± 1.2	0.219bc ± 0.015
MD 06-5433-3	677.8jk ± 24.7	99.5a ± 3.7	ND	2.3de ± 0.3	60.6d ± 1.4	0.286cd ± 0.009
MD 06-5445-5	121.0a ± 8.4	256.9cd ± 6.9	ND	2.4de ± 0.2	86.8e ± 2.7	0.608g ± 0.016

* Values based on triplicate readings. Mean ± SD shown. Values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). ND = not detected.

The oils from soybeans with different color coats were also evaluated for their α-tocopherol contents (**Table 3.5)**. All oils contained α-tocopherol, with the low value coming from the yellow MD 05-6077 at 109.9 μg/g oil and the highest value coming from the black MD 06-5440-2 at 1083.8 μg/g oil. No specific trend was apparent between α-tocopherol content and seed coat color, although the average level of α-tocopherol was 572, 341, 318, and 274 μg/g oil for black, green, yellow, and brown soybean genotypes, again with high standard deviations (352, 101, 232, and 27 μg/g oil, respectively. Soybean oil with higher carotenoid content did not necessarily contain higher α-tocopherol. These data indicated that soybeans with different coat color generally contain comparable levels of carotenoids and α-tocopherol, however, black soybeans contained the highest average amounts of both.

Some carotenoids (including beta-carotene and cryptoxanthin) are forms of pro-vitamin A, though the soybeans in the present study were low in these components. The predominant carotenoid detected was lutein, and consumption of lutein has protective effects against a variety of ocular diseases. Tocopherols are members of the Vitamin E family. Both carotenoids and tocopherols are lipophilic antioxidants and may have protective effects against oxidative damage for oils during storage and cellular components after ingestion/absorption.

3.4.4 Isoflavone Composition

Isoflavone compositions of these soybeans with different seed coat colors were compared using the hydrolyzed 50% acetone extracts of the de-fatted soy.

Genistein, daidzein and glycitein were detected in all soybean flours with glycitein as the predominating isoflavone in each sample (**Table 3.6)**. The black soybeans Peking, Pi 88788 and Pi 90763 had significantly higher total isoflavones among all soybeans tested ($P \leq 0.05$). Soybeans with a black seed coat had an average level of total isoflavone content of 2.1 μmol/g whole soybean, which was much higher than that of 1.0, 0.8, and 0.7 μmol/g whole bean observed in the green, brown, and yellow soybeans, respectively (**Table 3.6**). This observation was supported by a trend seen in two previous reports using 50% acetone as the extraction solvent, where total flavonoid contents were 3-to-4-fold higher in black soybeans than yellow (Xu et al, 2007; Xu & Chang, 2007). However, two other previous studies reported black soybeans to contain fewer isoflavones than yellow soybeans, though these studies used a different solvent system and detection method (acetonitrile-water and HPLC), which limits their relevance for direct comparison to this study (Xu and Chang, 2008a & b*)*. It is recognized that the isoflavone values of this study may be lower than ranges of values presented in other analyses, a difference believed to be mainly due to the extraction conditions.

It was also noted that soybeans with same seed coat color may differ significantly ($P \leq 0.05$) in their total isoflavone contents and isoflavone compositions (**Table 3.6**). For instance, Pi 88788 and Peking black soybean samples had significantly higher total isoflavone content than the other three black soybeans ($P \leq 0.05$). Peking black soybeans had about same amount of glycitein and daidzein, whereas Pi 88788 black soybeans had twice as much glycitein than daidzein, although total isoflavone contents did not differ between the two (**Table 3.6**). Though overall

trends may exist in isoflavones based on color, it cannot be assumed that any one color contains a specific level. Consequently, the genotype of soybean for use in a high-isoflavone food must still be evaluated individually and not solely by seed coat color.

Table 3.6. Isoflavones and Cyanidin-3-glucoside Detected in Soybeans by Seed Coat Color.*

	Daidzein	Genistein	Glycitein	Total Isoflavones	Cy-3-Glc
	(μmol/g flour)				(mg/g flour)
			Black		
MD 06-5437-1	0.18bc ± 0.00	0.16ab ± 0.00	0.47def ± 0.00	0.80cd ± 0.00	0.03a ± 0.01
MD 06-5440-2	0.30f ± 0.00	0.32c ± 0.00	0.53gh ± 0.01	1.15g ± 0.01	0.25c ± 0.02
Peking	1.18i ± 0.03	0.81e ± 0.08	1.39k ± 0.03	3.38j ± 0.12	0.57e ± 0.02
Pi 88788	0.81g ± 0.01	0.91f ± 0.01	1.69m ± 0.02	3.41j ± 0.03	0.22b ± 0.01
Pi 90763	0.94h ± 0.01	0.78e ± 0.00	1.41k ± 0.01	3.13i ± 0.01	0.30d ± 0.02
			Brown		
MD 0304 WN46-1	0.11a ± 0.00	0.20b ± 0.01	0.45de ± 0.01	0.76bc ± 0.02	ND
MD 0304 WN46-2	0.19bcd ± 0.00	0.28c ± 0.00	0.52hi ± 0.00	0.98f ± 0.01	ND
MD 0304 WN46-3	0.19cd ± 0.00	0.28c ± 0.01	0.48efg ± 0.01	0.95ef ± 0.02	ND
MD 0304 WN46-4	0.19cd ± 0.00	0.27c ± 0.00	0.44cde ± 0.01	0.90ef ± 0.01	ND
			Green		
Emerald	0.32f ± 0.00	0.46d ± 0.02	0.59i ± 0.01	1.37h ± 0.01	ND
Verde	0.26e ± 0.01	0.31c ± 0.01	0.38ab ± 0.01	0.95ef ± 0.03	ND
			Yellow		
MD 0304 WN-46	0.24e ± 0.00	0.30c ± 0.00	0.80j ± 0.01	1.34h ± 0.01	ND
MD 05-6073	0.12a ± 0.01	0.13a ± 0.00	0.34a ± 0.05	0.60a ± 0.05	ND
MD 05-6077	0.12a ± 0.00	0.17ab ± 0.00	0.36ab ± 0.00	0.65ab ± 0.00	ND
MD 05-6079	0.19cd ± 0.00	0.20b ± 0.00	0.50fgh ± 0.01	0.89ef ± 0.01	ND
MD 06-5433-1	0.22d ± 0.00	0.21b ± 0.00	0.50efg ± 0.01	0.93de ± 0.01	ND
MD 06-5433-3	0.17b ± 0.00	0.17ab ± 0.00	0.41abc ± 0.01	0.75bc ± 0.00	ND
MD 06-5445-5	0.17b ± 0.00	0.20ab ± 0.00	0.42bcd ± 0.00	0.79bcd ±	ND

* Values based on triplicate readings. Mean ± SD shown. Values in the same column marked by the same letter are not significantly different ($P \leq 0.05$). ND = not detected

These data indicated that black soybeans may serve as an excellent dietary source of isoflavones, which have been shown to slow or inhibit the development of a variety of chronic diseases (cardiovascular disease, various cancers, among others), in addition to their ability to lessen the symptoms of menopause. Because of this, black soybeans may serve as a valuable ingredient in functional foods targeting these diseases and life stage.

3.4.5 Cyanidin-3-Glucoside Contents

Recently, nine anthocyanins were characterized in the seed coat of black soybeans, and cyanidin-3-glucose (Cy-3-glc) was shown to be the predominant anthocyanin compound in black soybean seed coats and was detected in whole black soybeans (Xu & Chang, 2008a; Lee et al, 2009; Choung et al, 2001). In the present study, all soybean samples were examined for their Cy-3-glc contents, which were detected in all soybeans with black seed coats, but not in other colors. As shown in **Table 3.6**, Peking contained the highest amount at 0.57 mg/g flour and was almost double the next nearest sample, Pi 90763. Though Peking contained the most Cy-3-glc of the soybeans tests, Choung and others (Choung et al, 2001) reported its anthocyanin content to be in the middle of a range of ten black soybeans tested with acidified MeOH as the extraction solvent. Data in **Table 3.6** also suggested the huge possible variation of anthocyanins among black soybean cultivars and lines.

Soybeans with black-colored seed coats have long been recognized by various Eastern cultures to have health beneficial properties, with attributions of anti-inflammation and anti-toxic activity (Kim et al, 2008). In various studies,

anthocyanins of black soybean seed coats were able to suppress lipid peroxidation in human low density lipoproteins (LDL), inhibit the proliferation of HT-29 human colon cancer cells, and reduce cyclooxygenase-2 (COX-2) mRNA level and the number of aberrant crypt foci numbers in F344 rats (Astadi et al, 2009; Kim et al, 2008). Black soybean anthocyanins have also been shown to mediate the damage incurred by ischemic myocardial infarction in rats (Kim et al, 2006) and by ultraviolet B radiation in both human and mice skin cells (Tsoyi et al, 2008). Various components of black seed coat soybeans have been implicated as having anti-obesity properties, including peptides (Rho et al, 2007; Kim et al, 2007) and anthocyanins (Kwon et al, 2007). Taken together, identification and/or development of black soybean lines rich in anthocyanins may well be an approach to enhance their farm gate value through use in health beneficial functional foods.

3.4.6 Total Phenolic Content (TPC) and Antioxidant Activities

It is prudent to perform a variety of antioxidant activity tests on samples in order to gain a broad perspective of the antioxidant properties of the samples. TPC, ORAC, HOSC, and ABTS radical scavenging were chosen for this study because of their activities as discussed here: The TPC assay is used to compare the reducing power of a sample compared to a standard phenolic, in this case gallic acid. ORAC is perhaps the most commonly used antioxidant activity assay and utilizes competitive kinetics between a fluorescent probe and the sample's antioxidants to provide information on a sample's ability to scavenge peroxyl radicals through a hydrogen atom transfer. The HOSC assay is a relatively new assay compared to ORAC, but

uses a similar method to estimate a sample's ability to scavenge hydroxyl radicals. ABTS radical cation scavenging method measures the ability of a sample to reduce the non-physiological radical.

All soybeans sampled contained significant levels of phenolics, and the black seed coat soybeans exhibited much higher levels of TPC than soybeans of the other seed coat colors (**Figure 3.1).** Peking had the highest TPC value of 12.1 mg gallic acid equivalents (GAE)/g flour, and MD 06-5437-1 with black seed coat had about 2.7 mg GAE/g flour, which was the lowest among all black soybeans. The soybeans with brown, green and yellow seed coats had values between 0.8 and 2.2 mg GAE/g flour, with most values clustered between 1.2 and 1.8 g GAE/g flour.

