ABSTRACT

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Soybeans have diverse uses in foods and are known for their health-beneficial properties. Research has shown that consumption of soybeans or their components may help to prevent or alleviate chronic illnesses including heart disease, some cancers, and osteoporosis. Foods made from soybeans can develop flavors or odors that are unfavorable, in part because of lipid oxidation. Approaches to prevent lipid oxidation include modification of soybean traits such as fatty acid composition or lipoxygenase enzyme levels. Soybeans with modified α-linolenic acid (18:3n3) and soybeans with reduced lipoxygenase were analyzed to determine if there was an unintentional effect of modification on health-beneficial components. The effects of genotype and growing environment on nutraceutical components were analyzed for the modified 18:3n3 soybeans. Additionally, an assay for measurement of lipoxygenase-1content in soybean meal was developed for high-throughput analysis using fluorescein as a fluorescent probe. The results showed a significant effect of genotype of at least $P < 0.05$ for fatty acid composition, isoflavone levels, lutein, and

tocopherol levels in the modified 18:3n3 soybeans. Environment had significant effect on certain fatty acids, lutein, tocopherols, and oxygen radical absorbance capacity (ORAC). The effect of genotype × environment was significant for total phenolic content (TPC) and antioxidant activity against hydroxyl radical, as well as isoflavone composition, fatty acid composition, lutein, and tocopherol composition. Soybean lines with modified-lipoxygenase content contained similar or higher isoflavone levels compared to non-modified soybeans. The results of this research demonstrated that soybean modifications for improved oil stability did not adversely affect the health-enhancing components. Additionally, genotype or growing environment may be a factor in selecting the best soybean lines for nutraceutical development.

NUTRACEUTICAL PROPERTIES OF SOYBEANS WITH MODIFIED TRAITS

By

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Introduction

Soybean is a legume with diverse uses in foods and several health-enhancing components. This study aims to examine the health-beneficial properties of Maryland-grown soybeans that have been modified for specific traits. The first objective is to study low 18:3n3 soybean lines for antioxidant capacity and phytochemical composition, and identify effects of genotype, grown environment, and their interaction on these properties. The second objective is to study modified-lipoxygenase soybean lines grown in Maryland for nutraceutical properties including antioxidant capacity and phytochemical composition. Soybean lines with notable composition may be selected for further study or development of functional foods. The third objective is to investigate an improved chemical assay for identification of the soybean LOX enzyme. This will help to determine the effectiveness of LOX-modification efforts and identify soybean lines that may be useful for further breeding.

Chapter 1: Literature Review

1.1. Overview

Soybeans and their components comprise a considerable portion of animal and human food products in the United States. Soybeans comprise 90% of oilseeds grown in the U.S., and in 2005 were the second-largest crop in area and value (Ash et al., 2006). The majority of soybean meal (98%) is used for animal feed, with the remaining meal used in human food products. Soybean oil, however, is extracted and used extensively in human foods. The U.S. Food and Drug Administration allowed a health claim for soy protein in 1999 (Ash et al., 2006) and this helped to increase public awareness of soy as a health beneficial food. The current American use of soy includes traditionally Asian foods (tofu, tempeh, natto, miso, and soymilk) (Golbitz & Jordan, 2006), as well as vegetarian foods and mock meats. Additionally, soy components such as oil, protein, lecithin, fiber, and sterols are used in processed foods for both functionality and nutrition (Sugano, 2006).

In addition to the health benefits, soy food consumption can be promoted because increased emphasis on plant-based proteins in place of animal-based proteins may reduce the environmental impact of current agricultural practices. According to Carlsson-Kanyama & Gonzalez (2009), when comparing the overall grams protein obtained per kg of greenhouse gases produced, cooked soybeans yield 12 times more protein per kg than beef, and 2 times that of chicken. It may therefore be economically and environmentally efficient to increase consumer demand for soy foods.

Despite increased consumer interest in functional foods, many soy-based foods are considered "health" foods, and appeal to certain groups but not to the general American population. As reported by the consumer research group Mintel, the market for soy foods decreased from 2008 to 2010 and is expected to continue decreasing through 2012. While some consumers like the taste of soy foods, 45% of those that avoid soy say they dislike the flavor (Mintel, 2011).

Some soy foods have odors or flavors that are disagreeable to consumers, although food processing can help to reduce them (Macleod & Ames, 1988; Yuan & Chang, 2007). High content of polyunsaturated fats and oxidative enzymes found naturally in soybeans may partially contribute to the perceived poor flavor of soy products. Consumers usually consider flavor first when deciding to purchase a food. However, consumers are also more likely to purchase a functional food if they know its health benefits (Wansink et al., 2004). Increased knowledge of the health components of soy, as well as improved flavors, may help to increase the consumption of this versatile food.

1.2. Soybean Composition, Use in Foods, and Nutritional Properties

1.2.1. Soybean Oil

Soybean oil is consumed more than any other type of edible oil in the United States and the rest of the world (Eckel et al., 2007). It is used to produce cooking oils and margarines, and is used in all types of packaged and processed foods. The ubiquitous use of soybean oil draws concerns over its health effects. Soybean oil consists of a high amount of polyunsaturated fats, which are generally considered

more healthful than the saturated fats found in animal products. However, these polyunsaturated fats decrease the oxidative stability of the oil in processed foods. Hydrogenated soybean oil is more resistant to oxidation, so it is included in packaged foods to improve the shelf-life (DiRienzo et al., 2008).

1.2.1.1. Soybean Oil Composition

Oil consists of triacylglycerol molecules that contain 3 fatty acids attached to a glycerol backbone. Fatty acids contain a carboxylic acid and an aliphatic tail (Nawar, 1996). Short-chain fatty acids have 2-6 carbons, medium-chain fatty acids have 8-12 carbons, and long-chain fatty acids have 14 or more carbons. During exposure to heat or enzymes, the triacylglycerol may be hydrolyzed, and the fatty acids released. The free fatty acids are more susceptible to oxidation than the triacylglycerol (Nawar, 1996).

Soybean contains on average 15 to 20% oil by weight. The major fatty acids consist of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic (18:3) (Sugano, 2006). The percentage of each fatty acid may vary depending on the genotype of soybean. Some genotypes are bred to contain altered quantities of certain fatty acids. The typical soybean that has not been modified contains 10% palmitic, 4% stearic, 23% oleic, 53% linoleic, and 8% linolenic fatty acids (Snyder & Kwon, 1987). Unprocessed soybean oil also contains phytosterols, phospholipids, tocopherols, carotenoids, and chlorophyll (Snyder & Kwon, 1987). Oil processing includes degumming and bleaching steps that remove many of the phytochemicals.

1.2.2. Soy Protein

Soy protein is available in multiple forms for use in processed foods. Commercial soy protein products are available as soy flour, soy protein concentrate, and soy protein isolate, and protein content of each product depends on the production method (Paulson et al., 2006; Singh et al., 2008). Soy flour is higher in protein and lower in carbohydrate than wheat flour. The flour can be produced in full-fat or defatted lines. Soy flour is added to baked goods to improve moisture content and texture. Soy flour added to wheat products can also help to bleach undesirable colors due to activity from the soybean lipoxygenase enzyme. Soy protein concentrate and isolate are added to products such as nutritional bars, powdered protein shakes, and meat analogs (Singh et al., 2008)

1.2.2.1. Soy Protein Nutritional Properties

Soy protein contains all of the essential amino acids for humans, which makes it unique among plant-based proteins (Singh et al., 2008). Protein makes up 40% dry weight of the soybean. Soy protein prior to processing is not highly bioavailable due to inhibition factors such as trypsin inhibitors, urease, and hemagglutitin. Even traditional soy foods such as tofu or edamame are processed with heat before they are consumed. Soy protein isolate can be processed to have protein digestibility amino acid score comparable to animal protein (Singh et al., 2008). These factors make soy protein a good choice for addition to vegetarian food products and nutritional supplements.

1.2.2.2. Soy Protein Effect on Serum Cholesterol

Observational studies have shown a reduced incidence of cardiovascular disease (CVD) correlated with soy protein intake (Vega-Lopez & Lichtenstein, 2005). Elevated levels of total serum cholesterol and LDL-cholesterol (LDL) are traditional risk factors for CVD. A 1995 meta-analysis of randomized controlled trials by Anderson et al. showed a significant reduction in total cholesterol, LDL, and triglycerides from soy protein compared with animal protein (Anderson et al., 1995). Intake of 25 g per day is recommended to meet the U.S. Food and Drug Administration-approved a health claim for soy protein and risk of coronary disease (FDA, 2011). This amount is equivalent to 3 cups of soy milk or 10 ounces firm tofu. Interestingly, a review by Messina et al. (2006) indicates that typical intake of soy protein in Japan is 8-11 g per day, and this population still appears to obtain health benefits from dietary soy. However, this data reflects the total population on average. Older people in Japan have more traditional diets with higher levels of soy protein (Messina et al., 2006). Since the approval of the health claim, a large number of clinical trials have been conducted on dietary intake of soy protein (Sirtori & Johnson, 2006; Xiao, 2008; Zhan & Ho, 2005). Many studies have found a larger LDL reduction in hypercholesterolemic subjects as opposed to those with normal serum cholesterol (Anderson et al., 1995; Zhan & Ho, 2005).

The mechanism of soy protein effect on LDL reduction has been attributed to reduced absorption of intestinal cholesterol, reduced cholesterol synthesis, or stimulation of LDL receptor transcription (Cho et al., 2008). An in vitro study of soy

protein hydrolysate on human liver cell lines showed significant increase of LDL receptor transcription (Cho et al., 2007). This would then increase uptake of LDL-cholesterol in the liver and reduce serum levels. The same study did not find a high bile acid binding capability in soy protein hydrolysate, and found that at high levels the soy protein hydrolysate actually stimulated cholesterol synthesis. Thus more research is needed to identify how soy protein exerts an effect on serum cholesterol.

A 2006 analysis by the American Heart Association nutrition committee found that the effect of soy protein on cholesterol levels was not as significant as previously thought (Sacks et al., 2006). The authors found that in well-controlled studies, LDL cholesterol reduced only 2 to 7 percentage points with intake of soy protein compared with animal protein. The announcement of the results cast doubt upon the previous health claim. The AHA nutrition committee concluded that soy remains a heart-healthy food due to its low-saturated fat content. However, the cholesterol-reducing effects may not be sufficient to warrant high consumption of soy protein for that purpose. Soy protein intake has also not shown clear effect on hypertension, or on the high-density lipoprotein (HDL) (Xiao, 2008).

Some evidence from in vitro and animal studies shows that soy protein can reduce adiposity and promote greater percentage of lean body mass (Bhathena & Velasquez, 2002). Mechanisms for the impact of soy protein on obesity include increased satiety, improved insulin sensitivity, lipid metabolism, and hormonal effects. Studies in humans have not shown a clear reduction in body weight or fat mass with soy protein when compared with animal protein. However, the soy protein

diets in the studies did improve serum LDL and triglycerides more effectively than animal protein diets (Bhathena & Velasquez, 2002).

1.2.3. Phenolics

Soybeans contain several types of phenolic compounds, including phenolic acids and isoflavones (Sakthivelu et al., 2008; Snyder & Kwon, 1987). Phenolics are plant compounds consisting of an aromatic ring and hydroxyl group. Phenolic acids are known to possess anti-radical and antioxidant activity, due to donation of the hydrogen from the hydroxyl group (Sroka & Cisowski, 2003). In fact, they are one of the most effective antioxidants from natural sources, more potent in vitro than other dietary antioxidants such as ascorbic acid, α-tocopherol, and β-carotene (Rice-Evans et al., 1996). The number and position of the hydroxyl groups on the phenolic ring has been correlated with the antioxidant activity of the particular phenolic compound (Sroka & Cisowski, 2003).

Polyphenols in plants are composed of aromatic rings with 2 or more hydroxyl groups, and are associated with plant coloring and defense against environmental stress. Polyphenols in the human diet are linked to health benefits such as prevention of cardiovascular disease (Hooper et al., 2008; Manach et al., 2005). Most initial evidence for the health benefits of polyphenols was from epidemiological studies observing dietary intake of polyphenol-rich foods (Manach et al., 2005). Some proposed mechanisms for the effect of these compounds are inhibition of LDL-cholesterol oxidation, inhibition of blood platelet aggregation, improved endothelial wall function, and reduction of oxidative stress and inflammation (Manach et al., 2005; Rice-Evans et al.,1996). Flavonoids are the largest subclass of polyphenols.

Flavonoids in particular have shown to improve endothelial elasticity and to improve flow-mediated dilation of arteries (Duffy & Vita, 2003). While many in vitro and animal studies have confirmed the benefits of polyphenols, it is unclear what the actual impact dietary intake of polyphenols confers to humans. There is wide variation in the bioavailability of ingested polyphenols, and often they are metabolized and excreted quickly in the urine. Often the levels of phenolic compounds used in animal or in vitro studies are not feasible for dietary intake in humans (Manach et al., 2005). Metabolites of the polyphenols are not always measured, and these may have their own biological activity, such as through cell signaling pathways (Crozier et al., 2009).

1.2.4. Isoflavones

Isoflavones are flavonoids found in mainly in legumes, and in highest amounts in soybeans. Soybeans typically contain from 0.1 to 5 mg total isoflavones per gram of whole bean (Jian, 2009; Larkin et al., 2008). Many health effects of soybeans are speculated to be related to the isoflavones. Isoflavone levels are associated with the protein content of the soybean. After protein extraction from soy meal, the isoflavone level of the protein is similar to that of the whole bean prior to extraction (Larkin et al., 2008).

There are three forms of soy isoflavones: genistein, daidzen, and glycitein. In whole soybeans and many soy foods, isoflavones exist as hydrophilic glucoside conjugates: 6”-O-malonylglucosides, 6”- O-acetylglucosides, and β-glucosides (Larkin et al., 2008). During digestion, these are hydrolyzed to the more bioavailable aglycone form (Cassidy et al., 2006; Koh & Mitchell, 2007). Fermentation and some

types of food processing also produce the aglycone form prior to food consumption (Villares et al., 2011).

The specific isomers of isoflavones may have different bioactive properties (Lakshman et al., 2008; Pavese et al., 2010). For example, in several studies, the isoflavone genistein has shown activity against hormone-related cancers in vitro (Pavese et al., 2010). Additionally, different levels of each are noted within soybean seeds. For example, Sakthivelu et al. (2008) found that as total isoflavones increased, the percentage of genistein increased compared to daidzein. Riedl et al. (2007) found that the Ohio soybeans had increased daidzein over genistein when total isoflavones increased. This increase in one isomer over the others was proposed to be related to the soybean growing conditions such as moisture and temperature. Identification of the isoflavone profile is important in understanding the potential health benefits of different soybean lines.

1.2.4.1. Health Benefits of Isoflavones – Cardiovascular

The isoflavone content of foods varies by type of food and processing conditions (Villares et al., 2011). In studies of isoflavones separated from soy protein, the isoflavones do not have significant effect on reducing serum LDL (Sirtori & Johnson, 2006). However, soy protein without isoflavones retains its hypocholesterolemic properties (Sirtori & Johnson, 2006). Most evidence shows a synergistic effect of soy isoflavones and soy protein on reduction of LDL cholesterol (Sirtori & Johnson, 2006). A meta-analysis of flavonoids by Hooper et al. (Hooper, et al., 2008) found that soy protein isolate significantly reduced LDL cholesterol and diastolic blood pressure, while other soy products did not.

Soy isoflavones can, however, provide antioxidant activity that may protect against cardiovascular disease. LDL oxidation (ox-LDL) is known to promote aggregation of fatty streaks in arteries (Stocker & Keaney, 2004). In vitro, animal, and human studies indicate that inhibition of lipid peroxidation by soy isoflavones may reduce risk of atherosclerosis (Fritz et al., 2003; Sirtori & Johnson, 2006). Kapiotis et al. (1997) reported that genistein is capable of inhibiting LDL oxidation in vitro under initiation by two separate oxidation systems: copper (2^{+}) and a superoxide/nitric oxide radical system ($O_2^{\bullet-}/NO^{\bullet}$). Genistein was also able to prevent oxidation of LDL in endothelial cells, and prevent damage to the cells by ox-LDL. Yamakoshi et al. (2000) fed New Zealand rabbits a cholesterol-containing diet supplemented with two doses of isoflavone-rich extracts of fermented soy (0.33 and 1g isoflavone aglycones/100 g food). After 8 weeks, HPLC analysis of cholesteryl ester hydroperoxides demonstrated an increase in resistance to Cu-induced LDL oxidation with the isoflavone-supplemented diets. The high dose produced a significant reduction in hydroperoxides (-94%), while the low dose produced a reduction that was not significant (-37%). Additionally, atherosclerotic lesions in the aortic arch were significantly decreased by both isoflavone treatment levels, but no difference in serum cholesterol levels was seen. In human studies, supplementation of the diet with a soy food resulted in a significantly prolonged lag phase of LDL oxidation as compared to control diets (Ashton et al., 2000; Scheiber et al., 2001; Tikkanen et al., 1998; Wiseman et al., 2000), with effective doses ranging from 15 g soy protein/50 mg isoflavones per day (Wiseman, et al., 2000) to 33 g soy protein/86 mg isoflavones per day (Kendall et al., 2002). The length of dietary interventions

ranged from 2 to 12 weeks. Based on the above studies, the antioxidant effect of soy isoflavones against LDL-cholesterol may be their largest benefit against cardiovascular disease.

1.2.4.2. Health Benefits of Isoflavones – Cancer Prevention

Isoflavones are classified as phytoestrogens that can bind to estrogen receptors in the body. Much of their unique biological activity is related to the ability to act as an estrogen agonist or antagonist (Setchell, 1998). Isoflavones have been shown to lower serum estrogen and lengthen menstrual cycles in premenopausal women (Yamamoto & Tsugane, 2006).

Research has focused on soy in the prevention of some types of cancer, primarily breast, prostate, and colon cancers (MacDonald et al., 2005; Yamamoto & Tsugane, 2006). The initial association was based on epidemiological studies that showed societies with higher soy intake had lower incidence of certain cancers (Wu et al., 2008). For example, it was reported that the incidence of breast cancer in Japan was 32 per 100,000 women, while it was 107.5 per 100,000 among Japanese women living in Hawaii (Messina et al., 2006). It is known that the traditional Japanese diet is much higher in soy than Western diets and this may have partially explained the difference in cancer incidence in that study.

Breast cancer risk related to intake of isoflavones focuses on the estrogen-binding activity of isoflavones. In vitro studies have shown anti-cancer effects at supraphysiological levels (Yamamoto & Tsugane, 2006), however not at normal physiological levels. Studies of animals have shown dietary isoflavones reduced formation of tumors (Setchell, 1998). Evidence from human studies remains

inconclusive (Xiao, 2008; Yamamoto & Tsugane, 2006). While many studies have shown an inverse relationship between soy intake and premenopausal breast cancer, the association between soy and postmenopausal breast cancer is unclear (Yamamoto & Tsugane, 2006). Animal studies and epidemiological study have indicated that intake of soy early in life predicts lower risk of breast cancer as an adult (Yamamoto & Tsugane, 2006).

In prostate cancer, isoflavones have shown to delay the growth of tumors in vitro and in animal studies (Messina, 2003; Pavese et al., 2010). Genistein has shown the largest effect (Jian, 2009). Lakshman et al.(2008) showed inhibition of prostate cancer metastasis in mice with dietary intake of genistein at physiological levels. Some epidemiologic studies have shown inverse relationship of soy intake and prostate cancer, however very few populations have a significant intake of soy foods. It is difficult to conduct case-control studies in Western cultures, for example. However, studies of Seventh-Day Adventists in the United States, and others of Japanese and Chinese men have shown a reduced risk of prostate cancer with intake of soy foods (Jian, 2009).

In relation to colon cancer, case-control studies in Japan showed inverse correlation between dietary isoflavone intake and risk of disease (Akhter et al., 2009; Budhathoki et al., 2011). However, studies of animal models have not shown a conclusive effect from intake of isoflavones (Cooke, 2006). In an in vitro study, Slavin et al. (2009b) demonstrated an antiproliferative effect of soy meal aglycone extracts against HT-29 colon cancer cells. The soy extracts showed stronger

inhibition compared to an aglycone mixture of the same concentration, suggesting that the extract contained inhibitory compounds beyond isoflavones.

1.2.4.3. Soy Isoflavones as a Hormone Replacement Alternative

Isoflavone-rich foods have been studied as an alternative to hormone therapy in menopausal women. Decreasing estrogen levels at menopause can lead to bone density reduction, and many studies have investigated the effect of phytoestrogens on bone health. There has been epidemiological correlation between soy intake and increased spinal bone density in Asian countries. It is not known if the effects are from soy protein, isoflavones, or both (Messina, 2003). Recent research has shown that dietary isoflavones can reduce markers of bone resorption and preserve spinal column bone mass (Ma et al., 2008). A meta-analysis by Liu et al. (2007) reported that diet supplementation with soy isoflavones significantly increased spinal bone mineral density, and the effect was greatest with consumption of more than 90 mg/ day. A study by Piastowska-Ciesielska et al. (Piastowska-Ciesielska & Gralak, 2010) found that a genistein supplement equivalent to 1% soybean in the diet increased the femoral bone mineralization in growing rats. Most human intervention studies of soy isoflavones and bone mass have been inconclusive, possibly due to variation in the dose and composition of the isoflavones.

1.2.5. Carotenoids

Carotenoids are pigment compounds in plants, and serve as protective antioxidants to plant tissues (von Elbe & Schwartz, 1996). β-carotene is a precursor to vitamin A in animals. Other carotenoids present in mammalian serum include

lutein, lycopene, and β-cryptozanthin. The carotenoid structure consists of an isoprene backbone. A large number of double bonds make the structure susceptible to oxidation. However, this double bond allows scavenging of free radicals through acceptance of a hydrogen molecule (Kiokias & Oreopoulou, 2006).

Carotenoids have been researched in prevention of chronic disease with mixed results. Lutein and zeaxanthin have been associated with prevention of age-related macular degeneration and cataracts (Ma & Lin, 2010; Ribaya-Mereado & Blumberg, 2004). Epidemiological studies show that intake of high-carotenoid foods can reduce cardiovascular disease risk, but randomized control trials with dietary supplements have not verified this. In some cases, carotenoid supplementation increased incidence of lung cancer and cardiovascular disease. These results may be related to high levels of supplementation and may not reflect the effects of natural levels of dietary carotenoids (Lichtenstein, 2009).

The carotenoid content of soybeans can vary with the genotype and color (Simmone et al., 2000). Previous studies of mature yellow soybeans showed lutein as the main carotenoid. β-carotene has been detected in green colored soybeans and in immature soybeans. However, few lines of yellow soybeans have been found to contain β-carotene (Monma et al., 1994; Slavin et al., 2009a). Crude soybean oil is processed to eliminate carotenoids due to color preferences (Snyder & Kwon, 1987). However, foods made from whole soybeans will contain carotenoids that may be beneficial to human health.

1.2.6. Tocopherols

Tocopherols are lipophilic compounds in biological membranes that are produced in photosynthetic organisms (Gregory, 1996; Lampi et al., 2008). They are known for antioxidant activity, especially in maintaining oil stability. They contain a chromanol ring and an isoprene side chain. Tocopherols are able to donate hydrogen atoms to lipid peroxyl radicals and thus interrupt the propagation of lipid oxidation (Lampi et al., 2008).

α-Tocopherol is the primary tocopherol responsible for vitamin E activity in animals (Gregory, 1996; Kim et al., 2007). Tocopherol levels in soybean oil have a maximum concentration at which optimum stability is maintained. Above this level, the tocopherols can form peroxyl and oxy radicals, hydroxyl radicals, and singlet oxygen. Thus they become pro-oxidants and the oil can oxidize more rapidly (Kim et al., 2007). Up to 50% of tocopherols may be lost with during deodorization of crude soybean oil (Snyder & Kwon, 1987). While the tocopherol level helps to maintain oxidative stability of oil during frying, over time tocopherol levels are dramatically reduced due to the high temperature (Normand et al., 2003).

The tocopherol content of soybeans is also important nutritionally, because Vitamin E is an essential human nutrient. In the United States, the average dietary intake of vitamin E has been shown to be less than the recommended amount (Maras et al., 2004). Some studies have shown vitamin E intake to be preventive against cardiovascular disease and certain cancers. However, other studies have shown that high levels of vitamin E supplementation may have little effect, or even adverse

health effects (Dutta & Dutta, 2003; Saremi & Arora, 2010). For example, in persons that smoke cigarettes, Vitamin E supplementation along with a high PUFA diet has shown increased LDL oxidation (Weinberg et al., 2001). However, levels of tocopherols in soybean are not sufficient to cause the same effects as dietary supplementation.

As discussed above, soybeans provide a nutritious food source and many of the components have potential added health benefits. It is therefore of interest to further develop soybeans for use as functional foods.

1.3. Oxidative Stability of Soybean Oil

1.3.1. Lipid Oxidation

Lipid oxidation is a major concern in food production as it can cause poor odor and flavor and reduce shelf-life. Lipid oxidation leads to formation of hydroperoxides which break down to further undesirable compounds such as aldehydes, ketones, and dienals (Cherrak et al., 2003; Choe & Min, 2006; Wilson, 2004). Autoxidation is a self-catalytic reaction with molecular oxygen (Choe & Min, 2006; Nawar, 1996). This is thought to be the primary reaction in breakdown of lipids. Other mechanisms for oxidation include exposure to elevated temperature, photochemical reactions, catalysis by metal ions, and enzymatic reactions. The process of lipid autoxidation involves 3 steps: initiation of a free radical, propagation, and termination (Nawar, 1996).

Antioxidants inhibit the propogation reaction by acting as hydrogen atom donors or acceptors. Tocopherols in soybean oil are considered the most important compound to prevent lipid oxidation (Choe & Min, 2006). Phenolic structures are

also useful antioxidants because they form stable resonance structures and prevent attack of the free radical by oxygen (Nawar, 1996).

The high α-linolenic (18:3n3) content of regular soybean oil makes it susceptible to autoxidation. The 18:3n3 molecule contains 3 double bonds, and thus it can deteriorate easily during processing and storage. 18:3n3 oxidizes twice as quickly as linoleic acid (18:2n-6) in stable conditions. At high temperatures during cooking, it can degrade even more rapidly (Wilson, 2004).

