ABSTRACT

Title of Thesis: EFFECT OF PLANTING TIME AND LOCATION ON THE ISOFLAVONE CONTENT AND PROFILE OF DIFFERENT SOYBEANS [*Glycine max* (L.) MERRILL] CULTIVARS

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Isoflavones found in high concentrations in soybeans have received great attention due to their potential beneficial effects in human health. The effects of soybean planting time, location and seed type on isoflavone content and profile were evaluated using 40 cultivars.

Isoflavones were extracted with 80% methanol and analyzed by HPLC. Conditions were optimized to separate all reported soybean isoflavones plus an additional acylated genistein-related peak. Genistein derivatives were most abundant, followed by daidzein and glycitein-related isoflavones. Total isoflavone content varied greatly among cultivars, from 212 (Black Jet) to 3056 mg/Kg seed (Stressland). Planting time and location affected the isoflavone content. Soybeans planted at Poplar Hill in a double crop system exhibited higher isoflavone concentrations than soybeans planted during the full season at Wye Farm. Plants exposed to low temperatures and precipitation exhibited

higher isoflavone content. It may be possible to select cultivars and growing conditions to obtain isoflavone rich seeds.

EFFECT OF PLANTING TIME AND LOCATION ON THE ISOFLAVONE CONTENT AND PROFILE OF DIFFERENT SOYBEANS [*Glycine max* (L.) MERRILL] CULTIVARS

by

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DEDICATION

This Thesis is dedicated to Carolina, my beloved wife, and to my parents Víctor and Norma.

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My education could never be accomplished without the endless support and care from my wife and parents. Each single piece of my progress, in University of Maryland or thereafter, should all be attributed to their sacrifice and love to me. This thesis is dedicated to them.

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LIST OF ABREVIATIONS

AB	Double Crop (After Barley)
ASA	American Soybean Association
BLUPS	Best Linear Unbiased Predictors
CE	Capillary Electrophoresis
CGC	Canadian Grain Commission
CRBD	Completely randomized block design
CZE	Capillary Zone Electrophoresis
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ER	Estrogen Receptor
FSH	Follicle-Stimulating Hormone
GC	Gas Chromatography
HPLC	High performance liquid chromatography
ISB	Indiana Soybean Board
LOX	Lipoxygenase
LH	Luteinizing Hormone
MSU	Michigan State University
MS	Mass Spectrophotometry
NCDC	National Climatic Data Center
PH	Poplar Hill Facility
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
RR	Roundup Ready Tolerant
nRR	Non-Roundup Ready Tolerant (Standard)
SPE	Solid Phase Extraction
TVP	Textured Vegetable Protein
UMCP	University of Maryland at College Park
USDA	United States Department of Agriculture

CHAPTER 1: INTRODUCTION

The Soybean (*Glycine max* L.), is one of the oldest crops of China, and has been used by Chinese and other Oriental cultures for centuries in many forms as one of the most important source of protein and oil. Soybeans are also unique because of the presence of isoflavones. These compounds are very similar in chemical structure to the human estrogens, which are hormones that the human body makes and requires for normal growth and development. It has been reported that isoflavones may play an important role in cancer prevention by inhibiting tumor initiation, in repair of oxidative damage, in moderation of menopausal symptoms, and other health effects.

Nowadays, consumers are interested in foods that may help prevent or reduce the incidence of illness, for this reason, the soybean has caught the attention of the world and now it is seen as a crop that could help to combat world hunger and also contribute to the prevention of chronic diseases. This could be observed by the increase on US consumer consumption of soy foods and soy-based food ingredients, having the last ones practically doubled from 1998 to 2000, and it has been attributed to reported health benefits of soy including prevention of cancer, osteoporosis and coronary heart disease.

Due to the characteristics of isoflavones and their important biological effect, different studies have been undertaken in the fields in order to gather more information about the incidence of these compounds. Genetic and agronomic studies are of crucial importance in order to determine how genetics, breeding and environmental factors could affect the composition of isoflavones in the soybean. High levels of soybean isoflavones

are desired for nutraceutical purposes. However, lower levels of isoflavones are desired for food products, because isoflavones have been linked with the undesirable flavors in soybeans.

Researchers have studied different environmental factors in order to evaluate their effect on the isoflavone content in soybeans. High variability within and among cultivars has been reported, with isoflavone content varying from cultivar to cultivar, from year to year, from location to location, also due to the planting date. These suggest that different climate conditions and other environmental factors might be contributing factors to variation in isoflavone contents. However, the effects of other factors, such as the genetic factor (Standard and Glyphosate tolerant cultivars) and differences among type of cultivar (grain and food cultivars), on isoflavone concentration have not been previously reported. Also, there is limited information on the effect of environmental factors such as water availability and growing temperature.

This thesis presents the study of the effect of planting time and location on the isoflavone content and profile of 24 soybeans grain cultivars. From these grain cultivars the genetic factor was evaluated, studying two types of grain cultivars: Standard and Glyphosate (Roundup Ready) tolerant cultivars. Also was studied how the environmental factors (temperature and precipitation) affected the isoflavone content in the seeds.

The importance of this study was to understand how the effect of planting time and location, and the different environmental factors present, affected the isoflavone content, and hence, how to select the adequate combination of cultivar and growing conditions to favor the desired level of isoflavones.

In addition, 16 different standard cultivars were evaluated, to contrast the isoflavone content according to the soybean type: green, food and grain type. This information may be helpful to classify, according to the level of isoflavones, which cultivars would be the better ones for edible use, and also to select promising grain cultivars that could be used for direct consumption or as soy-based foods.

The objectives of this study were to determine the differences in the isoflavone content among different soybean cultivars, taking in to account the genetic factor for glyphosate tolerance (Roundup Ready and Standard cultivars) and according to the seed type (food and grain cultivars). In addition, we evaluated the effect of planting time and location in the qualitative and quantitative composition of isoflavones in different soybean cultivars to determine how environmental factors such as air temperature and precipitation affected their isoflavone content.

CHAPTER 2: LITERATURE REVIEW

2.1 Soybeans

The Soybean (*Glycine max* L.), is one of the oldest crops of China, and has been used by Chinese and other Oriental cultures for centuries in many forms as one of the most important source of proteins and oil (Wilson 1991). For this reason and due to many others (high protein yield per unit area than other crop), the soybean has caught the attention of the world and now it is seen as a crop that could help to combat world hunger. Most recently, interest in soybean has increased due to the presence of isoflavones, compounds that may play an important role in preventing and treating chronic diseases (Messina and Messina 1991; Messina and others 1994; Kennedy 1995; Setchell 1998; Munro and others 2003).

Wild types of soybeans were first introduced in the United States as a hay crop. Introductions from China, Manchuria, Korea and Japan have been important in developing cultivars for the United States. Modern breeding efforts to improve the agronomic traits, such as more erect growth, reduced lodging and increased seed size, have been primarily responsible for the development of soybeans into a crop of world-wide importance (Mullen 2003).

2.1.1 Agronomic Characteristics.

The soybean belongs to the family Leguminosae, subfamily Papilionoideae, and genus Glycine, L. The cultivated form called *Glycine max* (L.) Merrill, grows as a summer annual. However, some related species are perennial in nature (Mullen 2003). The plant is bushy with height ranging 0.75 to 1.25 m, branching sparsely or densely, depending on cultivar and growing conditions (Liu 1997b).

The flowers are white or violet (Howell and Cadwell 1978; Mullen 2003). The beans grow in pods that develop in clusters of 3 to 5 cm with each pod usually containing 2 or 3 beans. These beans are sometimes big or small, long, round or oval. The color can also vary. Some are yellow, others are green but can also be brown and some are even black or with spots (Howell and Caldwell 1978; Mullen 2003).

One of the most important agronomic characteristics of soybeans is that it can take nitrogen from the air and convert it to a form usable by the soybean plant (ASA 2003), characteristic that will be explained later in more detail.

2.1.1.1 Seed Morphology

Hicks (1978) described clearly the seed morphology. The soybean seed is essentially devoid of endosperm and consists of a seed coat and a large, well-developed embryo that contains two pieces of cotyledons that function as food reserve structures. The seed coat is marked with a hilum or seed scar that varies in shape from linear to oval. The coat protects the embryo from fungi and bacterial infection before and after planting. Besides cotyledons, the embryo has three other parts: radicle, hypocotyl, and epicotyl (Figure 2.1). The radicle and hypocotyl are located under the seed coat at one end of the hilum, just below the micropyle, which is a tiny hole formed by the integuments during

seed development. The third part, the epicotyl, is very small and placed between the pair of cotyledons (Figure 2.1). During germination, the radicle becomes the primary root, whereas the hypocotyl lifts the cotyledons above the soil surface. The epicotyl is the main stem and growing point (Liu 1997b; Hicks 1978).

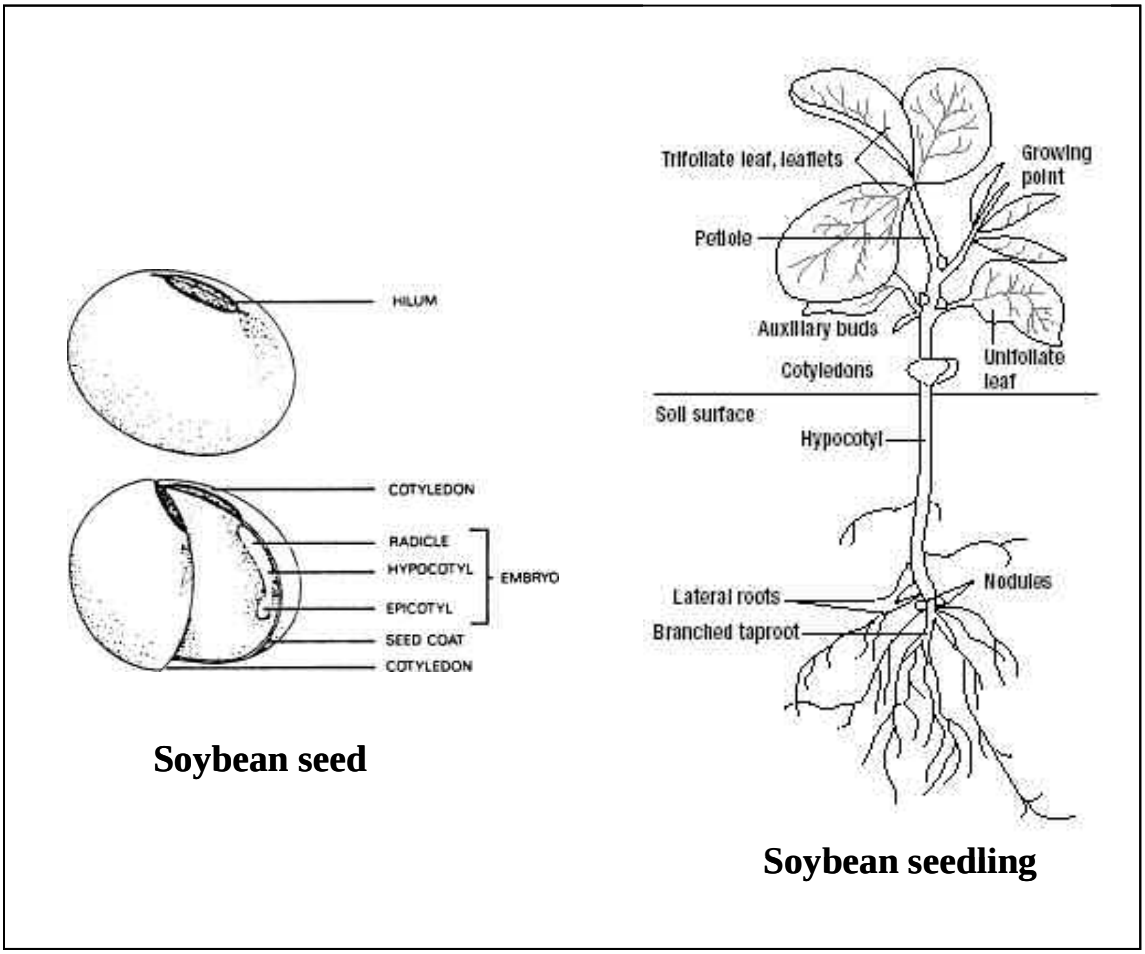


Figure 2.1: Structure of a soybean seed, and a soybean seedling.
Source: MSU 2000, Roth and others 2003.

The seed of various soybean types can be yellow, green, black and several shades of brown, but most commercial cultivars are yellow. The seed colors are genetically controlled and, except for green, are carried in the testa, a leathery textured seed coat.