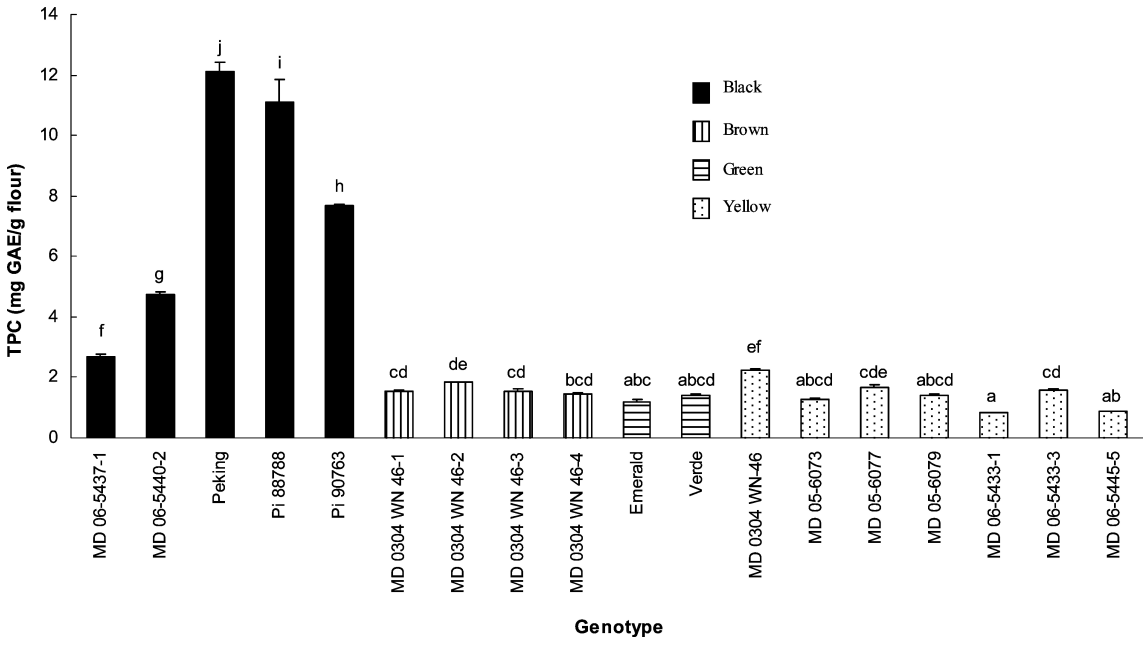

Figure 3.1. Total Phenolic Contents of Colored Seed Coat Soybean Extracts. Values are based on triplicate tests and expressed as mg gallic acid equivalents per g soybean flour. Means and SD are shown (n = 3). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

All soybeans sampled also contained scavenging capacities against peroxyl (ORAC), hydroxyl (HOSC), and $ABTS^{+}$ radicals, and black seed coat soybeans exhibited significantly higher antioxidant activities ($P \leq 0.05$) than soybeans of the other seed coat colors in all three assays (**Figures 3.2, 3.3 & 3.4)**. Peking black soybeans, which had the highest TPC, also had the highest $ABTS^{\cdot+}$ scavenging capacity of 102 μmol trolox equivalents (TE)/g flour, while Pi 88788 had the greatest ORAC value of 255 μmol TE/g flour and HOSC value of 238 μmol TE/g flour. MD 0304 WN 46 yellow soybeans had significantly stronger radical scavenging capacities against all three tested radicals than other yellow, or green, or brown soybeans. The values for TPC, ORAC, HOSC and $ABTS^{\cdot+}$ scavenging capacity were in very strong agreement—each of these four tests was significantly (P <0.01), strongly, [(r) > 0.92] and positively correlated to each other test.

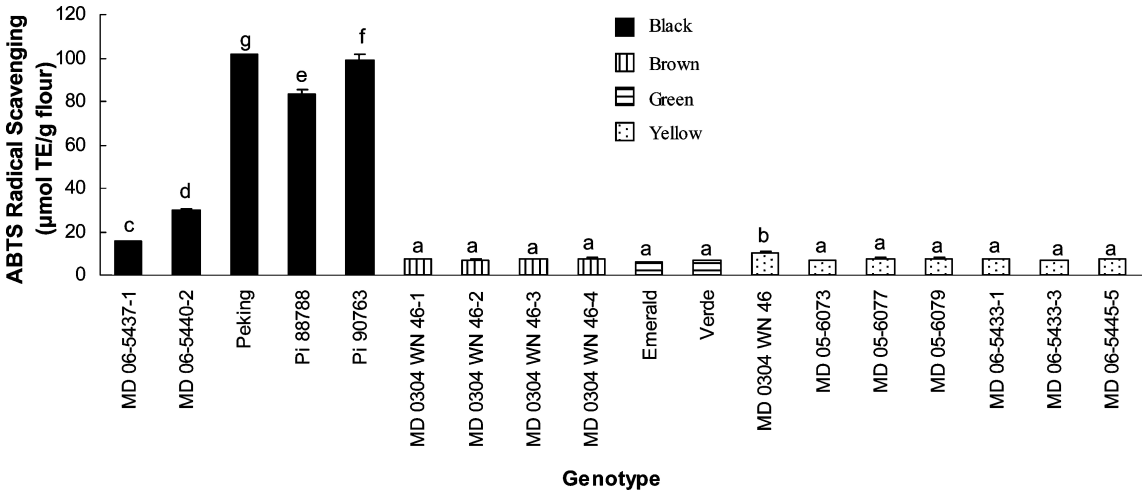

Figure 3.2. ABTS Radical Scavenging Capacity of Colored Seed Coat Soybean Extracts.
Values are based on triplicate tests and expressed as μmol trolox equivalents/g soybean flour. Means + SD are shown. (n = 3). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

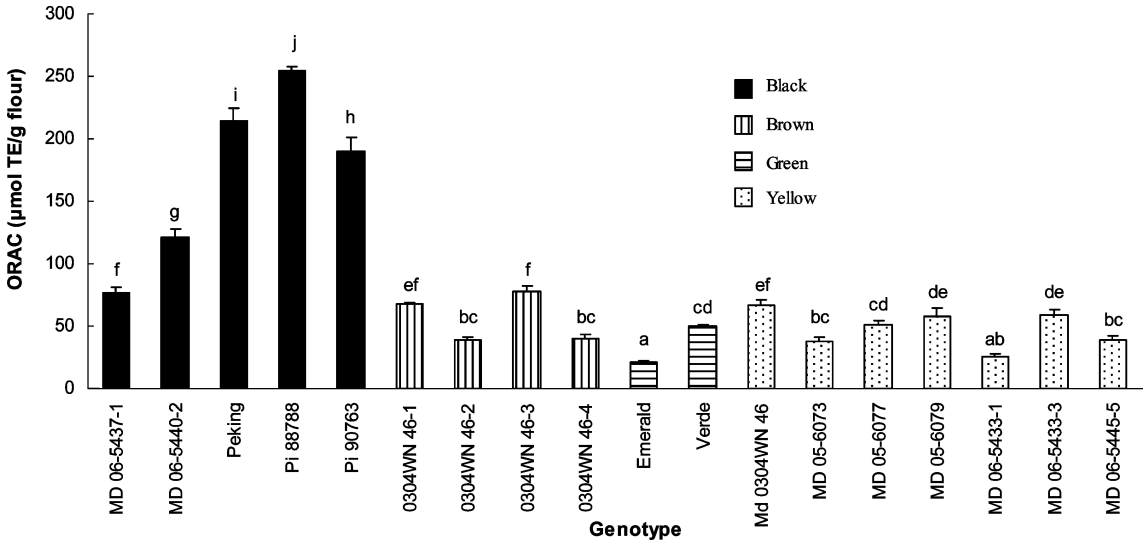

Figure 3.3. Oxygen Radical Absorbance Capacity of Colored Seed Coat Soybean Extracts.

Values are based on triplicate tests and expressed as µmol trolox equivalents/g soybean flour. Means + SD are shown. (n = 3). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

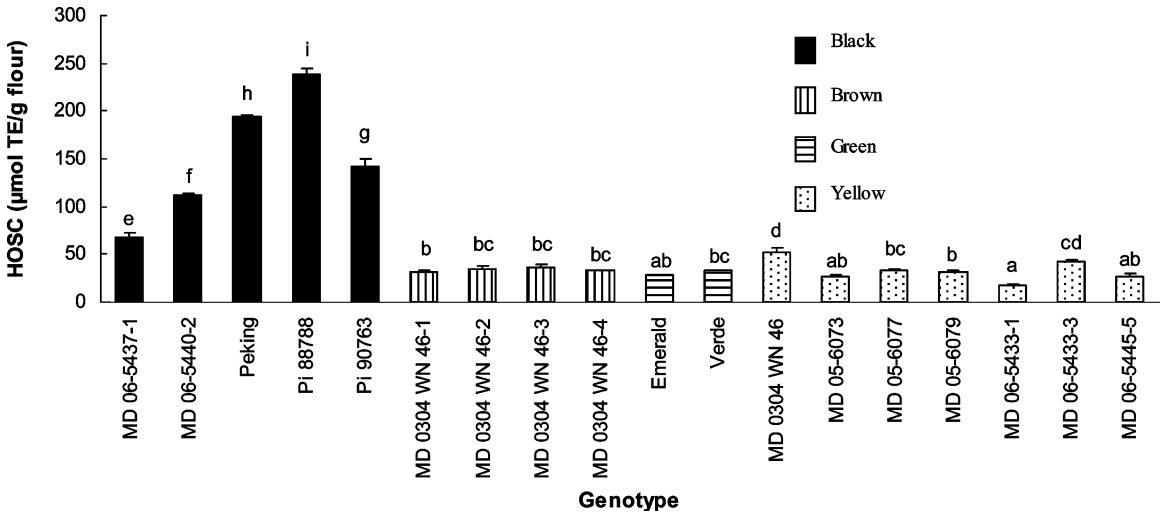

Figure 3.4. Hydroxyl Radical Scavenging Capacity of Colored Seed Coat Soybean Extracts.

Values are based on triplicate tests and expressed as µmol trolox equivalents/g soybean flour. Means + SD are shown. (n = 3). Genotypes marked by the same letter are not significantly different ($P \leq 0.05$).

These data agreed with previously published studies in both approximate values and the magnitude of differences seen between black soybeans and other color soybeans for both TPC and antioxidant activity, measured in other studies by oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging capacity, and ferric reducing antioxidant power (FRAP) assays (Xu et al, 2007; Xu & Chang, 2007, 2008a & b; Furuta et al, 2003). Limited data was available on values of $ABTS^{\cdot+}$ scavenging capacity and HOSC for soybeans. The HOSC assay was chosen in this study because it is the only radical scavenging capacity assay validated for radical purity and concentration consistency, and it is also performed under the physiological pH of 7.4 (Moore et al, 2006).

Soybeans with black seed coats have repeatedly been shown to have higher antioxidant contents than those with other seed coat colors in almost every antioxidant measurement that is commonly used: total phenolic contents (TPC), oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging capacity, and ferric reducing antioxidant power (FRAP) (Xu et al, 2007; Takahata et al, 2001; Xu & Chang, 2008b; Furuta et al, 2003). Worthy of note, the vast majority of isoflavones have been shown to be found in the cotyledon and germ, not the seed coat (Xu & Chang, 2008a), and some studies have found fewer isoflavones in black seed coat soybeans as compared to other colors (Xu & Chang, 2008a & b). The difference in antioxidant activity arises at least in part from the presence of anthocyanins in the seed coats, which only black seed coats contain (Todd & Vodkin, 1993). They serve as both pigment and antioxidant. In addition, previous research has shown that brown and black seed coat soybeans contain

proanthocyanins (Todd & Vodkin, 1993), which may contribute to the overall health properties of black and brown soybeans, though they were not measured in the present study.

3.4.7 Anti-proliferative Activity Against HT-29 Cells

In the present study, two black soybean samples (Peking and Pi 88788), one brown soybean (MD 0304 WN 46-1), one green soybean (Emerald), and one yellow soybean sample (MD 0304 WN-46) were selected because of their higher antioxidant activities within their same seed coat color groups and investigated for their potential anti-proliferative effect using HT-29 human colon cancer cells. The anti-proliferative effects of five soybean extracts (50% acetone extracts evaporated and re-dissolved in DMSO) were compared to the control containing only solvent and to the isoflavone mixture, at three different concentrations shown in **Table 3.1**. The isoflavone mixture contained daidzein, glycitein, and genistein at the same levels that were present in the Pi 88788 soybean extract, which had the highest total isoflavones among all tested soybean samples.

Figure 3.5A shows the comparison of cell number after 48 h treatment by unhydrolyzed extracts, whose isoflavones are therefore mostly in their original glycoside forms on a same per soybean flour weight basis. **Figure 3.5B** shows cell number after 48h treatment by the hydrolyzed extracts, which are therefore in their aglycone forms. As seen in **3.5A**, Peking soybean extract at the high dose of 15 mg soy flour equivalents/mL is the only unhydrolyzed extract to significantly ($P \leq 0.05$) suppress HT-29 cell proliferation after 48 h of treatment, whereas the isoflavone

mixture and unhydrolyzed Pi 88788 black soybean extract at the high testing dose did not produce a statistically significant reduction in cell number until 72 h treatment time (data not shown). Among the hydrolyzed extracts, the highest treatment level (15 mg flour equiv/mL) of black Peking and Pi 88788, yellow MD 0304 WN-46, and brown MD 0304 WN-46-1 soybeans, and the medium treatment level (5 mg flour equiv/mL) of Peking and MD 0304 WN-46 significantly ($P \leq 0.05$) reduced cell number at 48 h treatment (**Figure 3.5B**). Data in **Figure 3.5** suggests that soybeans with different seed coat color may differ in their antiproliferative components. Data in **Figure 3.5** also suggested that the hydrolyzed, aglycone forms of soybean extracts might be more effective in suppressing HT-29 colon cancer cell growth than their corresponding glycosides on the same per molar concentration basis. This may be partially explained by the greater cellular availability of the aglycones because of the reduced water solubility and polarity, rendering them better able to cross the cell membrane and exert effects intracellularly.