The poor stability of natural soybean oil creates a need for hydrogenation in order for the products to remain stable on the shelf or at the high temperatures used in frying (Eckel et al., 2007). The process of hydrogenation changes the double bond structure of the fatty acid, from *cis* to *trans*. The resulting structure is more stable in food products against oxidation and hydrolysis during cooking.

1.4. Modified Soybeans

Soybeans used for human foods have been bred to select for specific traits, especially in the fatty acid profile. Some are bred for low-saturated fat content, some for low α-linolenic acid (18:3n3) content, and others for high-oleic (18:1n-9) content (Ash et al., 2006; Fehr, 2007; Sugano, 2006). Soybean fatty acids may be modified through traditional cross-breeding or genetic engineering. Low levels of unstable polyunsaturated fatty acids are desirable to help reduce the need for hydrogenation (Fehr, 2007). In 2006, the FDA mandated labeling of *trans*-fats in foods, and demand for soybeans with reduced 18:3n3 is increasing (Fehr, 2007).

1.4.1. Value of Low α-Linolenic Soybeans

Low α-linolenic soybeans contain less than 3% 18:3n3 versus the 7% in non-modified soybeans (Sugano, 2006). Some low α-linolenic cultivars contain less than 1% 18:3n3. In commercial baked and fried food products, the desired 18:3n3 content is 2% of total fatty acids to maintain stability without hydrogenation (Wilson, 2004) (Table 1). In order to compete with other stable oils, low α-linolenic soybean oil should be used in food production. A 2009 study compared expeller pressed low α-linolenic soybean oil to other common cooking oils in the stability of tortilla chips over time (Warner, 2009). Chips were fried for 5 and 35 hours and then stored for 4 months. Hexanal formation was measured to determine fatty acid oxidation. With 5 hours of frying, low α-linolenic soybean oil performed as well as high-oleic sunflower oil, corn oil, and hydrogenated soybean oil. With 35 hours of frying, low α-linolenic soybean oil had significantly less hexanal formation than regular soybean oil, however had more than high oleic sunflower oil and hydrogenated soybean oil.

Table 1.1. Preferred Fatty Acid Composition of Cooking Oils*

Fatty Acid	Unmodified Oil	Frying	Baking
Saturated (16:0 + 18:0)	15%	7%	42%
Oleic (18:1)	23%	60%	19%
Linoleic (18:2)	53%	31%	37%
α-Linolenic (18:3)	9%	2%	2%

Table modified from: Wilson, R. Seed composition. In Boerma & Specht (eds.), Soybeans: Improvement, Production, and Uses, 2004, 621.

1.4.1.1. Safety of *Trans* Fat

When compared with unsaturated fatty acids, *trans* fats have an undesirable effect on low-density lipoprotein (LDL) cholesterol and high-density lipoprotein

(HDL) cholesterol. The ratio of total cholesterol to HDL cholesterol is a marker of cardiovascular disease risk. Multiple studies have shown an unfavorable ratio of cholesterol:HDL when *trans* fats are increased in the diet, even compared to high-saturated fat diets. It has been estimated that increasing dietary *trans* fat by 2% may increase coronary disease risk by 23% (Eckel et al., 2007). In recent years, restaurants and food manufacturers have taken measures to reduce *trans* fatty acids in their food products.

The reduction of 18:3n3 content naturally increases the relative levels of other fatty acids in the soybean. The 18:3n3 may be replaced with oleic (18:1n-9), linoleic (18:2n-6), stearic (18:0), or palmitic (16:0) fatty acids. For health considerations, an increase in the monounsaturated oleic acid would be desirable. However, when 18:3n3 is reduced, there is often an increase in saturated fatty acids. This contributes to the stability of the oils, but a higher intake of palmitic acid may have deleterious health effects (Warensjo et al., 2008). An increase in dietary stearic acid intake has been evaluated as non-significant to cardiovascular risk, even though it is a saturated fatty acid (Baer et al., 2003; Warensjo et al., 2008). Cultivars have been developed with both low palmitic and low α-linolenic traits (Cherrak et al., 2003). This is a good combination of traits from the perspective of health value, because saturated fats as well as *trans* fats are reduced in the final food product.

1.4.1.2. Health Effects of Lipid-Modified Soybeans – Long Chain Polyunsaturated Fatty Acids (PUFA)

α -Linolenic acid is an omega-3 fatty acid, as the first double bond is at the third carbon from the methyl group on the carbon backbone. Omega-3 fats are the

subject of much research due to their potential for reducing inflammatory diseases in humans (Kris-Etherton, 2010). The recommended ratio of omega-6 to omega-3 fats in the human diet is approximately 3 to 1. This ratio in non-modified soybean oil is 7 to 1. When modified to 3% 18:3n3, the ratio becomes approximately 18 to 1. From this perspective, reduction of 18:3n3 will further decrease the intake of beneficial omega-3 fats, particularly in light of the large intake of soybean oil in the American diet. However, it has been reported that the benefit of reducing *trans* fats likely outweighs the harm of reducing a-linolenic acid intake (Lichtenstein et al., 2006).

1.4.1.3. Health Effects of Lipid Modified Soybeans – Tocopherols

Previous research has shown that low α-linolenic soybeans have reduced tocopherol content, and indicated that there is a linear correlation between tocopherols and 18:3n3 levels (Almonor et al., 1998; Dolde et al., 1999; McCord et al., 2004). Normand et al. (2003) reported a lower initial level of tocopherols in low α-linolenic soybean oil compared to regular soybean oil. Additionally, after frying, the tocopherols in the low α-linolenic oil degraded more quickly, and the oil produced more polar compounds (oxidation products) than the regular soybean oil. However, McCord et al. (2004) reported that some low- α-linolenic genotypes did have an equivalent tocopherol level as standard soybeans, and that selection for high levels of tocopherol in these soybeans may be possible.

1.4.2. Additional Lipid-Modified Soybeans Through Breeding Efforts

Decreasing saturated fatty acids (18:0 and 16:0) through breeding efforts and genetic modification has been a goal for some soybean producers. Foods must

contain 1 gram or less saturated fat per serving to be labeled as low in saturated fat (Fehr, 2007). Soybean oil should contain less than 7% 16:0 and 18:0 combined to meet this guideline (Fehr, 2007). The first low-saturated soybean oil available commercially was sold in 1997, and was used by the USDA National School Lunch Program. However, canola oil remains lower in total saturated and is less costly to produce. Thus the use of low-saturated soybean oil has not gained commercial popularity (Fehr, 2007).

Increased oleic acid (18:1n-9) is also a goal of soybean breeding programs, due to the stability of this monounsaturated fatty acid (Fehr, 2007). Soybean oil with 50% 18:1n-9 and 1% 18:3n3 was developed by Iowa State University. This oil demonstrated oxidative stability of 15 hours, while the reduced 18:3n3 with normal 18:1n-9 content had only 9 hours of oxidative stability (Fehr, 2007). High oleic (18:1n-9) soybeans have also been produced with 80-90% oleic acid content. This oil has demonstrated good stability, but Warner & Gupta (2005) found it had poor flavor in sensory tests of fried potato chips. Mixing high-oleic and low- α-linolenic in a 1:1 ratio proved the best combination for stability and flavor in that study. The Pioneer seed brand has produced a high-oleic soybean with 75% 18:1n-9, and 3% 18:3n3, which was approved for cultivation in 2010 (Pioneer Hi-Bred International, 2011). The company reports extended frying time of 2 to 3 times that of conventional soybean oil and extended shelf life measured by peroxide value.

1.4.3. Lipoxygenase-Modified Soybeans

Soybean lipoxygenase catalyzes the oxidation of unsaturated fatty acids that contain a cis, cis-1,4-pentadiene moiety (Robinson et al., 1995). The presence of this

enzyme is sometimes considered desirable in soy flour, as it can bleach wheat flour to improve the color in baking (Wolf, 1975). The unfavorable result of the LOX-fatty acid reaction is the formation lipid hydroperoxides. These hydroperoxides are broken down by hydroperoxide lyase into hexanal and hexanol compounds that have an undesirable odor and flavor (Wang et al.,1997). These sensory characteristics are detectable in products when soy flour is greater than 2 to 4 % of the ingredients (Wolf, 1975). The beany or grassy flavor is easy to recognize in unprocessed soy foods, and may discourage consumers from using soy products (Wolf, 1975). The free radical compounds that are produced by the LOX reaction can also destroy the beneficial health components of the soy flour such as tocopherols and carotenoids (Nishiba & Suda, 1998). The soybean LOX enzyme can be inactivated by heat processing or acid treatment. Typically soy meal or flour is treated with heat to inactivate anti-nutritional factors such as trypsin inhibitor, and this will also reduce the lipoxygenase activity. However, the same processes may reduce the phytochemical content of soy flour or reduce the protein quality (Wolf, 1975).

There are three major LOX isoenzymes (LOX-1, 2, and 3) known in soybeans (Axelrod et al., 1981). LOX-1 is most active at pH 9, while LOX-2 and LOX-3 are active between pH 6 and 7. LOX-1 is the most prevalent isoenzyme in soybeans and is the most heat-stable (Matoba et al., 1985). Although LOX-1 is most prevalent, LOX-2 has demonstrated higher production of hexanal than other isoenzymes (Matoba et al., 1985). Interestingly, Hildebrand et al. (1990) reported that presence of LOX-3 reduced the formation of hexanal in soybean homogenate. These authors

suggested that LOX-3 converts hydroperoxylinoleic acid into products that are not available to hydroperoxide lyase, so that hexanals cannot be formed.

The level of LOX in soybean has been positively correlated with the level of hexanal volatiles detected in soymilk (Achouri, et al., 2008). There has been an effort to breed soybean cultivars that are null for one or more LOX isoenzymes in the hope of improving the taste of soy foods. Some studies have indicated that LOX-null products do have reduced hexanal content and improved sensory acceptability (Furuta et al., 1996; Tran et al., 1992; Yuan et al., 2008). Others have indicated that the off-flavors persist even in LOX-null soy products. Iassonova et al. (2009) reported the presence of another enzyme that uses phospholipids as a substrate and produces volatile compounds similar to the LOX products. Additionally, Matoba et al. (1985) found continued hexanal production in soy homogenate after the heat-deactivation of LOX. Lei & Boatright (2008) reported that hexanal was formed in soy protein isolate without LOX activity when reducing agents were present. They also found that transition metals, and iron in particular, acted as catalysts for hexanal formation. The combined evidence suggests that inactivation or elimination of LOX may improve soy food flavor, but may not completely eliminate hexanal volatiles. Additional enzymes, such as peroxidase, and other soy protein components may be partly responsible for volatile components in soy foods.

The soybean line, food ingredients, and processing conditions may all affect the particular activity of LOX in a food product (Kumar et al., 2003; Yuan et al., 2008). For example, cultivar and environmental conditions have shown to effect LOX activity in soybeans (Kumar et al., 2003). The processing pH may also affect

the isozyme activity and subsequent hexanal production. Additionally, the LOX enzyme prefers free fatty acids as a substrate, while soybeans contain mostly triacylglycerols (Matoba, et al., 1985). The addition of free fatty acids as food ingredients can increase the production of hexanal in a soy-based food.

1.4.3.1. Health Aspects of Lipoxygenase-Modified Soybeans

There is little published data about the effect of lipoxygenase modification on the nutrient and phytochemical profile of soybeans. Oliveira et al. (2007) reported that LOX-null soybeans in Brazil contained higher levels of total isoflavones than the non-modified parent soybean cultivar. Nishiba & Suda (1998) reported that non-modified soybeans lost vitamin and antioxidant capacity much more rapidly than LOX-null soybeans when homogenated in water. Based on these findings, it may be possible that LOX-null soybeans have higher initial levels of health-beneficial components. It is also possible that the phytochemicals are better preserved against degradation in LOX-null soybeans. It is of interest to study Maryland-grown soybean lines with modified LOX content to determine variation in antioxidant capacity and chemical composition. These lines could be useful for improved flavor and stability in functional soy foods.

1.5. Influence of Genotype and Environment

It is accepted that the traits of agricultural crops vary by genotype and by the environmental conditions in which they are grown (Moore et al., 2006a; Wang & Zheng, 2001). Additionally, there is frequently an interaction shown between genotype and environment. Moore et al. (2006a) described the effect of genotype and

environment on the antioxidant properties of winter wheat bran. They found that genotype, environment, and their interaction had significant influence on the antioxidant capacity. In soybeans, there have been several studies on the effects of genotype and environment on isoflavones (Hoeck et al., 2000; Riedl et al., 2007; Seguin et al., 2004; Wang & Murphy, 1994). The effects of these variables on protein and oil content, as well as fatty acid composition, have also been investigated. Additional studies have discussed tocopherols, lutein, and antioxidants (Dolde et al., 1999; Kanamaru et al., 2006; Lee et al., 2004; Prakash, Upadhyay, Singh, & Singh, 2007).

Eldridge and Kwolek (1983) described variation in the isoflavone levels of defatted soy flour in different lines grown at the same location. They also reported variation in the same line grown at different locations, and also variation by growing season. Wang and Murphy (1994) found that growing season had more effect on isoflavone levels than growing location. Several groups have reported interactions between genotype, location, and year that affected isoflavone levels (Hoeck et al., 2000; Lee et al., 2003; Riedl et al., 2007; Seguin et al., 2004). In most cases it was still possible to identify genotypes that typically produced high or low isoflavone levels.

The differences between locations and years are often related to the effects of temperature and precipitation. Tsukamoto et al. (1995) studied 7 lines with four different planting dates within one season. They reported that earlier planting dates resulted in lower levels of total isoflavones. This early planting corresponded to higher temperatures during seed development. Britz et al. (2011) also found that

soybeans from early maturity groups were most likely to be influenced by growing season and location compared to late maturity groups (due to more fluctuation in temperature or precipitation at critical growth stages). Rasolohery et al. (2008) demonstrated that soybeans grown at low temperature (13-23 °C) in a controlled environment had significantly higher isoflavone levels than those grown at 18-28 °C or 23-33 °C. Riedl et al. (2007) did not report a significant effect of temperature, but showed that increasing precipitation levels correlated with increased isoflavones. Bennett et al. (2004) found that applied irrigation increased isoflavone levels by as much as 2.5 times. Overall, literature supports the findings that soybean isoflavone levels increase with lower temperatures and higher levels of irrigation or precipitation during the seed development.

Several studies have reported the variation in antioxidant activity by genotype of soybean (Lee et al., 2004; Prakash et al., 2007; Tepavcevic et al., 2010). Few have discussed antioxidant activity in relation to growing environment or year. However, isoflavones are a strong predictor of some measures of antioxidant activity and much data already exists for these as indicated above. Riedl et al. (2007) measured DPPH radical scavenging capacity as well as total phenolic content (TPC) in Ohio soybeans, and compared them among locations. TPC did show significant variation by growing locations, which is expected because the TPC was also highly correlated to isoflavone level. That study did not find a significant effect of growing location on the DPPH radical scavenging capacity.

The total oil and individual fatty acid levels are also affected by temperature during development of the soybean. Ray et al. (2008) found that early planting date

(higher temperature during seed fill) resulted in higher oil content and higher palmitic acid (16:0), yet lower levels of 18:3n3 fatty acid. Genotype × planting date interactions were also observed. Previous studies have also confirmed the relationship between growing temperature and 18:3n3 level (Oliva et al., 2006; Wilcox & Cavins, 1992).

The tocopherol composition of soybeans is known to be variable by both genotype and environment. Dolde et al. (1999) reported that genotype and growing location had an effect on tocopherol levels, and the genotype had a stronger effect than location. They found that total tocopherols were elevated at lower growing temperatures. Almonor et al. (1998) found opposite results in low-linolenic modified soybeans. Britz and Kremer (2002) found that soybeans grown at higher temperatures and in drought conditions increased the level of α-tocopherol compared to δ-tocopherol. Seguin et al. (2010) reported an increase in α-tocopherol levels in seeds with an early planting date (mid-May), and higher δ-tocopherol at later planting dates (mid- to late June). Results were still highly variable over the 6 environments in the study. While there is a correlation between environment and tocopherol levels, it has not been consistent in previous research. This may be explained by an interaction between genotype and environment.

The variation of lutein content of soybeans has been described in some studies. Kanamaru et al. (2006) found significant variation in lutein among different soybean genotypes. Lee et al. (2009) reported significant variation in the same genotypes across 4 environments, although year and planting date did not have significant effect. The proposed reason for environmental variation was not

addressed in that study. The same group also found that lutein level was positively correlated with 18:1n-9 and negatively correlated with 18:3n3. Seguin et al. (2011) studied lutein content of soybeans among 4 environments and over 2 growing seasons. Seeding date and environment were significant factors in lutein variation, but no consistent trend was identified. The specific environmental effects on lutein level of soybeans have yet to be determined.

In summary, soybeans contain several bioactive components that may positively affect human health. The literature has shown that multiple factors may influence the composition of soybeans. Soybeans modified for specific traits such as reduced LOX and 18:3n3 may have improved stability and sensory properties compared to non-modified soybeans. It is of interest to determine the factors that affect nutritional quality and health benefits of soybeans grown in Maryland. Previous research has not specifically addressed the potential effect of genotype and environment on isoflavone composition and antioxidant capacity of reduced 18:3n3 soybeans. There is also little previous research on nutraceutical composition of modified-LOX soybeans. Study of these modified soybean lines is necessary to determine how they can be effectively used for development health beneficial foods.

1.6. Methods for Soybean Research

1.6.1. Gas Chromatography for Fatty Acid Characterization

Gas chromatography (GC) is a commonly used technique for identification of fatty acids in oil. Helium is the carrier gas, and a fused silica capillary column is the stationary phase. A flame ionization detector (FID) fueled by hydrogen gas detects compounds as they are eluted from the column. The FID is best suited to detect

carbon-carbon or carbon-hydrogen bonds (Reineccius, 2003). To create a volatile sample, the triacylglycerols are saponified and esterified to form fatty acid methyl esters (FAMEs) (Reineccius, 2003). Compounds analyzed through gas chromatography must be volatized at high temperatures of 250 degrees C. Lipids are suited to this method after preparation.

An advantage of GC with FID is high sensitivity in analysis. A disadvantage is that time-consuming sample preparation is required, which may alter the sample components (Reineccius, 2003).

1.6.2. High Performance Liquid Chromatography for Isoflavone Analysis

High performance liquid chromatography (HPLC) is a pressurized system that separates compounds based on partition between the stationary phase in the column and liquid solvent(s) (Rounds & Gregory, 2003). In normal phase HPLC, the stationary phase is polar and the mobile phase is non-polar. Reversed-phase HPLC uses a non-polar stationary phase (such as an octadecyl chain), and a polar mobile phase Solutes are retained in the column based on hydrophobicity. Increasing the percentage of organic solvent in the mobile phase will decrease retention time (Rounds & Gregory, 2003). For isoflavone separation, reversed-phase HPLC with ultraviolet-visible (UV-vis) detection is typically used. Water and a polar solvent such as acetonitrile or methanol compose the mobile phase (Collison, 2008; Klump et al., 2001; Penalvo et al., 2004).

Advantages of HPLC analysis are speed of analysis, and high sensitivity if the appropriate detector is used. A disadvantage is the requirement for volatile solvents

in the mobile phase. Another disadvantage is that samples usually require preparation and filtering prior to analysis (Rounds & Gregory, 2003).

Extraction conditions for isoflavone analysis have been thoroughly investigated by several authors (Lin & Giusti, 2005; Luthria et al., 2007; Murphy et al, 2002). As isoflavones exist in different forms, the solvent choice can affect the quantification of each form depending on hydrophobicity. Hydrolysis with base will cleave acetyl- and malonyl- groups, leaving only β-glucosides and aglycones. Complete acid hydrolysis will leave only aglycones present. The current preference is to avoid hydrolysis and measure all 12 isoflavone forms (Collison, 2008). However, the hydrolysis step simplifies quantification and reflects the composition of isoflavones after digestion.

Murphy et al. (2002) reported that aqueous acetonitrile (53%) was the most favorable extraction solvent for isoflavones compared to the same concentration of acetone, ethanol, or methanol. Lin and Giusti (2005) found that 58% acetonitrile was better than 80% methanol or 83% acetonitrile. However, Luthria et al. (2007) reported that dimethyl sulphoxide:ethanol:water (5:75:25, v/v/v) was an optimal extraction solvent. The method of extraction also significantly influenced total isoflavone recovery, with pressurized liquid extraction or sonication producing the highest levels. The choice of extraction method and solvent may depend on the food matrix and the overall purpose of the analysis.

1.6.3. Tocopherol and Carotenoid Analysis

Reversed-phase HPLC is also described in the literature for detection of tocopherols and carotenoids. Often samples are saponified to reduce extraneous

compounds in the oils prior to analysis. However, the process of saponification exposes the samples to heat and a strong base, which may result in loss of some of the carotenoids. Tocopherols and carotenoids are not soluble in water, so a polar organic solvent is more suitable for the mobile phase (Gimeno et al., 2000). Methods for simultaneous detection of tocopherols and carotenoids have been described (Darnoko et al., 2000). If UV detection is used, tocopherols are detected at 295 nm and carotenoids at 450 nm. However, some UV detectors cannot read both wavelengths simultaneously and another detection system is required.

1.6.4. Antioxidant Analysis

Antioxidant capacity has been defined as the ability of a substance to prevent or delay deterioration by oxygen (Huang et al., 2005). There are multiple types of free radicals and different sources of antioxidants within a biological system. While there are several assays that can determine aspects of antioxidant capacity, but there is not one single assay to determine total antioxidant capability (Prior et al., 2005). There is also lack of standardization in antioxidant assays between laboratories. The results of one assay reported by a laboratory are only true under specific conditions, which may not correlate to a biological system (Huang et al., 2005). Several antioxidant assays are required to determine the scope of antioxidant capacity of a single compound.

Assays for antioxidant capacity typically measure either single electron transfer reactions (SET) or hydrogen atom transfer reactions (HAT). HAT reactions occur during the chain-breaking reactions during the propagation stage of autoxidation, and thus can inhibit formation of peroxyl radical. Assays involving

HAT reactions include oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), Crocin bleaching, and LDL oxidation inhibition. SET reactions include Trolox equivalent antioxidant capacity (TEAC), total phenolic content assay (TPC), and 2,2-diphenyl-1-picryhydrazyl (DPPH) (Huang, et al., 2005). Assays have been developed to detect scavenging of ROS or reactive nitrogen species (RNS) using mechanisms other than those previously described. These include hydroxyl radical scavenging capacity (HOSC), singlet oxygen scavenging capacity, and peroxynitrite scavenging capacity (Huang et al., 2005).

In measuring antioxidant capacity of food compounds, it is recommended to choose assays that quantify physiological radicals. In this respect, ORAC and HOSC, and LDL oxidation inhibition assays are advantageous. Huang et al. (2002) identified the area under the curve (AUC) quantification method as a recommended measure in some antioxidant assays.

1.6.4.1. Total Phenolic Content

The total phenolic content is measured in an assay with the Folin-Circlet (FC) reagent (phosphomolybdate and phosphotungstate). The TPC assay is an electron transfer reaction; under basic conditions the phenol becomes a phenolate anion and reduces the FC reagent (Huang et al., 2005). The reagent color turns from yellow to blue upon reduction. After 2 hours, the reaction is measured with a spectrophotometer at 765 nm.

Singleton and Rossi developed a standardized TPC method to produce consistent results (Singleton & Rossi, 1965). The method includes reaction time,

temperature, and ratio of reagent volumes. The phenolic content of the samples is calculated based on a standard curve using gallic acid. The TPC assay is widely performed in antioxidant studies and has good correlation with other assays such as Trolox Equivalent Antioxidant Capacity (TEAC) (Huang et al., 2005). However, many other organic and inorganic compounds can interfere in the reaction (Prior et al., 2005).

1.6.4.2. Hydroxyl Radical Scavenging Capacity (HOSC)

The hydroxyl radical (·OH) is a highly reactive physiological molecule that can damage lipids, proteins, and DNA (Huang et al., 2005). The HOSC assay developed by Moore et al. (2006) measures antioxidant capacity against OH radical generated by the Fenton reaction of Fe(II) and H_2O_2. Fluorescein is the fluorescent probe, and the reaction can be measured in a 96-well plate reader. The antioxidant prevents degradation of the fluorescein by the ·OH. The antioxidant activity is calculated by the area under the curve of fluorescein degradation compared with the blank (Cheng et al., 2006).

The method described by Moore et al. (2006) has been verified by ESR. The HOSC method is a hydrogen atom transfer assay (HAT), which is similar to the physiological lipid peroxidation reaction. Additionally, the HOSC assay is conducted at a physiologic pH and may be a good reflection of radical scavenging under physiologic conditions (Cheng et al., 2006).

1.6.4.3. Oxygen Radical Absorbing Capacity (ORAC)

The oxygen radical absorbing capacity assay was first developed by Cao et al. (1993). The method detects the radical scavenging ability of a chain-breaking antioxidant against peroxyl radical. The radical is generated by 2,2' -azobis(2-amidinopropane) dihydrochloride (AAPH) (Ou et al., 2001). Cao and Cutler used B-phycoerythrin (B-PE) as a fluorescent probe. Later, Ou et al. developed a method using fluorescein as the fluorescent probe that is accurate when conducted with a 96-well plate reader (Ou et al., 2001). AAPH degrades the fluorescent capacity of fluorescein over time at 37 degrees C. The antioxidant capacity of a sample is measured by the area under the curve of fluorescein degradation compared with the blank (Ou et al., 2001). Trolox, a vitamin E analog, is used as the standard to compare antioxidant activity of the sample.

The ORAC assay measures scavenging of a physiological radical, which makes it more relevant than some other antioxidant testing methods (Moore & Yu, 2008). It is also conducted at a physiological pH (7.4). The described method measures only hydrophilic antioxidant capacity, whereas another method would be needed to measure hydrophobic antioxidant capacity (Ou et al., 2001).

1.6.4.4. Relative DPPH· Scavenging Capacity (RDSC)

2,2-diphenyl-1-picryhydrazyl (DPPH) is a commercially available, stable free radical. When reduced by an antioxidant compound, the color of the DPPH· solution decreases. The decreased light absorbance is measured by a spectrophotometer over

time to quantify the rate of the reaction (Huang et al., 2005). DPPH radical scavenging value has typically been reported as percent DPPH· scavenged over time, however there is little consistency between laboratories due to differences in methods (Cheng et al., 2006). Cheng et al. (2006) reported a high-throughput DPPH· scavenging assay measured in Trolox equivalents that can be compared between laboratories. Absorbance of standards and samples is measured at equal time points and the AUC is determined. Trolox equivalent is determined by a standard curve of known Trolox concentration (Cheng et al., 2006).