The green color of seeds may be carried in the cotyledons and/or testa. The outward appearance of the testa can vary from shiny to dull. Sometimes, depending on cultivar and environment, seed coats are etched, and partial cracks appear in the top layers of the seed coat (Mullen 2003).

Hilum color is genetically controlled and is often used as an aid in identifying cultivars. Distribution of color in the hilum and the seed coat varies genetically and is influenced by the environment. Near one end of the hilum is a small round scar (micropyle), which appears to be a hole through the seed coat. This was the opening through which the pollen tube entered the embryo sac, during fertilization (Mullen 2003).

2.1.1.2 Germination and Seedling Development

Germination is a complex metabolic and physiologic process that begins with the seed and result in a plant capable of completing a normal life cycle. Under favorable environmental conditions, food reserves in the seeds are utilized to develop the root and shoot, the seedling emerges from the soil and becomes self-sufficient, and the plant perpetuates the species by developing viable seeds for the next generation (Hicks 1978; Howell and Caldwell 1978).

Soybean seeds are usually planted at depths between 2 and 5 cm, depending on soil type and moisture conditions. A good supply of soil moisture during germination is critical, because the seed must reach a moisture content of 50% before the germination starts. However, excessive moisture is unfavorable for germination too, probably due, in part, to restriction of the oxygen supply (Howell and Caldwell 1978).

After the soybean seed is planted in the soil, the radicle is the first part of the embryo to penetrate the seed coat. It develops rapidly into a root, which must become firmly anchored for the seedling to develop enough leverage to force its way to the soil surface (Liu 1997b; Mullen 2003). Lateral roots form soon after the radicle begins to elongate. Within 4 or 5 days after planting, root hairs appear on the laterals. These hairs are the main absorbing surface of the root system. The roots branch and rebranch. By the end of the growing season, they penetrate to a depth of 5 ft or more in a well-drained, good prairie soil. However, the bulk of the root is found in the upper 12 in. of the soil, with extensive growth in the topmost 6 in. (Carlson 1973).

After the radicle emerges, the hypocotyl begins to elongate. It forms an arch that is pushed upward through the soil. As the arch breaks the soil surface, it pulls the cotyledons and epicotyl upward. The uppermost cells of the hypocotyl stop growing as cells on their underside continue to grow until the arch is straightened. This process lifts the cotyledons into an upright position (Howell and Caldwell 1978; Mullen 2003). As soon as the epicotyl is exposed to the sunlight, the first two leaves begin expanding from it. They unfold and develop rapidly thereafter. Known as unifoliate, the two leaf blades are opposite each other and located at the same node. All later-formed leaves are trifoliate (three leaf blades). They are located only one at a node and are alternate in position on the stem. Soon after exposure to sunlight, the cotyledons and other plant parts develop chlorophyll and turn green. However, the food reserve in the cotyledons remains the main source of nourishment for about one to two weeks after emergence. The cotyledons drop thereafter (Liu 1997b; Mullen 2003).

2.1.1.3 Growing Stages and Maturity Groups

Development of the soybean plant begins at germination and ends when mature seeds are ready for harvest. All aspects of soybean development including length of vegetative growth, timing of flowering, and maturity date are greatly influenced by photoperiod and temperature (Wiebold 2002).

Most crop plants have two major growth stages: the vegetative and the flowering or reproductive stage. In the soybean plant, the period between emergence and the appearance of the first flower is the vegetative stage, which usually takes 6-8 weeks (Mullen 2003).

The actual days of vegetative growth as well as the ultimate size of the plant before flowering depend on many factors, including genotypes, planting date, geographic locations, and environmental conditions. The soybean plant is also photoperiod sensitive, which means that it makes the transition from vegetative to flowering stages in direct response to day length (Liu 1997b; Howell and Caldwell 1978; Wiebold 2002).

Latitude plays a major role in the adaptation of soybeans to various geographic regions. On the American continent, soybean cultivars have been divided into 12 maturity groups. Those adapted for flowering at the highest latitudes are labeled 000. As the latitude decreases, the number of the maturity group increases from 0 to IX. In the northern latitudes where day lengths change rapidly as the year proceeds, the bands of latitudes for maturity groups are narrow, whereas in the south day length changes are much less pronounced, the bands of latitudes for maturity groups are wider (Whigham and Minor 1978).

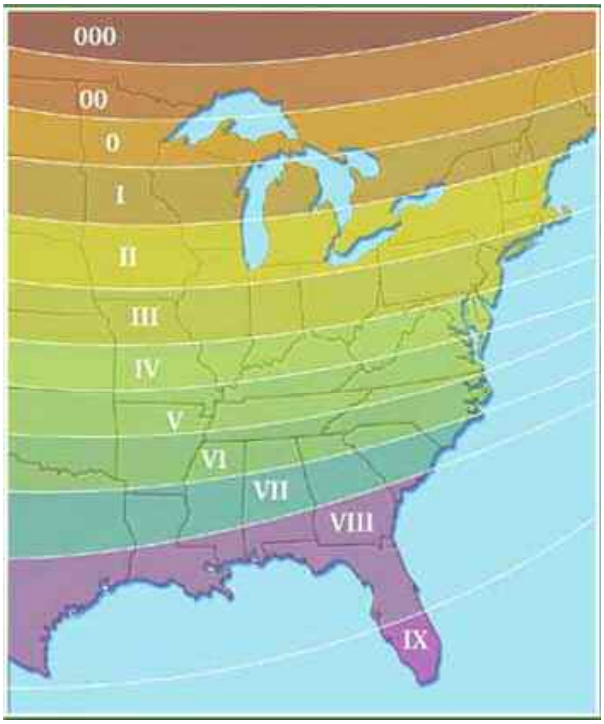


Figure 2.2: Soybean seeds cultivar adaptation (Wiebold 2002)

Cultivars adapted to southern latitudes will often grow taller and mature later than normal when planted in northern latitudes, encountering risk of early frost damage before seeds become mature, which could lead to reduced or no yield. Cultivars adapted to northern latitudes will often be shorter and mature earlier than normal when planted in southern latitudes, flowering and setting pods before plants have reached their full vegetative growth, leading to reduced yield (Liu 1997b; Wiebold 2002).

2.1.1.4 Seed Development

The early stages of seed development are characterized by a period of rapid cell division. (Nielsen 1996).

In each axil (junction of a stem and a branch or leaf) an axillary bud is present. This bud may develop into a branch, a flower cluster, or fail to develop, depending on the environment (Mullen 2003).

Flowering usually begins in the lower part of the plant (commonly at the fourth node) and then progresses toward the bottom and top. Flower petals are white or violet and are borne in racemes (flowers arranged on a stem-like branch arising from the nodes). The flowers are normally self-pollinated with less than one percent natural crossing. Artificial hand pollinations are made by plant breeders in the process of developing new cultivars. In a 2690 Kg/Ha yield only about 25 percent of the flowers develop into mature pods containing seed. In a field planting, the normal number of pods per plant ranges from 30 to 100 with 2 to 3 seeds per pod. However, lines and cultivars with narrow leaflets often have 4 seeds per pod. Plant spacing can influence pod number. Widely spaced plants have produced more than 400 pods per plant. The flowering period of indeterminate Corn Belt cultivars is 3 to 4 weeks. This period usually begins 6 to 8 weeks after seedling emergence. High temperatures and drought stress during seed development (especially during rapid seed fill) can reduce germination and vary the protein and oil content of the seed (Mullen 2003).

Rapid cell elongation in cotyledons, elevated metabolic activity and a swift increase in seed dry weight are characteristics on the next 35 to 40 day period in seed development. This stage in development is characterized by the rapid proliferation of oil and protein bodies. Generally the onset of oil accumulation can be demonstrated to slightly precede that of protein, and the oil accumulation plateaus before protein deposition (Nielsen 1996).

Depending on the maturity group, genotype and growth conditions, soybean plants require 108 to 144 days from seed germination to the recovery of mature seeds (Nielsen 1996).

2.1.1.5 Diseases and Pests

According to Svec (1997), Bowers and Russin (1999), Fuderburk and others (1999) and Lawrence and McLean (1999), soybeans are susceptible to the attack of different pests and diseases during their growing season. Approximately one hundred bacterial, fungal, viral and nematode pathogens are known to attack soybeans. Within the major fungal diseases could be found brown leafspot (*Septoria glycines* Hemmi), frogeye leafspot (*Cercospora sojina* Hara), phytophthora root rot (*Phytophthora megasperma* Drechs), stem canker (*Diaporthe phaseolorum*), purple seed stain (*Cercospora kikuchii* T. Tomayasu) and stem blight (*Phomopsis soja* Lehman) (Bowers and Russin 1999). In addition, the major bacterial diseases are bacterial blight (*Pseudomonas syringae subsp. glycinea*), pustule (*Xanthomonas campestris pv glycinea*), and wildfire (*Pseudomonas syringae subsp. tabaci*). Major viral diseases include soybean mosaic (*Potyvirus* (SMV)), bud blight (*Nepovirus* (TRSV)), and bean pot mottle (*Comovirus* (BPMV)). Species of nematodes that cause major problems are cyst nematode (*Heterodera glycines* Ichinohe) and root knot nematode (*Meloidogyne arenaria* (Neal) Chitwood, *Meloidogyne hapla* Chitwood, *Meloidogyne incognita* (Kofoid & White) Chitwood, *Meloidogyne javanica* (Treub) Chitwood) (Lawrence and McLean 1999).

Other pests that attack soybeans include birds, rodents, insects and weeds, having the ability of decrease soybean yields. The two major groups of insects that attack

soybeans are larvae of lepidoptera (moths) and colepotera (beetles). Four major species of the Lepidopterous Larvae that cause problems are the cloverworm (*Plathypena scabra*, Fabricius), soybean looper (*Pseudoplusia includens*, Walker), velvetbean caterpillar (*Anticarsia gemmatalis*, Hubner) and corn earworm (*Heliothis zea*, Boddie). Major pod and stem feeding insects are stink bugs (*Acrosternum hilare*, Say), alfalfa hopper (*Spissistilus festinus*, Say) and lesser cornstalk borer (*Elasmopalpus lignosellus*, Zeller) (Fuderburk and others 1999).

According to Liu (1997b), Reddy and others (1999), and Reddy (2001), another problem that soybeans face during the growing period are both broadleaf and grass weeds. They could affect seriously the soybean yield if they are left after mid-season. In fact, weeds constitute the greatest hazard to soybean production in terms of the magnitude of losses they can cause (Reddy and others 1999). They compete with plants for nutrients, moisture and sunlight, and also reduce the trading value of the crop due to their presence in the harvested seed. Rotating crops, spraying chemicals, and choosing resistant cultivars brought about by plant breeding have been the major tools used by farmers to control diseases and pests (Gasser and Fraley 1992). But, due to environmental concerns about the wide use of pesticides, herbicides and other chemicals, biological control has become increasingly popular. During the past decade, advances in biotechnology coupled with plant breeding have led to the development of plants that withstand insects, viruses and herbicides (Gasser and Fraley 1992; Reddy and others 1999).

a. Disease Resistance

An herbicide-resistant crop is made to tolerate a specific herbicide. Roundup Ready soybeans (tolerant to Glyphosate) have been recently commercialized. Glyphosate (Roundup) is a nonselective herbicide that kills most annual and perennial grasses and broadleaf weeds. Recent advances in plant biotechnology have made it possible to insert a gene into soybeans to provide crop tolerance specifically to the herbicide Glyphosate (Reddy 2001; Padgette and others 1996).

According to Reddy (2001), tolerance to glyphosate in soybean represents a revolutionary breakthrough in weed control technology, it is the most widely grown of all transgenic crops produced commercially in the world. In 2000, Roundup Ready soybeans were planted to 25.8 million hectares globally, which amounts to 58% of the total transgenic crop area. The United States soybean area planted with Roundup Ready soybean has increased from 2% in 1996 to 68% in 2001.

Roundup Ready soybeans remain unaffected when treated with the herbicide because of the expression of a glyphosate-resistant EPSP synthase with a high catalytic activity in the presence of glyphosate. The continued action of the glyphosate-resistant EPSP synthase enzyme helps to maintain aromatic amino acids levels in plants (Reddy 2001).

In addition, some phenols such a genistein and daidzein can be elicited in response to biotic and abiotic stresses (unfavorable conditions in plants like damage by pests). Signal molecules are assumed to link elicitor or stress receptors with transcription or downstream response genes. (Dixon and Paiva 1995; Somssich and Hahlbrock 1998). According to Sanderman and coworkers (1998), the level of isoflavones in non-

glyphosate-tolerant plants might be increased when treated with glyphosate. Taylor and others (1999) suggested that this increase in isoflavones is a result of stress-induced response to the application of glyphosate to non-glyphosate-tolerant soybeans. The application of glyphosate to the Roundup Ready lines did not cause a stress response. The composition of glyphosate-treated Roundup Ready soybeans was substantially equivalent to the composition of soybean seeds from plants that were not treated with glyphosate.