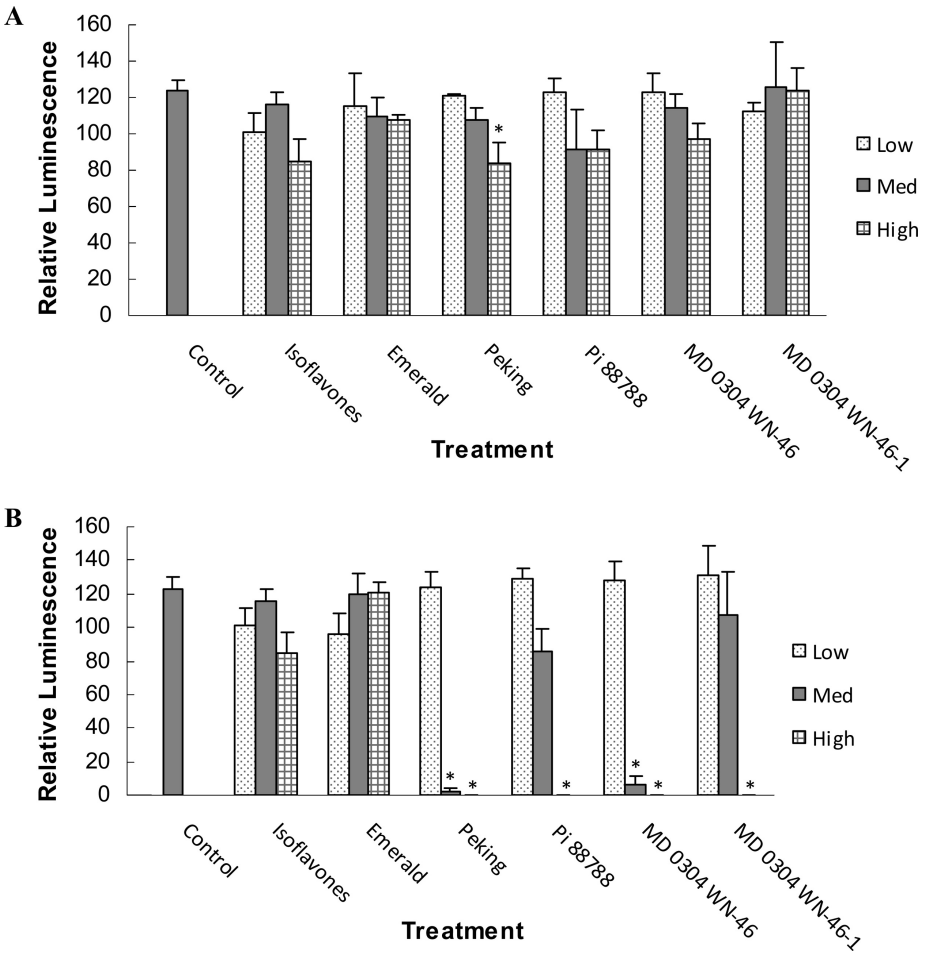

Figure 3.5. Comparison of Cell Treatments at 48 Hours.
(A) Treated by glycoside forms of soybean extracts; (B) Treated by aglycone forms of soybean extracts (hydrolyzed). Isoflavones treatment contains genistein, daidzein and glycitein. Low, medium and high doses of soybean extracts represent 1.5, 5, and 15 mg flour equivalents/mL treatment, with isoflavone concentrations of each shown in **Table 3.1**. The isoflavone standard was designed to mimic the concentration of isoflavones in Pi 88788. Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean + SD shown (n = 3). Columns marked by (*) are significantly different from the control ($P \leq 0.05$).

The hydrolyzed, aglycone form of the Peking, Pi88788, MD 0304 WN-46 and MD 0304 WN 46-1 extracts, and the soy isoflavone mixture suppressed HT-29 cancer cell proliferation in a dose and time dependent manner (**Figure 3.6).** At the high treatment dose, the aglycone forms of black Peking and Pi 88788, brown MD 0304

WN46-1, and yellow MD 0304 WN-46 soybean flour extracts significantly ($P \leq 0.05$) reduced cell number after 3 h of treatment (**Figures 3.6B, 3.6C, 3.6D and 3.6E)**. The aglycone forms of Peking soybean extracts also reduced cell number significantly ($P \leq 0.05$) in the medium dose (5 mg defatted flour equiv/mL) at 3 h of treatment, whereas the only other medium dose to significantly ($P \leq 0.05$) impact antiproliferative capacity was the yellow MD 0304 WN-46 after 24 h of treatment (**Figures 3.6B and 3.6E)**. No treatment displayed any inhibitory effect at the low dose at any time under the experimental conditions.

The isoflavone standard mixture **(Figure 3.6A)** did not produce as effective a reduction in cell number as Pi88788, whose isoflavone concentration it was designed to mimic (**Figure 3.6C**), nor were its effects as fast. The isoflavone standard mixture produced a statistically significant reduction in cell number by 72 h, as compared to 3 h by the actual Pi88788 aglycone extract. Also, by 72 h the Pi88788 aglycone extract had essentially eliminated viable cells to 0% of the vehicle, while the isoflavone mixture only reduced cell numbers to 60% of the vehicle.

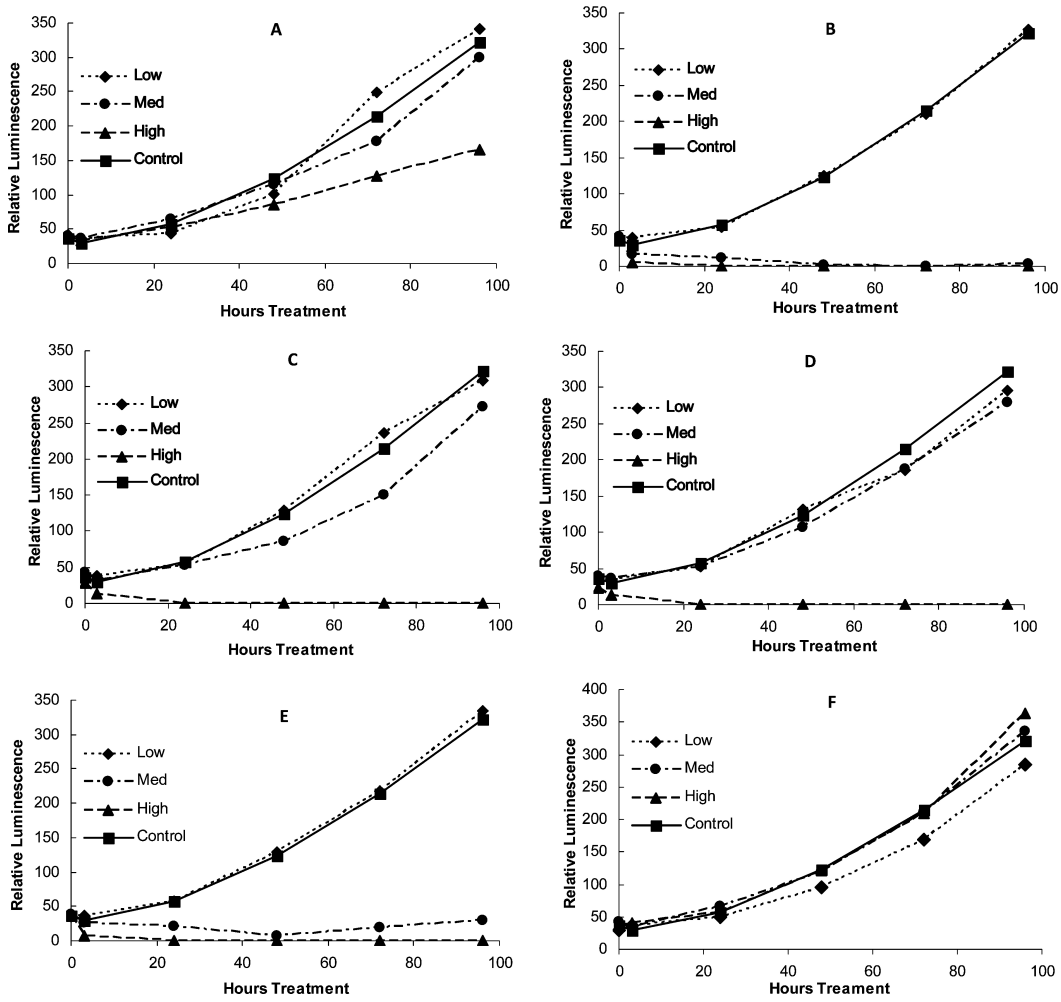

Figure 3.6. Time and Dose Effects of Soy Isoflavones and Hydrolyzed Soybean Extracts on HT-29 Cell Proliferation.
Relative luminescence is proportionate to the number of viable cells. Values are based on triplicate tests, with mean values shown (n = 3). (A) Soy isoflavones consisting of genistein, daidzein and glycitein. (B) black Peking, (C) black Pi 88788, (D) brown MD 0304 WN46-1, (E) yellow MD 0304 WN-46, and (F) green Emerald extracts were hydrolyzed with base prior to cell treatment to convert all isoflavones to aglycone form. Low, medium and high doses of soybean extracts represent 1.5, 5, and 15 mg flour equivalents/mL treatment, with isoflavone concentrations of each shown in **Table 3.1**. The isoflavone standard was designed to mimic the concentration of isoflavones in Pi 88788.

Interestingly, the green Emerald soybean had isoflavone levels similar to or greater than those of the yellow MD 0304 WN-46 and brown MD 0304 WN-46-1 soybeans, but had no detectable anti-proliferative activity at any dose at any time under the experimental conditions (**Figure 3.6F)**, whereas both yellow MD 0304 WN-46 and brown MD 0304 WN-46-1 soybean extracts significantly ($P \leq 0.05$) suppressed HT-29 cell growth (**Figures 3.5B, 3.6D and 3.6E**). In addition, the MD 0304 WN-46 soybean extract had the lowest total isoflavones, which was approximately 25% of that in the isoflavone mixture, but its aglycone form elicited significant reduction in cell number after 3 h of treatment at both the medium and high doses, whereas the isoflavone mixtures did not significantly ($P \leq 0.05$) inhibit cell proliferation until 72 h of treatment at the high dose. These data suggested soybean lines and cultivars with the same seed coat color might differ in their anti-proliferative activities, and that other factors in soybeans beyond isoflavones and cyanidin may contribute to the overall anti-proliferative activity of soybean flour extracts.

Finally, the isoflavone mixture was broken down into its individual components to test which may be contributing to the antiproliferative activity. Additionally, cyanidin was introduced to the mixture to mimic the concentration of the predominant anthocyanidin in addition to the isoflavones of the two black soybean extracts present after hydrolysis. Concentrations of treatment media are shown in **Table 3.2**. Results at 96 hours are represented in **Figure 3.7**. Every combination of two or three isoflavones produced significant reduction in HT-29 cell proliferation at 96 hours. Glycitein was the only individual isoflavone to produce

significant cell reduction in 96 h. This is likely because the glycitein concentration was approximately twice that of genistein or daidzein in the treatments (designed to mimic the concentrations seen in Pi 88788). However, it may be important to note that while all the combination treatments were successful at inhibiting cell proliferation versus the control, they were not significantly different from each other ($P \leq 0.05$). In other words, the glycitein treatment contained less total isoflavones than any of the combination treatments, though it was similarly effective at reducing cell proliferation. A difference was seen in the time required to significantly ($P \leq 0.05$) reduce cell proliferation—the Peking and Pi88788 mimics with isoflavones and cyanidin elicited a significant inhibition of cells by 48 hours, whereas the mixture of three isoflavones and glycitein alone exhibited significant inhibition in 72 h. These data suggested that variations within a certain range of isoflavone type and dose, in combination with cyanidin, may elicit the same ultimate response by cells.