The DPPH· assay can be measured in different solvents, both polar and non-polar (Cheng et al., 2006). It is also a simple and quick assay to perform, if a micro-plate reader is used. A disadvantage is that it is not a physiological radical, so results do not correlate well with assays such as ORAC or OH radical scavenging. It may also have a reversible reaction with some phenols, such as eugenol, and so the accurate reaction rate may not be determined (Huang et al., 2005).

1.6.5. Lipoxygenase Measurement in Soybean

Programs that breed modified LOX seeds require efficient methods for LOX testing. Most of the popular methods measure the color change of a probe after reaction with linoleic hydroperoxide. In the method described by Axelrod et al.(1981), purified LOX is combined with linoleic acid in a buffer solution. The hydroperoxide formation is measured by a spectrophotometer at 234 nm. The pH of the reaction mixture is 9.0 for LOX-1, 6.1 for LOX-2, and 6.5 for LOX-3. Suda et al. (1995) developed a method to measure the bleaching of methylene blue and β-carotene by linoleic hydroperoxide. This method uses soybean aqueous extract and

linoleic acid. Methylene blue is used as a probe for LOX-1 and LOX-2, and β-carotene is used for LOX-3. The time is measured until bleaching begins to quantify the strength of the LOX. Other colorimetric methods have been reported, such as measurement of the Fe^{3+} - xylenol orange complex (Waslidge & Hayes, 1995) or the DMAB-MBTH method (Anthon & Barrett, 2001). These methods are not quantitative and impurities in the samples may interfere with absorbance. The uptake of O_2 as measured by electrode is also described by Axelrod et al. (1981). Although it is quantitative, the authors note that this method is not always accurate in measuring LOX-2 and LOX-3.

Chapter 2: Effect of Genotype and Environment on Nutraceutical Properties of Low α-Linolenic Soybeans

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2.1. Abstract

Soybeans contain several health-enhancing nutrients and phytochemicals, including isoflavones, carotenoids, and essential fatty acids. Previous research has shown that these properties may vary by soybean genotype and growing conditions. Eight soybean genotypes grown in 3 environments in Maryland were analyzed for total phenolic content (TPC), antioxidant capacity, isoflavone composition, lutein, tocopherols, and fatty acid composition. Soybean samples consisted of 7 low α-linolenic acid (18:3, n-3) genotypes and 1 standard genotype for comparison. Fatty acid composition, isoflavones, lutein, tocopherols, and specific antioxidant assays had significant variation by genotype ($P < 0.05$). Environment had a significant effect on fatty acids, lutein, individual tocopherols, oxygen radical absorbing capacity (ORAC), and the isoflavone glycitein ($P < 0.05$). Analysis of interaction between genotype and environment showed a significant effect on antioxidant capacity, isoflavones, lutein, tocopherols, and fatty acids ($P < 0.05$). 18:3 n-3 content was positively correlated with palmitic acid (16:0) ($r = 0.519$, $P < 0.001$), and with total isoflavones ($r = 0.422$, $P < .001$). The results of this study show that the genotype, growing environment, and their interactions in Maryland-grown low α-linolenic acid soybeans may alter the levels of specific health-enhancing properties.

2.2. Introduction

Soybeans contain numerous compounds that are beneficial to human health. In recent years, soy compounds have been evaluated for their role in prevention of cardiovascular disease, cancer, osteoporosis, and other diseases (Xiao, 2008). Soy is used in a line of food products, and many people select soy foods out of desire for a healthier diet (Schyver & Smith, 2005).

Soybean oil composes a large portion of the American diet, mainly due to its presence in processed foods (Ash et al., 2006). Soy oil contains 7% α-linolenic acid (18:3n3), an unstable fatty acid that can be easily oxidized (Wilson, 2004). With this level of 18:3n3, soy oil is hydrogenated during processing to prevent the off-flavors associated with autoxidation (Wilson, 2004). Hydrogenation of oil may produce harmful *trans* fatty acids that are associated with the increased risk of cardiovascular diseases (Eckel et al., 2007). Through cross-breeding and genetic modification, soybeans have been developed with reduced levels of 18:3n3. The oil of these soybeans can be used in processed foods without the need for hydrogenation (Fehr, 2007). Because the FDA has mandated labeling of *trans*-fats in foods, demand for soybeans low in 18:3n3 has increased (Fehr, 2007). Our previous research has demonstrated that low 18:3n3 soybean genotypes grown in Maryland may possess similar antioxidant capacity, isoflavone, tocopherol, and carotenoid composition to the non-modified genotypes (Slavin et al., 2009).

It has previously been shown that the nutrient composition in food crops is affected by genotype (G), environment (E), or interaction between G and E (G × E) (Wang & Murphy, 1994; Moore et al., 2006; Riedl et al., 2007). Moore et al. (2006)

reported an effect of G, E, and G × E on phenolic acid composition and antioxidant capacity in hard winter wheat grain. They also observed an effect of elevated temperature on total phenolic content in wheat lines grown in Colorado. G, E, and interaction between G and E are known to cause variation in soybean components (Wang & Murphy, 1994). In 1994, Wang & Murphy found that the ratio of isoflavone isomers in soybeans varied due to differences in G. They also found that growing season and location had an effect on the levels of isoflavones. Britz et al. (2008) reported variation in soybean tocopherol levels related to growing season, genotype, and location. Lee et al. (2009) reported the effect of environmental conditions on lutein content in soybeans. However, no previous study has examined the effect of G, E, and their interaction on antioxidant properties and other health-enhancing components of soybeans bred for low α-linolenic acid.

Individual environmental factors such as temperature and precipitation/irrigation have been shown to affect the isoflavone composition of soybeans (Riedl et al., 2007). Furthermore, the effects of growing conditions on soybean isoflavone contents depended on the soy genotype, and genotype of soybean accounted for the potential for isoflavone production. The study of Ohio soybeans by Riedl et al. (2007) found that precipitation rather than temperature was correlated with isoflavone levels. Soybean fatty acid composition may also vary by exposure to environmental conditions. It was found that warmer growing temperatures might increase α-linolenic acid (18:3n3) and linoleic acid (18:2n6), but might decrease the levels of oleic acid (18:1n9) (Wilson, 2004). Ray et al. (2008) found that soybeans with an earlier planting date had reduced 18:3n3 compared to those with a later

planting date. These previous research data indicated possible effects of G, E, and G × E on chemical compositions in soybeans with reduced α-linolenic acid content. Therefore, the present study was conducted to determine whether and how G, E, and G × E may alter the selected health components and antioxidant properties of Maryland-grown low α-linolenic soybeans. This research is part of our continuous effort to enhance the value-added production of Maryland-grown soybeans.

2.3. Materials and Methods

2.3.1. Materials and Chemicals

Whole soybeans from the 2007 growing season were collected by Dr. William Kenworthy of the Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park. Seven genotypes were modified for reduced 18:3n3 and one was a non-modified cultivar commonly grown in Maryland. The soybeans were products of a traditional breeding program. The selected soybeans were grown at two locations in Maryland, one of which had both a full season and a double crop planting. Thirty percent ACS-grade hydrogen peroxide was purchased from Fisher Scientific (Fair Lawn, NJ). 2,2'-azobis(2-aminodopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Fluorescein (FL), iron (III) chloride, 2,2-diphenyl-1-picryhydrazyl radical (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (St. Louis, MO). 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 to particle size 20-mesh using a handheld coffee bean grinder. Five grams of ground soybeans were combined in a tube with 10 mL of petroleum ether. Tubes were vortexed for 15 seconds, and held 20 hours at ambient temperature in the dark. The supernatant was removed and stored. The extraction was repeated twice. The petroleum ether was evaporated overnight under nitrogen, and the remaining oil was weighed. The oil samples were stored in the dark until further testing.

2.3.3. Antioxidant Extraction

The defatted soy flour that remained following oil extraction was air-dried overnight at ambient temperature. One gram of each soy flour sample was combined in a test tube with 10 mL 50% acetone. The tubes were vortexed 3 times for 15 seconds each, and kept in the dark at ambient temperature overnight. The supernatant was removed and filtered, and stored in the dark until further testing.

2.3.4. Fatty Acid Composition

The soybean oil was prepared for gas chromatography (GC) analysis according to a previously described procedure (Yu et al., 2002). The soybean oil was saponified and methylated to form fatty acid methyl esters (FAME), and dissolved in iso-octane. GC analysis was performed with a Shimadzu GC-2010 with FID. Helium was the carrier gas at a flow rate of 2.2 mL/min. The stationary phase was 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 started at 136 ºC, increased by 6 ºC/min until 184 ºC and held for 3 minutes, then increased by 6 ºC/min to a final temperature of 226 ºC. Fatty acids were identified by comparing FAME retention time with that of known standards. The FAMEs were quantified by calculating the area under the curve of each identified peak.

2.3.5. Total Phenolic Content (TPC)

The total phenolic content of each soy flour extract was determined according to a previously described laboratory procedure (Moore & Yu, 2008). The final reagent mixture contained 50 µL of soy flour extract or standard, 250 µL of Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL of ultra-pure water, using gallic acid as a standard. After 2 hours reaction time at ambient temperature, absorbance was read at 765 nm. The reactions were conducted in duplicate and results reported in mg gallic acid equivalents (GAE) per gram of whole soybean.

2.3.6. Isoflavone Composition

Three mL of soy flour extracts in 50% acetone were combined with 0.75 mL 36% hydrochloric acid and heated for 2 hours in a water bath at 55 °C. This step was performed to hydrolyze isoflavones to the aglycone form. The acetone was then evaporated under nitrogen. The remaining solution was extracted 3 times with 4 mL ethyl ether/ethyl acetate (1:1, v/v), and washed with 3 mL distilled water. The ethyl ether/ethyl acetate was removed using a nitrogen evaporator. The remaining soy extract was quantitatively re-dissolved in 0.5 mL methanol and filtered through a 0.45

μm filter prior to HPLC analysis. HPLC was performed according to a previously described method (Lee et al., 2007), using a Shimadzu LC-20AD with autosampler. The column was a Phenomenex C18 (150 × 4.6 mm, 5 μm). The eluent consisted of 99.9% distilled deionized water with 0.1% acetic acid (v/v) (Solvent A) and 99.9% acetonitrile with 0.1% acetic acid (v/v) (Solvent B). The gradient progressed from 25% to 32% solvent B over 20 minutes. The detection wavelength was set at 254 nm. Oven temperature was 30 °C. Peak area of samples was compared to that of known standards to quantify isoflavone content.

2.3.7 Lutein Content

The soybean oil samples and standards were diluted in methanol/acetonitrile/chloroform (7:7:6, v/v/v) and filtered through a 0.45 μm filter. Soybean oil was diluted 1:5 to fall within the standard curve with lutein concentration range of 1-10 μg/mL. HPLC analysis was performed according to a previously described method (Su et al., 2002) using a Phenomenex C-18 column (250 × 4.6mm, 5μm) with a Phenomenex security guard cartridge. The mobile phase was isocratic, using methanol/acetonitrile/chloroform (45:45:10, v/v/v) with 0.05% ammonium acetate (w/v) in the methanol and 0.1% triethylamine (v/v) in the acetonitrile. 50 μL of each standard or sample was injected and run time was 10 minutes, with each sample conducted in duplicate. A standard curve was developed from the known standards, and peak area of unknown samples was compared to this for quantification.

2.3.8. Tocopherol Composition

Soybean oil and tocopherol standards were diluted 1:10 in methyl-tert-butyl ether and filtered through a 0.45 μm filter. Reversed-phase HPLC with UV detection was performed according to a previously described procedure (Darnoko et al., 2000) with modifications. The stationary phase was a Waters C-30 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of methanol/MTBE/water, (81:15:4, v/v/v) (solvent A), and MTBE/methanol (91:9, v/v) (solvent B). The mobile phase was run from 0 to 16% solvent B in 13 minutes, 100% solvent B from 13 to 23 minutes, and re-equilibrated with 100% solvent A from 23 to 32 minutes. Flow rate was 1.0 mL/min., and injection volume was 30 μL. The UV detector wavelength was 295 nm. Each standard and sample was run in duplicate.

2.3.9. Relative DPPH Radical Scavenging Capacity (RDSC)

DPPH scavenging capacity was determined according to a previously described laboratory procedure (Cheng et al., 2006), using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). DPPH radical solution (0.2 mM) was prepared in 50% acetone and filtered through a P5 grade filter paper (Fisher Scientific). Trolox standards were prepared in 50% acetone at concentrations of 6.25 μM, 12.5 μM, 25 μM, 37.5 μM, and 50 μM. Each final reaction mixture contained 100 μL soybean extract, Trolox standard, or 50% acetone (control), and 100 μL 0.2 mM DPPH solution. The absorbance was read at 515 nm. The radical scavenging capacity (RDSC) was calculated from the area under the curve and reported in μmol Trolox equivalents (TE) per gram whole soybean.

2.3.10. Oxygen Radical Absorbing Capacity (ORAC)

The ORAC values were determined using a previously reported laboratory procedure with fluorescein (FL) as a fluorescent probe (Moore & Yu, 2008). Trolox standards were prepared in 50% acetone at concentrations of 20, 40, 60, 80 and 100 μM. The other reagents were prepared in 75 mM phosphate buffer. In the initial reaction, 225 μL 8.16×10^{-8} M FL was combined with 30 μL of sample, standard, or blank in a 96-well plate. The plate was heated at 37 °C for 20 minutes in a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). 25 μL of 0.36 M AAPH was added to each well and the fluorescence of the mixture was recorded every 2 minutes over a 40-minute period at 37 °C. Excitation and emission wavelengths were 485 and 535 nm, respectively. The results were reported as micromoles of Trolox equivalents (TE) per gram whole soybean, based on area under the curve calculations (Ou et al., 2001).

2.3.11. Hydroxyl Radical Scavenging Capacity (HOSC)

The HOSC assay was conducted using a previously reported laboratory procedure (Moore et al., 2006). Trolox prepared in 50% acetone was used as the standard at concentrations of 20, 40, 60, 80, and 100 μM. Fluorescein was used as a fluorescent probe and the assay was performed using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). 3.43 M iron (III) chloride and 0.1999 M hydrogen peroxide were prepared in ultra-pure water. 9.28×10^{-8} M FL was prepared in 75 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 170 μL of 9.28×10^{-8} M FL, 30 μL of sample, standard, or blank, 40 μL of 0.1999 M hydrogen peroxide, and 60 μL of 3.43 M iron (III) chloride. The fluorescence was recorded

every 4 minutes for 4 hours. Antioxidant capacity was calculated by area under the curve (AUC) described by Moore et al. (2006). Results were reported as micromoles of Trolox equivalent per gram of whole soybean.

2.3.12. Data on Environmental Conditions

The precipitation at each location during the growing season was reported by Kenworthy and others of the Maryland Cooperative Extension in "Agronomy Facts No. 32" (Kenworthy et al., 2008). Daily temperature highs, lows, and averages were obtained from records kept by National Oceanic and Atmospheric Administration (NOAA) weather stations in the vicinity of the soybean fields.

2.3.13. Statistical Analysis

Data was analyzed using SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL). Factorial design analysis of variance (ANOVA) was performed on the data using a general linear model (GLM) with three replicates, using genotype and environment as fixed effects. Replicates were samples from each test plot at each location. Tukey's post-hoc test was used to determine differences between means after ANOVA analysis. Correlation was analyzed using a two-tailed Pearson's correlation test. Statistical significance was noted for values of $p < 0.05$ ($\alpha > 0.95$).

2.4. Results and Discussion

The soybeans used in this study were grown in 3 environments in Maryland: the Wye Research Center near Queenstown (full season crop), and 2 environments at the Poplar Hills field near Salisbury, MD (both full season and double crop soybeans

were analyzed and considered different environments). The present study evaluated the chemical compositions and antioxidant properties of eight soybean cultivars grown at the three different environmental conditions. The effects of environmental conditions (E), genotype (G), and the interaction between G and E (G × E) on chemical compositions and antioxidant properties of soybeans were also investigated. The environmental conditions including the precipitation and temperature highs, lows and averages at each location during the growing season are summarized in **Table 2.1**. In addition, the correlation between each examined chemical component and antioxidant property was calculated.

2.4.1. Chemical Compositions of the Eight Soybean Cultivars Grown in Three Maryland Environments

2.4.1.1. Oil Content and Fatty Acid Composition

Oil content of the soybeans ranged from 14.0 to 18.2 g/100 g among all genotypes from the 3 growing locations under the experimental conditions (**Table 2.2**). The fatty acid profiles of the soybeans under the different growing conditions might differ significantly (**Table 2.2**). MD 05-6377 had lowest 18:3n3 concentration ranging from 1.04 to 1.20%, which was significantly lower than all other soybean cultivars from all growing locations. AG2091V, AG3521V, and MD 05-6381 soybeans had 18:3n3 content between 2.1-2.5%, which was significantly higher than that in MD 05-6377 cultivar at all three locations. This range was significantly lower than that in MD 04-6006, MD 05-5656, and MD 04-5217 soybeans grown at Poplar Hills (double cropped) in Salisbury (PD), and that in MD 04-6006, MD 05-5656 at the Wye Research Center location in Queenstown, Maryland (**Table 2.2**). MD 05-6377

Table 2.1. Environmental Conditions During Soybean Growing Season by Genotype and Environment*

	Abs. High (°C)	Abs. Low (°C)	Avg. High (°C)	Avg. Low (°C)	Overall Avg. (°C)	Precipitation (in.)
AG2091V /PF	36.7	6.1	29.4	15.0	22.3	9.39
AG2091V /PD	36.7	6.1	29.6	15.2	22.5	8.68
AG2091V/ W	37.8	11.7	28.9	20.2	24.5	9.14
AG3521V/ PF	36.7	6.1	29.4	15.0	22.3	9.39
AG3521V /PD	36.7	6.1	29.6	15.2	22.5	8.68
AG3521V/ W	37.8	11.7	28.9	20.1	24.5	9.14
MD 04-6006/ PF	36.7	6.1	29.4	15.0	22.3	9.39
MD 04-6006 /PD	36.7	6.1	29.6	15.2	22.5	8.68
MD 04-6006/ W	37.8	11.7	28.9	20.1	24.5	9.14
MD 05-5656/ PF	36.7	6.1	29.4	15.0	22.3	9.39
MD 05-5656/ PD	36.7	6.1	29.6	15.2	22.5	8.68
MD 05-5656 /W	37.8	11.7	28.9	20.2	24.5	9.14
MD 05-6377/ PF	36.7	6.1	29.4	15.0	22.3	9.39
MD 05-6377/ PD	36.7	6.1	29.6	15.2	22.5	8.68
MD 05-6377/ W	37.8	11.7	28.9	20.2	24.5	9.14
MD 05-6381/ PF	36.7	6.1	29.4	15.0	22.3	9.39
MD 05-6381/ PD	36.7	1.7	29.1	14.6	22.0	9.95
MD 05-6381/ W	37.8	11.7	28.7	19.9	24.3	9.14
MD 04-5217/PF	36.7	6.1	29.4	15.0	22.3	9.39
MD 04-5217/ PD	36.7	6.1	29.6	15.2	22.5	8.68
MD 04-5217/ W	37.8	11.7	28.9	20.1	24.5	9.14
Manokin/ PF	36.7	6.1	29.4	15.0	22.3	9.39
Manokin/ PD	36.7	1.7	29.1	14.6	22.0	9.95
Manokin /W	37.8	11.7	28.7	19.9	24.3	9.14

*Temperatures reported for each location and genotype represent absolute high, absolute low, average high, average and overall average in ° C during 2007 season from planting to harvest. Precipitation is reported in inches. Differences among genotypes at the same location are due to differing number of days to maturity. PF: Poplar Hills full season crop (Salisbury, MD), PD: Poplar Hills double cropped (Salisbury, MD), W: Wye Research Center (Queenstown, MD).

Table 2.2. Oil Content and Fatty Acid (FA) Composition of Soybeans*

	Oil	16:0	18:0	18:1n-9	18:2n-6	18:3n3
AG2091V /PF	18.2e ± 0.1	10.7j-l ± 0.1	4.6b-d ± 0.2	31.0ef ± 2.1	51.4d ± 2.12	2.3bc ± 0.0
AG2091V /PD	17.3de ±1.3	11.1lm ± 0.4	6.1f-h ± 0.3	26.9cd ± 1.4	55.0gh ± 1.4	2.5cd ± 0.1
AG2091V/ W	16.6b-e ±0.5	10.8j-l ± 0.1	4.2ab ± 0.1	32.3fg ± 0.5	50.6d ± 0.5	2.2b ± 0.0
AG3521V/ PF	16.9c-e ± 0.2	10.5jk ± 0.2	5.0d ± 0.6	26.0c ± 1.4	56.5hi ± 2.2	2.5cd ± 0.1
AG3521V /PD	17.5de ± 2.0	10.6jk ± 0.1	4.8d ± 0.0	22.0ab ± 0.3	59.7h ± 0.2	2.5de ± 0.1
AG3521V/ W	16.7b-e ± 0.5	10.5j ± 0.1	3.8a ± 0.0	26.5c ± 0.6	56.7hi ± 0.6	2.5cd ± 0.1
MD 04-6006/ PF	14.6a-c ± 1.3	6.8f ± 0.1	6.3g-i ± 0.1	36.5 i ± 0.7	47.2c ± 0.7	3.1ef ± 0.1
MD 04-6006 /PD	14.6a-c ± 0.4	7.6g ± 0.2	5.5e ± 0.1	27.9cd ± 1.2	55.1gh ± 1.1	3.5fg ± 0.1
MD 04-6006/ W	16.1a-e ± 3.7	6.8f ± 0.1	4.6b-d ± 0.1	41.4l ± 1.2	43.9a ± 0.9	3.6hi ± 0.2
MD 05-5656/ PF	14.0a ± 1.0	6.0e ± 0.1	5.9e-h ± 0.3	37.4jk ± 1.5	47.6c ± 1.6	3.1ef ± 0.1
MD 05-5656/ PD	15.7a-e ± 2.3	5.8c-e ± 0.2	6.7l ± 0.1	39.9l ± 0.3	44.1ab ± 0.2	3.5fg ± 0.1
MD 05-5656 /W	14.3ab ± 0.2	6.0e ± 0.1	4.6b-d ± 0.1	39.3kl ± 0.8	46.5c ± 0.9	3.6hi ± 0.2
MD 05-6377/ PF	16.3a-e ± 0.2	4. 3a ± 0.1	5.0ef ± 0.3	34.3gh ± 0.1	54.7f-h ± 0.3	1.0a ± 0.04
MD 05-6377/ PD	17.2de ± 0.4	4.7b ± 0.2	5.0d ± 0.0	27.0 c ± 0.2	62.2k ± 0.2	1.0a ± 0.05
MD 05-6377/ W	15.2a-d ± 0.5	4.2a ± 0.2	4.6b-d ± 0.1	37.7jk ± 0.5	52.3de ± 0.5	1.2a ± 0.17
MD 05-6381/ PF	16.3a-e ± 0.2	5.5c ± 0.3	5.7ef ± 0.3	30.2ef ± 0.5	56.6hi ± 0.6	2.1b ± 0.2
MD 05-6381/ PD	17.2de ± 0.4	5.9de ± 0.3	4.8d ± 0.1	26.5c ± 0.5	60.6jk ± 0.8	2.2bc ± 0.2
MD 05-6381/ W	15.2a-d ± 0.5	5.6cd ± 0.1	4.2ab ± 0.1	29.2de ± 0.6	58.7ij ± 0.3	2.3bc ± 0.3
MD 04-5217/PF	17.2de ± 0.7	9.5l± 0.2	6.3hi ± 0.5	34.0hi ± 1.2	46.2bc ± 1.5	3.0de ± 0.1
MD 04-5217/ PD	17.0c-e ± 0.4	9.8 ± 0.2	5.8e-g ± 0.5	26.8c ± 2.0	54.0efg ± 2.1	3.4f-h ± 0.2
MD 04-5217/ W	16.2a-e ± 0.5	9.2h ± 0.2	4.3a-c ± 0.1	39.3kl ± 1.6	44.2ab ± 1.4	3.3ef ± 0.2
Manokin/ PF	16.6b-e ± 0.6	10.9k-m ± 0.1	4.8cd ± 0.1	23.2ab ± 0.2	54.1e-g ± 0.1	7.0j ± 0.1
Manokin/ PD	15.3a-d ±0.4	10.7jk ± 0.2	5.5e ± 0.1	23.7b ± 0.3	52.6d-f ± 0.1	7.5k ± 0.1
Manokin / W	15.4a-d ±0.5	11.2m ± 0.1	4.5b-d ± 0.1	21.1a ± 1.0	55.2gh ± 0.5	8.1l± 0.4

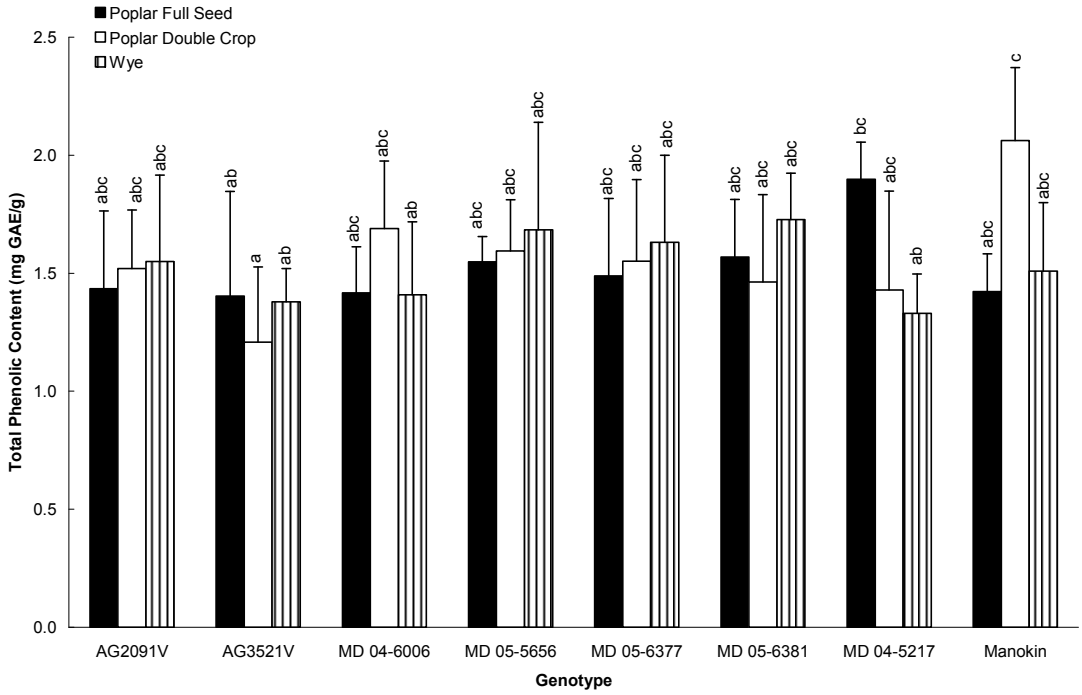
*Data are expressed as mean of 3 replicate plots, each tested in duplicate, ± SD (N= 6). Oil is expressed as g/100 g of whole soybean. Fatty acids are expressed as g/100 g oil. All genotypes are low 18:3n3, except Manokin, which is a non-modified genotype. Values marked by the same letter within each fatty acid group are not statistically different ($p < 0.05$). PF: Poplar Hills full season (Salisbury, MD); PD: Poplar Hills double cropped (Salisbury, MD); W: Wye Research Center (Queenstown, MD).

soybean from all three locations also had significantly lower palmitic acid (16:0) level, with a range of 4.2-4.7 g/100g fatty acids, than the other soybeans grown at all tested locations. Interestingly, all seven low-linolenate soybeans grown at Wye Research Center had higher or same concentration of oleic acid (18:1n9) compared to the same genotype grown at the other two locations (**Table 2.2**). These data suggested that both genotype and growing environment could alter oil content and fatty acid composition in soybeans.