In addition, some researches have found that isoflavones possess antifungal activity (Wyman and VanEtten 1978; Weidenborner and others 1990). Also isoflavones are reported to perform a number of important physiological functions involved in the growth and development of soybeans. For example, soybean isoflavones induce *nod* genes in *Bradyrizobium japonicum* (Cho and Harper 1991; Kape and others 1991; Smit and others 1992), and they are associated with the response of soybeans to infection by *Phytophthora megasperma* (Graham and others 1990).

Graham and others (1990) and Graham and Graham (1991), proposed that pre-existing pools of daidzin and malonyl-daidzin, and of genistin and malonyl-genistin, might contribute to resistance to infection in soybean seeds and seedling tissues. The glucosides appeared to have limited microbial toxicity (Naim and others 1974; Fett and Jones 1984) but potentially their hydrolysis could yield isoflavones with greater fungitoxicity (Naim and others 1974; Kramer and others 1984). Daidzin and malonyl-daidzin provide precursors for the biosynthesis of glyceollins which are a type of phytoalexins; phytoalexins are pathogen induced defense related compounds in plants and hence contribute to the restriction of the spread of the fungus (Morris and others 1991).

Morris and others (1991) evaluated the accumulation of isoflavones in soybean leaves and hypocotyls in response to *Phytophthora megasperma*, finding that isoflavones accumulated in much higher concentrations in leaves and hypocotyls following inoculation. In leaves, daidzin, malonyl-daidzin, glycitein –7-O-β-glucoside, genistin, malonyl-genistin and the glyceollins accumulated in the border zones surrounding necrotic lesions, but only the glyceollins were found in abundance in the lesions themselves. Also, other isoflavonoids like formononetin and medicarpin, function as defense compounds in alfalfa (*Medicago sativa*) (Dixon and others 1999).

Isoflavonoids can act as stimulatory as well as inhibitory factors in interactions of legumes with fungi. The isoflavones daidzein and genistein, released in soybean root exudates, act at nanomolecular concentrations as chemo-attractants of zoospores of *Phytophthora sojae*, and also induce their encystment and germination (Morris and Ward 1992).

According to Dixon and others (1999), another manner in which isoflavonoids may impact plant health is through their role in the establishment of symbiotic nitrogen fixation. Flavonoid and isoflavonoid compounds play critical roles as activators of *Rhizobium nod* genes, leading to the formation by the bacteria of substituted lipochitoolisaccharide signal molecules (*Nod* factors) that in turn induce root hair curling and the cortical cell divisions that characterize the early development of the nitrogen fixing legume root nodule (Dénarié and others 1996). Flavones in alfalfa and red clover root exudates potentially activate *nod* gene expression (Peters and others 1986; Redmond and others 1986) whereas the major inducers of the *nod* genes of the soybean symbiont *Bradyrhizobium* are the isoflavones daidzein and genistein (Kosslak and others 1987).

Reduced synthesis of daidzein in soybean roots at suboptimal temperatures limits *Rhizobial* colonization (Zhang and Smith 1996).

2.1.2 Harvesting, Drying and Storage

Harvesting soybean seeds after their development and maturation is a critical step in profitable soybean production. Although most soybeans are harvested at the dry mature stage, a very small portion is harvested at the immature stage in certain regions. The immature seed is used as a vegetable or an ingredient for recipes (TeKrony and others 1987; Keith and Delouche 1999).

Soybeans are considered dry mature when seed moisture reduces to less than 14% in the field. At this stage, seeds are ready for harvesting. The exact harvesting date depends on the cultivar, growing regions, planting date, and local weather conditions. In the United States, planting may start as early as May 1 or as late as July 15, and harvest may begin as early as September 15 and as late as mid-December. The most active harvest periods are the months of October and November (Liu 1997b; Keith and Delouche 1999).

The moisture level in the seeds plays an important role for the seed preservation and quality. If moisture content after harvest is more than 14%, soybeans need to be dried in order to meet the quality standard of soybean trading, and retain the maximum quality of the grain. It is important to reach the desired moisture level not allowing the growth of bacteria and fungi, and also preventing germination of seeds (Keith and Delouche 1999).

Soybeans are stored at farms, elevators, and processing plants in various types of storage structures before being channeled to the next destination, and finally to

processing. In the United States, soybeans are usually stored in steel tanks or concrete silos. Loss in quality of soybeans during storage results from the biological activity of seeds themselves, microbial activities, and attack by insects, mites and rodents. Quality loss is characterized by reduced seed viability and germination rate, coloration, reduced water absorption, compositional changes, and ultimately reduced quality of protein and oil (TeKrony and others 1987; Keith and Delouche 1999).

Storage conditions also affected isoflavone content in soybean seeds. Results indicated that soybeans stored at 84% relative humidity (RH) and 30 °C, present significant interconversion between aglycones and β-glucosides. The percentage of β-glucosides and malonylglucosides in total isoflavones decreased from 99% to 3% in 9 months. In contrast, the aglycones increased from 1% to 97% (Hou and Chang 2002). At 57% relative humidity (RH) and 20 °C, and ambient conditions, the glucoside forms increased with storage time, but malonylglucosides tend to decrease. At refrigeration conditions (4 °C), isoflavone distribution had no significant changes during storage (Hou and Chang 2002).

2.1.3 Grades, Standards and Inspection

As with any natural product, there is a great variability in soybean quality. Factors contributing to this variation include growing conditions, growing regions, cultivars, production practices, storage, and handling (Keith and Delouche 1999).

To promote fair-trading of soybean commodity and provide a medium of communication between buyers and sellers at national and international levels, each country has set up rules regarding trading procedures, grades and standards. Although

standards around the world have distinct differences, they possess many basic similarities (Hill and Shonkwiler 1989).

In the United States, the United States Department of Agriculture (USDA) establishes the standards for soybean trading. Table 2-1 shows the most current standards.

In general, soybean grades are based on the minimum test weight per bushel, maximum percent limits of damaged kernels, foreign material, splits, and colors others than yellow (proportion of green, brown or black beans), and maximum count limits of other material (Berk 1992; Liu 1997b). Soybeans are sold by grade and the price is adjusted accordingly. The purchaser may include additional quality parameters according to the end use (Berk 1992).

Table 2.1: The U.S. Grades and Grade Requirements for Soybeans

Grading Factors	Numerical Grades			
	1	2	3	4
Minimum limits of				
Test weight (lbs/Bu)	56.0	54.0	52.0	49.0
Maximum Percent limits of				
Damaged kernels				
Heat damaged	0.2	0.5	1.0	3.0
Total damaged	2.0	3.0	5.0	8.0
Foreign material	1.0	2.0	3.0	5.0
Splits	10.0	20.0	30.0	40.0
Soybean of other colors	1.0	2.0	5.0	10.0
Maximum count of				
Other materials				
Animal filth	9	9	9	9
Castor beans	1	1	1	1
Crotalaria seeds	2	2	2	2
Glass	0	0	0	0
Stones	3	3	3	3
Unknown substance	3	3	3	3
Total	10	10	10	10

Source: From Official United States Standards for Grains, Item No. 810.1604 (1995)

2.1.4 Cultivar Identification

Selecting the correct cultivar is essential for successful soybean production. Soybeans have great genotypic variations in terms of agronomic performance and characteristics, chemical composition, and physical appearance. Yield, disease and stress resistance, and maturity group are among the other variations relating to agronomic performance and characteristics (Liu 1997b;Roth and others 2003).

Compositional differences are reflected mainly in oil and protein content, fatty acid composition, and types of storage proteins. Physical appearance of the seeds includes seed size and shape, seed coat and hilum color. Although some of these differences are determined by growing environments, others are genetic and stable and therefore can be used for cultivar identification (Liu 1997b)

The shape of the soybean seed varies from almost spherical to elongated and flat. The industrial cultivars grown for oil are nearly spherical while the elongated cultivars are the ones used as a vegetable (Berk 1992; Liu 1997b). The surface of soybean seeds is often smooth, with variations from dull to shiny. Seed size is expressed as the number of seeds per unit volume or weight. Soybean seed weights vary greatly among cultivars, generally ranging in size from 7.6 to 30.3 g/100 seeds (Liu and others 1995a). Industrial soybeans weigh from 18 to 20 g/ 100 seeds, and the seeds of "vegetable" cultivars are considerably larger (Berk 1992). The seed size is also controlled by environmental conditions. In general, soybeans plants grown under environmental stresses tend to have smaller seeds than those grown under normal conditions. These stresses may come from nutrient deficiencies in the soil, water availability, diseases and pests (Liu 1997b).

In the United States commercial and industrial cultivars are yellow or yellow brown; and the presence of seeds of other colors in a lot are excluded by grading standards and considered a defect (Berk 1992; Liu 1997b).

Hilum color is also a major factor in cultivar identification, and as well as other physical appearances have become important factors in determining the type of food application for a particular soybean cultivar (Liu 1997b).

2.1.5 Food Beans and Oil Beans

There are distinct differences in how the East and the West use soybeans. In the Far East, traditionally, soybeans are made into various foods for human consumption whereas in the West, most soybeans are crushed into oil and defatted meal (Liu 1997b).

Because of this difference, in the U.S. soybean market, two major types of soybeans have emerged: oil beans and food beans; since there are two distinct types of soybean end uses and there is a strong effect of raw soybeans on yield and quality of final soy products (Liu 2001; Liu and others 1995b; Orthoefer and Liu 1995; Wilson 1995).

2.1.5.1 Oil Beans

Oil beans include most of the commonly produced soybeans in the United States (ISB 2003). These beans are crushed for the oil and meal market. Although most soybean oil is refined for human consumption, defatted soy meal is mainly used as animal feed. A small portion of the meal is processed into soy protein products, including soy flour, concentrates, isolates, and textured soy protein. These products serve as functional and

nutritional ingredients in various types of food, including baked goods, dairy and meat products, infant formula, and meat analogs (meat alternatives) (ISB 2003).

In general, most oil beans have medium seed size, ranging between 3000 and 4000 seeds per pound, yellow or yellow brown seed coat, with hilum color predominantly being black and imperfect black. Most oil beans have high oil content, medium-to-low protein content, and high field yield potential (Liu 2001, ISB 2003).

Key quality factors for oil beans are oil content, protein content, and the fatty acid composition. Oil and protein content give quantitative estimates of the beans as a source of oil, and of the defatted meal as a source of protein for animal feed. The fatty acid composition provides information about the nutritional, physical and chemical characteristics of the oil extracted from the beans (CGC 2002).

2.1.5.2 Food Beans

Food beans have been selected and bred over the past several decades for making into various types of soyfoods for direct human consumption. They are called specialty or identity-preserved soybeans, and usually carry a premium price in the market for a trade-off in yield or other agronomic characteristics (ISB 2003). They do not differ fundamentally from oil beans, except that they have a lighter seed coat and a clear hilum and are higher in protein and lower in oil (Liu 1997b).

Food beans are further classified into tofu beans, natto beans, sprout beans, and green vegetable soybeans. Most often, these beans are extra clean, with superior seed quality (U.S. Grade 1 or higher) (Liu 2001; ISB 2003).

Tofu beans are bred for soymilk and tofu production. In general, they are higher in protein content (40% or higher, dry matter basis) and lower in oil content. Most tofu beans have medium-to-large seed size (larger than 3600 seeds per pound or 12.6 grams per 100 seeds). However, large seeded soybeans are preferred because they are visually appealing and have less hull in proportion to the whole soybean weight (Wang and Chang 1995; Liu 2001; ISB 2003).

Because seed color affects visual appearance of soyfoods, most tofu beans have a clear hilum, light yellow to yellow seed coat, and light yellow cotyledons that result in a whiter soymilk or tofu product, which is visually more appealing to most consumers (Wang and Chang 1995). In contrast, manufacturers of natto, an ethnic Japanese food of fermented whole soybeans, prefer small to extra small soybeans for better fermentation. These beans are called, appropriately, natto beans (Liu 2001; ISB 2003). For sprout production, soybeans with medium seed size and high germination rate are preferred (Orthoefer and Liu 1995; ISB 2003). For consumption as a green vegetable, immature soybeans with large seed size, clear hilum, thin seed coat, high contents of sugar and free amino acids (to impart sweet and delicious taste), and tender texture (to have a better mouthfeel) are preferred. They are known as edamame beans (Rackis and others 1972; Orthoefer and Liu 1995; Liu 2001).