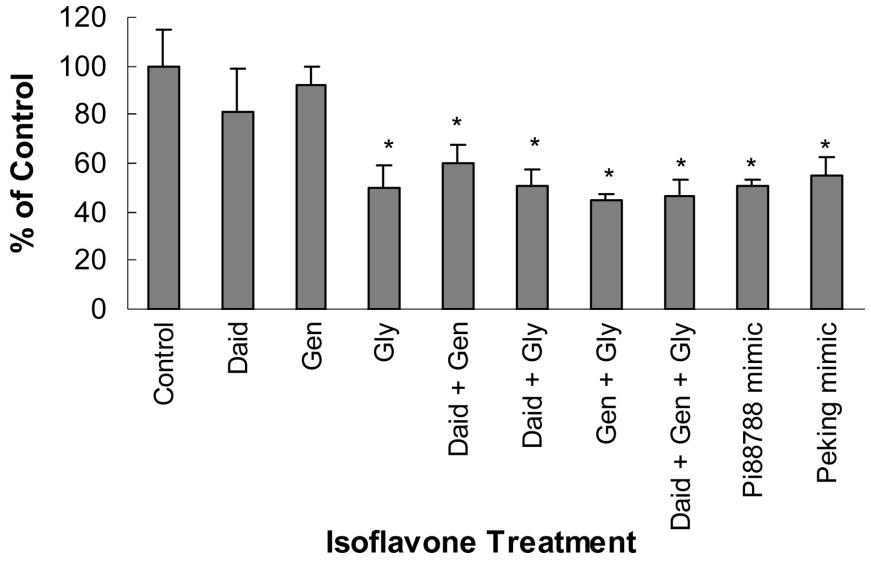

Figure 3.7. Effects of Cell Isoflavone Treatments at 96 Hours.
Daid = daidzein, Gly = glycitein, Gen = genistein. Treatment concentrations are shown in **Table 3.2,** with high doses shown here. All treatments, including control contained 1.2% DMSO. Values are based on triplicate tests, with mean + SD shown. Columns marked by (*) are significantly different from the control ($P \leq 0.05$).

Previous work has suggested that isoflavones and anthocyanidins independently inhibit cancer cell growth in their aglycone form in previous in vitro studies (Zhang et al, 2005; Yu et al, 2004; Guo et al, 2004). Taking into account the data in **Figures 3.5, 3.6 and 3.7,** the present study suggested that soybeans contain anti-proliferative components, though their anti-proliferative activities may not be fully explained by their isoflavone content, cyanidin content, and seed coat color.

This study is fundamentally limited in its analysis of only one crop year. It is well-accepted that environmental conditions (temperature, solar radiation levels, soil composition, water availability, etc.) affect the nutritional and phytochemical composition of crops, including soybeans. For example, soy isoflavone levels have been shown to be depressed during growth at higher temperatures (Tsukamoto et al, 1995). For this reason, additional studies analyzing multiple crop years may serve to identify if the trends noted here hold true across a variety of growing conditions.

There has been question if the bioactivity and prevention of diseases by soybeans have been falsely attributed to any individual phytochemical or component. Data from this study suggests that while black soybeans may have higher levels of isoflavones, anthocyanins, and antioxidant activity, their health beneficial property as a whole may not increase proportionately with these levels. More studies are necessary comparing the effects of isolated soy components with those of the whole bean or bean part.

3.6 Acknowledgements

This research was supported by grants from the Maryland Soybean Board in 2007, 2008, and 2009, grants from the Maryland Grain Producers Utilization Board (MGPUB) with MGPUB grant proposal numbers of 208198 and 209198, a grant from National Science Foundation with a federal grant number of CBET-0650650, and a grant from JIFSAN. MD lines with reduced 16:0 and 18:3 were developed as part of a separate grant funded by the United Soybean Board and Maryland Soybean Board to William Kenworthy.

Chapter 4: A Single Extraction and HPLC-UV-ELSD Procedure for Simultaneous Quantification of Phytosterols, Tocopherols and Lutein in Soybeans

4.1 Abstract

An isocratic high performance liquid chromatography method (HPLC) and saponification/extraction procedure were developed and validated for simultaneous quantitation of phytosterols, tocopherols and lutein in soybeans. Separation was achieved on a phenyl column with a ternary, isocratic solvent system of acetonitrile, methanol and water (48:22.5:29.5, v/v/v). Evaporative light scattering detection (ELSD) was used to quantify β-sitosterol, stigmasterol, campesterol, α-, δ- and γ-tocopherols, while lutein was quantified with ultraviolet (UV) absorption at 450 nm. Peak identification was verified by retention times and spikes with external standards. Quantification was performed through fitting of regression lines with concentrations verified by molar extinction coefficients to standard curves for each compound ($R^2 >$ 0.99). Accuracy of the saponification and extraction was demonstrated via recovery analysis of spiked samples, and the quantitative results of four soybeans using the described saponification and HPLC analytical method were validated using the existing methods. This method offers a more efficient alternative to individual methods for quantifying lutein, tocopherols and sterols in soybeans.

4.2 Introduction

The quantification of food phytochemicals is integral to understanding the complex relationship between diet composition and disease prevention. Quantifying food phytochemicals is particularly interesting when studying soy due to the variety of health-beneficial compounds known to exist in the legume. Isoflavones and soy protein are the best known, but health-beneficial, lipophilic compounds also abound in soy: tocopherols, carotenoids, sterols, and lecithin. Making the task of accurately quantifying phytochemicals in our food particularly onerous, the levels of bioactive compounds in soybeans are known to vary among genotypes and growing conditions, such as water availability, soil composition, and temperature (Wang et al, 2008; Riedl et al, 2007; McCord et al, 2004). In order to analyze sufficient samples to better understand these differences, analytical methods with greater cost- and time-effectiveness are necessary. Analytical methods that quantify multiple phytochemicals in a single procedure would therefore be valuable tools.

Sterol analysis has traditionally been performed via gas chromatography (GC), and some GC methods have also been adapted to also quantify tocopherols (AOCS, 2003; Du and Ahn, 2002). GC offers strong resolving power to identify and quantify the wide range of sterol structures, including glycosidic forms (see reviews by Abidi, 2001 and Lagarda et al, 2006). However, quantitative GC has several drawbacks, including complex sample preparation procedures—sometimes including column or thin-layer chromatography to first isolate compounds before derivatization to achieve volatility—followed by the necessity to use both internal and external

standards due to the losses inherent to the sample preparation methods, as well as high temperature of operation and destructive detection techniques (Abidi, 2001; Lagarda et al, 2006).

Valid RP-HPLC separation methods for soy phytosterols are limited in the literature. Many methods have been reported for their separation, but problems continue to plague sterol HPLC. Adequate separation of the three main soy phytosterols—campesterol, stigmasterol, and β-sitosterol—in a reasonable timeframe has yet to be achieved reliably. Two methods developed in the 1980s and 90s (Indyk, 1990; Holen, 1985) show reasonable separations of these three. Bedner et al (2008) also showed successful separation on a phenyl column, but analysis-time was approximately 50 minutes when the method was repeated. Most importantly, none of the methods could analyze carotenoids in addition to sterols.

Quantitation of sterols via HPLC has also proven difficult due to their low absorbance at UV wavelengths and interferences at their λ_{max} of approximately 205 nm. Evaporative light scattering detectors (ELSD) and flame ionization detectors (FID) are gaining ground as viable replacements for UV and diode array detectors in the detection of sterols, given their stance as universal detectors that do not require absorption in the UV region. HPLC combined with atmospheric pressure chemical ionization mass spectrometry (APCI-MS) is also increasingly popular in the detection of sterols (see Lagarda et al, 2006), but cost makes it prohibitive for widespread applications. Regardless, the use of UV and diode array detection (DAD) at ~205 nm remains common in the HPLC detection of sterols, given its affordability and omnipresence in analytical labs (Abidi, 2001; Lagarda et al, 2006).

RP-HPLC separations of tocopherols and carotenoids are more easily achieved than sterols and are fairly commonplace in the literature, particularly since the advent of the carotenoid (C_{30}) column in the 1990s (Sander et al, 2000; Puspitasari-Nienaber et al, 2002). Alpha, gamma and delta-tocopherols have also been successfully quantified on C_{18} columns (Britz and Kremer, 2002; Kurilich and Juvik, 1999), but C_{30} columns and normal phase separations offer the ability to separate the β and γ isomers. However, soybeans are recognized to contain only minimal β-tocopherol (Yoshida et al, 1998), and its co-elution with γ-tocopherol is therefore not generally considered a problem in soy analysis. Detection is also generally easy for these two families of compounds, both having strong UV and visible absorbance at wavelengths of relatively low interference of 295 and 450 nm, for tocopherols and carotenoids, respectively. Fluorescence is also commonly used to detect tocopherols. Good separation and quantification of the major soy carotenoids and tocopherols are easily achieved by applying the method Darnoko et al. reported in 2000 (Moros et al, 2002).

Extraction conditions vary within and between each of these three classes of compounds, but the basic procedures are the same: sample homogenization, optional saponification, solvent extraction, and sometimes purification. The purpose of saponification is to free the esterified sterols, tocopherols, and xanthophylls including lutein of their side chains, which simplifies analysis by allowing quantification of only the core molecules. Saponification may be used directly on a homogenized sample or on its extraction. When directly on the sample, saponification is commonly performed in alcohol. However, the harsh conditions of saponification may reduce

the recovery of labile carotenoids and tocopherols (Muzhingi et al, 2008; Feltl et al, 2005; Oliver and Palou, 2000; Ryyanen et al, 2004). Thus, a protective antioxidant is an essential component of the saponification procedure. Extraction solvents differ somewhat between these chemical classes, but converge on hexane and ether. Methylene chloride and chloroform are also sometimes used in combination with methanol for carotenoid and sterol extractions, but fairly common contaminants in these solvents may react with carotenoids (Feltl et al, 2005), whereas a hexane:ethyl acetate solution is frequently used for tocopherols.

To our knowledge, only one published report separates these three classes of compounds in a single HPLC run. Manzi et al (1996) reported the detection of cheese carotenes, tocopherols, and sterols in a single run using a diode array detector; however, the method is not applicable to soy products because the forms of each of these classes of compounds differ greatly between cheese and soy. The current work therefore reports for the first time the simultaneous quantitative extraction and LC separation of the major carotenoid, tocopherols and phytosterols in soy. Furthermore, it achieves this separation in an isocratic elution, with all peaks eluting in 40 minutes.

4.3 Materials and Methods

4.3.1 Materials and Chemicals

Soybeans grown in Maryland during 2009 were obtained from Dr. William Kenworthy, Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD 20742.

HPLC-grade solvents were obtained from Fisher Scientific (Pittsburgh, PA), and were specifically *not* filtered due to the sensitivity of the ELSD to filter residues. All other solvents were also obtained from Fisher, and were of ACS-grade or higher purity. Na_2CO_3, NaCl, and tert-butylhydroquinone (TBHQ) were obtained from Sigma (St. Louis, MO). HPLC tocopherol and sterol standards were also obtained from Sigma, and were purchased in the highest available purity. Lutein HPLC standard was obtained from Indofine Chemical Company (Hillsborough, NJ). 0.20 μm PTFE syringe filters were manufactured by National Scientific (Rockwood, TN).

4.3.2 Saponification and Extraction Procedure

Saponification was used to convert carotenoid, tocopherol and sterol esters to their free forms prior to HPLC analysis for quantification. The complete procedure is depicted in **Figure 4.1**. The method of Kurilich and Juvik (1999b) was used as a starting point, and was modified according to preliminary results as follows.

Due to the well-recognized loss of xanthophylls and tocopherols during saponification conditions discussed above, tert-butyl hydroquinone (TBHQ) was used as an antioxidant to protect these analytes of interest. The use of butylated hydroxytoluene (BHT) was specifically avoided due to its production of an errant

peak overlapping with campesterol on the ELSD during elution, and butylated hydroxyanisole (BHA) was found to be less effective at protecting analytes in preliminary trials (data not shown). Additional sample protection was achieved by preparing samples under minimal light conditions and holding them on ice during extraction procedures after saponification. The Na_2CO_3 wash step was added to remove fatty acids due to the column's predisposition to clog upon their presence in samples.