2.4.1.2. Total Phenolic Content

Phenolics are potential antioxidative components (Sroka & Cisowski, 2002). Total phenolic content of the soybeans was between 1.6 and 2.1 mg GAE/g whole soybean (**Figure 2.1**). These values are consistent with previously reported levels of soybean TPC value of 1.5 - 5.4 mg GAE/g (Riedl et al., 2007; Slavin et al., 2009). AG3521V and Manokin grown at the Wye Research Center location significantly differed in their TPC values, suggesting the possible effect of genotype on TPC. No difference in TPC was observed in any tested soybean genotype grown at the different locations.

Figure 2.1. Total Phenolic Content (TPC) of Soybeans*



*Data are expressed as mg gallic acid equivalent (GAE)/g soybean. Values represent mean of 3 replicate plots ± SD (n = 6). Values marked by the same letter are not statistically different ($p < 0.05$). Poplar full season, Poplar double crop, and Wye indicate growing environment.

2.4.1.3. Isoflavone Composition

The total and individual isoflavones were estimated and reported in their aglycone levels. Total isoflavones in the soybean samples ranged from 0.37 to 0.90 µmol/g soybean among all genotypes grown at different environments (**Table 2.3**). This total isoflavone content is lower than that reported previously. Riedl et al. (2007) found total isoflavones in Ohio soybeans in a range of 1.6 and 7.1 µmol/g when extracted from soy flour with acidic acetonitrile. Slavin et al. (2009) found 0.9-2.4 µmol/g in Maryland-grown soybeans from the 2005 growing season. This difference might be partially due to the different extraction solvent, extraction procedures, growing seasons, and different soybean cultivars or lines.

Table 2.3. Isoflavone Composition of Soybeans*

	Daidzein	Glycitein	Genistein	Total Isoflavones
AG2091V/ PF	31.2ab ± 9.6	68.4d-g ± 16.2	21.6ab ± 6.4	0.45a ± 0.01
AG2091V/ PD	38.5b-d ± 9.4	58.2b-g ± 17.6	39.8a-d ± 9.8	0.50ab ± 0.12
AG2091V/ W	34.2ab ± 3.2	95.8g ± 7.6	30.9a-d ± 4.4	.59a-c ± 0.05
AG3521V/ PF	34.1ab ± 7.6	84.6 ± 7.8	31.6a-d ± 10.0	0.55 a-c ± 0.08
AG3521V/ PD	31.2ab ± 1.3	44.6a-f ± 3.9	33.1a-d ± 2.1	0.40a ± 0.01
AG3521V/ W	33.2ab ± 3.2	61.0c-g ± 3.1	34.4a-d ± 1.4	0.47a ± 0.02
MD 04-6006/ PF	41.0a-d ± 1.6	75.1fg ± 11.0	25.3ab ± 2.7	0.52ab ± 0.03
MD 04-6006 /PD	63.8a-e ± 4.4	33.7a-c ± 1.12	46.5a-d ± 5.3	0.54ab ± 0.04
MD 04-6006/ W	53.8a-e ± 11.8	74.3e-g ± 32.9	38.3a-d ± 13.0	0.62a-c ± 0.21
MD 05-5656/ PF	78.2d-f ± 7.5	49.9a-g ± 5.4	52.9b-e ± 6.7	0.68a-c ± 0.07
MD 05-5656/ PD	58.3a-e ± 18.6	41.7a-d ± 6.3	43.0a-d ± 14.7	0.54ab ± 0.14
MD 05-5656 / W	84.2ef ± 22.9	78.2g ± 10.5	61.9a-d ± 18.9	0.83bc ± 0.18
MD 05-6377/ PF	29.3a ± 8.4	61.2c-g ± 25.9	15.3a ± 5.3	0.39a ± 0.14
MD 05-6377/ PD	38.8a-d ± 3.5	35.3a-c ± 4.5	36.0a-d ± 3.0	0.41a ± 0.04
MD 05-6377/ W	32.4ab ± 3.7	46.3a-f ± 10.5	22.2ab ± 2.2	0.37a ± 0.04
MD 05-6381/ PF	44.2a-d ± 6.4	55.6a-g ± 12.2	18.9a ± 3.8	0.44a ± 0.06
MD 05-6381/ PD	33.7ab ± 5.1	35.3ab ± 4.5	30.0a-d ± 2.6	0.34a ± 0.03
MD 05-6381/ W	39.2a-c ± 5.4	46.3a-c ± 7.5	26.7a-d ± 2.2	0.36a ± 0.03
MD 04-5217/PF	39.0a-c ± 16.0	88.4g ± 10.3	28.8a-d ± 11.4	0.57a-c ± 0.09
MD 04-5217/ PD	40.0a-c ± 9.9	43.9a-e ± 9.8	36.1a-d ± 7.7	0.45a ± 0.10
MD 04-5217/ W	45.1a-d ± 1.8	61.6c-g ± 12.4	39.3a-d ± 3.1	0.54ab ± 0.05
Manokin/ PF	107.7e ± 76.0	47.8a-g ± 36.2	83.0e ± 61.1	0.90c ± 0.66
Manokin/ PD	70.5b-e ± 12.2	25.8a ± 4.3	58.9c-e ± 6.2	0.56a-c ± 0.08
Manokin/ W	69.5b-e ± 11.2	41.2a-d ± 23.4	51.4b-e ± 8.9	0.61a-c ± 0.10

*Data are expressed as mean of 3 replicate plots, each tested in duplicate, ± SD (N= 6). Daidzein, genistein, and glycitein are expressed as μg/g whole soybean. Total isoflavones are expressed as μmol/g whole soybean. All genotypes are low 18:3n3, except Manokin, which is a non-modified genotype. Values marked by the same letter within each component are not statistically different ($p < 0.05$). PF: Poplar Hills full season (Salisbury, MD); PD: Poplar Hills double cropped (Salisbury, MD); W: Wye Research Center (Queenstown, MD).

Also noted was that there were high standard deviations in isoflavone levels among replicates of the same location and genotype, suggesting that possible effects of other factors might have contributed to the variation. Glycitein, daidzein, and genistein were detected in all tested soybean samples with a concentration of 25.8-95.8 μg/g for glycitein, 29.3-107.7 μg/g for daizein, and 15.3-83.0 μg/g for genistein (**Table 2.3**). On a per weight basis, glycitein was the primary isoflavone compound in AG2091V, AG3521V, and MD 04-5217 soybeans, but not in MD 05-5656 and Manokin genotypes grown at all three tested locations in Maryland (**Table 2.3**). Furthermore, glycitein was the primary isoflavone compound in MD 04-6006 and MD 05-6377 soybeans grown at Poplar Hills (full season) and the Wye Research Center locations, but not necessarily the major one of these two soybean lines grown at Poplar Hills (double cropped). On the other hand, daidzein was the major isoflavone compound in MD 05-5656 and Manokin soybeans.

The ratio of isoflavones also varied by genotype and environment. In the AG2091V soybean, the ratio of daidzein/glycitein/genistein was 1:2:1.5 in the Poplar Hills full season environment, but was 1:2.8:0.9 in the Poplar Hills double cropped and 1:3:1 in the Wye Research Center environments, respectively. In the MD 05-6377 double cropped soybeans, the ratio of daidzein/glycitein/genistein was approximately 1:1:1. Taken together, these results indicated the possible effects of genotype and growing environment on soybean isoflavones, providing background for further investigation into the effects of each and their potential interaction on soybean phytochemicals.

2.4.1.4. Lutein Content

Lutein has been identified previously as the predominant carotenoid in soybeans (Slavin et al., 2009). In the present study, lutein levels ranged from 10.4 to 27.2 μg/g oil (**Table 2.4**). Lutein was highest in the MD 05-6381 and MD 04-5217 genotypes, and lowest in MD 05-5656 soybeans across all tested locations, suggesting the effect of genotype on soybean lutein concentration. A trend in environment was seen in five of the eight soybean lines, with the highest lutein levels in the Poplar Hills full season environment followed by the double cropped, and with lowest levels in the Wye environment (**Table 2.4**). In addition, the highest lutein level in the full season and double cropped environment was in the MD 05-6381 genotype, whereas the highest level found in the at the Wye Research Center location was in the MD 04-5217 line. These results showed the possible effect of genotype and environment interaction on lutein content in soybeans.

2.4.1.5. Tocopherol Composition

There have been several studies on the tocopherol levels of soybeans, including those with modified fatty acids (Almonor et al., 1998; Dolde et al., 1999; McCord et al., 2003; Britz et al., 2008;). In the present study, α, γ, and δ-tocopherols were detected in all soybean samples (**Table 2.4**). Total tocopherols ranged from 2.3 to 3.1 μmol/g oil, and α-tocopherol ranged from 259.5 and 317.7 μg/g oil.

The α-tocopherol levels and total tocopherols were generally lower than those reported for Maryland-grown soybeans by Slavin et al. (2009), but are consistent with levels reported for Indian soybeans by Rani et al. (2007). The soybeans evaluated by

Slavin et al. were grown in Maryland during the 2005 season, and this may partially account for differences in tocopherol levels. Other studies have reported tocopherols on per gram of soybean basis, so are not necessarily comparable due to different extraction procedures.

MD 04-6006 soybeans had similar or higher levels of total tocopherol, while the MD 04-5217 genotype contained the highest α-tocopherol across all tested environments (**Table 2.4**). Four soybean lines had the highest amount of total tocopherols in the Poplar Hills full season environment, and the other three soybean lines produced greatest total tocopherols in the Wye Research Center environment. Furthermore, α-tocopherol was most abundant in four of the soybean lines at Poplar Hills full season, while three others had higher levels in the double cropped environment. MD 05-6377 was the only line that produced the highest level of α-tocopherol in the Wye Research Center location.

Table 2.4. Lutein Content and Tocopherol Composition of Soybeans*

	Lutein	α-tocopherol	γ-tocopherol	δ-tocopherol	Total tocopherol
AG2091V/ PF	15.6de ± 2.4	286.9a-e ± 21.6	384.7a-d ± 55.9	403.4c-f ± 46.9	2.67a-e ± 0.17
AG2091V/ PD	17.0de ± 1.2	290.1a-e ± 14.2	368.2a-d ± 38.0	347.2a-e ± 38.2	2.49a-c ± 0.12
AG2091V/ W	17.5de ± 0.6	272.0ab ± 23.2	326.2a ± 45.4	394.6b-f ± 42.5	2.48a-c ± 0.21
AG3521V/ PF	18.2de ± 1.1	291.1b-e ± 32.6	400.5a-e ± 66.0	339.9a-e ± 32.2	2.55a-c ± 0.25
AG3521V /PD	15.2c-e ± 1.0	306.0c-e ± 13.2	429.2a-f ± 66.3	301.3a ± 48.0	2.55a-c ± 0.28
AG3521V/ W	14.0a-d ± 0.6	268.9ab ± 5.4	371.1a-d ± 20.5	362.7a-f ± 35.8	2.49a-c ± 0.09
MD 04-6006/ PF	18.6c-e ± 1.1	296.1b-e ± 11.0	524.6f ± 44.8	419.7ef ± 46.2	3.08e ± 0.22
MD 04-6006/ PD	18.0c-e ± 1.0	276.9a-c ± 12.0	471.0c-f ± 6.9	360.0a-f ± 44.6	2.74b-e ± 0.07
MD 04-6006/ W	14.9a-d ± 0.6	280.1a-d ± 8.3	414.1a-f ± 43.9	441.1f ± 63.9	2.83b-e ± 0.22
MD 05-5656/ PF	12.3a-c ± 1.9	269.4ab ± 10.5	352.1ab ± 83.5	403.7d-f± 51.8	2.56a-c ± 0.30
MD 05-5656/ PD	10.6ab ± 0.6	281.7a-d ± 13.5	390.5a-e ± 47.1	445.6f ± 51.0	2.79b-e ± 0.15
MD 05-5656/ W	10.4a ± 1.5	269.5ab ± 3.7	391.3a-e ± 27.6	427.5ef ± 42.4	2.71a-e ± 0.08
MD 05-6377/ PF	17.5de ± 1.4	277.1a-c ± 6.8	498.0ef ± 26.2	357.8a-f ±39.6	2.80b-e ± 0.12
MD 05-6377/ PD	17.1de ± 2.2	273.9ab ± 12.1	478.9d-f ± 53.1	337.3a-e ± 36.3	2.69a-e ± 0.18
MD 05-6377/ W	14.3a-d ± 1.6	289.1a-e ± 6.4	501.0ef ± 38.3	406.3d-f ± 43.2	2.97de ± 0.13
MD 05-6381/ PF	25.0h-j ± 1.0	283.1a-d ±13.0	442.5b-f ± 48.2	311.8a-c ± 35.9	2.56a-c ± 0.18
MD 05-6381/ PD	27.2ij ± 0.9	274.7ab ± 13.0	401.9a-e ± 71.3	305.8ab ± 29.3	2.43ab ± 0.22
MD 05-6381/ W	19.9c-e ± 0.8	279.1a-d ± 18.0	412.9a-e ± 25.3	347.4a-e ± 37.2	2.57a-d ± 0.11
MD 04-5217/ PF	24.8ij ± 2.8	317.7e ± 18.4	472.3c-f ± 71.5	370.6a-f ± 53.3	2.87c-e ± 0.25
MD 04-5217/ PD	23.0g-j ± 5.8	308.4de ± 11.2	383.8a-d ± 93.9	336.9a-e ± 37.9	2.54a-c ± 0.27
MD 04-5217/ W	22.8f-j ± 3.0	294.7a-d ± 10.5	374.8a-d ± 24.7	408.8d-f ± 43.2	2.68a-e ± 0.09
Manokin/ PF	21.1e-i ± 6.6	270.4ab ± 17.0	346.0ab ± 55.2	325.9a-d ± 33.8	2.33a ± 0.20
Manokin/ PD	17.0b-e ± 1.5	270.4ab ± 8.6	363.0a-c ± 23.5	397.1b-f ± 34.0	2.48a-c ± 0.08
Manokin/ W	19.4d-g ± 1.2	259.6a ± 7.5	383.0a-d ± 43.3	390.2a-f ± 26.9	2.54a-c ± 0.17

*Data are expressed as mean of 3 replicate plots, each tested in duplicate, ± SD (N= 6). Lutein, α-, γ-, and δ-tocopherol are expressed as μg/g oil. Total tocopherol is expressed as μmol/g whole soybean. All genotypes are low 18:3n3, except Manokin, which is a non-modified genotype. Values marked by the same letter within each component are not statistically different ($p < 0.05$). PF: Poplar Hills full season (Salisbury, MD); PD: Poplar Hills double cropped (Salisbury, MD); W: Wye Research Center (Queenstown, MD).

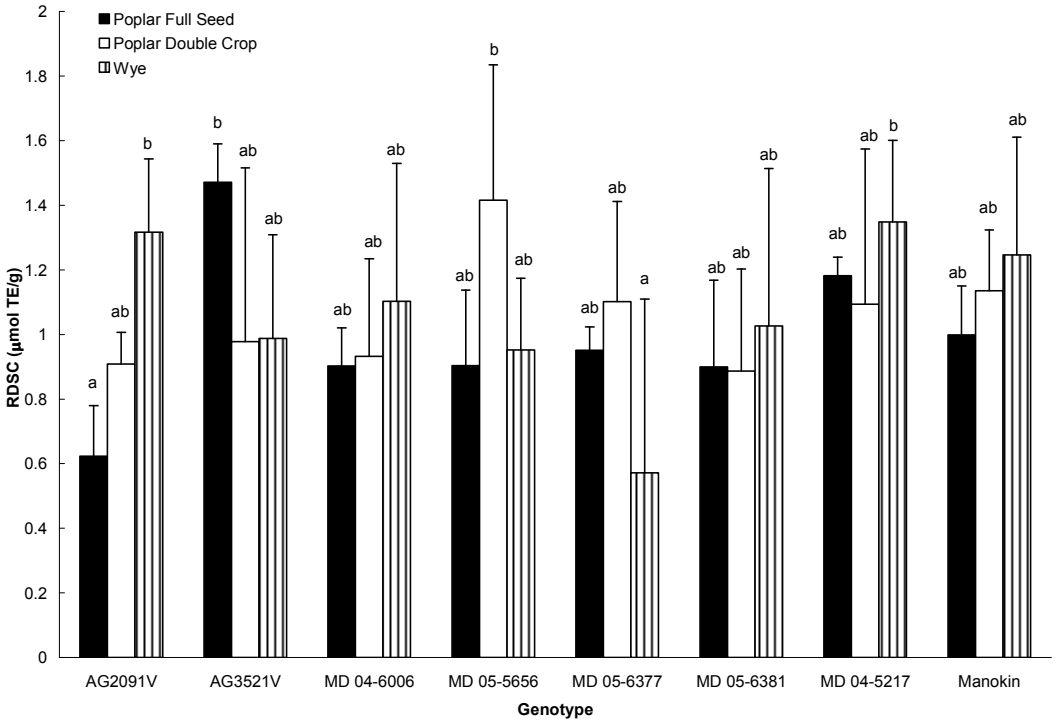
2.4.1.6. Antioxidant Properties

All genotypes under all growing conditions demonstrated scavenging capacity against DPPH (RDSC value), hydroxyl (HOSC), and peroxyl (ORAC) radicals (**Figures 2.2-2.4**). RDSC value ranged from 0.6 to 1.5 μmol TE/g among the genotypes at all locations (**Figure 2.2**). A greater RDSC value is associated to a stronger DPPH radical scavenging capacity. The soybean line that had the greatest RDSC value at one growing location did not necessarily show the highest DPPH radical scavenging capacity in a different growing environment (**Figure 2.2**). Other groups have previously reported DPPH radical scavenging capacity of soybean extracts (Xu & Chang, 2008), however it is difficult to compare the results from different laboratories since not all results were reported as relative DPPH radical scavenging capacity using a standard antioxidant such as Trolox in the present study.

Hydroxyl radical scavenging capacity (HOSC) values varied from 20.1 and 40.1 μmol TE/g whole soybeans under the experimental conditions (**Figure 2.3**). AG3521V soybean in the Poplar Hills full season environment showed a 11% stronger HOSC than its counterpart in the Poplar Hills double cropped environment, whereas MD 04-5217 line grown in the double cropped environment had about 90% higher HOSC value than that in the full season environment (**Figure 2.3**). ORAC values also varied by genotype and environment, as seen in **Figure 2.4**. ORAC values ranged from 22.4 to 58.4 μmol TE/g. These ORAC values were within the range previously reported level of 21.2-91.3 μmol TE/g for yellow soybean by Xu and Chang (2008). Interestingly, the soybean with the greatest ORAC value, which was Manokin in the Poplar Hills full season environment, did not necessarily exhibit strongest DPPH and hydroxyl radical scavenging capacities in the same environment (**Figures 2.2-2.4**). These radical scavenging capacity results suggested that each soybean line or cultivar may

respond to environment differently. These results also indicated that each antioxidant property may respond to individual environmental factors differently. Therefore, the contribution of genotype, environment, and their interaction were evaluated for their effect on chemical components and antioxidant properties.

Figure 2.2. Relative DPPH· Scavenging Capacity (RDSC) of Soybeans*



*Data are expressed as µmol Trolox equivalent (TE)/g soybean. Values represent mean of 3 replicate plots ± SD (n = 6). Values marked by the same letter are not statistically different ($p < 0.05$). Poplar full season, Poplar double crop, and Wye indicate growing environment.

Figure 2.3. Hydroxyl Radical Scavenging Capacity (HOSC) of Soybeans*

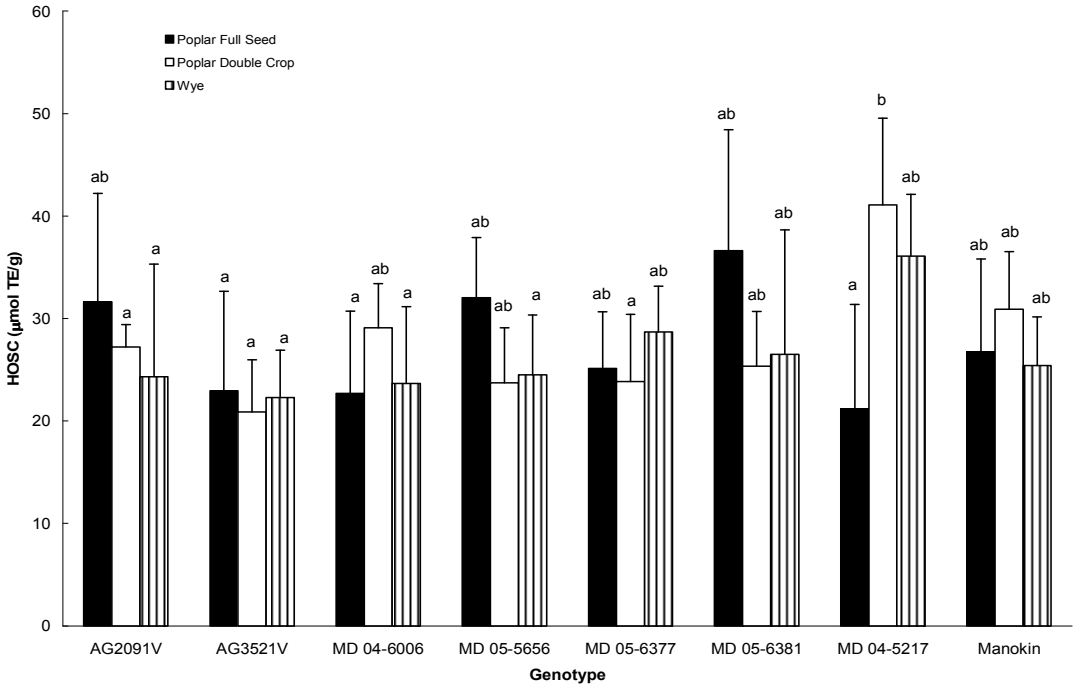
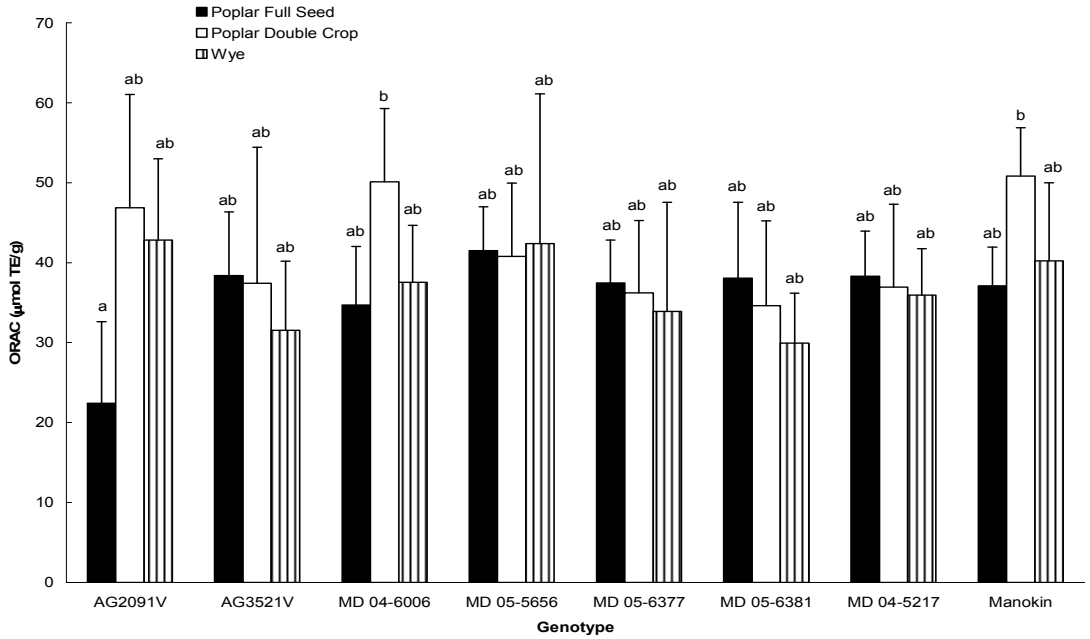


Figure 2.4. Oxygen Radical Absorbing Capacity (ORAC) of Soybeans*



*Data are in Fig. 2.3 and 2.4 are expressed as µmol Trolox equivalent (TE)/g soybean. Values represent mean of 3 replicate plots ± SD (n = 6). Values marked by the same letter are notstatistically different ($p < 0.05$). Poplar full season, Poplar double crop, and Wye indicate growing environment.