For mature soybeans consumed in the form of either cooked or roasted whole beans called soynuts, similar features would also be desirable, that is, large seed size, clear hilum, thin seed coat, and soft texture (Orthoefer and Liu 1995). Further improvements of food beans include low beany flavor, low lipoxygenase activities, high

soluble protein fraction, high ratio of 11S/7S storage protein, low levels of oligosaccharides, and white cotyledon tissues (Liu 1997b).

2.1.6 Herbicide-Resistant Soybeans

Advances in biotechnology have made it possible to transfer genes from organism to organism by means that bypass the normal sexual processes governing intraspecific inheritance. Isolated genes are moved into a crop plant in such a way that the genes are integrated into the chromosomes and expressed. A significant advantage is the fact that whole plants expressing a foreign gene can be regenerated from single transformed cells (Gianessi and Carpenter 2000). After being absorbed by plants, the herbicide glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Glyphosate binds to EPSPS resulting in EPSPS's inhibition, causing the plant to starve for EPSP and the metabolic products derived from EPSP (Padgette and others 1996).

Research to develop glyphosate tolerant plants began at Monsanto in the early 1980's. Simple selection as whole plants or cell cultures either with or without mutagenesis agents proved to be largely unsuccessful. Attempts to identify glyphosate tolerant plant EPSPS as well as extensive random mutagenesis efforts failed to identify EPSPS with adequate glyphosate tolerance (Wells 1994).

The ability to metabolize glyphosate is distributed widely among soil bacteria. Research demonstrated that EPSPS from a number of bacteria exhibited tolerance to glyphosate (Padgette and others 1996). Monsanto scientists collected bacterial cultures from diverse sources and analyzed them for their tolerance to glyphosate. One type of bacteria represented heavily in the screening was glyphosate-degrading bacteria. The

rationale being that perhaps organisms that can grow in the presence of glyphosate and degrade the herbicide may also express naturally glyphosate-tolerant EPSPS. The EPSPS with the highest tolerance to glyphosate found in the screening was CP4 EPSPS from *Agrobacterium tumifacien*, that demonstrated extremely high glyphosate tolerance (Padgette and others 1996).

The gene from CP4 EPSPS was cloned and introduced into soybeans (Padgette and others 1995). The lead soybean line with a Roundup Ready gene is denoted 40-3-2, and expresses the CP4 EPSPS gene product. Upon glyphosate treatment the transgenic plant remains unaffected because the continued action of the introduced glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic amino acids (Gianessi and Carpenter 2000). The endogenous EPSP is inhibited by glyphosate (upon glyphosate treatment); however, the plant relies on the introduced glyphosate-tolerant EPSPS for EPSP synthesis (Padgette and others 1996).

Line 40-3-2 and progeny from crosses between line 40-3-2 and other soybean cultivars were yield tested under weed-free conditions in 1992 and 1993. Data from these experiments showed that there is no yield penalty observed upon glyphosate treatment of this line with Roundup even at rates as high as twice the level to control most weeds (Delanney and others 1995). Line 40-3-2 has been used in various breeding programs to develop new cultivars with a Roundup Ready gene. (Roundup Ready is Monsanto's trademark for its genes conferring glyphosate tolerance). As a single dominant gene, the glyphosate-tolerant gene can be used very effectively in breeding programs. Over 150 seed companies offer more than 1,000 Roundup-Ready cultivars (Gianessi and Carpenter 2000).

2.1.7 Composition, Chemistry and Nutritional Value of Soybeans

2.1.7.1 Proximate Composition

The composition of soybeans may vary somewhat according to cultivar and growing conditions. On the average, oil and protein together constitute about 60% of dry soybeans. The remaining dry matter is composed of mainly carbohydrates (about 35%) and ash (about 5%) (Berk 1992; Liu 1997c).

For good storage stability and good viability as a seed, soybeans should have a moisture content of about 12% to 13%. Above this level, serious danger of mold attack exists, especially in hot weather. Below 12%, the beans tend to crack and split extensively in the course of handling. Too larger proportion of split beans is considered a defect as this may induce increased rancidity during storage (Berk 1992).

Since the water content of stored mature beans is usually about 13% on a wet basis, soybeans contain about 35% of protein, 17% of oil, 31% of carbohydrates and 4.4% of ash (Liu 1997c).

Through plant breeding it has been possible to obtain protein levels between 40% and 45%, and lipid levels between 18 and 20%. Usually, an increase of 1% in protein content is accompanied by a decrease of 0.5% in oil. Incidentally, this negative correlation between protein and oil is one of the reasons for the lack of interest in high-protein cultivars, since the production of these cultivars does not result in increased income per hectare cultivated (Berk 1992).

In general, cultivated soybeans comprise approximately 8% hull, 90% cotyledons, and 2% hypocotyl axis. Cotyledons contain the highest percentage of both protein and oil, whereas the hull has the lowest values of these components. In fact, only traces of oil are found in the hulls. The hypocotyl axis has a protein content similar to cotyledons but its lipid content is about the half that in cotyledons (Liu 1997c).

The actual composition of the whole soybean and its structural parts depend on many factors, including cultivars, growing season, geographic location, and environmental stress (Smith and Circle 1978; Liu 1997c). The proximate composition of soybeans is shown in Table 2.

Table 2.2: Proximate Composition of Soybeans and Their Structural Parts

Seed part	Percentage of whole seed weight	% (Moisture-free basis)			
		Protein Nx6.25	Lipid	Carbohydrate (includes fiber)	Ash
Cotyledon	90	43	23	43	5.0
Hull	8	9	1	86	4.3
Hypocotyl	2	41	11	43	4.4
Whole seed	100	40	20	35	4.9

Source: Cheftel and others (1985).

Liu and others (1995) reported that among the 10 selected soybean genotypes grown in Arkansas, on a dry matter basis, protein varied from 39.5% to 50.2%, oil 16.3% to 21.6%, and protein plus oil 59.7% to 67.5%.

Drought and air temperature also affect the chemical composition of soybeans. Dornbos and Mullen (1992) reported that severe drought increased protein content by 4.4 percentage points, whereas oil content decreased by 2.9 percentage points. As drought

stress increased, as measured by accumulating stress degree-days, protein content increased linearly with air temperature, while oil content decreased linearly.

2.1.7.2 Lipids

During seed development, soybeans store their lipids, mainly in the form of triglycerides, in an organelle known as oil bodies. Triglycerides are neutral lipids, each consisting of three fatty acids and one glycerol that link the three acids. The functional properties, oxidative stability, as well as the nutritional value of edible oils in general and soybean oil in particular are all determined by their fatty acid composition, geometric configuration, and positional distribution. Most of the fatty acids from soybeans are unsaturated. The highest percentage of fatty acids in soybean oil is linoleic acid, followed in decreasing order by oleic, palmitic, linolenic and stearic acid. Soybean also contains more minor fatty acids, including arachidic, behenic, palmitoleic, and myristic acid (Nawar 1996).

During processing, components extracted from soybeans by organic solvents such as hexane are classified as crude oil. Major components of crude oil are triglycerides (or triacylglycerols) (96%). Minor components include phospholipids (2%), unsaponifiables materials (1.6%), free fatty acids (0.5%), minute amounts of carotenoid pigments, and trace metals. Unsaponifiable material consists of tocopherols, phytosterols, and hydrocarbons. The concentration of these minor compounds is reduced after typical oil processing (Berk 1992; Liu 1997c).

There is a large genetic variation in fatty acid composition of soybean oil, mainly resulting from plant breeding (Hammond and Glatz 1989). Also, the fatty acid composition depends on the cultivar and growing conditions (Berk 1992).

Soybean oil is classified as a semi-drying oil in view of its high linoleic and linolenic acid content. The presence of three double bonds in the linolenic acid is responsible, in great part, for the stronger tendency of soybean oil to undergo oxidative deterioration, leading to the development of off-flavors (Berk 1992; Nawar 1996; Liu 1997c).

The phospholipids are surface-active substances located on the surface of the oil bodies. The relatively high content of phospholipids in soybean oil (two to three times higher than other common vegetable oils) is explained by the small size of the oil bodies, resulting in a larger surface per unit weight of lipids (Berk 1992).

Crude soybean oil contains 1to 3% of phospholipids. Among the total phospholipids in soybeans, phosphatidyl choline is about 35%, phosphatidyl ethanolamine is about 25%, and phosphatidyl inositol is about 15%, phosphatidic acid is 5 to10%, and the rest is a composite of all the minor phospholipid compounds (Liu 1997c).

Although the phospholipid fraction of soybeans contains a number of distinct substances, the technical term lecithin is used to name the entire fraction. Lecithin is a valuable emulsifier and has many food, medical and industrial uses. Because of their emulsifying power, the bulk of phospholipids must be removed from the crude oil before refining. This is done through a process known as degumming, because the phospholipids are separated as hydrated gums (Berk 1992; Nawar 1996; Liu 1997c).

The unsaponifiables contain mainly tocopherols and sterols. They are partially removed in the course of deodorization. The free fatty acids and pigments are removed in the process of refining and bleaching. Thus the concentration of non-triglycerides is reduced in the refined oil to less than one percent (Berk 1992; Nawar 1996).

2.1.7.3 Proteins

Based on biological function in plants, seed proteins are of two types: metabolic proteins and storage proteins. Metabolic proteins include enzymatic and structural, and are concerned in normal cellular activities, including the synthesis of the second type. Storage proteins, together with reserves of oils, are synthesized during seed development. The majority of soybean protein is storage protein (Liu 1997c).

a. Characterization

Based in solubility patterns, legume seed proteins are divided into albumins and globulins. Albumins are soluble in water, whereas globulins are soluble in a salt solution. As in all legumes, the bulk of soybean proteins are globulins (Berk 1992). Globulins in soybeans are further divided into two distinctive types: legumin and vicilin, commonly known as glycinins and conglycinins. Compared with vicilins, legumins have larger molecular size, less solubility in salt solutions, and higher thermal stability (Liu 1997c).

The solubility of soybean proteins in water is strongly affected by the pH. Close to 80 % of the protein in raw seeds or unheated meal can be extracted at neutral or alkaline pH. As the acidity is increased, solubility drops rapidly and a minimum is

observed at pH 4.2 to 4.6. This is the isoelectric region of soybean proteins taken as a whole (Berk 1992).

The pH dependence of solubility is used in the manufacture of isolated soybean protein, whereby defatted, unheated meal is extracted with water at neutral or slightly alkaline pH, and the protein is then precipitated from the filtered extract by acidification to the isoelectric region (Berk 1992).

A more precise means of identifying proteins has been based on approximate sedimentation coefficients using ultracentrifugation to separate seed proteins (Howard and others 1983). Under appropriated buffer conditions, soy protein exhibits four fractions after centrifugation. These fractions, known as 2S, 7S, 11S and 15S have been studied extensively (Berk 1992; Liu 1997c). The 11S and 7S fractions constitute about 70% of the total protein in soybeans. The ratio 11S/7S is a varietal characteristic and may vary from 0.5 to 3 (Berk 1992).

The 2S fraction consists of low molecular weight polypeptides (in the range of 8,000 to 20,000 Daltons) and comprises the Kunitz and Bowman-Birk trypsin inhibitors and cytochrome C (Berk 1992; Liu 1997c). The 7S fraction is highly heterogeneous. Its principal component is β-conglycinin, a sugar containing globulin with a molecular weight in the order of 150,000. The fraction also comprises enzymes (β-amylase and lipoxygenase) and hemagglutinins (Berk 1992; Liu 1997c). The 11S fraction consists of glycinin, the principal protein of soybeans. Glycinin has a molecular weight of 320,000 to 350,000 and is built of 12 sub-units, associated through hydrogen bonding and disulfide bonds. The ability of soy proteins to undergo association-dissociation reactions under known conditions is related to their functional properties and particularly to their

texturization. The 15S protein is probably a dimer of glycinin. Conglycinin and glycinin are storage proteins and they are found in the protein bodies within the cells of the cotyledons (Berk 1992).

b. Enzymes

Soybeans, as all seeds, contain the enzyme systems necessary for germination. The most important enzyme in soybeans is lipoxygenase (LOX), this enzyme catalyses the oxidation of poly-unsaturated fatty acids by molecular oxygen, producing conjugated unsaturated fatty acid hydroperoxides. The enzyme also has an ability to form free radicals, which can then attack other constituents (Berk 1992; Liu 1997c). In plants, they are present in various organs and the highest activities are found in legume seeds (Whitaker 1991).