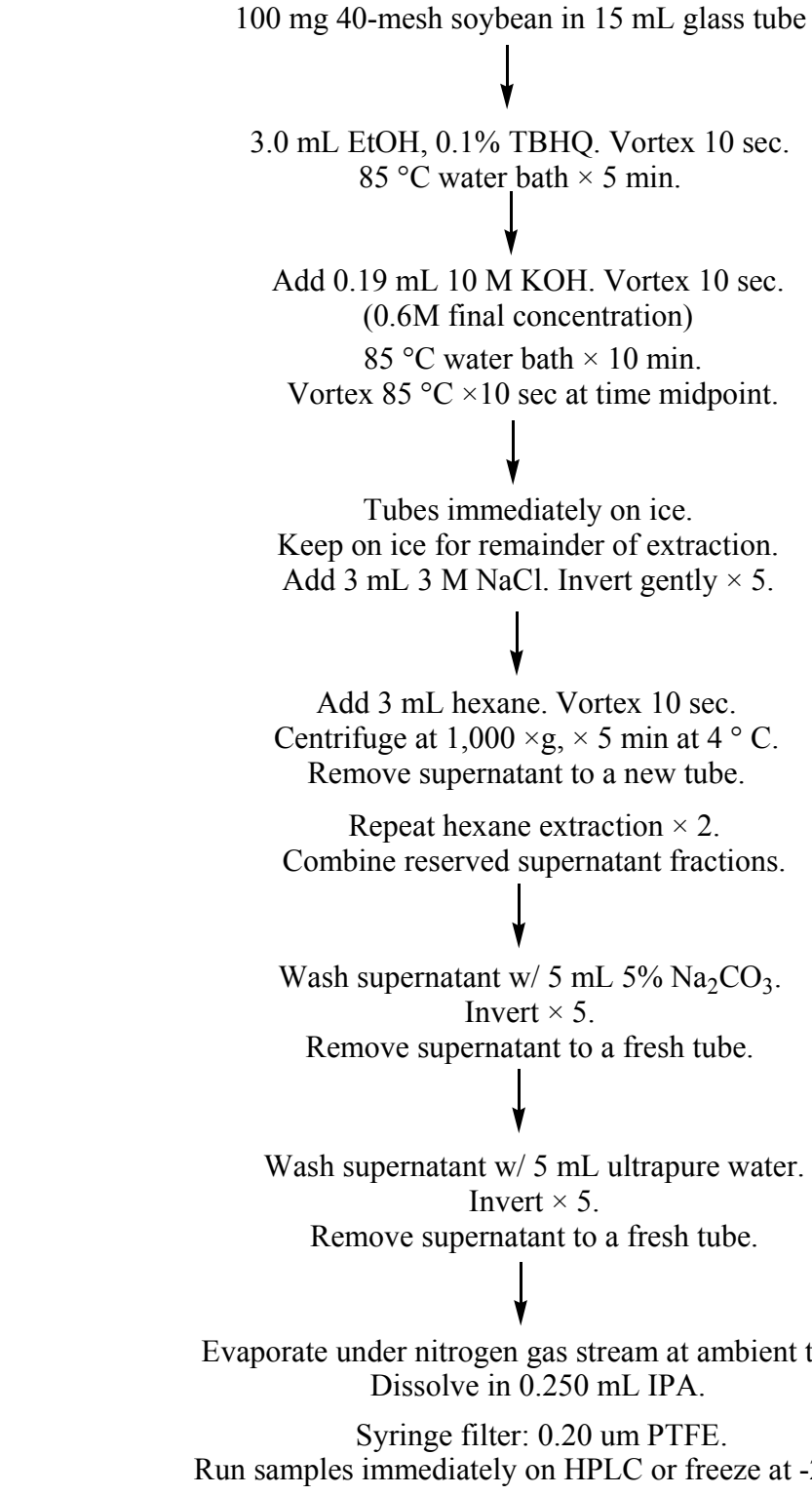

Figure 4.1. Soybean Saponification Conditions
EtOH = ethanol. TBHQ = tertiary-butyl hydroquinone. IPA = isopropyl alcohol. PTFE = polytetrafluoroethylene.

4.3.3 HPLC Conditions

A Shimadzu Prominence HPLC system (Columbia, MD) was used in the method development process, consisting of a LC-20AD quaternary pump, SIL-20A HT autosampler, CTO-20AC column oven, SPD-20A UV/Vis detector, and ELSD-LT II detector.

A range of reversed-phase columns and solvent conditions were tested prior to accepting the current method. Ultimately, an XTerra phenyl column (Waters, Milford, MA) with dimensions of 150 mm × 3.9 mm (3.5 μm) was used to achieve separation with an isocratic elution consisting of acetonitrile:methanol:water (48:22.5:29.5, v/v/v). The column was protected by a 20 mm × 3.9 mm guard column of the same packing material. The phenyl column produced complete separation of campesterol and stigmasterol, which was not achieved on C_{18} and C_{30} columns. The quaternary pump was used with 3 solvent reservoirs to precisely deliver the appropriate eluent mix due to fairly significant sensitivity of the method to changes in solvent composition as little as 0.5%. Column temperature was maintained at 30 °C. Isocratic conditions were held for 40 minutes, followed by a 4 minute linear gradient to 100% methanol, held for 7 minutes, then a subsequent 4 minute linear gradient to return to initial conditions, which were held for 6 minutes to re-equilibrate before the next run for a total run time of 61 minutes. The methanol flush procedure is strongly advised between runs, and operators are advised to be vigilant of increasing pressure and change guard columns frequently, as the column strongly retains the sterols and fouls easily if proper precautions are not taken.

The ELSD was operated at a drift-tube temperature of 40 °C with an ultra-pure (>99.999%) nitrogen gas flow at 350 kPa. The instrument gain was set to level 8, and no signal offset was used. Data points were collected at 10 Hz. Eluents were not filtered at the manufacturer's suggestion to reduce signal noise caused by filter residues. The UV/Vis detector collected data for lutein at 450 nm.

4.3.4 Method Validation

Peak identity was validated through the use of retention times of external standards, as well as the increase of peak area upon spiking with external standard.

4.3.4.1 Linearity and Range

Stock solutions were created from commercial standards, and concentrations were verified using Beer's law ($A = \varepsilon lc$) according to the molar extinction coefficients (ε) listed in **Table 4.1**. Absorbance was measured using a Shimadzu (Columbia, MD) UV-1601 spectrophotometer in a Suprasil quartz cuvette (Fisher Scientific, Pittsburgh, PA). The use of molar extinction coefficients with lutein and tocopherols provides a reasonable assurance of purity. Admittedly, the use of the coefficients with sterols is less assuring due to the high potential for interference at their λ_{max}, and is dependent upon the assurance of purity by the manufacturer. Values for all three sterols were similar to expected values based on standard weights and dilutions. Despite a significant literature review, an ε value for campesterol was not found. In the absence of this coefficient for campesterol, the coefficient for β-sitosterol was used, since the UV-absorbing group is similar to both molecules (same

double bond at C5 in the sterol ring structure), and the molecules' only difference lies in the extra methylene group in the acyclic chain (i.e., a non-UV-absorbing region).

The verified stock solutions were then diluted to the range of concentrations shown in **Table 4.2**. Standard curves were constructed with a minimum of 5 points.

Table 4.1. Molar Extinction Coefficients for Determination of Standard Curve Concentrations

	Solvent	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})	E (1%/cm)	Source
Lutein	MeOH	442	149,600	--	Craft and Soares (1992)
α- tocopherol	MeOH	292	--	0.0076	AOCS (2003)
δ-tocopherol	MeOH	298	--	0.0087	AOCS (2003)
γ-tocopherol	MeOH	298	--	0.0091	AOCS (2003)
β-sitosterol	EtOH	206	4,200	--	Wheeler and Mateos (1956)
Stigmasterol	EtOH	205	5,500	--	Wheeler and Mateos (1956)
Campesterol*	EtOH*	206*	4,200*	--	Wheeler and Mateos (1956)*

* Campesterol value could not be located, and β-sitosterol value was substituted. See text for full explanation.

4.3.4.2 Precision/Reproducibility

Reproducibility and precision of the combined extraction and analytical method were assessed by performing intrasample, intraday, and interday extractions and HPLC injections of saponified Manokin soybean samples, then comparing means and standard deviations. Three injections of the same sample (intrasample) were used to determine the variability of the HPLC method. Four samples of the same Manokin soybeans were extracted and injected on the same day to determine intraday variability, while three samples were extracted and injected on three separate sequential days to assess the interday variability.

4.3.4.3 Recovery

Recovery of the extraction method was determined through the use of three sets of spiked samples and was calculated as below (concentrations represent those of the injection solution). Concentrations of spikes are shown in **Table 4.4.**

$$\% \text{ Recovery} = \frac{\text{Pre-saponification spike (µg/mL)} - \text{No spike (µg/mL)}}{\text{Post-saponification spike (µg/mL)} - \text{No spike (µg/mL)}} \times 100\%$$

4.3.4.4 Accuracy

To ensure accuracy of the standard curves, concentrations of stock solutions were verified through the use of molar extinction coefficients (**Table 4.1**). Additionally, extracts were quantified via previously published methods for comparison to the current method.

To validate the consistency of the current method with other methods, four different soybean genotypes were saponified in triplicate according to the procedure in **Figure 4.1,** and were then subjected to analysis via the current method and that of another previously published method. For sterols, the liquid chromatographic method of Bedner et al (2008) was followed with UV detection at 205 nm. Tocopherols and carotenoids were detected via the liquid chromatographic method of Darnoko et al (2000), utilizing a C_{30} carotenoid column and UV-Vis detection at 295 and 450 nm, respectively.

4.4 Results

Figure 4.2 shows chromatograms of a mixture of standards and a representative sample. While baseline resolution was not achieved between campesterol, δ-tocopherol, and stigmasterol, peak shape is consistent and lends itself to quantification nonetheless. Baseline separation was achieved for lutein, β-sitosterol, and γ and α-tocopherols under the specified conditions.

4.4.1 Linearity and Range

Regression coefficients of best-fit standard curves and their coefficients of determination (R^2) are listed in **Table 4.2**. ELSD regression lines are logarithmic in nature and were fitted with the "power" fit in Microsoft Excel, while lutein's UV data received a linear fit. Ranges of concentrations that were used are also shown. The minimum quantitation limit for δ and γ- tocopherols was determined to be 7.5 μg/mL, sterols was determined to be 5 μg/mL, and α-tocopherol's lower quantification limit was deemed to be 10 μg/mL. Below these levels, relative standard deviations consistently less than 10% could not be achieved. Lutein quantitation, however, was reliable down to the lowest concentration tested (0.25 μg/mL) due to the higher sensitivity of UV/Vis detection.

Care should be taken if readings fall above the verified range, as solubility of the analytes is likely a problem above the concentration range listed in **Table 4.2**. Methanol was originally used as an injection solvent due to its production of sharper, nearly baseline-resolved peaks; however, the values of samples fell outside the linear range of the methanol standard curve.