2.4.2. Effects of Genotype (G), Environment (E), and the Interaction between G and E (G × E) on Soybean Composition and Antioxidant Property

2.4.2.1. Oil Content and Fatty Acid Composition

The percent of total mean square for each variable (G, E, and G × E) was determined to quantify the contribution of each variable to soybean components and antioxidant properties. G accounted for the most variations in soybean oil content (60%, $p < 0.001$). E accounted for 27% variation in soybean oil content ($p < 0.01$), while G × E contributed 13% of that ($p < 0.01$). For the majority of fatty acids, G had a larger effect on variation than E (**Table 2.5**). Genotype showed the largest effect of 98.8% and 97.5% ($p < 0.001$), respectively, on 16:0 and 18:3n3 contents. The line MD 05-6377 contained the lowest 18:3n3 levels at all locations (1.0-1.2 g/100 g oil). This line also contained the lowest level of 16:0 at all locations (4.20-4.72 g/100 g oil). This line may be noted for future analysis, because soybean oil with low 18:3n3 and low 16:0 is desirable for reduced-trans and saturated fat consumption (Fehr, 2007). G also was the major contributor for total saturated fat (79%, $p < 0.001$).

E had a large effect (84.3%) on stearic acid (18:0) ($p < 0.001$), while G had more effect on the other fatty acids. When comparing averages by environment, 18:3n3 and 16:0 were both lowest in the Poplar Hills full season environment (earlier planting date) and highest in the Poplar Hills double cropped environment (later planting date) at statistically significant levels. Ray et al. (2008) also found lower 18:3n3 levels in non-modified soybeans with an earlier planting date. The same study found that 16:0 was lower at a later planting date, which was not observed in our results. The double cropped soybeans

contained the lowest level of 18:1n9 and highest level of 18:2n6 compared to other environments. The differences observed by planting date likely reflect changes in temperature or other environmental conditions (Tsukamoto et al., 1995). In addition, E had significant effect on total saturated fat in soybeans (20%, $p < 0.001$) (**Table 2.5**).

Oleic acid (18:1n9) is a desirable component of edible oil due to its benefits to cardiovascular health and stability in foods (Tarrago-Trani et al., 2006). The full season soybeans in this study appeared to have a more desirable fatty acid profile compared to the double cropped soybeans, due to lower 18:3n3 and higher 18:1n9.

Table 2.5. Effect of G, E and G × E on Soybean Composition[a]

	% Genotype	% Environment	% G × E
Oil Content	60.15***	27.01**	12.84**
Total Sat	78.99***	20.01***	1.00***
16:0	98.81***	0.93***	0.26***
18:0	10.24***	84.27***	5.49***
18:1n-9	53.34***	40.82***	5.84***
18:2n-6	55.85***	36.93***	7.23***
18:3n3	97.45***	2.11***	0.44***
TPC	40.64	5.78	53.58**
Daidzein	88.52***	1.67	9.81*
Genistein	78.64***	8.99	12.37**
Glycitein	26.38***	64.82***	8.80***
Total ISF	67.19***	21.28*	11.52
Lutein	78.26***	17.63***	4.11**
α-Tocopherol	57.17***	30.81**	12.02**
γ- Tocopherol	70.25***	19.08*	10.67**
δ- Tocopherol	42.94***	49.04***	8.01*
Total Tocopherol	69.09***	16.70	14.21**
RDSC	38.56*	12.16	49.28***
HOSC	47.92**	5.96	46.12***
ORAC	21.37	55.77*	22.86

[a]Effect of genotype (G), environment (E), and genotype × environment (G × E) on soybean composition and antioxidant properties expressed as percent of total mean square. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Values without asterisks are not significant at $p < 0.05$.

The percent variation due to G × E was low in the fatty acids, ranging from 0.3 – 5.8% (**Table 2.5**) ($p < 0.001$). In both Poplar Hills environments, 18:1n9 was highest in the genotype MD 05-5656 (9.3-39.9 g/100 g oil). However, at the Wye Research center environment, 18:1n9 was highest in the MD 04-6006 genotype (41.4g/100 g oil). The line AG2091V produced the highest oil content in the Poplar Full season environment, but AG3521V contained the highest levels in the other 2 environments, though the differences are not statistically significant (**Table 2.2**).

2.4.2.2. Total Phenolic Content

There was not a significant effect of G or E individually on variation in soybean TPC; however there was a significant effect of G × E interaction ($p < 0.05$). For example, the genotype MD 04-5217 demonstrated a high TPC level in the Poplar Hills double cropped environment, while the AG2531V genotype had a significantly lower level in the same environment. The effect of G × E interaction accounted for 53.6% of variation in TPC levels ($p < 0.01$) (**Table 2.5**). When analyzing wheat lines from Colorado, Moore et al. (2006a) found that E accounted for most of the variation in TPC (79.5%). Riedl et al. (2007) reported significant variation in soybean TPC by environment. However, the present study did not find a similar effect.

2.4.2.3. Isoflavone Content

Others have reported significant differences in soy isoflavone level based on genotype (Wang & Murphy, 1994). In the present study, there was significant variation by G in the isoflavone levels ($p < 0.01$). Overall, the Manokin soybean with regular 18:3n3 concentration contained the highest levels of total isoflavones.

Among the reduced 18:3n3 genotypes, MD 05-5656 contained the highest average levels of total isoflavones, daidzein, and genistein across the different environments. Daidzein and genistein had the most variation attributed to G (88.5 and 78.6% respectively, $p < 0.001$). The variation in isoflavones due to environmental differences is also well documented in the literature (Wang & Murphy, 1994; Tsukamoto et al., 1995; Riedl et al., 2007). Our current study found that the total isoflavone levels and the isomer glycitein showed significant variation by environment ($p < 0.05$). This variation was demonstrated by reduced levels in the Poplar Hills double cropped environment. Of the isoflavone isomers, only glycitein had the majority of percent variation attributed to E (64.8%, $p < 0.001$) (**Table 2.5**).

There was a small effect of G × E, ranging from 8.8 to 12.4% ($p < 0.05$) for total and individual isoflavones. The G × E combination with the highest total isoflavone level was MD 05-5656 at the Wye Research Center location (0.83 μmol/g whole soybean), but G × E interaction was not statistically significant ($p = 0.069$).

2.4.2.5. Lutein Content

A few studies have reported that lutein content in soybeans might significantly vary across genotypes (Wang & Murphy, 1994; Kanamaru et al., 2006) and environments (Lee et al., 2009). Our results showed that G accounted for 78.6% of variation in lutein levels ($p < 0.001$) (**Table 2.5**). MD 05-6381 and MD 04-5217 were the lines with the highest overall lutein levels, ranging from 19.9 to 27.2 μg/g

oil. Environment also accounted for about 18% of the variation in lutein level ($p <$ 0.001), with higher mean levels at the Poplar Hills location than at the Wye Research center location. The combination of G and E that produced the highest lutein level was MD 05-6381 in the Poplar Hills double cropped environment (27.2 μg/g oil) (**Table 2.4**). Based on proportion of total mean squares, the effect of G × E accounted for only 4.1% of the variation ($p < 0.01$).

2.4.2.6. Tocopherol Composition

α-, γ-, δ-, and total tocopherols showed significant variation by G ($p < 0.01$) (**Table 2.5**). The genotype MD 04-5217 contained the highest α-tocopherol levels at all locations. G contributed to the largest amount of variance in α-, γ-, and total tocopherols based on proportion of mean squares (57.1-70.3%, $p < 0.001$) (**Table 2.5**). Individual tocopherol isomers also showed significant variation by E. E contributed to the majority of variance in δ-tocopherol (51.2%, $p < 0.001$), and about 30 % of that in α-tocopherol ($p < 0.01$) and 20% of that in γ-tocopherol ($p < 0.05$). An environmental effect on soybean tocopherols was also noted by Britz et al. (2008) and Dolde et al. (1999), although in both studies G was responsible for more variation than E. In addition, G × E showed significant contribution to α-, γ- and total tocopherols ($p < 0.01$), and to δ-tocopherol ($p < 0.001$).

2.4.2.7. Antioxidant Capacity

There was a significant effect of G on RDSC ($p < 0.05$) and HOSC ($p < 0.01$). The largest proportion of variation in the HOSC assay was attributed to G (47.9%)

(**Table 2.5**). G contributed to 38.6% of the variation in soybean RDSC levels. Moore et al. (2006a) examined variation in antioxidant capacity of hard winter wheat, and reported the variation of RDSC in winter wheat samples was attributed mainly to G (88.6%). Thus food crops may have varying factors that influence antioxidant capacity, suggesting the possibility of improving the antioxidant properties in food crops such as soybeans and wheat through breeding effort or genetic modification.

There was significant variation by environment in the ORAC levels when averaged among all genotypes ($p < 0.05$). The double crop soybeans had a higher ORAC level on average than the other environments. The largest variation in ORAC was attributed to E (55.8%, $p < 0.05$). The other antioxidant assays did not demonstrate significant variation by environment. This finding was in agreement with that for wheat by Moore et al. (2006a). They found that the ORAC value of winter wheat was more affected by E (51.8%) than G, which is similar to the effect found on soybeans in the current study.

In addition, G × E might significantly alter RDSC and HOSC ($p < 0.001$). The effect of G × E interaction contributed the most variation to RDSC (49.3%, $p < 0.001$) (**Table 2.5**). This differs from the findings of Moore et al. (2006a), who reported that either G or E had a larger effect than G × E for most antioxidant properties in hard winter wheat lines. No effect of G × E on ORAC was detected in the current study.

2.4.3. Effects of Individual Environmental Conditions on Soybean Composition and Antioxidant Properties

The effects of environment (E) on chemical composition and antioxidant properties in soybean were observed in the present and previous studies (Wang & Murphy, 1994). It is interesting to know whether and how individual environmental conditions may alter which chemical composition and antioxidant property in soybeans grown in Maryland. This information could be used to improve the agricultural practices to enhance the nutritional value of soybeans in Maryland and other locations worldwide.

2.4.3.1. Oil Content and Fatty Acid Composition

Oil content on soybeans was positively correlated with average high temperature with a Pearson's correlation coefficient (r) value of 0.199 ($p < 0.05$), and negatively correlated with overall average and average low temperatures, with r-values of -0.182 ($p < 0.05$) and -0.190 ($p < 0.05$). No correlation between oil content and precipitation was detected. In the present study, correlation analysis of air temperature and fatty acid levels showed a strong positive correlation between stearic acid (18:0) and average high air temperature (r = 0.690, $p < 0.01$), and the reverse for average low temperature (r = -0.699, $p < 0.01$) and overall average temperature (r = -0.689, $P < 0.01$) (**Table 2.6**). This finding may explain the large effect of E on 18:0 levels (**Table 2.5**). Small positive correlations were observed between overall average and average low air temperatures and 18:1n9 level in soybean oil ($p < 0.01$).

In contrast, 18:2n6 level was negatively correlated with overall average and average low air temperatures ($p < 0.01$), and 18:3n3 level was negatively correlated with average high air temperature ($p < 0.01$). Precipitation had a positive correlation with α-linolenic acid (r = 0.22, $p < 0.01$), but had no influence on other fatty acid concentrations.

2.4.3.2. Total Phenolic Content

Total phenolic content of soybeans had significant correlation with precipitation ($p < 0.05$). There were no significant correlations between TPC and individual environmental factors (**Table 2.6**).

2.4.3.3. Isoflavone Composition

Previous research has shown negative correlation between isoflavones and air temperature during seed development (Tsukamoto et al., 1995). In the present study, total isoflavones, genistein, and daidzein did not have a significant correlation with air temperature (**Table 2.6**). Only glycitein was positively correlated with overall average air temperature (r = 0.204, $p < 0.05$) and average low temperature (r = 0.204, $p < 0.05$). Other reports have indicated that irrigation or precipitation during seed fill may influence soybean isoflavone levels (Riedl et al., 2007). Overall precipitation levels did not have significant correlation with isoflavone levels in the present study, however precipitation is known only for the total growing season rather than seed fill dates.

2.4.4.4. Lutein Content

Lutein content was negatively correlated with overall average air temperature ($r = -0.243$, $p < 0.01$) and average low temperature ($r = -0.222$, $p < 0.01$), and was positively correlated with precipitation levels ($r = 0.312$, $p < 0.01$) (**Table 2.6**). In review of the literature, we did not find previous studies examining correlation of individual environmental factors with lutein accumulation in soybean oil. Based on our current results, further investigation of this relationship may be warranted.

2.4.4.5. Tocopherol Composition

α-tocopherol had a positive correlation with average high temperature ($r = 0.313$, $p < 0.001$), while δ-tocopherol had a positive correlation with overall average temperature ($r = 0.321$, $p < 0.001$) and average low temperature ($r = 0.320$, $p < 0.001$) (**Table 2.6**). No effect of precipitation has been observed for tocopherol composition in soybeans in the present study. This suggests that increased air temperature may increase the level of α-tocopherol, while reducing the δ-tocopherol concentration. Britz et al. (2008) also found elevated α-tocopherol levels in warmer temperatures and with full season planting dates. Low 18:3n3 soybeans have been previously shown to have higher α-tocopherol content in warmer temperatures (Wilson, 2004). As previously noted, the Poplar Hills locations had the highest α-tocopherol levels on average. This location did not have the highest average air temperatures, so there may be other factors involved in the production of α-tocopherol.

2.4.4.6. Antioxidant Capacity

Large effects of specific weather conditions on antioxidant capacity were not observed in the selected genotypes of soybeans. This may be due in part to the fact that selected growing locations were not exposed to extremely different weather conditions. The crops grown at Poplar Hills were exposed to lower temperatures than the crops at Wye Research Center, but high temperatures were similar throughout the growing season. The weather information used for analysis was collected, from data available in records. Concurrent observation of specified weather conditions during crop growth may provide more accurate data for specific crop locations.

Table 2.6. Correlation Between Soy Components, Antioxidant Capacity, and Weather Conditions*

	Precipitation	Average High Temp	Overall Average Temp.	Average Low Temp.
Oil Content	-0.009	0.199*	-0.182*	-0.190*
16:0	-0.024	0.011	-0.015	-0.017
18:0	-0.024	0.690**	-0.686**	-0.699**
18:1n-9	-0.055	-0.148	0.309**	0.298**
18:2n-6	-0.016	0.144	-0.247**	-0.241**
18:3n3	0.220**	-0.180**	-0.032	-0.009
TPC	0.167*	-0.068	-0.056	-0.040
Daidzein	0.098	-0.045	-0.018	-0.009
Glycitein	0.064	-0.119	0.204*	0.197**
Genistein	0.010	-0.021	0.002	0.004
Total Isoflavones	0.074	-0.093	0.098	0.101
Lutein	0.312**	0.024	-0.243**	-0.222**
Total Tocopherols	-0.113	0.036	0.078	0.067
α-Tocopherol	-0.157	0.313**	-0.192*	-0.211*
γ-Tocopherol	-0.054	0.182*	-0.140	-0.147
δ-Tocopherol	-0.094	-0.258**	0.321**	0.320**
RDSC	-0.068	-0.031	0.052	0.048
HOSC	0.010	0.052	-0.057	-0.059
ORAC	-0.035	0.100	-0.091	-0.094

Data expressed as Pearson's correlation coefficient (r-value), = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Values without asterisks are not significant at $P < 0.05$. Absolute high and low temperatures had similar correlations as the average high and lows so are not reported here. TPC = total phenolic content, RDSC = relative DPPH· scavenging capacity, HOSC = hydroxyl radical scavenging capacity, ORAC = oxygen radical absorbance capacity.

Additional environmental factors that were not measured in this study may have affected soybean composition. For example, statistical analysis showed that ORAC values had 55% variation due to E, but they did not have a significant correlation when compared with air temperature or precipitation. This may indicate that other environmental factors are responsible for ORAC variation. Soil conditions or solar radiation are factors not measured by this study that may be responsible for crop variation (Moore et al., 2006a).

2.4.5. Correlation Between Individual Chemical Compositions and Antioxidant Properties

Table 2.7 shows the Pearson correlation coefficients between each chemical composition and antioxidant properties conducted in this research. Interestingly, level of 16:0 was positively correlated with 18:3n3 concentration ($r = 0.519$, $p < 0.01$) and negatively correlated with 18:1n-9 ($r = -0.538$, $p < 0.01$). This suggested the possibility of obtaining soybean lines low in α-linolenic and palmitic acids through breeding effort to enhance shelf stability of soybean oil without hydrogenation and to improve its nutritional value. Level of 18:3n3 was positively correlated with daidzein, genistein, and total isoflavones ($P < 0.01$), but negatively correlated with glycitein content ($p < 0.01$), indicating the possible effect of reducing 18:3n3 on isoflavones in soybeans. Also noted was a significant high correlation between oleic acid (18:1n9) and δ-tocopherol. There was a negative correlation between 18:3n3 and α-, γ-, and total tocopherol, indicating that reducing the 18:3n3 level may be related to increased tocopherols in the selected genotypes. The results of Almonor et al. (1998) support the finding that reduced 18:3n3 soybeans produce relatively higher amounts of α-tocopherol

Table 2.7. Correlation Between Soybean Composition and Antioxidant Assays*

	Oil	16:0	18:0	18:1	18:2	18:3	TPC	RDSC	HOSC	ORAC	Daid.	Glyc.	Geni.	T. ISF	Lutein	α-Toco.	γ-Toco.	δ-Toco.
16:0	.305**																	
18:0	-.077	-.164																
18:1n-9	-.233**	-.538**	.196*															
18:2n-6	.220**	.037	-.270**	-.803**														
18:3n3	-.189*	.519**	.003	-.388**	-.133													
TPC	-.129	-.073	.189*	.032	-.089	.140												
RDSC	-.070	.195*	.009	-.018	-.130	.161	.097											
HOSC	.023	.002	.117	.015	-.039	.037	.232**	.005										
ORAC	-.123	.089	.225**	-.061	-.091	.202*	.673**	.123	.123									
Daid.	-.288**	.122	.031	.032	-.219**	.584**	.266**	.087	.082	.201*								
Glyc.	.159	.162	-.023	.381**	-.411**	-.221**	.046	.128	-.090	-.046	.045							
Geni.	-.220**	.287**	-.036	-.163	-.139	.591**	.269**	.107	.074	.272**	.936**	.025						
T. ISF	-.164	.225**	.008	.116	-.370**	.422**	.292**	.126	.069	.210*	.853**	.499**	-.222**					
Lutein	.238**	.106	.028	-.277	.247**	.021	-.007	.082	.141	-.041	-.240**	-.036	-.237**	-.222**				
α-Toco.	.206*	.091	.291**	.129	-.154	-.245**	-.014	.028	-.045	.007	-.387**	.258**	-.335**	-.179*	-.340**			
γ-Toco.	-.159	-.447**	.185*	.160	.084	-.318**	.061	-.050	-.154	-.097	-.259**	.068	-.358**	-.212*	.137	.487**		
δ-Toco.	-.326**	-.176*	.031	.710**	-.755**	.046	.072	.122	-.144	-.021	.182*	.289**	.090	.273**	-.485**	-.021	.176*	
T. Toco.	-.264**	-.380**	.196	.491**	-.350**	-.240**	.076	.032	-.187*	-.076	-.157	.239**	-.262**	-.040	-.088	.505**	.866**	.621**

*Data expressed as Pearson's correlation coefficient (r-value). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Values without asterisks are not significant at $p < 0.05$. TPC = total phenolic content, RDSC = relative DPPH· scavenging capacity, HOSC = hydroxyl radical scavenging capacity, ORAC = oxygen radical scavenging capacity, Daid. = daidzein, Glyc. = glycitein, Geni. = genistein, T. ISF = total isoflavone, Toco. = tocopherol.

than non-modified soybeans. However, later research by McCord et al. (2003) demonstrated proportional changes in tocopherol isomers with changes in 18:3n3. The relationship between tocopherols and 18:3n3 may be due primarily to similar environmental conditions that exert effects on both (Dolde et al., 1999).

Lutein also had negative correlation with α- and δ-tocopherols. Based on findings from Lee et al. (2009) and Dolde et al. (1999) these correlations may be primarily related to the effects of external conditions. These data suggest that in some cases, selection for one soybean component may occur under conditions that reduce levels of other desirable components. However, Wang et al. (2008) demonstrated that α-tocopherol and lutein are highly heritable in soybeans, and that through genetic manipulation soybeans may be produced that contain elevated levels of each component. Lutein and α-tocopherol were negatively correlated with the majority of isoflavones in the present study.

Among antioxidant properties, ORAC was highly correlated with TPC, which is consistent with the results of previous studies on agricultural products (2006a). TPC was also positively correlated with HOSC ($r = 0.232$, $p < 0.01$). ORAC and TPC were also positively correlated with daidzein, genistein, and total isoflavones, as isoflavones are phenolic compounds with known antioxidant activity (Lee et al., 2007).

In conclusion, the health components and antioxidant properties of soybeans were affected by genotype, environment, and the interaction between genotype and environment. Each chemical component or antioxidant property may respond to genotype, environment, and their interaction at different levels. Furthermore, each

soybean component and antioxidant property may respond to individual environmental factors differently. Among the soybeans studied, there was not one particular genotype or environment that produced outstanding levels of all health components. However, it may be possible to select the ideal genotype and environment for an enhanced level of a specific component. Continuation of this analysis over multiple growing seasons would provide a better indication of the best combination of genotype and environment for nutraceutical, chemical, and nutritional properties in these soybeans.

Chapter 3: Isoflavone Composition and Antioxidant Properties of Lipoxygenase-Modified Soybeans

Adapted from Whent et al., *J. Agric. Food Chem.* **2011**, *59, 12902-12909*

3.1. Abstract

Maryland-grown soybean lines modified for low lipoxygenase-1 (LOX-1) content and a traditional non-modified cultivar were analyzed for fatty acid composition, total phenolic content (TPC), isoflavone composition, relative DPPH· scavenging capacity (RDSC), and hydroxyl radical scavenging capacity (HOSC). Soybean lines included black, brown, and yellow soybeans. TPC of all soybean lines ranged from 2.84 to 4.74 mg Gallic acid equivalents (GAE)/g flour. Total isoflavones were between 2.78 and 8.66 μmol/g flour. RDSC of all lines was between 0.48 and 14.62 μmol Trolox equivalents (TE)/g flour, and HOSC ranged from 53.57 to 135.52 μmol TE/g flour. Some modified-LOX genotypes demonstrated antioxidant capacity and/or isoflavone content that was similar to or higher than the non-modified cultivar ($p < 0.05$). Black colored soybeans demonstrated higher TPC and RDSC than most yellow soybean lines, although did not have higher isoflavone content. The results demonstrate that modification of the LOX trait did not necessarily alter antioxidant capacity or chemical composition of the experimental soybean lines when compared with a non-modified cultivar. These soybean lines may be studied further for nutraceutical properties and use in functional foods.

3.2. Introduction

Soybeans and their food products are widely consumed by humans, and they provide a nutritious component of the diet. In addition to protein and essential fatty acids, soybeans contain nutraceutical components that may enhance human health. Soybeans are known sources of phenolics, including isoflavones. They also contain tocopherols and carotenoids, which provide antioxidants as well as vitamins to the diet. Oxidative stress is believed to be a factor in chronic disease, and previous research has shown soy foods to have high antioxidant activity *in vitro* and *in vivo* (de Santana et al., 2008; Wang et al., 2011). Soy foods have been studied for their role in chronic disease prevention, specifically against osteoporosis, coronary heart disease, and some types of cancer (Isanga & Zhang, 2008).

Although recognized as a health-enhancing food, soybeans and foods produced from them can develop sensory properties that make them less desirable to consumers (Wolf, 1975). Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a catalyst for oxidation of polyunsaturated fatty acids that contain a cis,cis-1,4-pentadiene moiety. This enzyme is contained in soybean seed, and after homogenization the reaction leads to formation of secondary volatile carbonyl compounds (Hildebrand & Kito, 1984). Soymilk is particularly susceptible to LOX-catalyzed reactions due to the homogenization of seeds with water during production (Torres-Penaranda et al., 1998). There are 3 iso-enzymes of lipoxygenase (LOX) that are active at different pH. The enzyme can be inactivated by heat, and this method is typically used in food processing as it also reduces anti-nutritional factors contained

in soybean. However, there is interest in reducing the lipoxygenase content in soybean seeds through breeding, in order to further reduce the possibility of poor odors or flavors (Robinson et al., 1995).

Soybeans have been modified through breeding for elimination of one or more LOX iso-enzymes. Studies of these modified soybeans in food products have shown mixed results for sensory improvement. Torres-Penaranda et al. (1998) reported that soymilk made with LOX-null soybeans had less astringency and cooked beany flavor and aroma compared with soymilk from non-modified soybeans. However, LOX-null soymilk was reported to be more yellow-colored by sensory panelists. Yuan & Chang (2007) reported that LOX-null soybeans produced soymilk with less odor-causing compounds as identified by gas chromatography, although it was not evaluated by a sensory panel. This effect was seen in both raw soymilk and after 20 minutes of boiling. Thus the LOX-null soymilk contained fewer volatile compounds, even after heat denaturation of the normal-LOX soymilk. There is also possibility of improved nutritional quality in modified LOX soybeans. Nishiba & Suda (1998) reported that soybeans with normal LOX content lost vitamins and antioxidant capacity much more rapidly than LOX-null soybeans when homogenated in water.

It is known that modification of soybean for a specific trait may affect other traits. For example, modification for fatty acid content has been associated in some studies with altered tocopherol levels (Dolde et al., 1999; Scherder et al., 2006). Oliveira et al. (2007) reported that reduced LOX soybeans contained greater levels of isoflavones than the parent non-modified soybeans. There is relatively little research on the chemical composition and antioxidant capacity of modified-LOX soybeans.

The purpose of this study is to evaluate modified-LOX soybeans grown in Maryland for chemical composition and antioxidant capacity, and to identify experimental lines that may provide improved sensory properties as well as nutraceutical benefits.

3.3. Materials and Methods

3.3.1. Plant Materials and Chemicals

Whole soybeans from the 2009 growing season were collected by Dr. William Kenworthy of the Department of Plant Sciences and Landscape Architecture, University of Maryland, College Park. Thirty-six lines were experimental genotypes that were modified for LOX-1 content through traditional breeding methods. An additional experimental line (08-5865) was of normal LOX-1 content. In addition, the low-LOX cultivar Japan-L1L2L3 and the traditional Manokin cultivar of Maryland were among the studied soybeans. Thirty percent ACS-grade hydrogen peroxide was purchased from Fisher Scientific (Fair Lawn, NJ). 2,2'-azobis(2-aminodopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Fluorescein (FL), iron (III) chloride, 2,2-diphenyl-1-picryhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), isoflavone standards, tocopherol standards, and carotenoid standards were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of the highest commercial grade and used without further purification.