LOX in soybeans are of particular interest because they have been implicated as the principal cause of undesirable flavors, commonly known as “greeny” or “beany”, associated with soybean products. Numerous articles have been published on the identification and characterization of soybean LOXs and their roles in producing flavor compounds in soybean products, among which are Axelrod and others (1981), Hildebrand and others (1988), MacLeod and Ames (1988), Robinson and others (1995), and Wilson (1996).

Soybean seeds are the richest known source of LOXs. Four LOX isozymes have been isolated and identified as L-1, L-2, L-3a and L-3b. The last two are so similar in behavior and composition that they are often considered a single type (L-3). All isozymes are monomeric proteins with a molecular weight in the range of 100,000, and contain one

atom of tightly bound nonheme iron per molecule (Berk 1992). L-1, the best characterized enzyme among the isozymes, differ from the others in being heat stable, having a pH optimum of approximately 9, and preferring anionic substrates (linoleic and linolenic acids). L-2 and L-3 are less heat stable, prefer esterified substrates, and have a pH optima close to neutrality (Berk 1992).

It has been reported that L-3 is the most abundant isoenzyme in mature soybeans on a protein basis. L-1 is almost as abundant as L-3. L-2 is less abundant but has the highest specific activity. Therefore, on the basis of enzymatic activity, similar amounts of L-2 are present in soybeans (Hildebrand and others 1988). Furthermore, weather conditions have been found to play a considerable role in influencing the activities of the LOXs isoenzymes in soybeans; the differences in activity between the generations of a cultivar were larger than those between the different cultivars from the same year (Marczy and others 1995).

The enzyme urease is frequently mentioned in connection with soybean protein products. With no technological importance to itself, this enzyme has served as an indicator for the adequacy of the heat treatment given to soybean meal. Although better tests now exist for this purpose, residual urease activity is still sometimes used as an evidence of insufficient heat treatment (Berk 1992).

c. Antinutritional Factors

Several of the soybean proteins have been found to exert specific physiological effects. These are the trypsin inhibitors and the hemagglutinins (lectins) (Berk 1992).

Protease inhibiting proteins are widespread in nature, but the trypsin inhibitors of soybeans are the best known and most thoroughly studied. Soybeans contain two types of trypsin inhibitors. Both bear the names of scientists who first isolated and characterized them. They are respectively known as the Kunitz inhibitor with a molecular weight in the range of 20,000, and the Bowman-Birk inhibitor, which is a much smaller polypeptide in the 8,000 Dalton range (Berk 1992; Liu 1997c). Both types consist of a number of differentiable proteins. The amino acid sequence and spatial structure of these proteins have been elucidated (Koide and others 1973; Odani and Ikenaka 1973).

Researchers found that raw soybeans or unheated soybean meal impaired growth when fed to young rats or chicks (Berk 1992). This effect is completely eliminated when the soybean component is properly heated. Since trypsin inhibitors are also heat labile, it was concluded that their presence in the diet is responsible for the suppression of growth. In fact, growth is retarded if the inhibitors are added to diets containing heat-treated soybean meal (Kadake and others 1973; Berk 1992).

Inhibition of trypsin is not the only physiological effect of the trypsin inhibitors. It has been observed that their ingestion can result in increased pancreatic secretion and hypertrophy of the pancreas (Chernick and others 1948). Increased secretion of enzymes into the digestive tube represents an internal loss of protein (Green and Lyman 1972). Since the proteins excreted by the pancreas are particularly rich in sulphur containing amino acids, this internal loss could be especially important if the diet is marginal in methionine/cystine (Berk 1992).

The lectins, formerly known as hemagglutinins, are proteins that possess the ability to agglutinate red blood cells. The lectin found in raw soybeans has, apparently,

no observable dietary effect, good or bad. Furthermore, it too is easily inactivated by heat (Berk 1992).

d. Functional Properties

Many of the food uses of soybean products are based on the functional properties of soybean proteins. The functional characteristics include the ability of the proteins to thicken (viscosity), emulsify, form gels, foam, produce films and sulphur, absorb water and/or fat and create meat-like texturized structures (Berk 1992).

Functional properties are related to the amino acid composition and sequence (primary structure) as well as the spatial configuration of the protein molecule and the inter-molecular forces (secondary and tertiary structures). Soybean protein products with unique functional properties are available and constitute important tools in the formulation of the so-called "fabricated foods" (Berk 1992).

e. Nutritional Quality of Soy Protein

Proteins in soybean seeds contain all the essential amino acids required for human or animal nutrition, namely isoleucine, leucine, lysine, methionine, and cysteine, phenylalanine and tyrosine, threonine, tryptophan, valine and histidine, although they are limited in two major amino acids: methionine, followed by tryptophan (Zarkadas and others 1993).

Like proteins of most leguminous plants, soy protein is low in sulfur-containing amino acids, with methionine being the most significant limiting amino acid, followed by

cystine and threonine (Eggum and Beames 1983). However, soy protein contains sufficient lysine, which is deficient in most cereal proteins. This makes it particularly valuable to be combined with cereal proteins, as they are complementary with lysine and methionine.

Zarkadas and others (1993) measured the amino acid profiles of soybean cultivars, and they obtained some general findings, like that glutamic amino acid is the most abundant amino acid. The acidic amino acids (glutamate and aspartate) constitute approximately one-fourth of the total amino acids present, compared to the basic amino acids (lysine, arginine, and histidine), which constitute one-fifth. The amino acids with hydrophobic side chains (glycine, alanine, valine, leucine, and isoleucine) account for a further 19 – 20% of the total protein compared the mean values of 9.1 – 9.8% for total aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Mean values for proline accounts for a further 5.2-5.3%.

The amino acid composition of soybean protein does not differ considerably from one cultivar to another. Attempts to develop, genetically, soybean cultivars with a higher content of sulphur containing amino acids have not been successful (Berk 1992).

2.1.7.4 Carbohydrates

Carbohydrates are the second largest component in soybeans. However the economical value of soy carbohydrates is considered much less important than soy protein and oil.

Soybeans contain about 30% carbohydrates. These can be divided into two groups: soluble sugars (sucrose 5%, stachyose 4%, raffinose 1%) and insoluble fiber

(20%)(Berk 1992). Raffinose is a trisaccharide composed of galactose, glucose and fructose linked in that order. Stachyose is a tetrasaccharide with the following structure: galactose-galactose-glucose-fructose. Raffinose and stachyose are not broken down by the enzymes of the digestive track but are fermented by the microorganisms present in the intestine, resulting in the formation of intestinal gas. Flatulence, an inconvenience associated with the ingestion of pulses in general, is a factor that must be considered, sometimes, in the use of soybean products in human nutrition (Berk 1992; Liu 1997c).

The insoluble fraction is a complex mixture of polysaccharides and their derivatives. The major part of this fraction consists of cell wall carbohydrates: cellulose, hemicelluloses and pectic substances. The insoluble carbohydrates are not digested by the enzymes of the gastro-intestinal track and can be characterized as dietary fiber. They absorb water and swell considerably. Unlike other legumes, soybeans contain very little starch (less than 1%)(Berk 1992; Liu 1997c).

2.1.7.5 Minor Components

In addition to lipids, proteins and carbohydrates, soybean also contains various minor components including minerals, vitamins, phytin, and phenolics.

The mineral content of soybeans, determined as ash, is about 5 %. Among the major mineral components in soybean, potassium is found in highest concentration, followed by phosphorus, magnesium, sulfur, calcium, chloride, and sodium. The content of these minerals range from 0.2 to 2.1% on average. The minor minerals present in soybeans and soy products include silicon, iron, zinc, manganese, copper, molybdenum, fluorine, chromium, selenium, cobalt, cadmium, lead, arsenic, mercury and iodine. The

contents of these minor minerals range from 0.01 to 140 ppm. Like other components, minerals in soybeans are also influenced by cultivar, growing location and seasons (O'Dell 1979; Perkins 1995). When soybeans are processed, most of the mineral constituents go with the meal and few stay with the oil (Berk 1992; Liu 1997c).

Soybean contains both water-soluble and oil-soluble vitamins. The water-soluble vitamins present in soybeans mainly include thiamin, riboflavin, niacin, pantothenic acid, and folic acid. The oil- soluble vitamins present in soybeans are vitamin A and E with essentially no vitamins D and K (Liu 1997c).

Phytate is the calcium-magnesium-potassium salt of inositol hexaphosphoric acid commonly known as phytic acid. Phytate has an effect on the mineral bioavailability and protein solubility when present in animal feed and human food. It is well documented that the requirement for certain metals in laboratory animals is increased when soybeans are used as a source of protein in their diet (Weaver and others 1984).

Regarding to the phenolic compounds found in soybean, during the past several years, there has been much interest among clinicians and researchers in the potential role of soyfoods in the preventing and treating chronic diseases. Increasing evidence has suggested that the isoflavones in soybeans might be the contributing factors (Akiyama and others 1987; Adlercreutz and others 1992a; Cassidy and others 1994a; Anthony and others 1996).

2.2 Isoflavones

Isoflavones belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a

pyran ring. The structure is generally simplified as C_6-C_3-C_6 (Robinson 1991; Liu 1997c). This group of compounds is known as flavonoids which include by far the largest and most diverse range of plant phenolics. Besides isoflavones, other subclasses of flavonoids include flavones, flavonols flavanols, aurones and chalcones (Liu 1997c).

As shown in Figure 2.3, isoflavones differ from flavones in that the benzyl ring B is joined at the position 3 instead of position 2 (Anderson and Garner 2000).

Isoflavones are also classified as phytoestrogens due to their weak estrogenic activity in mammalian systems (Song and others 1999).

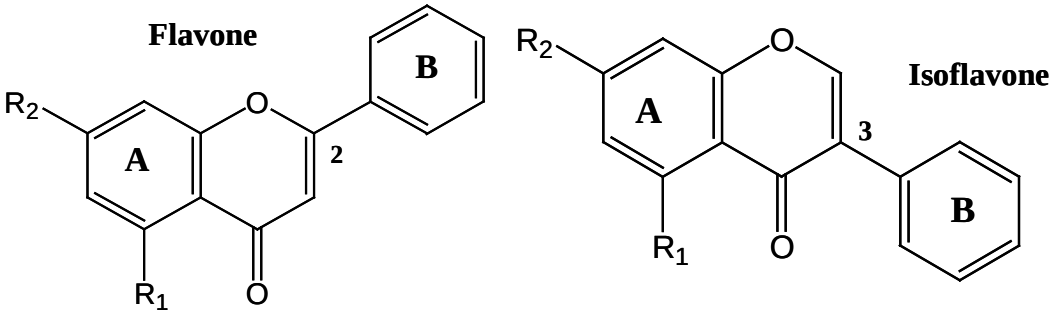
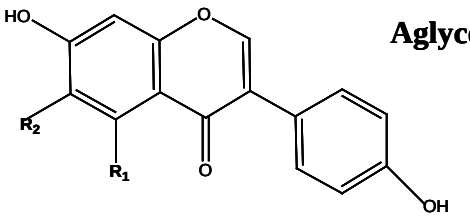


Figure 2.3: General structures of flavones and isoflavones (Anderson and Garner 2000)

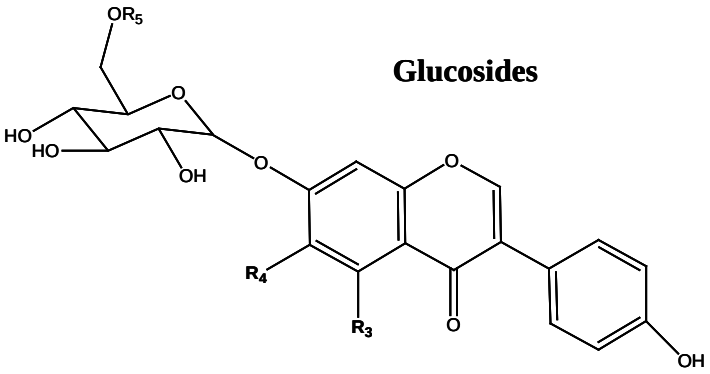
2.2.1 Isomers, Structure and Occurrences.

There are 12 isoflavone isomers (Figure 2.4): aglycones (genistein, daidzein and glycitein), β-glucoside derivatives (genistin, daidzin and glycitin), acetylglucoside derivatives (6”-O-acetylgenistin, 6”-O-acetyldaidzin and 6”-O-acetylglycitin), and malonylglucoside derivatives (6”-O-malonylgenistin, 6”-O-malonyldaidzin, 6”-O-malonylglycitin) (Setchell and Cassidy 1999).