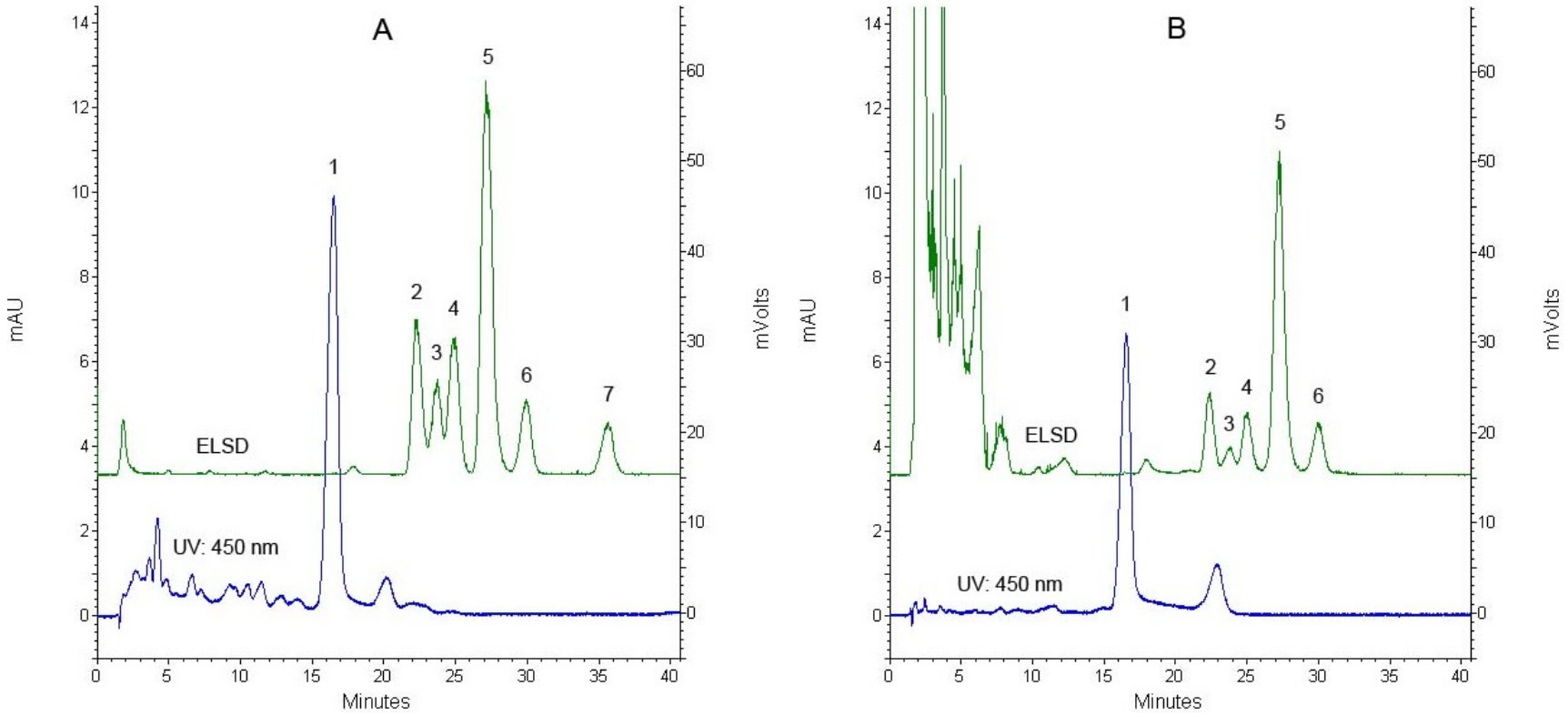

Figure 4.2. Chromatograms of standard mixture and saponified soybean.
(A) Standard mixture and (B) Saponified Manokin soybean. Peak identities: (1) Lutein, (2) Campesterol, (3) δ-tocopherol, (4) Stigmasterol, (5) β-sitosterol, (6) γ-tocopherol, and (7) α-tocopherol. The left y-axis on both figures represents the milli absorbance units detected at 450 nm, while the right y-axis represents the millivolts detected by the ELSD.

For this reason, the more nonpolar isopropyl alcohol was instead tested as injection solvent.

With isopropanol, the range was found to be appropriate for all the soybean samples and spikes tested (with the exception of α-tocopherol falling below the quantifiable limit). Additionally, all R^2 values were deemed appropriate for the detectors (see Discussion for further coverage of this topic).

Table 4.2. Concentration range and regression relationships of sterols, tocopherols and lutein as detected by ELSD or UV.

Analyte	Concentration Range (μg/mL)	R^2	ELSD*	
			a	b
Campesterol	5 - 200	1.000	731.1	1.513
Stigmasterol	5 - 200	0.999	570.3	1.563
β-sitosterol	10 - 400	0.999	950.0	1.462
α-tocopherol	15 - 150	0.998	206.0	1.725
δ-tocopherol	7.5 - 150	0.998	314.0	1.711
γ-tocopherol	7.5 - 150	0.995	471.8	1.598
			UV at 450 nm#	
			m	b
Lutein	0.25 - 10	0.999	109,224	-25,066

Standards run in triplicate. Best-fit lines determined with a minimum of 5 standard concentrations.
* logarithmic: $y = ax^b$
# linear: $y = mx + b$

4.4.2 Precision/Reproducibility

Results of reproducibility tests are shown in **Table 4.3**. Intra-sample injections were used to validate the consistency of the HPLC method. Relative standard deviations (RSD) of intra-sample injections (n = 3) were all below 3.0% (with the exception of β-sitosterol), which is the maximum limit recommended by the

manufacturer of the ELSD. Meanwhile, intra-day (n = 4) and inter-day (n = 3) extraction/injections had slightly more variability due to the added variable of sample preparation, but still remained acceptable below 10%. Lutein was the only exception at 12.3%. It is considered that the chemical instability of lutein may play a role in its inter-day variability.

Table 4.3. Precision of the Analytical Method and Reproducibility of the Combined Extraction and Analysis.

	Intra-sample (n = 3)	Intra-day (n = 4)	Inter-day (n = 3)
	μg/g soybean ± SD (RSD)		
Campesterol	165.9 ± 4.2 (2.5%)	156.8 ± 4.8 (3.1%)	157.7 ± 3.9 (2.4%)
Stigmasterol	150.4 ± 4.4 (2.9%)	139.5 ± 13.6 (9.7%)	145.1 ± 3.0 (2.1%)
β-sitosterol	474.8 ± 20.6 (4.3%)	439.7 ± 13.6 (3.1%)	447.1 ± 7.8 (1.8%)
α-tocopherol	BQL	BQL	BQL
δ-tocopherol	84.5 ± 1.5 (1.8%)	86.7 ± 2.4 (2.7%)	82.5 ± 1.5 (1.8%)
γ-tocopherol	129.1 ± 2.3 (1.8%)	132.6 ± 3.9 (2.9%)	131.2 ± 5.4 (4.1%)
Lutein	8.24 ± 0.22 (1.4%)	8.96 ± 0.64 (7.1%)	8.1 ± 1.0 (12.3%)

RSD: relative standard deviation, calculated as RSD = Standard Deviation / Mean * 100%
BQL: below quantification limit.

4.4.3 Recovery

The results of the recovery analysis of three doses of spiked samples are shown in **Table 4.4**. Data are presented as the concentration of the injection solution of the spiked samples in μg/mL. Recovery of all three sterols is excellent and consistent (approximately 100%).

Table 4.4. Recovery of Analytes from Spiked Samples Following Saponification.

	Lutein	α-tocopherol	δ-tocopherol	γ-tocopherol	Campesterol	Stigmasterol	β-sitosterol
Spike 1							
[Spike] (μg/mL)[a]	2	30	30	30	40	40	80
Intramean – before[b] (μg/mL)	1.82 ± 0.09 (4.8%)	23.8 ± 1.2 (5.1%)	22.0 ± 0.51 (2.3%)	28.8 ± 2.2 (7.8%)	43.6 ± 4.4 (10%)	47.8 ± 5.4 (11%)	86.6 ± 11.6 (13%)
Intramean – after[c] (μg/mL)	1.94 ± 0.33 (17%)	30.9 ± 2.3 (7.6%)	32.2 ± 3.3 (10%)	32.9 ± 4.2 (13%)	45.4 ± 6.3 (14%)	50.7 ± 6.3 (12%)	90.7 ± 12.6 (14%)
% Recovery (n=3)	94.0	76.9	68.3	87.7	96.1	94.3	95.5
Spike 2							
[Spike] (μg/mL)	3	45	45	45	60	60	120
Intramean – before (μg/mL)	2.77 ± 0.34 (12%)	33.1 ± 3.7 (11%)	35.5 ± 2.4 (6.9%)	44.1 ± 2.0 (4.5%)	70.1 ± 2.2 (3.2%)	76.0 ± 2.8 (3.7%)	137.6 ± 8.5 (6.2%)
Intramean – after (μg/mL)	3.14 ± 0.10 (3.2%)	46.1 ± 2.7 (5.8%)	48.0 ± 2.2 (4.6%)	47.0 ± 5.1 (11%)	68.5 ± 0.2 (0.3%)	72.3 ± 1.3 (1.8%)	127.8 ± 2.0 (1.5%)
% Recovery (n=3)	88.0	71.8	73.9	93.9	102.3	105.0	107.7
Spike 3							
[Spike] (μg/mL)	4	60	60	60	80	80	160
Intramean – before (μg/mL)	3.52 ±0.34 (9.7%)	36.0 ± 1.5 (4.1%)	38.8 ± 0.7 (1.9%)	49.5 ± 5.5 (11%)	79.7 ± 4.1 (5.2%)	87.1 ± 7.4 (8.5%)	157.0 ± 11.3 (7.2%)
Intramean – after (μg/mL)	3.88 ± 0.14 (3.5%)	56.2 ± 1.2 (2.1%)	61.7 ± 2.6 (4.3%)	59.9 ± 3.3 (5.5%)	83.5 ± 4.9 (5.9%)	89.6 ± 5.8 (6.5%)	160.1 ± 11.2 (7.0%)
% Recovery (n=3)	90.6	64.0	62.9	82.6	95.5	97.3	98.0
Pooled Runs							
% Recovery (n = 9)	90.8	70.9	68.4	88.1	97.0	98.9	100.4

[a] Intended concentration of spike.
[b] Mean of concentration recovered from pre-saponification spike.
[c] Mean of concentration recovered from post-saponification spike.
[b,c] Values represent mean (μg analyte/mL injection solution)± standard deviation (% relative standard deviation).

Lutein recovery is approximately 90% at all three spike levels, indicating good recovery, especially given lutein's susceptibility to degradation under saponification conditions. Tocopherol recovery is lower (γ-tocopherol averaging 88%, δ-tocopherol at 68%, and α-tocopherol at 71%), but recoveries are fairly consistent across the three spike levels. This consistency allows for comparison across samples, even without optimal recovery percentages.

4.4.4 Accuracy

Samples saponified according to the procedure in **Figure 4.1** were subjected to analysis by previously published analytical methods to allow for comparison with the current method. Results of the various methods are presented below in **Table 4.5**. The results from these methods are in strong agreement for lutein and γ-tocopherol. All others are within the appropriate range, and demonstrate the same trends (with the exception of δ-tocopherol) among the four soybean genotypes, with variations between methods being mostly below 15%. It is suspected that the less-than-ideal chromatographic separation of δ-tocopherol may hinder accurate quantitation. Furthermore, α-tocopherol values produced by the reference method (Darnoko et al, 2000) are shown. These values correspond to injection solution concentrations of 3 to 7 μg/mL, validating that they are indeed below the determined limit of quantification of the current method.

An evaluation of the accuracy of the analytical method is also evident in **Table 4.4**, in viewing the similarities between mean post-saponification spike concentration as analyzed versus the intended amount added. The agreement between

the spiked amount and concentration of post-analysis spikes was less than 10% deviation for all levels of lutein and the three tocopherol spikes. Agreement for sterols was less ideal, but remained below 15% for campesterol and β-sitosterol. Stigmasterol varied by as much 27% in the low dose of the spike.

Table 4.5. Comparison of Current Method to Previously Published Methods.

		Current Method			Reference Method		
	Soybean	**µg/g dry bean**	**SD**	**CV (%)**	**µg/g dry bean**	**SD**	**CV (%)**
Lutein	MD03-5527	5.610	0.054	1.0	6.010	0.214	3.6
	MD03-6420	5.551	0.070	1.3	6.113	0.128	2.1
	Peking	16.705	0.285	1.7	19.219	0.692	3.6
	Manokin	7.390	0.319	4.3	7.756	0.289	3.7
δ-tocopherol	MD03-5527	62.53	1.50	2.4	49.94	1.17	2.3
	MD03-6420	58.16	4.93	8.5	52.49	1.60	3.1
	Peking	79.48	3.80	4.8	53.02	1.19	2.2
	Manokin	80.22	1.04	1.3	79.65	2.63	3.3
γ-tocopherol	MD03-5527	143.5	3.5	2.4	138.9	7.4	5.3
	MD03-6420	120.3	8.9	7.4	118.1	6.5	5.5
	Peking	165.6	0.5	0.3	171.1	2.9	1.7
	Manokin	133.8	3.2	2.4	130.8	4.8	3.6
Campesterol	MD03-5527	164.4	3.2	1.9	183.8	2.0	1.1
	MD03-6420	162.9	2.0	1.2	180.0	4.3	2.4
	Peking	229.2	1.1	0.5	230.9	3.3	1.4
	Manokin	156.9	2.8	1.8	181.4	5.4	3.0
Stigmasterol	MD03-5527	160.6	0.9	0.6	184.9	4.9	2.6
	MD03-6420	179.0	5.9	3.3	209.2	2.2	1.1
	Peking	167.6	1.1	0.7	186.7	4.0	2.1
	Manokin	144.9	2.1	1.4	155.9	5.5	3.5
β-sitosterol	MD03-5527	500.1	2.3	0.5	441.0	13.4	3.0
	MD03-6420	461.7	10.5	2.3	421.3	8.4	2.0
	Peking	598.9	8.7	1.5	529.3	7.5	1.4
	Manokin	442.0	8.4	1.9	401.7	6.1	1.5
α-tocopherol	MD03-5527		BQL		16.7	0.6	3.8
	MD03-6420		BQL		15.1	1.2	6.9
	Peking		BQL		10.6	0.5	4.4
	Manokin		BQL		8.8	0.4	5.1

*Lutein and tocopherol reference method: Darnoko et al (2000).
*Sterol reference method: Bedner et al (2008).
BQL: below quantification limit.