3.3.2. Lipoxygenase-1 Content

Lipoxygenase-1 (LOX-1) content of soybeans was determined according to the method described by Whent et al. (2010) using a Victor^3 multilabel plate reader

(PerkinElmer, Turku, Finland). Briefly, soybeans were ground to particle size 20-mesh and 0.020 g meal was mixed in 10 mL distilled water in a 15 mL tube. Tubes were held at 4 °C for 1 hour, then centrifuged for 5 min. 1 mL supernatant was removed and held on ice for testing. 15-lipoxygenase Type 1 (from soybean) was diluted with pH 9.0 sodium borate buffer to 5 concentrations between 5 and 25 kilo-units (KU). 100 µL of 200 nM fluorescein working solution or pH 9.0 buffer (blank) was added to each well using a multichannel pipet. 120 µL of pure LOX-1 enzyme, soybean extract, or buffer (control) was added. Finally, 80 µL sodium linoleate substrate was added to the wells using a multichannel pipet to initiate the enzyme reaction. Fluorescence was recorded continuously for 6 minutes. Excitation and emission wavelengths were 485 and 515 nm, respectively. AUC calculations were performed to determine % fluorescein remaining, which measures hydroperoxide formation. LOX-1 presence was determined based on % fluorescein remaining compared to the standard curve. The limit of detection to declare positive presence of LOX-1 was 30 KU/g meal based on standard curve of purified enzyme.

3.3.3. Oil Extraction

Whole soybeans were ground to particle size 20-mesh using a handheld coffee bean grinder. Five grams of ground soybeans were combined in a tube with 20 mL of petroleum ether. Tubes were vortexed 3 times each for 30 seconds, and held 20 hours at ambient temperature in the dark. The supernatant was removed and stored. The extraction was repeated twice with 10 mL petroleum ether. The petroleum ether was evaporated overnight under nitrogen, and the remaining oil was weighed. The oil samples were stored in the dark until further testing.

3.3.4. Antioxidant Extraction

The defatted soy flour that remained following oil extraction was air-dried overnight at ambient temperature. 0.5 gram of each soy flour sample was combined in a test tube with 5 mL 50% acetone. The tubes were vortexed 3 times for 15 seconds each, and kept in the dark at ambient temperature overnight. The tubes were centrifuged, and the supernatant was removed and filtered, and stored in the dark until further testing.

3.3.5. Fatty Acid Composition

The soybean oil was prepared for gas chromatography (GC) analysis according to a previously described procedure (Yu et al., 2003). The soybean oil was saponified and methylated to form fatty acid methyl esters (FAME), and dissolved in hexane. GC analysis was performed with a Shimadzu GC-2010 with FID. Helium was the carrier gas at a flow rate of 2.2 mL/min. The stationary phase was 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 started at 136 ºC, increased by 6 ºC/min until 184 ºC and held for 3 minutes, then increased by 6 ºC/min to a final temperature of 226 ºC. Fatty acids were identified by comparing FAME retention time with that of known standards. The FAMEs were quantified by calculating the area under the curve of each identified peak and reported as relative percent of total fatty acids.

3.3.6. Total Phenolic Content (TPC)

The total phenolic content of each soy flour extract was determined according to a previously described laboratory procedure (Moore & Yu, 2008). The final reagent mixture contained 50 µL of soybean extract, 250 µL of Folin-Ciocalteu reagent, 1.5 mL of 20% sodium carbonate, and 1.5 mL of ultra-pure water, using Gallic acid as a standard. After 2 hours reaction time at ambient temperature, absorbance was read at 765 nm. The reactions were conducted in triplicate and results reported in mg Gallic acid equivalents (GAE) per gram of soy flour.

3.3.7. Isoflavones Composition

0.5 grams of defatted soy flour were combined with 5 mL 58% acetonitrile, vortexed 3 times for 15 seconds each, and held overnight in the dark for 18 hours. The tubes were centrifuged, and the supernatant was collected and held for further testing. Based on the method of Klump et al. (2001) with modification, 300 µL of 2N NaOH was added to each tube and tubes rested for 15 minutes. 100 µL of pure acetic acid was added to each tube and gently mixed. 0.5 mL was collected and filtered through a 0.45 µm syringe filter and stored at -20 °C until HPLC analysis. HPLC was performed according to a previously described method (Achouri et al., 2005), using a Waters 600 HPLC, 996 photodiode array detector, and Waters 717 autosampler. The column was a Phenomenex C18 (250 × 4.6 mm, 5 µm) with a Phenomenex C18 guard cartridge. The eluent consisted of 99.9% distilled deionized water with 0.1% acetic acid (v/v) (mobile phase A) and 99.9% acetonitrile with 0.1% acetic acid (v/v) (mobile phase B). The gradient was 15% solvent A for 5 minutes, increasing to 30% A over 10 minutes, held at 30% A for 10 minutes, increased to 50% A over 10

minutes, held for 5 minutes, returned to 15% A over 5 minutes, and held for 10 minutes to re-equilibrate. Injection volume was 10 µL. The detection wavelength was set at 254 nm.

3.3.8. DPPH Radical Scavenging Capacity Estimation

Relative DPPH· scavenging capacity (RDSC) was determined according to a previously described laboratory procedure (Cheng et al., 2006), using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). DPPH radical solution (0.2 mM) was prepared in 50% acetone and filtered through a P5 grade filter paper (Fisher Scientific). Trolox standards were prepared in 50% acetone at concentrations of 6.25 µM, 12.5 µM, 25 µM, 37.5 µM, and 50 µM. Each final reaction mixture contained 100 µL soybean extract, Trolox standard, or 50% acetone (control), and 100 µL 0.2 mM DPPH· solution. The absorbance was read at 515 nm. The radical scavenging capacity (RDSC) was calculated from the area under the curve and reported in µmol Trolox equivalents (TE) per gram soy flour.

3.3.9. Hydroxyl Radical Scavenging Capacity (HOSC)

The HOSC assay was conducted using a previously reported laboratory procedure (Moore et al., 2006). Trolox prepared in 50% acetone was used as the standard at concentrations of 20, 40, 60, 80, and 100 µM. Fluorescein was used as a fluorescent probe and the assay was performed using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). 3.43 M iron (III) chloride and 0.1999 M hydrogen peroxide were prepared in ultra-pure water. 9.28×10^{-8} M FL was prepared in 75 mM sodium phosphate buffer (pH 7.4). The reaction mixture consisted of 170 µL of 9.28

$\times 10^{-8}$ M FL, 30 µL of sample, standard, or blank, 40 µL of 0.1999 M hydrogen peroxide, and 60 µL of 3.43 M iron (III) chloride. The fluorescence was recorded every 4 minutes for 4 hours. Antioxidant capacity was calculated by area under the curve (AUC) described by Moore and others (2006). Results were reported as micromoles of TE per gram of soy flour.

3.3.10. Statistical Analysis

Data was analyzed using SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL). One-way ANOVA was conducted using data obtained in triplicate. Tukey's post-hoc test was used to determine differences between means after ANOVA analysis. Correlation was analyzed using a two-tailed Pearson's correlation test. Statistical significance was noted for values of $p < 0.05$ ($\alpha > 0.95$).

3.4. Results and Discussion

3.4.1. Lipoxygenase-1 (LOX-1) Content

Experimental soybean genotypes were modified through traditional breeding methods for the absence of the LOX-1 enzyme. Presence of LOX-2 or LOX-3 was not determined in this study. The line 08-5865 and the Manokin cultivar were bred with normal LOX-1 content. Soybeans were tested to determine the presence of LOX-1, with results seen in **Table 3.1**. Although the lines were bred for absence of LOX-1, some individual seeds of the modified genotypes may not be missing the LOX-1 allele, and therefore LOX-1 was found to have activity in some of the

Table 3.1. LOX-1 Presence in Soybean [a] and Fatty Acid Composition[b] of Soybean Oil

Genotype	LOX-1	16:0	18:0	18:1n-9	18:2n-6	18:3n3	SFA	MUFA	PUFA
09-5876 Black	-	11.74h-m ± 0.10	2.69a-e ± 0.06	18.55a-c ± 1.24	54.79b ± 0.95	12.24p-v ± 0.31	14.43	18.55	67.02
09-5878 Black	+	12.05j-n ± 0.23	2.52ab ± 0.14	18.37-c ± 0.28	55.08ab ± 0.22	11.97o-v ± 0.03	14.57	18.37	67.05
09-5883 Black	-	12.05j-n ± 0.13	3.05c-k ± 0.12	19.15a-c ± 0.29	53.97b ± 0.21	11.78n-u ± 0.10	15.10	19.15	65.75
09-5880 Brown	-	12.15j-o ± 0.08	2.76a-i ± 0.05	16.19a-c ± 0.24	56.18b-f ± 0.24	12.72s-v ± 0.03	14.91	16.19	68.90
09-5880 Yellow	-	12.28k-o ± 0.15	2.79a-i ± 0.13	15.52ab ± 0.35	56.66b-g ± 0.18	12.75s-v ± 0.17	15.07	15.52	69.41
09-5877	-	11.82i-m ± 0.17	2.61a-d ± 0.08	17.57a-c ± 0.27	55.11ab ± 0.23	12.90t-v ± 0.08	14.43	17.57	68.00
09-5879	-	6.65a ± 0.07	2.57a-c ± 0.06	19.92a-c ± 1.22	57.90b-g ± 0.79	12.96uv ± 0.37	9.22	19.92	70.86
09-5881	-	12.73no ± 0.15	2.86a-j ± 0.10	15.88ab ± 0.27	55.48b-d ± 0.20	13.05v ± 0.03	15.59	15.88	68.53
09-5882	-	12.49m-o ± 0.09	2.76a-i ± 0.13	21.62a-d ± 1.17	57.14b-g ± 0.88	5.99b ± 0.15	15.25	21.62	63.13
08-5424	-	7.70b ± 0.04	2.86a-j ± 0.17	27.23d ± 1.25	54.32b ± 0.80	7.89cd ± 0.26	10.56	27.23	62.20
08-5043	-	10.68de ± 0.08	3.44k-m ± 0.11	21.71b-d ± 1.16	54.66b ± 0.86	9.51fg ± 0.13	14.11	21.71	64.17
08-5083	-	9.90cd ± 0.05	2.65a-e ± 0.03	21.29a-d ± 0.20	55.86b-e ± 0.16	10.30g-l ± 0.05	12.55	21.29	66.16
08-5040	-	11.41e-j ± 0.12	3.12e-j ± 0.14	16.00ab ± 0.23	57.79b-g ± 0.17	11.69m-t ± 0.07	14.52	16.00	69.48
08-5059	-	11.74h-m ± 0.02	2.74a-i ± 0.08	17.93a-c ± 0.24	54.38b ± 0.24	13.20v ± 0.06	14.48	17.93	67.58
08-5053	-	12.04j-n ± 0.12	2.59a-d ± 0.03	16.28a-c ± 0.22	58.63b-g ± 0.11	10.46h-m ± 0.06	14.63	16.28	69.09
96-5978	+	11.14e-i ± 1.36	3.22h-m ± 0.46	15.67ab ± 0.19	61.18fg ± 0.14	8.27c-e ± 0.01	14.36	15.67	69.45
08-5865	+	9.47c ± 0.16	3.65lm ± 0.07	38.48e ± 1.21	44.04a ± 0.86	4.36a ± 0.15	13.12	38.48	48.40
08-5074	-	11.94j-n ± 0.15	2.89b-j ± 0.10	16.03ab ± 0.20	57.71 b-g ± 0.12	11.43l-r ± 0.04	14.83	16.03	69.14
08-5073	-	11.65h-l ± 0.11	2.40a ± 0.06	17.90a-c ± 0.26	57.30b-g ± 0.13	10.75i-o ± 0.09	14.05	17.90	68.06
08-5089	+	10.84e-g ± 0.10	3.67m ± 0.60	16.23a-c ± 0.25	58.23b-g ± 0.36	11.03j-p ± 0.36	14.51	16.23	69.26
08-5069	-	11.43e-j ± 0.12	2.83a-j ± 0.05	17.56a-c ± 0.23	58.03b-g ± 0.13	10.14g-k ± 0.11	14.26	17.56	68.18
08-5076	+	11.52f-k ± 0.14	2.52ab ± 0.02	14.79a ± 0.20	60.09c-g ± 0.10	11.07j-p ± 0.04	14.05	14.79	71.16
08-5075	-	11.42e-j ± 0.01	2.73a-g ± 0.13	17.50a-c ± 0.52	55.78b-d ± 0.51	12.56q-v ± 0.18	14.15	17.50	68.34
08-5082	-	11.10e-i ± 0.14	2.71a-f ± 0.04	15.54ab ± 0.18	58.86b-e ± 0.09	11.80n-u ± 0.06	13.81	15.54	70.66
08-5052	-	11.65h-l ± 0.18	2.55ab ± 0.11	16.25a-b ± 0.24	56.89b-g ± 0.23	12.66r-v ± 0.11	14.20	16.25	69.55
08-5085	-	10.77ef ± 0.09	2.73a-g ± 0.07	18.17a-c ± 0.20	57.66b-g ± 0.12	10.66h-n ± 0.07	13.50	18.17	68.33
08-5042	-	11.44e-j ± 0.04	2.83a-j ± 0.11	16.37a-c ± 0.13	57.10b-g ± 0.13	12.26p-v ± 0.04	14.27	16.37	69.36
08-5078	-	11.41e-j ± 0.10	3.06d-k ± 0.04	19.73a-c ± 0.44	55.93b-f ± 0.27	9.87f-j ± 0.14	14.47	19.73	65.81
08-5071	-	10.96e-h ± 0.10	2.49ab ± 0.05	16.96a-c ± 0.14	58.22b-g ± 0.15	11.37k-q ± 0.08	13.45	16.96	69.59
08-5050	-	10.97e-h ± 0.03	2.49ab ± 0.06	16.90a-c ± 0.18	58.30b-g ± 0.14	11.34k-q ± 0.07	13.46	16.90	69.64
08-6166	-	11.69h-m ± 0.14	3.24i-m ± 0.19	20.73a-d ± 0.22	54.58b ± 0.19	9.75f-i ± 0.06	14.94	20.73	64.33
03-6610	-	12.42l-o ± 0.08	2.75a-h ± 0.06	16.41a-c ± 0.20	55.49b-d ± 0.15	12.93uv ± 0.06	15.16	16.41	68.42
07-08WN 8	+	12.07j-n ± 0.03	3.18f-l ± 0.02	15.45ab ± 0.16	57.84b-g ± 0.13	11.46l-r ± 0.02	15.25	15.45	69.30
07-08WN 44	+	9.83c ± 0.10	3.20g-m ± 0.02	21.69b-d ± 0.36	61.03fg ± 0.27	4.26a ± 0.04	13.03	21.69	65.28
96-5981	+	11.55f-k ± 0.05	3.28j-m ± 0.03	14.91ab ± 0.24	61.61g ± 0.23	8.66d-f ± 0.01	14.82	14.91	70.27
96-5979	-	11.60g-k ± 0.11	3.18f-m ± 0.14	16.23a-c ± 0.23	60.96e-g ± 0.26	8.02c-e ± 0.06	14.79	16.23	68.99
96-5980	-	11.40e-j ± 0.10	3.23i-m ± 0.05	16.95a-c ± 0.16	60.48d-g ± 0.19	7.94cd ± 0.12	14.63	16.95	68.42
Japan-L1L2L3	-	12.88o ± 0.12	2.82a-j ± 0.09	17.29a-c ± 0.25	55.41b-d ± 0.15	11.60m-s ± 0.05	15.70	17.29	67.01
Manokin	+	11.74h-m ± 0.07	3.45k-m ± 0.09	18.31a-c ± 0.11	57.64b-g ± 0.26	9.19e-g ± 0.13	15.19	18.31	66.83

[a]Data represent detection of LOX-1 in mean of 3 replicates. + indicates LOX-1 present, - indicates LOX-1 not detected. [b]Results are reported as mean ± SD (n = 3). Individual fatty acids are expressed as a relative percent of the total fatty acids. SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids. Genotypes are yellow soybeans unless otherwise indicated. Manokin is a non-modified cultivar. Significant difference between genotypes is noted by different letters within each column as indicated by ANOVA with Tukey's HSD post-hoc test ($p < 0.05$).

genotypes. As the lines were experimental, in some cases the elimination of LOX-1 was not complete.

3.4.2. Fatty Acid Composition

Fatty acid composition of soybean oil is shown in **Table 3.1**. Linolenic acid (18:3n3) is an essential fatty acid in the human diet. However, it is usually desired for soybean oil to have reduced levels of 18:3n3 to improve stability in processing and to reduce the requirement for hydrogenation (Fehr, 2007). Typical non-modified soybeans contain an average of 8% 18:3n3 out of total fatty acids (Sugano, 2006). Soybeans modified for fatty acid may contain as little as 1% 18:3n3 of total fatty acids. The desirable levels of 18:3n3 for baking and frying oil are usually less than 3% (Wilson, 2004). The modified LOX soybeans in this study contained 18:3n3 ranging from 4.26 to 13.20% of total measured fatty acids. Genotypes 0708WN 44 and 08-5865 contained significantly lower 18:3n3 ($p < 0.05$) than the other soybean genotypes at 4.26 and 4.36% of total fatty acids, respectively. Our research found that both of these lines contained measurable LOX-1 (this is expected in line 08-5865 that was bred as normal-LOX). Of modified-LOX soybeans, line 09-5882 contained the lowest level of 18:3n3 (5.99% of total fatty acids), however this level is higher than the desired level for improved oxidative stability. In the soybean lines overall, there was a negative correlation between LOX-1 presence and 18:3n3 ($r = -0.231$, $p < 0.05$) (**Table 3.2**).

Oleic acid (18:1n9) is considered a desirable fatty acid for soybean oil. As a monounsaturated fatty acid it is beneficial for heart health (Bermudez et al., 2011). It also contributes to improved stability of soybean oil if it replaces the polyunsaturated

fatty acids. The 18:1n9 content of the studied soybeans was between 14.79 and 38.48% of total fatty acids. The experimental line 08-5865 contained the highest level, followed by 08-5424 (27.23%). The genotype 08-5865 also contained a low level of 18:3n3, and the combination with higher 18:1n9 may produce oil of higher stability than the other experimental lines. That particular line, however, is normal in LOX-content. Low 18:3n3 and elevated 18:1n9 is a desirable trait in oilseeds and some breeding programs have specialized in this combination (Fehr, 2007), although those seeds can achieve 18:1n9 levels of 80% of total fatty acids. Overall there was a negative correlation among all studied soybean lines between 18:1n9 and 18:3n3 ($r = -0.641$, $p < 0.01$) (**Table 3.2**). There was a positive correlation between LOX-1 and 18:1n9 ($r = 0.243$, $p < 0.01$), indicating that modification of the LOX-1 trait may have affected the level of certain fatty acids.

Palmitic acid (16:0) is a saturated fatty acid in food oils. Although it provides stability to foods, dietary intake may increase risk of cardiovascular disease (Warensjo et al., 2008). The 16:0 level of the soybeans ranged from 7.70 to 12.88 % of total fatty acids, which is near the reported average level of 10 % (Sugano, 2006). Genotype 08-5424 contained the lowest level of 16:0, although this level is not as low as that obtained in soybeans modified for saturated fatty acid content (Fehr, 2007). In this study, 16:0 levels were negatively correlated with 18:1n9 ($r = -0.536$, $p < 0.01$) (**Table 3.2**) and positively correlated with 18:3n3 ($r = 0.304$, $p < 0.01$). A similar correlation was seen in a previous study of low 18:3n3 soybeans (Whent et al., 2009).

Table 3.2. Correlations Between Soybean Antioxidant Capacity, Isoflavones, and Fatty Acids[a]

	TPC	RDSC	HOSC	Total ISF	Daidzein	Glycitein	Genistein	16:0	18:0	18:1n9	18:2n6	18:3n3
RDSC	0.645**											
HOSC	0.471**	0.443**										
Total ISF	0.220*	-0.133	0.353**									
Daidzein	0.227*	-0.130	0.297**	0.908**								
Glycitein	-0.132	-0.357**	0.115	0.484**	0.461**							
Genistein	0.212*	-0.073	0.326**	0.829**	0.657**	0.384**						
16:0	0.106	0.212*	0.202*	0.014	-0.011	0.058	0.109					
18:0	-0.134	-0.072	-0.051	-0.048	-0.126	-0.065	-0.061	-0.104				
18:1n9	0.018	-0.012	-0.165	-0.142	-0.120	-0.263**	-0.347**	-0.536**	0.383*			
18:2n6	-0.239**	-0.189*	0.260	0.040	0.060	0.246**	0.181	0.163	-0.215*	-0.787**		
18:3n3	0.251**	0.176	0.182*	0.224*	0.181	0.152	0.379**	0.304**	-0.537**	-0.641**	0.115	
LOX-1	0.073	-0.030	-0.030	0.088	-0.113	-0.079	-0.004	-0.106	0.394**	0.248**	-0.187*	-0.231*

[a]Data represents r-value of Pearson's two-tailed test. * = $p < 0.05$, ** = $p < 0.01$. ISF = Isoflavone.

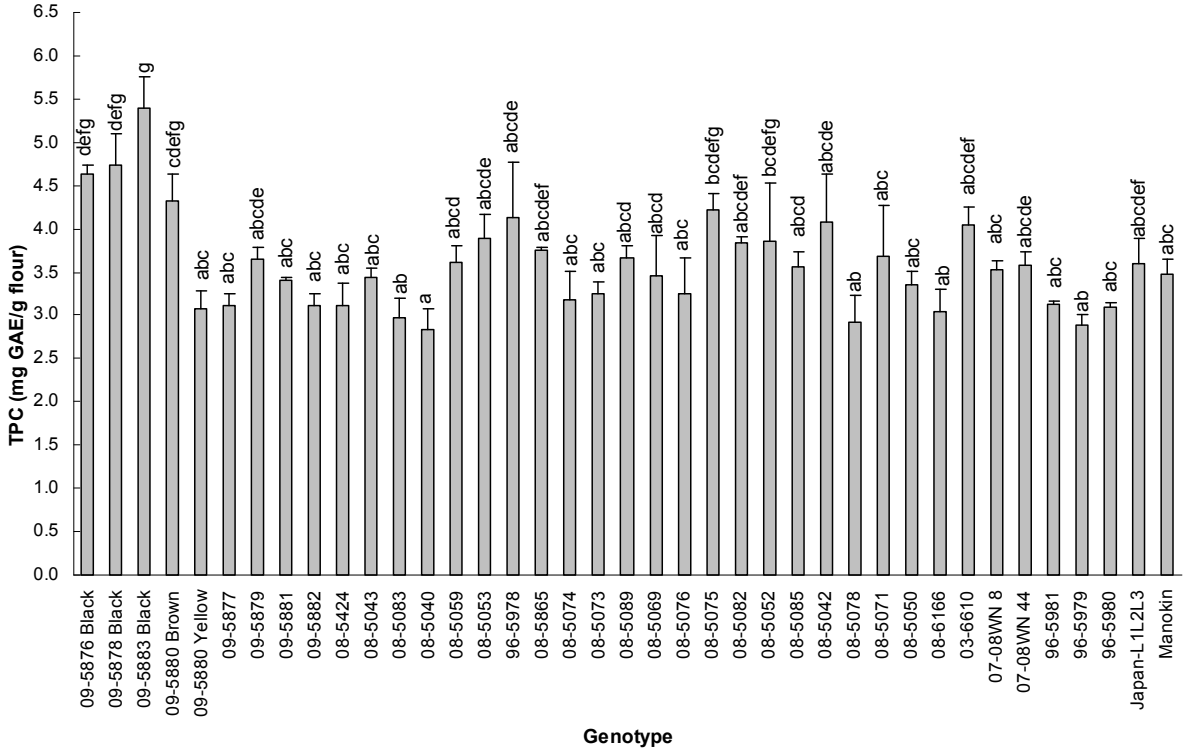
3.4.3. Total Phenolic Content (TPC)

Phenolic compounds are known to contribute to the antioxidant capacity of a food. Diets high in phenolics are associated with reduced risk of heart disease and some types of cancer (Crozier et al., 2009). Phenolic compounds found in soybeans include benzoic acids, cinnamic acids, flavonols, and anthocyanins (black soybeans) (Xu & Chang, 2008a). The TPC of the modified soybeans ranged from 2.84 to 4.74 mg Gallic acid equivalents (GAE)/g flour (**Figure 3.1**). Of yellow soybeans, genotypes 08-5075 and 08-5052 contained the highest TPC, at 3.69 and 3.66 mg GAE/g, respectively. These soybeans were also confirmed to be null for LOX-1. However, there was no significant correlation identified between LOX-1 presence and TPC. Black colored soybeans contained significantly higher levels than yellow soybeans ($p < 0.05$), which is consistent with the literature. According to previous studies, cyanidin-3-glucose is the major anthocyanin found in the black soybeans and contributes to the TPC level (Xu & Chang, 2008a; Slavin et al., 2009a).

3.4.3. Isoflavones Composition

Although many solvent systems have been described for isoflavone extraction, 58% acetonitrile was selected for this study as it was previously determined to be an effective extraction solvent by Lin & Giusti (2005). Murphy et al. (2002) and Collison (2008) have also described aqueous acetonitrile as a good solvent for determining isoflavone composition. Extracts were hydrolyzed under basic conditions to measure β-glucosides and aglycones, which were available as commercial standards. The total isoflavone content (**Table 3.3**) is described in

Figure 3.1. Total Phenolic Content (TPC) of Soybean*



*Data expressed as mg Gallic acid equivalent (GAE)/g soy flour. Each bar represents mean ± SD (n = 3). Significant difference between genotypes is noted by different letters above each bar as indicated by ANOVA with Tukey's HSD post-hoc test ($p < 0.05$). Genotypes are yellow soybeans unless otherwise indicated. Manokin is a non-modified cultivar.

µmol/g flour due to differing molecular weights of the individual isoflavone forms.