Aglycones

Compound	R_1	R_2
Daidzein	H	H
Genistein	OH	H
Glycitein	H	OCH_3



Glucosides

Compound	R_3	R_4	R_5
Daidzin	H	H	H
Genistin	OH	H	H
Glycitin	H	OCH_3	H
6”-O-Acetyldaidzin	H	H	$COCH_3$
6”-O-Acetylgenistin	OH	H	$COCH_3$
6”-O-Acetylglycitin	H	OCH_3	$COCH_3$
6”-O-Malonyldaidzin	H	H	$COCH_2COOH$
6”-O-Malonylgenistin	OH	H	$COCH_2COOH$
6”-O-Malonylglycitin	H	OCH_3	$COCH_2COOH$

Figure 2.4: Different forms of soybean isoflavones

The isoflavones occur predominantly as β-glucoside forms in plant and foods derived from these plants. Soybeans and soy foods can contain β-glucosides, 6”-O-malonylglucosides, and 6”-O-acetylglucosides in addition to the aglycones (Hendrich and Murphy 2001).

Isoflavones have an extremely limited distribution in nature and soybean and soyfoods can be considered the only natural dietary sources of these compounds (Coward and others 1993; Messina 1997). It is not surprising therefore that some researchers found that blood and urinary isoflavone levels of Asian (Adlercreutz and others 1993) and Western vegetarians (Adlercreutz and others 1995) are 10-100 fold higher that those of individuals consuming typical Western diets.

2.2.2 Isoflavone Content in Soybean and soybean related foods

Raw soybeans typically contain 2000 to 4000 mg of isoflavones/Kg whereas the isoflavone content in soyfoods, on a dry weight basis ranges from about 1000 to 3000 mg/Kg (Messina 1997).

Eldridge and Kwolek (1983) measured the anatomical distribution of soybean isoflavones in seeds. They found that different isoflavone concentrations are found in different parts of the seed; showing hypocotyls with the highest concentration (14,000 to 17,500 mg/Kg) followed by cotyledons (1580 to 3190 mg/Kg), whereas hulls showed the lowest concentration (100 to 200 mg/Kg). Later, Tsukamoto and others (1995) calculated the percentage of these compounds in the different parts of the seed, finding that 80 to 90% of the total seed isoflavone content were located in the cotyledons, with the

remainder 10 to 20% in the hypocotyls. However, hypocotyls had a higher isoflavone concentration on a weight basis compared with cotyledons. They also found that levels of total isoflavones in whole seed ranged from 199 to 3510 mg/Kg.

Additionally, Kudou and others (1991) found that glycitin and its derivatives only occur in the hypocotyl part of the soybean seed, whereas the genistin and daidzin and their derivatives occur both in cotyledons and hypocotyls.

Other researchers studied the variability in the isoflavone content of different soybean cultivars, finding that American cultivars ranged from 1176 to 4216 mg/Kg whereas, Japanese cultivars ranged from 1261 to 2343 mg/Kg. They found that the major constituents of American cultivars were 6”-*O*-malonylgenistin, genistin, 6”-*O*-malonyldaidzin and daidzin, whereas, Japanese cultivars had higher 6”-*O*-malonylglycitin concentration levels and higher ratios of 6”-*O*-malonyldaidzin to daidzin and 6”-*O*-malonylgenistin to genistin (Wang and Murphy 1994a)

Results from Aussenac and others (1998) differ from the other researchers (Wang and Murphy 1994a; Tsukamoto and others 1995). Aussenac and others (1998) used capillary zone electrophoresis for the quantification of soybeans, and the range of total isoflavone content obtained was from 3714 to 9540 mg/Kg, being higher than the other results obtained.

Many researchers also evaluated the effect of different processing methods on the isoflavone composition of diverse soy products. In general, processing of soybeans for the manufacture of soy-containing food products increases the hydrolysis of isoflavone glucosides, resulting in higher concentrations of aglycones (Hutchins and others 1995; Zhou and Erdman 1997).

Different processing methods are applied to soybeans to end up in different final products. For example, soymilk is produced by soaking finely ground soybeans in water. Another commonly consumed soy product is tofu, which is made by curdling fresh soymilk with a coagulant. Other commonly consumed foods are miso, a paste used in soups and sauces made by aging soybeans with rice or barley for 1 to 3 years; tempeh, a cake made by fermenting soybeans with rice or millet; and natto, a topping for rice and vegetables made by fermenting cooked whole soybeans and fried tofu. The fermentation of soybeans for products, such as tempeh and natto, will partially hydrolyze isoflavone glucosides giving them proportionally higher concentrations of aglycones (Hutchins and others 1995).

Other researchers studied the mass balance of isoflavones during processing conditions. They found that manufacturing steps caused significant losses of isoflavones (Wang and Murphy 1996). For example, in tempeh production, 12 and 49% of loss was due to soaking and heat processing respectively. In tofu processing, 44% loss was due to coagulation. Soy protein isolate production resulted in 53% loss by alkaline extraction. They also found that during tempeh, soymilk, and tofu production, malonyldaidzin and malonylgenistin decreased after soaking and cooking. In contrast, acetyldaidzin and acetylgenistin were generated during heat processing. They also found that, after the fermentation process in tempeh, daidzein and genistein concentrations increased as a result of fungal enzymatic hydrolysis. In the case of protein isolate processing, alkaline extraction caused the generation of daidzein and genistein, probably through alkaline hydrolysis.

Toda and others (2000) also studied the effect of processing methods in soybeans. They found that mild heating conditions (soaking) applied to soybeans showed similar isoflavone profile than raw soybeans, having the highest proportion of malonyl forms. On the other hand, stronger heating conditions (boiling and steaming) showed lower proportion of malonyl forms and higher non-acetylated β-glucoside forms. They also discovered that for a roasting process, a dry heating method, the proportion of acetyl forms increased.

2.2.3 Environmental Factors that Affect the Soybean Isoflavones Development

Environmental conditions could modify the expression of characteristics like days to flower, plant height, leaf area, oil and protein content, seed quality, and many others. (Whigham and Minor 1978). Any major variation in the environment may result in a stress on the plant. Shading, extremely high temperatures, water availability, inadequate nutrients, high density, wind and damage by pests are types of stress (Whigham and Minor 1978).

The importance of the changes in the plant characteristics by the environmental factors have leaded researchers to evaluate how these changes affect the metabolism and biochemistry of the plant. As a result, different researchers have evaluated changes in isoflavones due to different environmental factors.

Eldridge and Kwolek (1983) conducted a study on the effects of cultivar, location and crop year on the amount of isoflavones. It was found that the isoflavone content varied from cultivar to cultivar, from year to year, and there were also differences when

cultivars were grown at different locations; thus, involving the effect of the environment (imparted by the location and crop year) as one of the factors of change. Similar results were found by Tsukamoto and others (1995) where changes in the isoflavone content in response to different sowing dates were observed, suggesting that unknown climatic and environmental factors contribute to the isoflavone contents in soybean seeds. They studied the isoflavone content of seven Japanese soybeans grown at different locations, planting dates and under different temperatures during seed development. They found that the isoflavone content significantly decreased in seeds harvested after growth at a high temperature for all soybean cultivars tested.

In a comparable study with Japanese and American soybean seeds, Wang and Murphy (1994) obtained similar results, suggesting that climate conditions might be the attributing factor to variation in isoflavone content. Their results shown that there was a significant difference in the concentrations of isoflavones among different crop years, and, according to their results, the effect of crop year seemed to be more influential than that of location.

Aussenac and others (1998) also suggested that environment had a significant effect on isoflavone content in soybeans. They showed that total isoflavone content varied among different cultivars and with sowing dates, having the interactions between the cultivar and the sowing date significant effect in the isoflavone profile. Hoeck and others (2000) found similar results where genotype, environment, and genotype environment interactions had a significant effect on isoflavone content in soybeans from six cultivars grown at different locations in different.

In addition, due to the discovery of glyphosate tolerant soybean cultivars, researchers are also interested in understand how a genetic change could affect the different pathways in the plant metabolism, for example the phenolic and flavonoid synthesis in the plant. For example Duke and others (2003) evaluated the effect of non-phytotoxic levels of glyphosate and other herbicides on soybeans. They tested how these products affect the nutraceutical and phenolic compound biosynthesis in the glyphosate-resistant (GR) soybeans. The results obtained suggest that glyphosate had little or no effect on shikimate or isoflavones in GR soybeans.

2.2.4 Absorption and Metabolism

Isoflavones from soy are entering the food chain in processed foods and as supplements for their potential health benefit. However, their absorption varies considerably among individuals (Kelly and others 1995; Setchell 1998). This has been attributed in part to differences in the microbial population in the intestine because naturally occurring isoflavones are conjugated as glycosides that must be cleaved for absorption to take place. The differing composition of the preparations is a further issue because some supplements contain isolated isoflavones in their aglycone form, and the isoflavone content of soy products can also vary substantially (Tsunoda and others 2002).

Knowledge about their respective absorptions is therefore important. Absorption is usually measured as the excretion in urine of the ingested forms and their major metabolites. The amounts excreted in urine correlate with isoflavone intake (Nestel and others 1999; Arai and others 2000) although excreted isoflavones underestimate absorption because of the magnitude of unmeasured metabolites (Setchell 1998).

Unconjugated isoflavones (aglycones) may be absorbed quantitatively to a greater extent than glycosides because the excretion of isoflavones after eating fermented soybean, in which most of the isoflavone is present as aglycones, has been reported to exceed that after the consumption of soybean glycosides (Hutchins and others 1995). Izumi and others (2000) found higher levels of isoflavones in plasma after aglycone consumption than after glycoside ingested in pill form. By contrast, Setchell and others (2001) reported greater bioavailability from glycosides than from aglycones on the basis of plasma areas under the curve kinetics.

Coward and others (1996) noted that the isoflavones from soy protein isolate are more slowly absorbed than from soymilk, perhaps because in soymilk the isoflavones are present as β-glucosides whereas in isolate, they are present in large proportion as 6*”-O-*malonylglucosides and 6*”-O-*acetylglucosides. Isoflavone β-glucosides are readily hydrolyzed by b-glucosidase, whereas 6*”-O-*malonylglucosides and 6*”-O-*acetylglucosides are not. Thus, differences in isoflavone forms might affect the rate at which isoflavones are absorbed without necessarily affecting the total absorption.

The mix of isoflavones consumed may also influence absorption, metabolism or both because single-dose pharmacokinetics suggests that the bioavailability of genistein and daidzein differs in different mixes of the two isoflavones (Busby and others 2002).

Setchell and others (2002) suggest that isoflavone glycosides are not absorbed intact across the enterocyte of healthy adults, and their bioavailability requires initial hydrolysis of the sugar moiety by intestinal β- glucosidases for uptake to the peripheral circulation.

2.2.5 Estrogenic / Antiestrogenic Activity

Phytoestrogens are a broad group of plant-derived compounds of non-steroidal structure that can behave as estrogen mimics (Setchell 1998). A noticeable feature of the chemical structure of phytoestrogens is the presence of a phenolic ring that, with few exceptions, is a prerequisite for binding to the estrogen receptor (ER) (Leclerq and Heuson 1979). When the structures of isoflavones and estradiol (Figure 2.5) are overlaid, the can be virtually superimposed; the distance between the hydroxyl groups at each end of both molecules is virtually identical. On the basis of structure alone, it is not surprising that isoflavones bind the ER (Setchell and Cassidy 1999). For this reason, phytoestrogens can act as estrogen agonists or antagonists (Makela and others 1994; Barnes and Peterson 1995; Makela and others 1995); their actions at the cellular and molecular level are influenced by many factors, including but not limited to concentration dependency, receptor status, presence or absence of endogenous estrogens, and the type of target organ or cell (Setchell 1998).

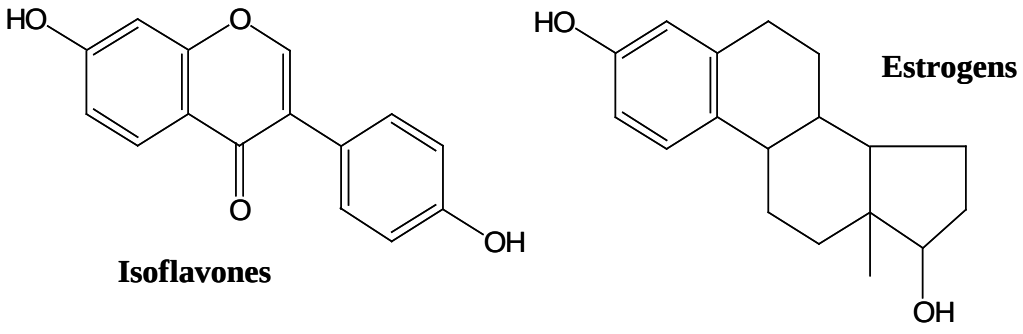


Figure 2.5: Comparison of the chemical structure of isoflavones with that of mammalian estrogen estradiol (Setchell 1998)

Kuiper and others (1996) recently identified and cloned a second and novel estrogen receptor, referred to as ERβ. This receptor has a unique anatomical distribution in tissues such as bone, brain, vascular endothelia, and bladder and it has a ligand specificity toward phytoestrogens (Kuiper and others 1997).