4.5 Discussion

The data presented here demonstrate that the current method is concise, precise, and accurate for quantifying lutein, tocopherols and sterols in soybeans. The use of evaporative light scattering detection (ELSD) in combination with UV detection at 450 nm makes this possible.

Inherently, ELSD's operating principles introduce additional variation beyond that observed with in-line liquid phase detectors: mobile phase coming off the column is nebulized, carried in a flow of nitrogen and evaporated in a heated drift tube, causing a phase transition of the mobile phase prior to reaching the detector. The dissolved solutes therefore transition from flow in a 0.1 μm tubing to a centimeters-wide nebulization chamber, followed by a millimeters-wide, heated drift tube. For this reason, greater relative standard deviation (RSD) values of up to 3% are considered acceptable by the instrument manufacturer, as are R^2 values for regression curves of above 0.99. This method fell within these acceptable parameters in the construction of the standard curve (data not shown), and in the intrasample injections (**Table 4.3**), with the exception of β-sitosterol with intraday %RSD at 4.3%. Strangely, β-sitosterol varied less during intraday and interday assessments (3.1% and 1.8%), indicating that there is less cause for concern about the analytical method's precision in detecting β-sitosterol than the intrasample RSD value might suggest.

Furthermore, the nature of the ELSD (discussed above) precludes it from being as sensitive as in-line liquid phase detectors. For this reason and its inherent lack of selectivity, Abidi (2000) particularly dis-recommended the use of an ELSD

for detection of tocopherols. This is arguably true, particularly in light of the current method's inability to detect α-tocopherol at levels where UV detection is capable. However, this method produced standard curves of R^2 values of 0.995 and above for the three tocopherols (**Table 4.2**), indicating its reliability when tocopherols are within the detectable range. Additionally, ELSD technology has advanced since the publication of Abidi's review in 2000, and lower evaporation temperatures are possible on newer models, allowing for less thermal degradation of analytes. Lutein, however, was selected for detection by absorption at 450 nm due to the ELSD's inability to detect it at sample concentrations well below 10 μg/mL injection. Additionally, lutein is also unstable at elevated temperatures, even given the reduced operating temperatures of current ELSD drift tubes, and may experience some loss at the current method's drift tube temperature of 40 °C.

Understanding the above limitations of ELSD technology, the results of the current method demonstrate that it can be used effectively to measure both tocopherols and sterols. Lutein analysis by visible light absorbance at 450 nm is generally routine and also proved an effective method in this application.

Though robustness was not explicitly tested, it deserves mention that the method is highly dependent on correct solvent mixing. The quaternary pump was used to precisely deliver the necessary solvent ratio (MeOH:Acetonitrile:Water, 22.5:48:29.5, v/v/v), as opposed to pre-mixing by hand. As little as a 0.5% change in solvent concentrations produced noticeable change in peak retention times and could impact separation. Furthermore, injection solvent is crucial: analytes have too low a solubility in methanol, though it does produce a better separation at the lower

concentrations. Acetonitrile as an injection solvent provides neither adequate solubility nor adequate separation. Isopropyl alcohol provides adequate solubility at the compromise of peak shape and separation. Lastly, it should be noted that the use of UV/Vis at 205 nm detection wavelength did not result in baseline separation of sterols from the tocopherols and is not a viable detection option with this method.

Saponification conditions and subsequent extraction produced excellent recovery results for sterols and lutein. However, recovery of α and δ-tocopherols was lower than desirable (at approximately 70% instead of 90+%). The tocopherols may be forming complexes in their de-protonated state after saponification, thus preventing their complete extraction. Given the extreme conditions necessitated in saponification (heat + elevated pH) and precedent examples of tocopherol loss under these conditions (see Introduction), loss to oxidation is also possible. Ultimately, despite the lower overall recovery of tocopherols, they are more importantly consistent across all three spike concentrations. This characteristic allows for the method to be used to make comparisons across soybeans, with the understanding that only roughly 70% (α and δ) and 90% (γ) are being recovered. The method has not been tested for other food matrices.

The phenyl column was chosen for this separation due to its ability to distinguish between campesterol and stigmasterol, a trait with which other reversed-phase columns struggle. Phenyl columns offer interesting possibilities in HPLC separations because of their unique chemical nature as a stationary phase. While the nonpolar nature of the phenyl group allows it to function generally as a reversed-phase system through Van der Waals forces of attraction, the π electrons of the

phenyl ring structure offer the potential for further tweaking of separations through π-π interactions. These interactions are observed when two chemical entities containing π electrons (as in carbon double bonds) are attracted to each other to an extent that would not otherwise be predicted based on its chemical formula and structure. They are thought to be the result of a complex type of electron donor-electron acceptor reaction involving both π and σ electrons (Hunter and Sanders, 1990). When applied to chromatography, π-active solutes are preferentially retained on the phenyl column (Snyder et al, 2007). This interaction may explain the late elution time of tocopherols – their relative polarity would otherwise predict their elution before the sterols, as is observed in other instances where they have been resolved together on RP-HPLC systems (Manzi et al, 1996).

The baseline resolution of the sterols using the phenyl column may also be attributable to π interactions of sterols with the phenyl column. Their resolution is exceptional and wide enough to accommodate the δ-tocopherol peak between campesterol and stigmasterol (as compared to campesterol and stigmasterol overlapping, as routinely seen on C_{18} columns). This suggests the possibility that the additional degree of unsaturation in stigmasterol allows for potentially greater π-π interactions, which may contribute to a slightly longer retention time and therefore better separation on the phenyl column.

Yang et al. (2005) indicated that acetonitrile weakened π-π interactions in relation to methanol between a phenyl column and their pharmaceutical ingredient analytes. However, the opposite was suggested by observations in the development of the current method. When methanol was used as the primary organic modifier,

tocopherols were eluted before the sterols, indicating a possible weakening of π-π interactions.

4.6 Conclusions

This method offers a concise extraction and quantitative analysis of three lipophilic chemical groups in soy – physterols, tocopherols and lutein. No special equipment is needed for the extraction; however, ELSDs remain fairly rare in analytical laboratories. The use of the ELSD to quantify tocopherols and sterols makes the separation possible, but also sacrifices sensitivity. For those studies not requiring extreme sensitivity, the sacrifice is rewarded with an increase in efficiency resulting from combining three basic analyses into one procedure.

4.7 Acknowledgments

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Appendix A. Preparation of Food Samples for Liquid Chromatographic Analysis

Analyte	Food Source	Saponification			Extraction			Reference
		Y/N	Procedure	Oxidative Protectant	Solvent	Ratio (solvent: material)	Other conditions	
Tocopherols/ tocotrienols	Rye flour	Y	0.5g flour + 5 mL EtOH + 2 mL H_2O; Flush w/ N_2; Bring to 0.6M KOH; 100 °C x 25 min	Ascorbic acid (0.1g)	n-hexane : ethyl acetate (80:20)	0.5g : 10 mL (3x)	'subdued' light; evaporated; hexane for injection; filtered 0.45µm	Ryynanen et al. (2004) *J. Food Comp. Anal.*
Carotenoids/ Tocopherols	Corn, ground	Y	0.6g grain +6 mL EtOH; 85 °C x 5min; Bring to 0.3M KOH; 85 °C x 10 min	BHT (0.1%)	Hexane	0.6g : 3 mL (2x)	Under 'safe' light; dried in speed vac dryer; ACN:MeOH:MeCl (45:20:35) for injection	Egesel et al. (2003) *Crop Sci.*
Tocopherols	Soybeans, ground	N	---	---	Hexane : ethyl acetate (85:15)	N/A	Soxhlet x 24h; 0.01% BHT; yellow light; filtered 0.45µm	Lim et al. (2007) *Eur. J. Lipid Sci. Technol.*
Tocopherols	Soybeans, ground	Y	3.0g meal + 20 mL EtOH; Sonication x 5 min; Flush w/ N_2; Bring to 3.6M KOH; 70 °C x 50 min	Pyrogallol (6%)	Hexane : ethyl acetate (85:15), with 0.01% BHT	5g : 20 mL (3x)	No yellow light?; Filtered 0.45µm	Lim et al. (2007) *Eur. J. Lipid Sci. Technol.*
Carotenoids	Soybeans, Blanched and homogenized	Y	70 °C x 30 min; N_2 atmosphere	BHT (0.01%)	Hexane (0.01% BHT)	Not stated	N_2 evaporated; re-dissolved in mobile phase	Simonne et al. (2000) *J. Agric. Food Chem.*
Tocopherol	Soybeans, crushed w/ hydraulics	N	---	---	Hexane	6 seeds : 2 mL	Dried in vacuum; Re-dissolved in hexane	Scherder et al. (2006) *Crop Sci.*

Appendix A cont'd. Preparation of Food Samples for Liquid Chromatographic Analysis

Analyte	Food Source	Saponification			Extraction			Reference
		Y/N	Procedure	Oxidative Protectant	Solvent	Ratio (solvent: material)	Other conditions	
Retinol, α-tocopherol	Infant formula	Y	50g + 100 mL H_2O; 30g of this removed; starch degraded; + 7 g KOH + 50 mL EtOH + 1g Na_2S; 85 °C x 30 min under N_2. Followed CEN method	1g sodium ascorbate	Elution from SPE with n-hexane (0.5% BHT)	20 mL absorbed : 100 mL solvent	SPE (Chromabond XTR 70 mL cartridge); 20 mL x 15 min absorption; extracts re-dissolved in mobile phase	Heudi et al. (2004) *J. Chrom. A*
Tocopherols/ Tocotrienols	Oils	Y	10 g oil + 25 mL 25 EtOH; 1.5 M KOH; 56 °C x 30 min; Dessicated w/ Na_2SO4	Pyrogallol (50 mg)	Hexane	Ratio not stated (3x)	Evaporated in vacuum; MeOH for injection	Bonvehi et al. (2000) *JAOAC*
Tocopherols/ Tocotrienols	Oils	N	---	---	Hexane to load Sep Pak; MeOH to elute, stabilized with MeCl.	1.5 g oil : 10 mL	Silica Sep-Pak; 1 mL in 500 mg column (activated with isopropanol and hexane)	Bonvehi et al. (2000) *JAOAC*
Tocopherols/ Tocotrienols	Cereals	N	---	---	Hexane	1 g cereal: 30 mL	N_2 evaporated; re-dissolved in 3 mL hexane;	Nielsen et al. (2008) *Cereal Chem.*
Carotenoids/ Tocopherols	Freeze-dried, ground corn	Y	0.6g corn + 6 mL EtOH; 85 °C x 5min; Bring to 0.3M KOH; 85 °C x 10min	BHT (0.1%)	Hexane	0.6g corn : 3 mL (3x)	dried in vacuum evap; ACN:MeOH:MeCl (45:20:35) for injection	Kurilich and Juvik. (1999) *J. Liq. Chrom. & Rel. Technol.*
Xanthophylls	Ground corn	Y	.6g corn + 6 mL EtOH; 85 °C x 5min; Bring to 0.3M KOH; 85 °C x 10min	BHT (0.1%)	Hexane	0.6g corn: 3 mL (3x)	Dim yellow lighting; N_2 dried; Re-dissolved in mobile phase	Moros et al. (2002) *J. Agric. Food Chem.*

Appendix A cont'd. Preparation of Food Samples for Liquid Chromatographic Analysis