The individual aglycone equivalents were calculated and are reported as µg/g flour. Total isoflavones ranged from 2.78 to 8.66 µmol/g flour (730.99 to 2230.15 µg/g in aglycone equivalent). This is in the range of previously reported isoflavone levels in soy flour extracted with aqueous acetonitrile. Murphy et al. (2002) reported 7.3 µmol/ g, and Lin & Giusti (2005) reported 3.9 µmol/g. In this study, the highest total isoflavone levels were found in yellow-colored soybean genotypes as opposed to the black-colored soybeans, which differed from the TPC results. This finding has previously been

reported in literature (Xu & Chang, 2008a) and suggests that phenolic compounds other than isoflavones (likely anthocyanins) are contributing to the high TPC of black soybeans. In fact, a previous study of black soybeans demonstrated that most phenolics are contained in the seed coat while the isoflavones are contained in the cotyledon (Xu & Chang, 2008b). In the current study, TPC was weakly correlated with total isoflavones ($r = 0.220$, $p < 0.05$) (**Table 3.2**). Genotype 08-5052 and 08-5075 contained the highest total isoflavones at 8.66 and 8.22 μmol/g flour, respectively. These genotypes were null for LOX-1 and also contained the highest TPC of the yellow soybeans. The non-modified soybean (Manokin) contained 6.86 μmol/g flour, and the normal-LOX experimental line 08-5865 contained 3.3 μmol/g. The results show that some soybean genotypes with modified LOX contain the same or higher levels of isoflavones when compared with non-modified genotypes, although a statistically significant correlation was not identified.

Individual isoflavone content was positively correlated with the total isoflavones by genotype ($p < 0.05$). Of the individual isoflavones, genistein and daidzein are found in higher amounts than glycitein (Murphy et al., 2002; Lin & Giusti, 2005). Total daidzein ranged from 360.0 to1367.0 μg/g flour, glycitein was between 32.0 and 129.2 μg/g flour, and genistein was detected at 327.9 to 922.3 μg/g flour. Many genotypes demonstrated similar levels of genistein and daidzein by weight (in aglycone equivalents), while others had one isoflavone in much higher levels than the other. For example, genotype 96-5978 contained 1112.28 μg/g flour of total daidzein and only 649.03 μg/g flour of total genistein.

Table 3.3. Isoflavone Composition of Soybean*

Genotype	Daidzein (μg/ g flour)	Glycitein (μg/ g flour)	Genistein (μg/ g flour)	Total Isoflavone (μmol/ g flour)
09-5876 Black	611.9a-i ± 14.3	38.2ab ± 4.6	568.1a-g ± 51.0	4.6b-g ± 0.2
09-5878 Black	548.9a-f ± 54.6	37.7ab ± 3.1	427.0a-c ± 61.7	3.9a-e ± 0.3
09-5883 Black	502.3a-e ± 47.9	40.8a-c ± 3.5	598.8a-h ± 74.2	4.3a-g ± 0.2
09-5880 Brown	567.0a-g ± 42.6	75.0a-f ± 3.5	649.4c-j ± 20.9	4.9b-h ± 0.2
09-5880 Yellow	526.1a-f ± 51.8	66.7a-f ± 9.8	495.9a-f ± 103.1	4.1a-f ± 0.5
09-5877	535.9a-f ± 26.1	32.0a ± 10.4	487.4a-e ± 103.2	4.0a-f ± 0.5
09-5879	800.5c-k ± 69.1	87.7a-g ± 16.4	570.4a-g ± 74.5	5.6g-m ± 0.5
09-5881	694.4a-i ± 105.1	117.3e-g ± 23.8	592.6a-h ± 111.0	5.3d-k ± 0.8
09-5882	484.3a-e ± 73.5	85.6a-g ± 20.9	409.2a-c ± 24.8	3.7a-d ± 0.3
08-5424	580.7a-g ± 60.0	84.9a-g ± 1.7	491.6a-f ± 55.5	4.4a-g ± 0.3
08-5043	829.5e-k ± 33.9	113.7e-g ± 34.0	904.7ij ± 25.8	6.7i-n ± 0.6
08-5083	613.8a-i ± 16.4	70.7a-f ± 28.2	583.5a-h ± 97.2	4.9b-h ± 0.3
08-5040	336.6a ± 31.5	59.3a-e ± 10.7	539.8a-g ± 86.7	3.5a-c ± 0.2
08-5059	975.6i-k ± 130.0	167.4g ± 24.4	668.4a-j ± 42.8	6.8j-n ± 0.7
08-5053	719.4b-j ± 54.8	103.8b-g ± 21.0	739.3d-j ± 92.8	5.9h-m ± 0.5
96-5978	1112.3k ± 154.6	107.3c-g ± 58.9	799.8ij ± 64.5	7.7n ± 0.4
08-5865	525.5a-f ± 46.7	45.4a-d ± 16.3	320.8ab ± 18.0	3.3ab ± 0.1
08-5074	586.1a-g ± 30.8	128.3fg ± 21.1	526.7a-g ± 45.6	4.7b-g ± 0.0
08-5073	360.0ab ± 12.7	55.9a-e ± 8.2	553.5a-g ± 32.0	3.8a-d ± 0.1
08-5089	459.2a-d ± 19.5	97.8a-g ± 4.9	692.9c-g ± 107.7	5.1b-j ± 0.8
08-5069	594.1a-h ± 70.1	69.1a-f± 4.4	550.6a-g ± 72.5	4.6b-g ± 0.5
08-5076	529.5a-f ± 57.6	94.2a-g ± 23.7	498.0a-f ± 87.4	4.3a-g ± 0.5
08-5075	1122.9k ± 59.2	110.7d-g ± 10.8	922.3j ± 97.2	8.2n ± 0.4
08-5082	926.5a-i ± 63.8	110.9d-g ± 7.8	931.8j ± 142.5	7.5l-n ± 0.5
08-5052	1367.7k ± 113.2	118.3e-g ± 13.0	744.2d-j ±49.5	8.7n ± 0.5
08-5085	609.7a-i ± 80.1	101.7b-g ± 17.3	669.1c-j ± 99.2	5.2c-k ± 0.7
08-5042	512.5a-e ± 40.3	82.5a-g ± 26.1	615.8b-I ± 38.0	4.6b-g ± 0.2
08-5078	433.0a-c ± 18.1	93.1a-g ± 16.6	423.8a-c ± 16.9	3.6a-d ± 0.1
08-5071	601.6a-h ± 66.7	92.4a-g ± 10.4	682.1c-j ± 47.1	5.2c-g ± 0.4
08-5050	607.7a-i ± 73.8	103.7b-g ± 14.3	671.4c-j ± 43.8	5.2c-g ± 0.2
08-6166	551.0a-f ± 82.1	91.4a-g ± 12.3	554.5a-g ± 58.7	4.5a-g ± 0.6
03-6610	1077.2jk ± 163.3	94.6a-g ± 16.7	880.3 h-j ± 79.6	7.8n ± 0.9
07-08WN 8	743.3c-j ± 37.8	105.5c-g ± 31.1	901.9ij ± 117.7	6.6h-n ± 0.7
07-08WN 44	834.1d-k ± 119.9	84.9a-g ± 5.2	584.7a-h ± 33.4	5.7h-l ± 0.6
96-5981	548.3a-f ± 26.8	94.6a-g ± 12.2	443.6a-d ± 81.7	4.1a-f ± 0.4
96-5979	365.5ab ± 51.9	57.7a-e ± 10.6	307.9a ± 42.4	2.8a ± 0.3
96-5980	531.2a-f ± 41.0	71.9a-f ± 6.5	455.9a-d ± 44.7	4.0a-f ± 0.3
Japan-L1L2L3	544.9a-f ± 15.9	129.2fg ± 17.9	442.0a-d ± 56.2	4.5a-g ± 0.4
Manokin	961.3h-k ± 41.7	59.5a-e ± 5.4	782.1e-g ± 62.1	6.9k-n ± 0.1

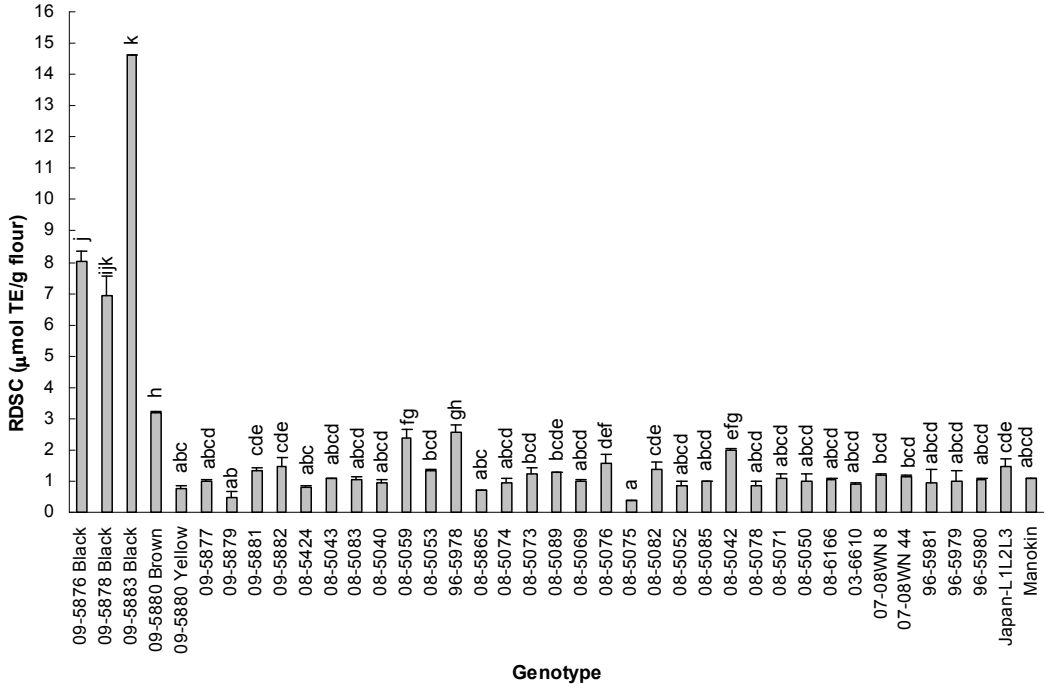
* Data represent mean ± SD (n = 3). Significant difference between genotypes is noted by different letters within each column as indicated by ANOVA with Tukey's HSD post-hoc test ($p < 0.05$). Daidzein, glycitein, and genistein are expressed in aglycone equivalents as μg/g flour. Total isoflavone is expressed as μmol/g flour. Genotypes are yellow soybeans unless otherwise indicated. Manokin is a non-modified cultivar.

3.4.4. Antioxidant Activity of Soybeans

3.4.4.1. Relative DPPH· Scavenging Capacity

DPPH$^{\bullet}$ is a stable free radical that can be used to measure single electron transfer (SET) reactions (Huang et al., 2005). RDSC was determined by the method of Cheng et al. (2006), which compares antioxidant activity to a standard curve of TE. Black soybean RDSC was between 6.93 and 14.61 μmol TE/ g flour, while the yellow soybean ranged from 0.48 to 2.38 μmol TE/g flour (**Figure 3.2**). These values are similar to those reported by Whent et al. (2009) for modified fatty acid yellow soybeans (0.6-1.5 μmol TE/g soybean) and Xu & Cheng (2008a) for North Dakota soybeans (0.36 – 1.16 μmol TE/g dry weight for yellow, 16.39-17.86 μmol TE/g for black soybeans). Other studies have reported DPPH$^{\bullet}$ scavenging capacity of soybean extracts, however cannot be directly compared due to varied experimental and reporting methods (Riedl et al., 2007; Slavin et al., 2009b). Several of the yellow soybean lines had similar or higher RDSC values compared to the non-modified Manokin line, which suggests that the antioxidant capacity was not altered by modification of the LOX enzyme. RDSC demonstrated positive correlation with TPC ($r = 0.645$, $p < 0.01$), however there was no significant correlation with total isoflavones. Phenolic compounds other than isoflavones are likely contributing to the RDSC in the studied soybeans.

Figure 3.2. Relative DPPH• Scavenging Capacity (RDSC) of Soybean*



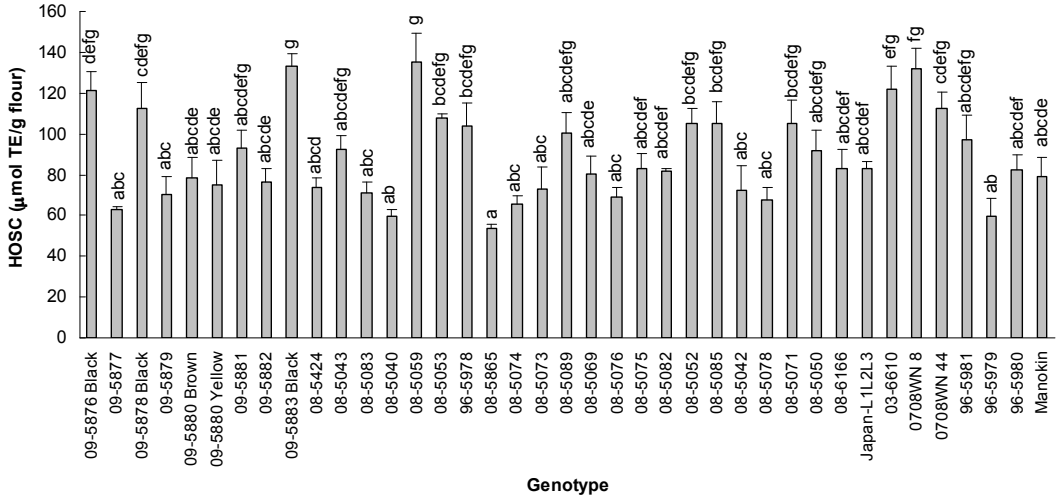
*Data expressed as µmol TE/g soy flour. Each bar represents mean ± SD (n = 3). Significant difference between genotypes is noted by different letters above each bar as indicated by ANOVA with Tukey's HSD post-hoc test ($p < 0.05$). Genotypes are yellow soybeans unless otherwise indicated. Manokin is a non-modified cultivar.

3.4.4.2. Hydroxyl Radical Scavenging Capacity (HOSC)

Hydroxyl radical (•OH) is a highly reactive physiological free radical that can damage cellular DNA (Jomova & Valko, 2011). HOSC of the soybean extracts was between 53.57 and 135.52 µmol TE/g flour (**Figure 3.3**). This is near the range reported by Slavin et al. (2009b) (68.3-109.5 µmol TE/g flour) for Maryland yellow soybeans. The black colored soybeans were in the higher range of HOSC levels, but were still similar to many of the yellow colored genotypes. Several of the yellow modified-LOX soybeans had similar HOSC levels as the Manokin non-modified soybean, and some were higher at statistically significant levels ($p < 0.05$). HOSC was positively correlated with TPC ($r = 0.471$, $p < 0.01$), RDSC ($r = 0.443$, $p < 0.01$),

and total isoflavones (r = 0.353, $p < 0.01$) (**Table 3.2**), indicating that phenolics such as isoflavones may contribute to scavenging of •OH.

Figure 3.3. Hydroxyl Radical Scavenging Capacity (HOSC) of Soybean*



*Data expressed as µmol TE/g soy flour. Each bar represents mean ± SD (n = 3). Significant difference between genotypes is noted by different letters above each bar as indicated by ANOVA with Tukey's HSD post-hoc test ($p < 0.05$). Genotypes are yellow soybeans unless otherwise indicated. Manokin is a non-modified cultivar.

In conclusion, this study has provided characterization of Maryland-grown soybeans with modified LOX content. The TPC, antioxidant activity, fatty acid, and isoflavone content were comparable to those previously reported for normal-LOX soybeans. Some specific genotypes were identified with isoflavone content similar to or higher than the Maryland-grown non-modified soybean. Soybean composition can be affected by growing location and year (Wang & Murphy, 1994; Lee et al., 2003), and studies over several growing seasons would be beneficial to confirm the findings for individual genotypes. Overall, these modified-LOX soybeans have been shown to

contain health-beneficial properties and may be useful as ingredients in functional foods. They also have potential to produce soy foods with improved oxidative stability and sensory properties, although further research needs to be conducted in that area with these selected genotypes.

Chapter 4: High-Throughput Assay for Detection of Soybean Lipoxygenase-1

Adapted from Whent et al., *J. Agric. Food Chem.* **2010**, *58*, 12602-12607

4.1. Abstract

A high-throughput assay was developed to detect soybean lipoxygenase-1 (LOX-1) activity using a multilabel plate reader. The assay was also adapted to a single cell fluorometer. Fluorescein is degraded by linoleic hydroperoxide produced from soybean lipoxygenase and linoleic acid. The decrease in fluorescence is measured over time and the area-under-the-curve (AUC) is used to quantify the LOX-1 content of soybean extract. A dose-dependent response is seen with varied dilutions of pure LOX enzyme or soybean extracts. Percent recovery was between 97% and 108%, and relative standard deviation was 4.3%. Advantages of the assay include the reduced preparation time of samples and reduced use of reagents in the high-throughput assay. Multiple samples can be measured in a single run with a multilabel plate reader.

4.2. Introduction

Soy foods are recognized for their health beneficial properties. Soy food consumption has been associated with the reduced risk of cardiovascular disease, osteoporosis, and some types of cancer (Isanga & Zhang, 2008). Replacing some dietary animal protein with soy protein has been recommended for lower risk of chronic disease (Anderson, 2008). Although soy foods are widely consumed in many Asian countries, they are less popular in the United States. One possible reason for

the dislike of soy products is the distinct "beany" flavors that they possess (Yuan & Chang, 2007). These undesirable flavor components are primarily the peroxidation products of polyunsaturated fatty acids catalyzed by lipoxygenase and hydroperoxide lyase.

Soybean lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a hydroperoxidase and catalyzes the oxidation of unsaturated fatty acids containing a cis,cis-1,4-pentadiene moiety. Conventional soybean seeds generally have three LOX isozymes including LOX-1, LOX-2 and LOX-3. The resulting products of the oxidation are converted to the undesirable odors and flavors associated with soy foods through a number of other reactions (Wolf, 1975). Processing treatments at high temperatures are used to inactivate the lipoxygenase (LOX) enzyme; however the heat may denature proteins and destroy other nutrients or health components such as isoflavonoids (Yuan et al., 2008; Euston et al., 2009). Through selective breeding, soybean lines have been developed that are null for one or more LOX isozymes. The goal for cultivation of these soybeans is to develop soy food products with reduced fatty acid oxidation and therefore improved flavor.

Rapid detection of the LOX enzymes is important to soybean breeding programs. Several colorimetric methods that can be measured visually or spectrophotometrically have been developed to detect the presence of lipid hydroperoxides that result from the LOX enzyme activity in soybeans or other plants (Suda et al., 1995; Waslidge & Hayes, 1995; Anthon & Barrett, 2001). One of the earliest methods involved the spectrophotometric measurement of conjugated diene generated from the LOX catalyzed peroxidation reaction of unsaturated fatty acid

substrates at 234 nm (Axelrod et al., 1981). A colorimetric method described by Suda et al. (1995) uses the bleaching of methylene blue or β-carotene as a spectrophotometric or visual indicator to determine the three isozymes of LOX . The methylene blue and β-carotene bleaching method was modified by Narvel et al. (2000) for use with small chips of soybean sample, which reduces sample preparation time**.** This modification of the method allows more rapid analysis, but can provide only a qualitative measure of the enzyme through a visual color change. The iodine-starch method and DMAB-MBTH method allow for relative quantification of LOX among samples. The iodine-starch method and DMAB-MBTH methods can measure activity of LOX in crude vegetable homogenate, and absorbance is read once after a specified time (Romero & Barrett, 1997; Anthon & Barrett, 2001). However, the presence of the LOX enzyme is measured by color change after a pre-determined time, which does not account for the benefits of the measuring reaction, and may not be as accurate as those accounting for reaction thermodynamics and kinetics such as the quantification using AUC. Measurement of blood platelet 12-lipoxygenase with ferrous oxidation of xylenol orange has been adapted to a high-throughput format (Waslidge & Hayes, 1995), and could possibly be adapted as a method for LOX detection in plants. But, one drawback of this method is that it may result in a false positive if the sample is able to chelate iron. This problem is more likely when using antioxidant-containing material such as soybeans. The presence of pigments may also interfere with the estimation of LOX using these colorimetric methods. In addition, measurement of O_2 consumption by electrode is another method that can quantify the LOX activity, although special equipment is required (Axelrod et al.,

1981). To date, the possibility to develop a fluorescence assay for LOX determination has not been investigated.

Rapid and efficient measurement of LOX activity is desirable for screening LOX-null soybean cultivars, and will be useful for soybean breeding programs. Despite the number of available detection methods, there is still a need for an efficient and quantitative method for measurement of LOX activity in soybeans. The purpose of this study was to develop a fluorescence assay for measurement of soybean LOX activity that can be adapted for high-throughput analysis and therefore improve the screening and quantification of LOX in modified soybeans.

4.3. Materials and Methods

4.3.1. Materials and Chemicals

Whole soybeans were provided by Dr. William Kenworthy in the Department of Plant Science and Landscape Architecture at the University of Maryland, College Park. Lines included Manokin, Japan 123 (Null for LOX-1, LOX-2, and LOX-3), and experimental lipoxygenase-null lines. Fluorescein, methylene blue, linoleic acid, and Tween 20 were obtained from Sigma Aldrich (St. Louis, MO). 15-lipoxygenase Type 1 (from soybean) was obtained from Cayman Chemical (Ann Arbor, MI). All other reagents were of highest commercial grade.

4.3.2. Soybean Extract

Soybeans were ground to 20-mesh using a household coffee grinder. For the methylene blue assay and single-cell fluorometric assay, 0.10 g of soy meal was combined in a 15 mL test tube with 10 mL ultra-pure water and vortexed twice for 15

seconds. For the 96-well high-throughput fluorescein assay, 0.020 g soy meal was combined with 10 mL ultra-pure water in a 15 mL test tube and vortexed twice for 15 seconds. Test tubes were held at 10 °C for 1 hour and then centrifuged at 1500 rpm for 5 minutes. The supernatant was collected and used immediately.

4.3.3. Lipoxygenase-1 Assay With Methylene Blue

The methylene blue assay for soybean lipoxygenase-1 activity was conducted according to the method previously described by Suda et al (1995) and Narvel et al (2000) with minor modification. Commercially purchased 15-lipoxygenase Type 1 (from soybean) was diluted in 200 mM pH 9.0 sodium borate buffer to 5 concentrations and held on ice until use. Sodium linoleate substrate was prepared with 90 mg linoleic acid, 40 μL Tween 20, 875 μL 0.5 M sodium hydroxide, and ultra-pure water in a final volume of 25 mL. The final reaction mixture contained 500 μL 200 mM pH 9.0 sodium borate buffer, 100 μL 200 mM methylene blue solution, 100 μL 12.6 mM sodium linoleate solution, 200 μL ultra-pure water, and 100 μL soybean extract or pure enzyme in a 1.5 mL cuvette. Absorbance was read at 660 nm and recorded at 10-second intervals for 6 minutes.

4.3.4. Lipoxygenase-1 Assay With a Single Cell Fluorometer Using Fluorescein as the Probe

Fluorescein stock solution was prepared at a concentration of 0.1 mM in pH 9.0 sodium borate buffer and stored at 0 ºC. Secondary stock solution was prepared by diluting the original stock solution to 0.01 mM. The secondary stock solution was diluted to 100 nM in pH 9.0 buffer to make the working solution. Pure soybean lipoxygenase was diluted in pH 9.0 sodium borate buffer to 5 concentrations and held

on ice until use. The final reaction mixture contained 600 µL 100 nM fluorescein working solution, 225 µL pH 9.0 buffer, 100 µL 12.6 mM sodium linoleate solution, and 75 µL soybean extract or pure enzyme in a 1.5 mL cuvette. A blank was prepared with buffer in place of fluorescein to determine the baseline fluorescence of the mixture. The fluorescence was read in a single-cell fluorometer and recorded at 10-second intervals for 6 minutes. The excitation and emission wavelength was 485 and 515 nm, respectively. Fluorescence of the blank was subtracted from the fluorescence of the standard or sample to obtain net fluorescence. Area under the curve (AUC) was calculated for net fluorescence as described by Moore et al. (2006):

$$AUC = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_{i-1}/f_0 + 0.5(f_i/f_0)$$

where f_0 is the fluorescence at 0 minutes and f_i is the final reading. The concentration of the commercially purchased enzyme dilutions was plotted against the calculated AUC and a standard curve was determined by linear regression. The concentration of LOX-1 in soybean extract was calculated using the equation derived from the standard curve.

4.3.5. High-throughput Lipoxygenase-1 Assay Using Fluorescein as the Probe

15-lipoxygenase Type 1 (from soybean) was diluted with pH 9.0 sodium borate buffer to 5 concentrations between 5 and 25 kilo-units (KU). The dilutions were prepared immediately before use and held on ice. 200 nM fluorescein working solution was prepared from the 0.01 mM secondary stock solution (described above). Sodium linoleate was prepared as described above. 100 µL 200 nM fluorescein working solution or pH 9.0 buffer (blank) was added to each well using a

multichannel pipet. 120 µL pure LOX-1 enzyme, soybean extract, or buffer (control) was added. Finally, 80 µL 12.6 mM sodium linoleate solution was added to each well using a multichannel pipet to initiate the enzyme reaction. Fluorescence was recorded continuously for 6 minutes using a Victor^3 multilabel plate reader (Perkin-Elmer). Excitation and emission wavelengths were 485 and 515 nm, respectively. AUC calculations were performed as described above.

4.3.6. Statistical Analysis

SPSS (version 10.0.5, 1999, SPSS Inc., Chicago, IL) was used to conduct statistical analysis. Means were compared by one-way analysis of variance with Tukey's post-hoc test. Statistical significance was declared at $p < 0.05$.

4.4. Results and Discussion

4.4.1. Development of LOX-1 Assay Using Fluorescein as the Probe

The first step in developing a high throughput method was determining the appropriate probe for LOX activity detection. Lipoxygenase has both dioxygenase and hydroperoxidase activity. Many assays currently used for LOX measurement were developed based on the lipid peroxidation reaction catalyzed by LOX. Peroxide radicals are generated during the oxidative chain reaction with or without LOX. Fluorescein ($C_{20}H_{12}O_5$) has been used as a probe for free radical scavenging capacity assays, as it is degraded by peroxyl radical ($ROO^{\bullet}$). This reaction is used as a probe in the oxygen radical absorbance capacity (ORAC) assay (Ou et al., 2001) and hydroxyl radical scavenging capacity (HOSC) assay (Moore et al., 2006b). According to Ou et al. (2001) and Moore et al. (2006b), fluorescein is stable and

resists degradation by light in the plate reader, so it may serve as an ideal probe for use in a fluorometric assay of LOX activity.