Dietary estrogens are weakly estrogenic (10-2- to 10-3-fold, depending on the system examined) when compared with estradiol or estrone, the principal circulating estrogens of most mammals (Tang and Adams 1980; Farmakalidis and others 1985; Markiewicz and others 1993; Molteni and others 1995). The preferential binding of non-steroidal estrogens to the ERβ receptor suggests that they may exert their actions through distinct and separate pathways from those of classical steroidal estrogens. Additionally, the lower affinity of several phytoestrogens for serum proteins would be expected to enhance the numbers of molecules available for receptor occupancy (Nagel and others 1998). More importantly, isoflavones possess numerous non-hormonal actions (Setchell and Adlercreutz 1988; Adlercreutz 1995; Setchell 1995). The finding that genistein is a potent inhibitor of protein tyrosine kinases (Akiyama and others 1987) and influences growth factors that regulate cell proliferation (Kim and others 1998) expands the biological potential for phytoestrogen action and may explain the effects of this isoflavone on signal transduction in cells lacking the estrogen receptor (Peterson and Barnes 1991, 1993; Barnes and Peterson 1995).

2.2.6 Isoflavones and Their Role in Disease Prevention and Treatment

Isoflavones found in high concentrations in soybeans have received great attention due to their potential beneficial effects in human health. The relative biological

activity of these compounds depends on their chemical structure, with genistein aglycone being the most potent. Soybeans are unique because of the presence of isoflavones, compounds very similar in chemical structure to the human estrogens, hormones that human body make and require for normal growth and development (Kurzer and Xu 1997; Setchell and Cassidy 1999). It has been reported that isoflavones play an important role in cancer prevention, inhibit tumor initiation, oxidative damage, moderation of menopausal symptoms, and other health effects (Kennedy 1995; Wei and others 1995; Setchell and Cassidy 1999).

Results from Cassidy and others (1994 b) in human studies of healthy pre-menopausal women indicate that 50 mg/day of aglycones is sufficient to have significant endocrine effects, whereas half this dose appears biologically inactive (Cassidy and others 1995). Dose and duration of intake will likely be the major factors that influence the clinical and biological outcome of a phytoestrogen-rich diet. A daily intake of textured vegetable protein (TVP) containing 45 mg of isoflavones modified characteristics of the menstrual cycle of healthy pre-menopausal women by prolonging its length, specifically the length of the follicular phase and suppressing the magnitude of the normal mid-cycle surge in follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This effect did not occur with an isoflavone-free soy protein, thus providing evidence that dietary soy protein–containing phytoestrogens have an endocrine-modulating effect and that this occurs at the level of the hypothalamic-pituitary-gonadal axis (Setchell and Cassidy 1999).

Interest in phytoestrogens as natural anticancer agents was stimulated from animal studies using the classical animal model of chemically induced breast cancer. In this

model, soy protein–containing isoflavones were found to reduce tumor formation significantly in a dose-dependent manner (Barnes and others 1990). This effect was completely abolished with soy protein that was devoid of isoflavones. These animal studies are supported by numerous in vitro studies that have shown that daidzein and genistein can inhibit cell growth (Adlercreutz 1995; Barnes 1995). The effects, however, may not be due entirely to their hormonal actions (Barnes and Peterson 1995). Soon after these animal studies were reported, genistein was shown to be a specific and quite potent inhibitor of many tyrosine kinases that are involved in the regulation of cell growth (Akiyama and others 1987). More recently, genistein has been found to augment transforming growth factor-β, an essential growth factor that inhibits the cell cycle (Kim and others 1998) and therefore progression of cell growth.

Theoretically, soy isoflavones have the potential to provide an exogenous source of estrogen, and the lower incidences of osteoporosis, breast cancer and menopausal symptoms for women in countries consuming soy as a staple have been attributed in part to the intake of isoflavones (Adlercreutz and others 1992b).

In reference to cardiovascular disease, animal studies show that substituting soy protein for dietary animal protein reduces serum total and LDL cholesterol concentrations (Sirtori and others 1993). Studies in rhesus monkeys show that soy has favorable effects on plasma lipid and lipoprotein concentrations (Anthony and others 1996, 1997). Similar responses are seen with acute loss of estrogen as in the ovariectomized rat model (Arjmandi and others 1997).

Isoflavones also are implicated in the prevention of osteoporosis, the bone-conserving effects of soy protein containing isoflavones were also confirmed in separate

studies using the ovariectomized adult rat. Bone density was shown to be greater in ovariectomized animals fed a soybean protein compared with animals fed a casein-based diet (Arjmandi and others 1996).

2.2.7 Role of Isoflavones in Food Off-Flavors

Although soybeans are an important food source, undesirable flavors and objectionable bitter and astringent tastes can be associated with soy products. These undesirable characteristics are considered to be due to isoflavones and other compounds (Tsukamoto and others 1995).

Matsuura and others (1989) studied the effect of soaking soybeans on the development of objectionable flavor or taste in soymilk. The found that during soaking, daidzin and genistin (glucoside isomers) decreased while daidzein and genistein (aglycones) increased. Later, Matsuura and Obata (1993) reported that during soybean processing the intensity of off-flavor in soymilk paralleled the concentration of isoflavone aglycones, such as daidzein and genistein, formed by the hydrolytic action of β-glucosidase on glucosides isoflavones (genistin and daidzin). Being the aglycones responsible for the objectionable after-taste as they increased during soaking of soybeans. Ha and others (1992) evaluated the effect of soaking treatment in the aglycone formation and discovered that soaking soybeans in mildly alkaline $NaHCO_3$ solutions at elevated temperatures, improved the flavor of soymilk during manufacturing, by inhibiting the formation of isoflavone aglycones.

Other cause of the off-flavors in soybean products are attributed to peroxidation of polyunsaturated fatty acids or esters catalyzed by an enzyme known as lipoxygenase

(LOX) producing ketones, aldehydes and alcohols, and many of them impart undesirable flavor (Nelson and others 1971, 1976). The genetic elimination of LOX from seeds has been attempted by several groups of investigators and has shown some promise in the off-flavors suppression. Mutants lacking of one or two of the individual LOX isozymes (Hildebrand and Hymowitz 1981; Kitamura and others 1983; Kitamura 1984) and even all three (Hajika and others 1991) have been reported. It has been shown that some of these mutants have reduced off-flavors in their seed products (Moreira and others 1993; Nishiba and others 1995). Kobayashi and others (1995) analyzed the volatile compounds extracted by simultaneous distillation and extracts from soybean homogenate (soymilk) of the normal and the LOX-lacking soybean cultivars by gas chromatography (GC) and GC-MS (mass spectrophotometry). Their results indicated that almost all the peaks of the volatile compounds obtained from soymilk of mutants lacking L-2 and L-3 or lacking all three LOXs were markedly lower than those of a normal cultivar. These data suggest that the concentration and composition of the flavor compounds of the LOX-lacking soybeans are quite different from those of the normal soybeans.

Wilson (1996) summarized studies on the comparison of LOX-null and LOX-containing soybean for such soyfoods as soymilk and tofu and concluded that that the LOX-null lines have the functional properties of soybeans but with less beany flavor.

2.3 Analytical Techniques Used in the Determination of Isoflavones

Several methods have been used for the isoflavone determination of soybeans, being High Performance Liquid Chromatography (HPLC) the most used method of

separation, and the selection of the extraction solvent the principal variation in the different procedures.

Extraction of isoflavones has been done in methanol, ethanol, acetone, and acetonitrile with water and/or dilute acid (Hendrich and Murphy 2001). Farmakalidis and Murphy (1984) used acetone-0.1M HCL for the extraction in the ratio 5:1, Wang and Murphy (1994a, 1994b) used acetonitrile and 0.1N HCl in the ratio 5:1, Klejdus and others (1999) used water, 3.5M of HCl and 80% ethanol in the ratio of 1:1:8 and Aussenac and others (1998) used 80% aqueous methanol.

Eldridge (1982) compared several extraction solvents, including 50%, 80%, and absolute ethanol, 50%, 80% and absolute methanol, ethyl acetate, and acetonitrile, and found that 80% methanol gave the most reproducible results and maximum extraction. Murphy (1981) also did study on extraction solvent to maximize extraction efficiency. The study found that solvents with 0.1 N HCl had increased extraction efficiency and acetonitrile with water or acid was superior to all other solvent systems examined. Because of these studies, 80% methanol and acidified 83% acetonitrile (10ml acetonitrile plus 2 ml 0.1 N HCl) have become the mostly used extraction solvents in isoflavone analysis.

Different HPLC columns have been used: a J.T.Baker 60-80 mesh, 50 x 2.5 cm silica gel column (Farmakalidis and Murphy 1984), a YMC-pack ODS-AM 303 column of 10 mm, 25 cm x 10 mm (Wang and Murphy 1994a, 1994b), a Zorbac SB C18 rapid resolution column of 3.5 mm, 75 x 4.6 mm.(Klejdus and others 1999). For the Capillary Zone Electrophoresis Analysis (CZE), Aussenac and others (1998) used a fused silica capillary column of 67 cm x 50 mm inside x 375 mm outside diameter.

For the elution of the isoflavones, Farmakalidis and Murphy (1984) used chloroform-methanol (50:50) for the elution of genistin and daidzin, and then fractionated by using semi-preparative HPLC. Wang and Murphy (1994a, 1994b) analyzed the samples in HPLC using a linear gradient of 0.1% glacial acetic acid in water, and 0.1% glacial acetic acid in Acetonitrile. Klejdus and others (1999) used a gradient of Acetonitrile with phosphate buffer, 0.5% acetic acid, and 0.1% of trifluoroacetic acid (TFA), in Reversed Phase High Performance Liquid Chromatography (RP-HPLC). In the case of Capillary Zone Electrophoresis Analysis (CZE), an electrophoretic buffer was used (Aussenac and others 1998).

From the different methods reported, we selected HPLC in our experiment for the isoflavone separation. This was because HPLC is the most common reported and recommended method for isoflavone separation. From the several solvents used for extraction, methanol (80%) was selected for the isoflavone extraction due to most of the researchers found it as a solvent that performs a maximum extraction with very reproducible results.

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant Material

Forty cultivars and genotypes were supplied by Dr. William Kenworthy, from the Department of Natural Resource Sciences and Landscape Architecture of the University of Maryland. Cultivars were compared and evaluated for isoflavone content and profile. A complete list of names and general characteristics of these cultivars is presented in Table 3.1. Cultivars evaluated belong to either the grain (glyphosate tolerant and standard), green seed, or food cultivars. The seeds included public and private brands available to Maryland farmers, and also promising new experimental lines.

Additionally, 24 of them were further evaluated to determine the effect of planting time and location on the soybean seed isoflavone content (Table 3.1). These cultivars were classified according to their resistance to glyphosate: fifteen (15) were glyphosate tolerant (Roundup Ready), and 9 belong to the standard form. In addition, cultivars fit in to three different maturity groups: III, IV and V. Some cultivars belonging to the maturity group IV were evaluated separately as IV-S due to their late maturing time. These maturity groups are the best adapted for growth in the Maryland area.

As well, the remaining cultivars (16 standard cultivars) were compared for isoflavone content according to the type of seed: grain, food or green type (Table 3.1). Grain cultivars are mainly used for oil extraction and animal feed; meanwhile, food cultivars are used for preparation of soybean-based foods like tofu, miso, and soymilk.