Analyte	Food Source	Saponification			Extraction			Reference
		Y/N	Procedure	Oxidative Protectant	Solvent	Ratio (solvent: material)	Other conditions	
Carotenoids/ Tocopherols	Oil	Y	0.4 g oil + 5 mL MeOH; 0.6M KOH; Sealed with N_2, 100 °C x 30 min	Pyrogallol	Diethyl ether	0.4 g oil : 10 mL (3x)	Several KOH washes, followed by five water washes. Evaporated under N_2; Re-dissolved in mobile phase	Darnoko et al. (2000) *J. Liq. Chrom. & Rel. Technol.*
Tocopherols	Oils, deodorized	N	---	---	---	---	Dissolved oils 1:10 in hexane	Warner and Mounts (1990). *JAOCS.*
Tocopherols	Lyophilized, ground soybeans	N	---	---	EtOH w/ 0.1% BHT	Not stated	Extracted 3xat 60 °C x 5 min. Washed w/ H_2O, fractionated w/ hexane. Dried under N_2.	Britz and Kremer. (2002) *J. Agric. Food Chem.*
Carotenoids/ Tocopherols/ Chlorophylls	Vegetable oils	N	---	---	---	---	Oil samples directly dissolved in mobile phase; filtered 0.2 µm nylon	Puspitasari-Nienaber et al. (2002) *JAOCS.*
Sterols	Oils, deodorized	Y	1 g oil + 100 mL 0.8 M ethanolic KOH, 80 °C x 30 min	No, but flushed with N_2	Ethyl ether	1 g oil : 100 mL	Dried over anhydrous $MgSO_4$., evaporated and re-dissolved in EtOH	Warner and Mounts (1990). *JAOCS.*
Sterols	Whole wheat flour	Y	Acid-hydrolysis first: 0.5 g flour + 1.0 mL EtOH + 5 mL 6 M HCl. 80 °C x 60 min. Then saponification of the dried organic phase: 8.0 mL EtOH + 0.01 M KOH, 80 °C x 30 min	Pyrogallol in saponification (3%)	Cyclohexane	0.5 g flour : 20 mL	Evaporated under nitrogen	Toivo et al. (2001) *J. Food Comp. Anal.*

Appendix A cont'd. Preparation of Food Samples for Liquid Chromatographic Analysis

Analyte	Food Source	Saponification			Extraction			Reference
		Y/N	Procedure	Oxidative Protectant	Solvent	Ratio (solvent: material)	Other conditions	
Sterols	Seaweed	Y	1 g seaweed + 50 mL ethanolic KOH (1M)	N	Hexane	1 g : 50 mL, then 25 and 25 mL	Dried with Na_2SO_4, evaporated in rotovap, redissolved in mobile phase, filtered 0.5 µm	Sanchez-Machado et al. (2004) *Biomed. Chrom.*
Sterols	Soybean oil	Y	0.2 g oil + 250 µL hexane w/ IS + 2 mL MeOH. 2 M KOH. 90 °C x 60 min.	N	Ethyl acetate	0.2 g oil : 4 mL	6-ketocholestanol internal standard. Dried with Na_2SO_4, evaporated under N_2. SPE used for further washing, then filtered 0.45 µm.	Careri et al. (2001) *J. Chrom. A.*
Sterols	Olive oil	Y	5 g oil + 50 mL EtOH, 2 M KOH. 100 °C x 60 min.	N	Diethyl ether	Not stated	Dried with Na_2SO_4, evaporated via rotovap, redissolved in $CHCl_3$. Sterols isolated on TLC, dissolved in MeOH for HPLC.	Canabate-Diaz et al. (2007) *Food Chem.*

Appendix B. Liquid Chromatographic Methods for Analysis of Carotenoids, Tocopherols and Sterols in Foods

Analyte	HPLC				Recovery	Source
	Mobile phase	Column	Detection method	Other conditions		
Carotenoids/ Tocopherols	A: MeOH:MTBE:H_2O (81:15:4) B: MTBE:MeOH (91:9) 1 mL/min	C_{30} with a guard column (5µm x 250 mm x 4 mm)	DAD: 295 and 450 nm	---	No data	Darnoko et al. (2000) *J. Liq. Chrom. & Rel. Technol.*
Carotenes/ Tocopherols/ Sterols	MeOH, 2 mL/min, isocratic	C_{18} (5µm x 250 mm x 4 mm)	UV/Vis: 208 nm (sterols), 405 nm (Carotenes). Fluorometer: $\lambda_{ex}/\lambda_{em}$ 325/475 nm (retinols), 280/325 (tocopherols)	---	Inc. saponification/extraction: 93-102% (tocopherols, retinols, carotenes and sterols)	Manzi et al. (1996). *Chromatographia*.
Carotenoids	A: MTBE B: MeOH 1.3 mL/min, gradient	C_{30} w/ 10 mm C_{18} guard (5µm x 250 mm x 4.6 mm)	DAD	Oven temperatures varied from 15 to 25 °C. 23 was concluded to be optimal.	No data	Bohm. (2001) *J. Sep. Sci.*
Carotenoids	ACN:MeOH:CH_2Cl_2:n-heptane (50:40:5:5) w/ 0.1% BHT, 0.05% TEA, 0.05M NH_4OAC in MeOH. 0.3 mL/min	C_{18} (5µm x 250 mm x 2 mm)	HPLC-MS-MS	21°C oven	Not reported	Huck et al. (2000). *J. Chrom. Sci.*
Tocopherols	ACN:THF:H_2O (60:25:15) 0.7 mL/min, isocratic	C_{18} (5µm x 150 mm x 3.9 mm)	ELSD Drift tube 120°C, exhaust 77°C, N_2 flow 14 psi	---	Not reported	Warner and Mounts (1990). *JAOCS.*

Appendix B cont'd. Liquid Chromatographic Methods for Analysis of Carotenoids, Tocopherols and Sterols in Foods

Analyte	HPLC				Recovery	Reference
	Mobile phase	Column	Detection method	Other conditions		
Tocopherols	ACN:THF:H_2O (60:25:10) 1 mL/min, isocratic	C_{18} w/ guard (7µm x 250 mm x 4.6 mm)	DAD: 290 nm	25°C oven	70-80%	Britz and Kremer. (2002) *J. Agric. Food Chem.*
Tocopherols/ tocotrienols	1,4-dioxane : n-hexane (3:97) 2 mL/min ; isocratic	Silica (5µm x 250 mm x 4.6 mm)	Fluorescence $\lambda_{ex}/\lambda_{em}$ 292/325 nm	30 °C oven	Tocopherols α: 94.3%; β: 93.6%; γ: 90.3%	Ryynanen et al. (2004) *J. Food Comp. Anal.*
Carotenoids/ Tocopherols	ACN:MeOH:MeCl (75:20:5); 0.1% BHT; 0.05% Et_3N; 1.8 mL/min; isocratic	C30 (5µm x 100 mm x 4.6 mm)	PDA λ_{abs}= 540 and 295	---	No data	Egesel et al. (2003) *Crop Sci.*
Tocopherols	Isopropanol:n-hexane (1.3:98.7) 1.0 mL/min; isocratic	Si60 (5µm x 250 mm x 4 mm)	Fluorescence $\lambda_{ex}/\lambda_{em}$ 290/330 nm	---	Tocopherols α: 103%; β: 110%; γ: 94%; δ: 107%	Lim et al. (2007) *Eur. J. Lipid Sci. Technol.*
Carotenoids	ACN:MeOH:THF (20:28:2); 1.0 mL/min; isocratic	C18 (5µm x 250 mm x 4.6 mm)	UV/Vis 450 nm; PDA scan 390-800 nm	30 °C oven	No data	Simonne et al. (2000) *J. Agric. Food Chem.*
Tocopherol	Isopropanol:hexane (1:99) 0.65 mL/min; isocratic	NP; Si60 (7µm x 250 mm x 3.1 mm)	UV λ_{abs}= 292 nm	---	No data	Scherder et al. (2006) *Crop Sci.*

Appendix B cont'd. Liquid Chromatographic Methods for Analysis of Carotenoids, Tocopherols and Sterols in Foods

Analyte	HPLC				Recovery	Source
	Mobile phase	Column	Detection method	Other conditions		
Retinol, α-tocopherol	Hexane:dioxan:2-propanol (96.7:3:0.3); 1.45 mL/min; isocratic	silica (Nucleosil 100-5) (250 mm x 4.6 mm)	UV/DAD, LC-MS APCI	---	Retinol: 102%; α-tocopherol: 96%	Heudi et al. (2004) *J. Chrom. A*
Tocopherols/ Tocotrienols	MeOH:H_2O (95:5) 1 mL/min; isocratic	C_{18} w/ guard column; (10µm x 300 mm x 3.9 mm	DAD: 292 nm Fluorescence: $\lambda_{ex}/\lambda_{em}$ 292/330 nm	---	HPLC method + direct injection only: α-tocopherol: 97-103%; γ-tocopherol: 94-108%; δ-tocopherol: 94-110%. No % Recovery for these extraction methods. Saponification generally produced values 65-80% of the non-saponified.	Bonvehi et al. (2000) *JAOAC*
Tocopherols/ Tocotrienols	MeOH:H_2O (95:5) 1 mL/min; isocratic	C_{18} w/ guard column; (10µm x 300 mm x 3.9 mm	DAD: 292 nm Fluorescence: $\lambda_{ex}/\lambda_{em}$ 292/330 nm	---		Bonvehi et al. (2000) *JAOAC*
Tocopherols/ Tocotrienols	Hexane:ethyl acetate:HAc (94.6:3.6:1.8) 1.0 mL/min; isocratic	Si60 w/ guard column (5µm x 125 mm x 4 mm)	Fluorescence: $\lambda_{ex}/\lambda_{em}$ 290/330 nm	25°C oven	96-100%	Nielsen et al. (2008) *Cereal Chem.*
Carotenoids/ Tocopherols	ACN:MeOH:MeCl (75:20:5) w/ 0.1% BHT and 0.05% Et_3N; 1.8 mL/min; isocratic	C_{18} w/ guard column (4µm x 150 mm x 3.9 mm)	UV/Vis: 450 nm and 290 nm	---	Lutein 99%; Zeaxanthin 85%; β-cryptoxanthin 116%; β-carotene 122%; α-tocopherol 103%; δ-tocopherol 85%; γ-tocopherol 115%	Kurilich and Juvik. (1999) *J. Liq. Chrom. & Rel. Technol.*
Xanthophylls	A: MeOH:MTBE:H_2O (81:15:4) B: MeOH:MTBE (9:91) 1.0 mL/min; gradient	C_{30} (250 mm x 4.6 mm)	DAD: 450 and 445 nm	---	No data	Moros et al. (2002) *J. Agric. Food Chem.*

Appendix B cont'd. Liquid Chromatographic Methods for Analysis of Carotenoids, Tocopherols and Sterols in Foods

Analyte	HPLC				Recovery	Source
	Mobile phase	Column	Detection method	Other conditions		
Carotenoids/ Tocopherols/ Chlorophylls	A: MeOH:MTBE:NH_4^+ acetate:H_2O (88:5:5:2) B: MeOH:MTBE:NH_4^+ acetate (20:78:2) Gradient	C_{30} w/ C_{18} guard column (5µm x 250 mm x 4.6 mm	Electrochemical (200-620mV)	25°C oven	No data on recovery; Between-day variation was <7.5% for each.	Puspitasari-Nienaber et al. (2002) *JAOCS.*
Carotenoids/ Tocopherols/ Chlorophylls	A: n-hexane:2-propanol (99:1); B: 2-propanol 1.2 mL/min; gradient	Si 60 (5µm x 250 mm x 4 mm)	UV/Vis DAD Fluorescence	---	No data	Psomiadou and Tsimidou. (1998) *J. Agric. Food Chem.*
Sterols	MeOH:H_2O (98:2) 0.7 mL/min, isocratic	C_{18} (5µm x 150 mm x 3.9 mm)	ELSD Drift tube 125°C, exhaust 82°C, N_2 flow 31 psi	25°C (room temp)	Not reported	Warner and Mounts (1990). *JAOCS.*
Sterols	MeOH:ACN (30:70) 1.2 mL/min, isocratic	C_{18} (5 µm x 150 mm x 4 mm)	UV: 205 nm And HPLC-MS	30 °C oven	Not reported	Sanchez-Machado et al. (2004) *Biomed. Chrom.*
Sterols	ACN:H_2O (86:14) 0.3 mL/min, isocratic	C_8 (5 µm x 150 mm x 2.1 mm)	UV: 208 nm And HPLC-MS-APCI	---	94-100%	Careri et al. (2001) *J. Chrom. A.*
Sterols	A: ACN B:MeOH 0.01% acetic acid 0.5 mL/min, gradient	C_{18} (5 µm x 150 mm x 2.1 mm)	HPLC-MS ESI and APCI	30 °C oven	Not reported	Canabate-Diaz et al. (2007) *Food Chem.*

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