Figure 4.1 (A) shows the dose-response of fluorescein at different levels of the pure LOX-1 enzyme, and measures degradation of fluorescein in the presence of hydroperoxides. The pattern of **Figure 4.1 (A)** is very similar to that of **Figure 4.1 (B)**, which shows the dose-response of methylene blue at the selected pure LOX-1 concentrations, indicating that fluorescein may be used as a quantitative probe for LOX-1. Another reaction was conducted with fluorescein and LOX-1 without the sodium linoleate substrate, and no change in fluorescence was observed over time (data not shown). This indicated that fluorescence was not affected by LOX-1 in the absence of the PUFA substrate. There are multiple LOX isozymes known, and LOX 1, 2, and 3 are most prevalent (Axelrod et al., 1981). These three isozymes are active at different pH levels. Assays to detect the presence of all enzymes must therefore be conducted at the three pH levels. Linoleic acid is commonly used as a substrate for lipoxygenase-1 (LOX-1). Lipoxygenase 2 and 3 (LOX-2 and LOX-3) are most active at pH 6-7, and it can be difficult to distinguish them based on pH alone (Axelrod et al., 1981).

The use of fluorescein for detection of soybean LOX-1 was modeled using the previous methylene blue bleaching method (Suda et al., 1995). The methylene blue LOX assay measures the bleaching of methylene blue by hydroperoxides formed by the radical-mediated enzymatic oxidation of linoleic acid (Toyosaki, 1992). The new fluorescein assay measures the decrease in fluorescence of fluorescein as it is degraded by hydroperoxide radical attacks. This test was conducted at pH 9.0 to

Figure 4.1. (A) Fluorescein LOX-1 Assay*

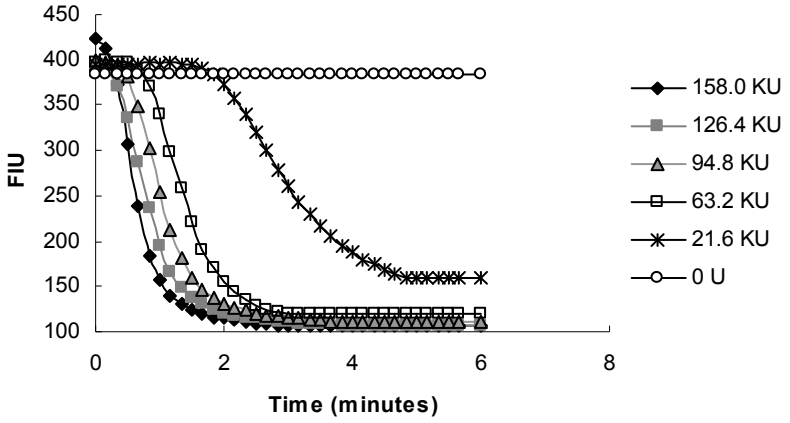
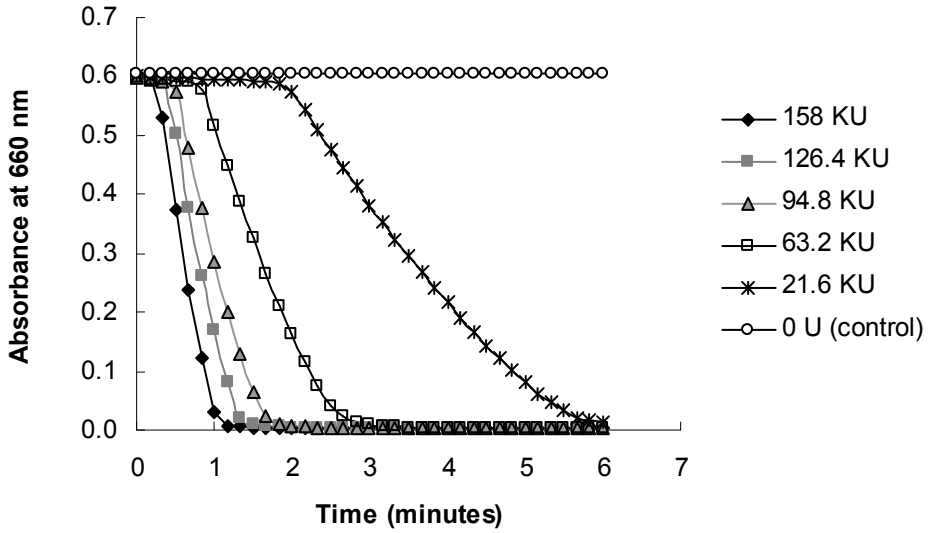


Figure 4.1. (B) Methylene Blue LOX-1 Assay*



*Comparison of A) fluorescein LOX-1 assay, and B) methylene blue LOX-1 assay. The reagents were A) 225 µL 200 mM pH 9.0 sodium borate buffer, 600 µL 100 nM fluorescein, 100 µL 12.6 mM sodium linoleate solution, 75 µL of 15-lipoxygenase (varied concentrations) and B) 500 µL 200 mM pH 9.0 sodium borate buffer, 100 µL 200 mM methylene blue, 100 µL 12.6 mM sodium linoleate solution, 100 µL of 15-lipoxygenase (varied concentrations) and 200 µL ultra-pure water. Absorbance or fluorescence were measured for 6 min., each assay was measured in triplicate. FIU = fluorescence intensity units.

measure the LOX-1 isozyme. This isozyme was chosen because it was available commercially while LOX-2 and LOX-3 were not. LOX-1 also has stronger catalytic activity than the other isozymes according the observations of Suda et al (1995). Ou et al. (2001) stated that a fluorescein solution will lose its fluorescence intensity below pH 7. Therefore, detection of the LOX-2 and LOX-3 isozymes with this fluorescein probe may have less definitive results than detection of LOX-1. Because lipoxygenase also has dioxygenase activity, a measurement of conjugated diene formation was performed according to the spectrophotometric method of Axelrod et al. (1981) and compared to the fluorescein lipoxygenase assay (figure not shown). The increase in absorbance at 234 nm coincided with the decay of fluorescein over time, and confirmed the LOX-1 activity.

Interestingly, it was discovered during assay development that Tween 20 contributed to fluorescence and therefore the amount added to the substrate was decreased to the point where there was sufficient emulsification in the solution but little fluorescence. Fluorescence of compounds in the blank solution was accounted by subtracting the blank reading from the sample readings.

Five minutes is the time recommended for bleaching of methylene blue by LOX-1 (Suda et al., 1995). Six minutes was an appropriate time for the degradation of fluorescein when using the quantities of reagents described above. After the initial degradation of fluorescein with peroxide radicals, products of lipid oxidation are formed which can increase the fluorescence of the solution. Therefore, the fluorescein assay has a cut-off point at which measurement was stopped. Higher enzyme concentrations begin to produce lipid oxidation products more quickly, and

the assay measurement was ended when the fluorescence of the strongest concentration reached its lowest level, which occurred after 6 minutes.

4.4.2. High-throughput Fluorescein LOX-1 Assay

A high-throughput quantitative assay is needed to rapidly screen the large soybean seed samples from the breeding program. The assay was modified for use in a Victor^3 multilabel plate reader. **Figure 4.2 (A)** shows dose-response of fluorescein at different levels of the pure LOX-1. An excellent linear relationship ($R^2 = 0.9906$) between LOX-1 concentration and the area under curve of the FIU-time plot was observed (**Figure 4.2 (B)**), indicating that the high-throughput fluorescence assay may be used to quantify LOX-1 activity. This facilitates the measurement of multiple soybean samples as well as the creation of the standard curve using the pure LOX-1 enzyme, since 4-5 dilution levels of the LOX-1 standard as well as 27-28 soybean samples can be tested in triplicates using one 96-well plate within 6 minutes. This is an advantage over previous methods that use a single-cell spectrophotometer, as they require up to 5 minutes for the measurement of each sample. These previous methods may take hours when measuring multiple samples. The high-throughput method also reduces the quantity of sample and reagents used. More importantly, the high-throughput assay is more sensitive and could detect a lower level of LOX-1 (**Figure 4.1** and **Figure 4.2 (A)**).

The addition of additional buffer solution and water was eliminated for the high-throughput assay. This reduced the reagents to only fluorescein (200 nM) in pH 9.0 buffer, pure enzyme or soybean extract, and substrate solution. The substrate

Figure 4.2. (A) High-throughput Fluorescein LOX-1 Assay*

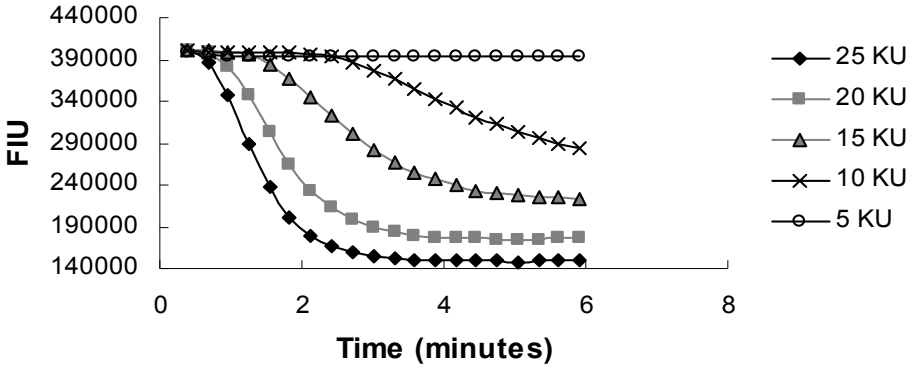
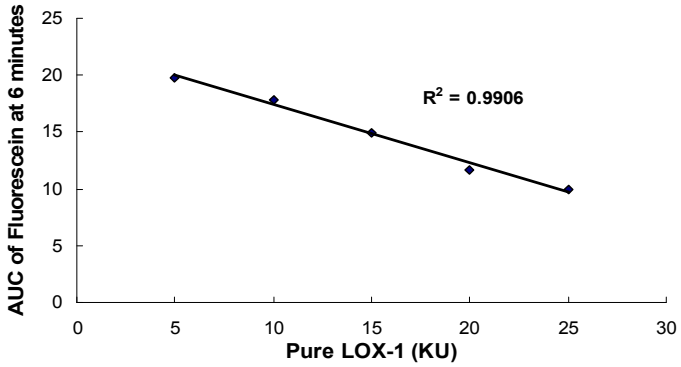


Figure 4.2. (B) Standard Curve of High-throughput Fluorescein LOX-1 Assay*



*A) High-throughput fluorescein LOX-1 assay. The reagents were 100 µL 200 nM fluorescein in pH 9.0 buffer, 120 µL varied concentration of 15-lipoxygenase, and 80 µL 12.6 mM sodium linoleate solution. Fluorescence was measured for 6 minutes. Each lipoxygenase concentration was measured in triplicate. B) Standard curve of high-throughput fluorescein LOX-1 assay. Concentration of 15-lipoxygenase (from soybean) is plotted against the area under the curve of fluorescence at 6 minutes of reaction time. All measurements were conducted in triplicate. FIU = fluorescence intensity units.

solution must be the last addition to the wells as the reaction will begin as soon as the substrate is added. The plate must then be placed in the plate reader quickly so that readings can begin before fluorescence begins to decrease. Six minutes was determined as the appropriate time for measurement in the multilabel plate reader. This allowed substantial degradation of fluorescein at the selected LOX-1 dilution levels.

4.4.3. Quantification

The use of the area under the curve (AUC) calculation for fluorescence allows for quantification of the LOX-1 enzyme based on the amount of hydroperoxide produced within the allotted time. The AUC of fluorescence of the samples over time was compared to a standard curve based on the fluorescence of pure LOX-1 enzyme of known quantity. There are other methods that quantify LOX activity. For example, the methylene blue assay quantifies the enzyme by measuring time until bleaching begins (Toyosaki, 1992). Measuring the AUC of fluorescence allows for more objective and accurate quantification. A potential difficulty in this quantification method is the instability of the pure LOX-1 enzyme that is used as a standard. The commercially purchased pure enzyme can lose activity quickly with time, even when held on ice. The enzyme was stored at -80 °C and used immediately after thawing in order to obtain a consistent result. The aqueous extract of soybean meal was less sensitive to changes in temperature and time.

4.4.4. Linearity and Range

For the high-throughput assay, the linear range of soybean LOX-1 (plotting concentration versus AUC) was determined at 5-25 KU (**Figure 4.2 (B)**). The results of 6 repeats of this assay are shown in **Table 4.1.** Soybean extract should be diluted to fall within this range if quantification is desired. Soybean extract from the Manokin line (normal LOX) at a concentration 2 mg/mL was appropriate to fit within this range upon measurement with the high-throughput fluorescein assay.

Table 4.1. Linearity of LOX-1 Fluorescein Assay*

	R^2
Day 1	0.9906
Day 2	0.9652
Day 3	0.9494
Day 4	0.9675
Day 5	0.9872
Day 6	0.9915

*Measurements were conducted under conditions for the high-throughput fluorescein LOX-1 assay in a 96-well plate. Measurements were conducted in triplicate.

4.4.5. Validity

Figure 4.1 demonstrates the validity of the fluorescein LOX-1 assay compared with the traditional methylene blue LOX-1 assay when using the pure LOX-1 enzyme. In **Figure 4.3,** the selected LOX-1-null soybeans as well as non-modified soybeans were tested and compared using the high-throughput fluorescein assay and the methylene blue assay. The results demonstrated the validity of the high-throughput fluorescein assay for measuring LOX-1 in soybean extract in

comparison with the methylene blue LOX-1 assay. It is clear that each soybean extract shows a similar reaction in the two assays.

Additionally, the assay has shown to be specific for the LOX-1 enzyme. In **Figure 4.1 (A)**, fluorescence does not decrease in the control reaction where there was no LOX-1 enzyme present in the mixture. In **Figure 4.3** the LOX-null soybean extracts do not have an effect on fluorescence when compared to the normal-LOX lines. Although experimental LOX-null lines are demonstrated in **Figure 4.3**, this reaction was also tested with the Japan 123 soybean that is known to be null for LOX (data not shown). This demonstrates that it is the LOX-1 enzyme and not another component of soybean extract that causes the degradation of fluorescein. However, it is acknowledged that LOX-2 or LOX-3 may have some activity under the conditions of this assay, so results should be expressed in terms of LOX-1 equivalents.

Figure 4.4 shows the calculated LOX-1 concentration per gram of flour when multiple concentrations of extract from one soy meal sample were analyzed. The calculated LOX-1 content of the soybean was not significantly affected by the concentration of the enzyme. Calculated values had a relative standard deviation (RSD) of 9.4%. These data indicated that the ratio of extraction solvent to soybean sample weight may not be very critical for LOX-1 activity estimation using the high-throughput fluorescein assay, and the high potential of this assay for practical utilization.

4.4.6. Reproducibility

The interday reproducibility of the high-throughput fluorescein LOX-1 assay was determined by measuring the AUC (at 6 minutes) of the reaction of an aqueous

extract of soybean meal from the Manokin line (2 mg/mL). This test was repeated 6 times within 6 days (**Figure 4.5**). Fresh soybean extract and sodium linoleate were freshly prepared each day. The RSD of 4.3% demonstrates an excellent reproducibility of the assay.

4.4.7. Accuracy and Precision

Soybean LOX-1 was analyzed at 3 different concentrations (10, 15, and 20 KU) on 3 separate days to determine the accuracy of the high-throughput assay (**Table 4.2**). The measured concentration was compared to expected concentration to determine the percent recovery. Average percent recovery varied from 97 to 108%, which demonstrates good accuracy. The RSD of pooled samples was between 4.62 to 7.35% which indicates excellent precision.

Figure 4.3 (A) High-throughput Fluorescein LOX-1 Assay*

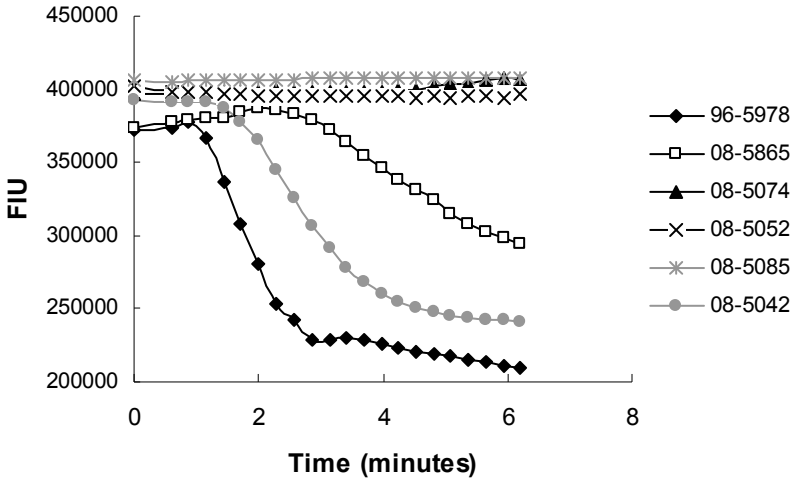
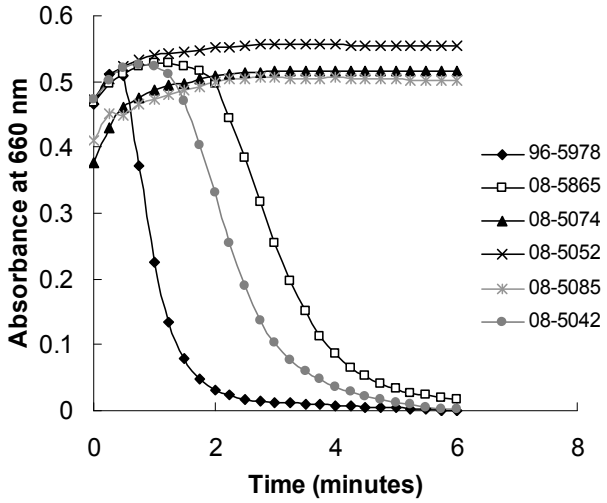
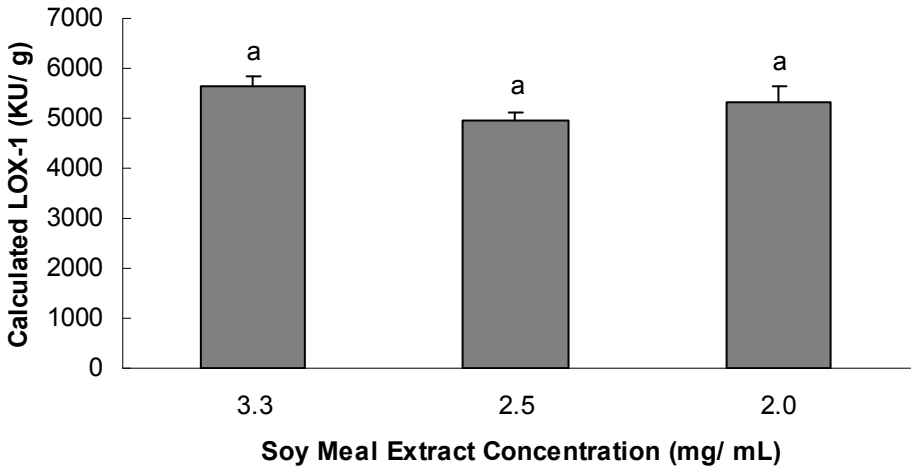


Figure 4.3 (B) Methylene Blue LOX-1 Assay*



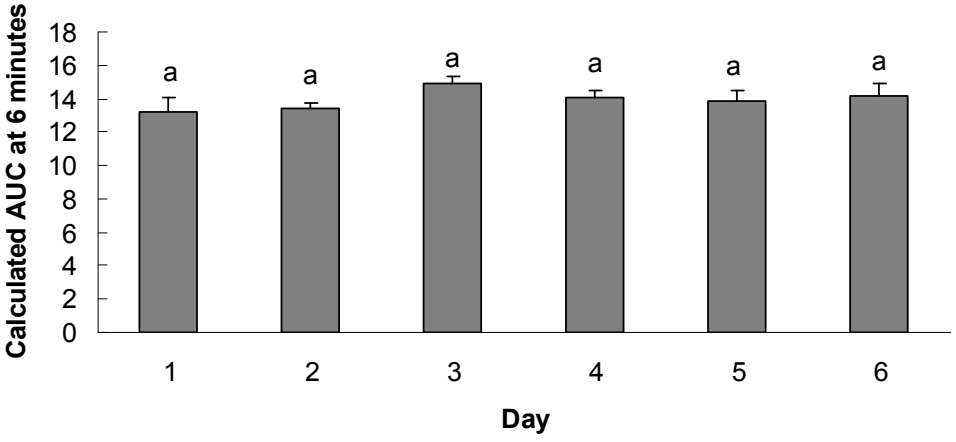
*Comparison of the A) high-throughput fluorescein LOX-1 assay and B) methylene blue LOX-1 assay using the extracts of six soybean lines. The reagents were A) 100 µL 200 nM fluorescein in pH 9.0 buffer, 120 µL of soybean extract, and 80 µL 12.6 mM sodium linoleate solution, and B) 500 µL 200 mM pH 9.0 sodium borate buffer, 100 µL 200 mM methylene blue, 100 µL 12.6 mM sodium linoleate solution, 100 µL soybean extract and 200 µL ultra-pure water. Fluorescence or absorbance at 660 nm was measured for 6 minutes. Each extract concentration was measured in triplicate. FIU = fluorescence intensity units.

Figure 4.4. Effect of the Ratio of Solvent to Soybean in Extraction on LOX-1 Activity Estimations*



*The LOX-1 equivalent of Manokin soybean was estimated using three ratios of solvent volume to soybean weight. The reagents were 100 μL 200 nM fluorescein in pH 9.0 buffer, 120 μL soybean extract, and 80 μL 12.6 mM sodium linoleate solution. Fluorescence was measured for 6 minutes. Each extract concentration was measured in triplicate. Columns with the same letter indicate no statistical difference at $p < 0.05$.

Figure 4.5. Reproducibility of the High-throughput LOX-1 Fluorescein Assay*



*0.020 g of Manokin soy flour was extracted in 10 mL distilled water and measured daily for 6 days. Measurements were conducted under conditions for the high-throughput fluorescein LOX-1 assay in a 96-well plate. Measurements were conducted in triplicate. Columns with the same letter indicate no statistical difference at $p < 0.05$.

Table 4.2. Accuracy and Precision of Quality Control (QC) Samples*

	QC1	QC2	QC3
Nominal Lipoxygenase Concentration (KU)	10	15	20
Run 1			
intra-mean	12.75	15.94	20.92
SD	1.11	1.50	0.33
% RSD	8.68	9.43	1.61
% Rec	127.5	106.3	104.6
N	3	3	3
Run 2			
Intra-mean	9.69	14.70	18.85
SD	0.85	0.42	0.85
% RSD	8.78	2.87	4.51
% Rec	96.1	98.0	94.3
N	3	3	3
Run 3			
Intra-mean	10.03	15.31	18.83
SD	0.46	0.70	1.63
% RSD	4.58	4.59	8.64
% Rec	100.3	101.9	94.1
N	3	3	3
Pooled Runs			
Inter-mean	10.82	15.15	19.53
SD	0.82	0.87	0.94
% RSD	7.35	5.63	4.92
% Rec	108.0	101.8	97.67
N	9	9	9

***** Measurements were conducted under conditions of the high-throughput fluorescein LOX-1 assay in a 96-well plate in triplicate.

4.4.8. Advantages and Limitations of the Fluorescein LOX-1 Assay

The purpose of the described method is to quickly measure the presence of LOX-1 in soybean extract. This method requires less preparation time than some other frequently used spectrophotometric methods. The direct soybean extract can be used without additional filtration and purification steps. Using a multilabel plate reader, multiple soybean samples can be tested within 6 minutes. The time is much reduced from the previous methylene blue method where each individual sample will require at least 15 minutes to be measured in triplicate. Sample extracts may be compared against each other, or quantified using the standard curve of the purified enzyme, which makes the comparison of cross-laboratory data possible.

No expensive reagents are required, and the high-throughput assay reduces the amount of reagents used. Additionally, only a small amount of ground soybean is required for the tests. This method can be used as a screening tool for soybean breeding programs as they select for seeds that are low in LOX-1. The method can potentially be modified for use with other soy foods and may assist in quality control during food processing. However, it needs to be pointed out that the assay depends on the peroxide radical attack to fluorescein, and presence of antioxidants such as reducing agents and radical scavengers in the sample may lead to under-estimation of LOX-1 activity or level.

Chapter 5: Summary and Significance

The first objective of this study was to evaluate the effect of genotype and environment and their interaction on the selected nutraceutical components in low 18:3n3 soybeans. The results showed a significant effect ($p < 0.05$) of genotype on nearly every measured component, except for TPC and ORAC. Genotype also had a larger effect than environment on many of the soybean components. Environment showed the largest effect on certain fatty acids, tocopherols, the isoflavone glycitein, and ORAC. The interaction between genotype and environment was significant for nearly every measured component, and the largest effect was seen for antioxidant capacity assays such as TPC, HOSC, and RDSC. There was correlation between temperature and precipitation during the growing season and certain soybean components such as fatty acids, lutein, and tocopherols. It is important to note that the soybean samples were all grown within the state of Maryland, which has low climate variability, so the full effect of environmental differences on these soybean lines may not have been demonstrated in this study.

The second objective was to evaluate selected nutraceutical components of modified lipoxygenase (LOX) soybeans to determine if these soybeans may be of benefit for functional foods. The study showed no significant difference overall between among the LOX-modified or non-modified soybeans in relation to isoflavone content or antioxidant capacity. In this respect, the LOX-modified soybeans may be useful for value-added foods, but further study of these soybeans as a food ingredient is required. Additional study over several growing seasons is also required.

The final objective was development of an improved assay for detection of LOX content in soybean. A preliminary assay was developed which detected hydroperoxide formation (an indicator of lipid oxidation) using fluorescein as a fluorescent probe. The assay was developed for use in a 96-well plate reader in order to analyze several soybean samples at one time. Using a purified LOX-1 enzyme as a standard, the qualitative content and the quantitative activity of LOX-1 in the soybean sample can be measured. The assay is currently developed to detect LOX-1, and further research is needed to use this method for LOX-2 or LOX-3 specifically.

In summary, modifications to fatty acid or enzyme levels in soybeans can be achieved without major changes to the health-enhancing components. These components can potentially be affected by differences in genotype or growing environment to select for desired traits. Further studies are recommended to evaluate the stability of these soybeans in functional food products.

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