Table 3.1: Classification of Different Soybean Cultivars According to Maturity Group, Brand and Type

	Brand - Cultivar	Maturity Group	Company	Seed Type
1	GENERAL	III	Public	Standard - grain
2	IA-3010	III	Public	Standard - grain
3	MACON	III	Public	Standard - grain
4	LS-93-0375	IV	Public	Standard - grain
5	STRESSLAND	IV	Public	Standard - grain
6	KS-4694	IV-S	Public	Standard - grain
7	MANOKIN	IV-S	Public	Standard - grain
8	MD-97-6491	IV-S	Experimental line	Standard - grain
9	CLIFFORD	V	Public	Standard - grain
10	AGWAY-APK397RR	III	Agway Farm Seeds	Roundup Ready –grain
11	ASGROW-AG3902	III	Monsanto	Roundup Ready –grain
12	DEKALB-DKB38-51	III	Monsanto	Roundup Ready –grain
13	M-ATL-MA3955RR	III	Mid Atlantic Seeds	Roundup Ready –grain
14	NK-S39-Q4	III	Hoffman Seeds, Inc.	Roundup Ready –grain
15	S.ST.-RT3799N	III	Southern States Cooperative, Inc	Roundup Ready –grain
16	ASGROW-AG4403	IV	Monsanto	Roundup Ready –grain
17	DEKALB-DKB44-51	IV	Monsanto	Roundup Ready –grain
18	M-ATL-MA4355RR	IV	Mid Atlantic Seeds	Roundup Ready –grain
19	MD-99-0198-3	IV	Experimental line	Roundup Ready –grain
20	DPL-DP4690RR	IV-S	Delta & Pine Land Co.	Roundup Ready –grain
21	USG 7489RR	IV-S	UniSouthGenetics, Inc	Roundup Ready –grain
22	VIGORO-V472NRR	IV-S	Royster-Clark, Inc.	Roundup Ready –grain
23	MD-99-0687-3	V	Experimental line	Roundup Ready –grain
24	USG 7509nRR	V	UniSouthGenetics, Inc.	Roundup Ready –grain
1	Harosoy	II	Public	Standard –grain
2	Harosoy Magenta	II	Public	Standard –grain
3	Kunitz	III	Public	Standard –grain
4	Clark	IV	Public	Standard –grain
5	Clark Magenta	IV	Public	Standard –grain
6	Clark Non-Nod	IV	Public	Standard –grain
7	Corsica Black	IV	Public	Standard –grain
8	Corsica Breeders	IV	Public	Standard –grain
9	Corsica Brown	IV	Public	Standard –grain
10	Verde	III	Public	Standard –green seed
11	Emerald	IV	Public	Standard –green seed
12	Black Jet	000	Johnny's Selected Seeds	Standard – food type
13	Envy	0	Johnny's Selected Seeds	Standard – food type
14	Butterbean	I	Public	Standard – food type
15	Jack	II	Public	Standard – food type
16	Japan L1l2l3	N.A.	Public	Standard – food type

N.A.: not available

The cultivars that are used for direct consumption are called green seed cultivars. For the comparison of isoflavones content according to the seed type we used 9 cultivars belonging to the grain type, 2 to the green type and 5 to the food type.

Within the 9-grain cultivars, we compared the parent cultivars Clark, Corsica and Harosoy, with variants of these cultivars that presented a single gene difference with the parent cultivar. The characteristics of these variants were: Clark non-nod, with a gene that prevents nodulation with most strains of *Rhizobium* under most environmental conditions; and Clark Magenta and Harosoy Magenta, that have reduced flavonol glycoside content in the leaves and lower photosynthetic rate. Also, the Kunitz cultivar was evaluated. This cultivar lacks of the Kunitz trypsin inhibitor, an antinutritional component in the seed. Japan L1L2L3, a food cultivar that lacks of the three of the lipoxygenase isozymes (L1, L2 and L3) was also evaluated, and is considered as lipoxygenase free.

3.2 Locations and Planting Dates

Soybeans were grown in two different locations of the Maryland Agricultural Experiment Station: Lower Eastern Shore Research and Education Center, Poplar Hill Facility (Quantico, MD) and Wye Research and Education Center (Queenstown, MD).

Two different planting dates were evaluated for both locations: single crop system (full season), planted in late May to early June and double crop system (after barley), planted in late June / early July.

3.3 Materials and Reagents

HPLC grade acetonitrile, glacial acetic acid, and methanol, as well as the internal standard flavone, were purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide A.C.S (KOH) and hydrochloric acid A.C.S. (HCl), were purchased from Fisher Chemicals (Fair Lawn, NJ). Isoflavone standards (genistein, daidzein, glycitein, genistin, daidzin and acetylgenistin) were obtained from LC Laboratories (Woburn, MA).

3.4 Equipment

Isoflavones were separated and analyzed using a high-pressure liquid chromatograph (HPLC Waters Delta 600 system) equipped with a photodiode array detector (Waters 996), autosampler (Waters 717plus) and Millennium 32 software (Waters Corp.). We also used a rotary evaporator (Buchi REIII Rotovapor, Switzerland) with chilling system (Lauda WKL 330, Lauda, Germany).

3.5 Methods

3.5.1 Extraction of Isoflavones

Isoflavone extraction was based on the procedures described by Barnes and others (1994). For each of the soybean cultivars, 2 g of soybean was ground in a Braun KSM2 coffee grinder (Germany) and extracted with 10 mL of 80% methanol. Flavone (50 μL of a solution containing1g flavone / 100 mL 80% methanol) was added in each flask before

the extraction as internal standard. Samples were stirred for 2 hr, filtered through Whatman #41 filter paper (Whatman International Ltd., England), and then concentrated in a rotary evaporator at 40°C.

Each concentrate was taken to 5 mL with 16% acetonitrile in a volumetric flask. The extracts were filtered through polypropylene Whatman 0.45 μm syringe filter (Whatman International Ltd., England), put into vials and analyzed by HPLC.

3.5.2 Alkaline Hydrolysis

Isoflavones were hydrolyzed with alkali by using the method described by Giusti and Wrolstad (1996). Five milliliters of soybean isoflavone extract was dissolved in 10 mL of 10% (w/v) KOH and allowed to react at room temperature for 10 minutes in the dark. After neutralization with HCl, samples were applied into an activated Sep-Pak® C_{18} cartridge (Waters Corp, Milford, MA). The cartridge was rinsed twice with water, and isoflavones eluted with methanol. Samples were concentrated in a rotary evaporator and taken to 5 mL with 16% acetonitrile in a volumetric flask. All samples were filtered through a polypropylene Whatman 0.45 μm syringe filter (Whatman International Ltd., England), then placed in vials for HPLC analyses.

3.5.3 HPLC Analyses

Soybean isoflavones were separated on a 150 x 4.6 mm silica base Symmetry® C18 column (Waters Corp, Milford, MA), 5 um pore size, using a linear gradient of A: 0.1% acetic acid and 5% acetonitrile in water and B: 0.1% acetic acid in acetonitrile. Solvent B increased from 10% to 14% over 10 min, to 20% in 2 min, maintained at 20%

for 8 min, increased to 70% over 10 min, maintained at 70% for 3 min, returned to 10% in 1 min and maintained at 10% until the end of 35 min run time. The injection volume was 25 µl, and the flow rate was 1 ml/min. Spectral data were collected over the run and isoflavone elution was monitored at 254nm.

Cultivars were analyzed for isoflavone content and profile. Retention times and spectra were compared with those of pure standards. Calibration curves were prepared by using different concentrations of authentic standards of aglycones, glucosides and acetylated isoflavones and used for isoflavone quantification in the samples. Experiments were done by duplicate.

3.5.4 Stability of Isoflavones Under Different Storage Conditions

3.5.4.1 Extraction of Isoflavones

Manokin, Macon and Corsica cultivars were used for this experiment. For each cultivar, 20 g of ground soybean were extracted with 100 ml of 80% methanol. Flavone (500 µL of 1g / 100 mL 80% methanol) was added in each flask before the extraction as an internal standard. The samples were stirred for 2 hours, filtered through Whatman #41 filter paper (Whatman International Ltd., England), then evaporated or concentrated in a rotary evaporator at 40°C.

Each concentrate was taken to 50 mL in a volumetric flask using one of two different solvent strengths: the first one with 16% acetonitrile in water, and the other with 25% acetonitrile in water. Each solution was homogenized and divided into 8 vials,

storing 3 under refrigeration and 3 under freezing temperatures. The other 2 were taken to HPLC analysis.

Samples of Macon seeds and flour were stored at room temperature for further analysis after a year, to compare the stability of the extracts with the stability of the isoflavones in the flour and intact tissues. Extracts were analyzed for isoflavone content and profile using the HPLC analysis described in step 3.5.2. Experiments were done by duplicate.

3.5.4.2 Evaluation of Stability Under Different Storage Conditions

The extracts stored under refrigeration and freezing temperatures were evaluated at four different time points of storage: time zero, 1 week, 1 month, and 10 months.

After the desired time of storage, samples were taken to room temperature, filtered through polypropylene Whatman 0.45 μm syringe filters (Whatman International Ltd., England) and analyzed by HPLC as described in step b. Extracts were analyzed for isoflavone content and profile. Experiments were done by duplicate.

3.5.5 Statistical Analysis

The experiment was carried out as a Completely Randomized Block Design (CRBD). Cultivars, and effects of location and planting time were evaluated using Best-Linear Unbiased Predictors (BLUPS) based on a two-way analysis-of-variance (ANOVA) model. Mixed and correlation procedures were conducted. Statistical analysis was done by using SAS package (version 8.1, 1999, SAS Institute Inc., Cary, NC).

CHAPTER 4: RESULT AND DISCUSSION

4.1 HPLC Separation of Soybean Isoflavones

HPLC conditions were optimized to achieve separation of daidzein, glycitein and genistein and their 9 derivatives reported in soybeans. Figure 4.1 shows the separation of all 12 reported isoflavones from soybeans, in a sample that was spiked with pure standards, to best illustrate the peak separation. In addition, Figure 4.1 shows the presence of an additional isoflavone peak, labeled as Genistein related peak. This peak matched the spectral characteristics of genistein (Figure 4.2), but did not match the retention time of any of the 12 reported isoflavones in soybeans. This indicates that this peak was different from all the genistein derivatives reported previously (genistein, genistin, 6”-*O*-malonylgenistin and 6”-*O*-acetylgenistin) and its configuration was different.

Alkaline hydrolysis was performed on the samples to determine the presence of acylating groups. Acylated isoflavones generally have acetic and malonic acids as acylating groups. If the genistein related isoflavone is an acylated compound, an alkaline hydrolysis would remove the acylated group(s) from the moiety (Giusti and Wrolstad 1996), generating a new peak in the chromatogram, or increasing the area of an existing peak. A typical example would be the elimination of the acylations of malonyl and acetyl genistin by alkaline hydrolysis, increasing the genistin peak. If the genistein related isoflavone is not an acylated compound, the alkaline hydrolysis would not remove the group and the peak would not disappear from the chromatogram after the hydrolysis.

After alkaline hydrolysis of the soybean extract, the genistein related peak disappeared from the chromatogram, indicating its acylated characteristics. The identity of the peak was not determined due to the low concentration of this compound, however, from the results obtained, we know that this genistein related peak is an acylating derivative from genistein.

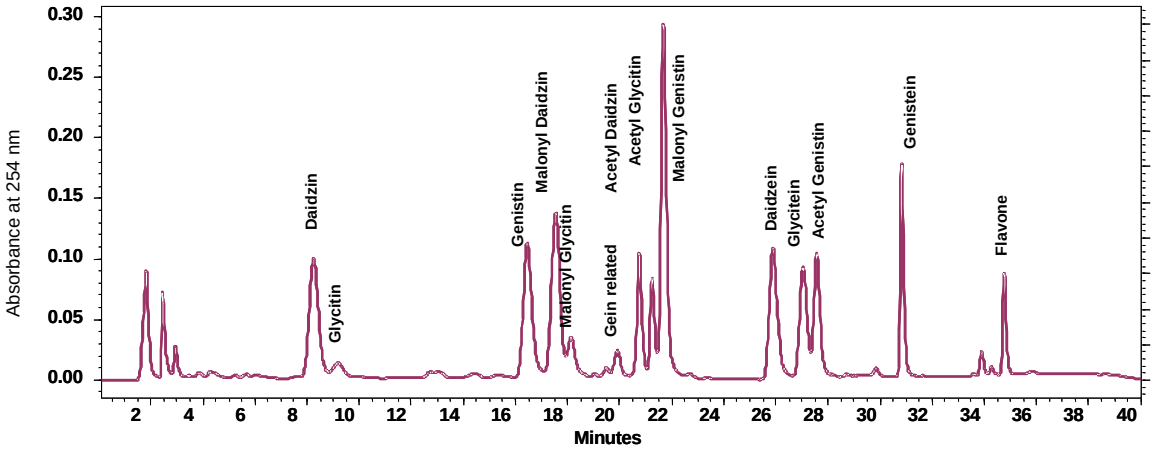


Figure 4.1: HPLC separation of isoflavones reported in soybeans.

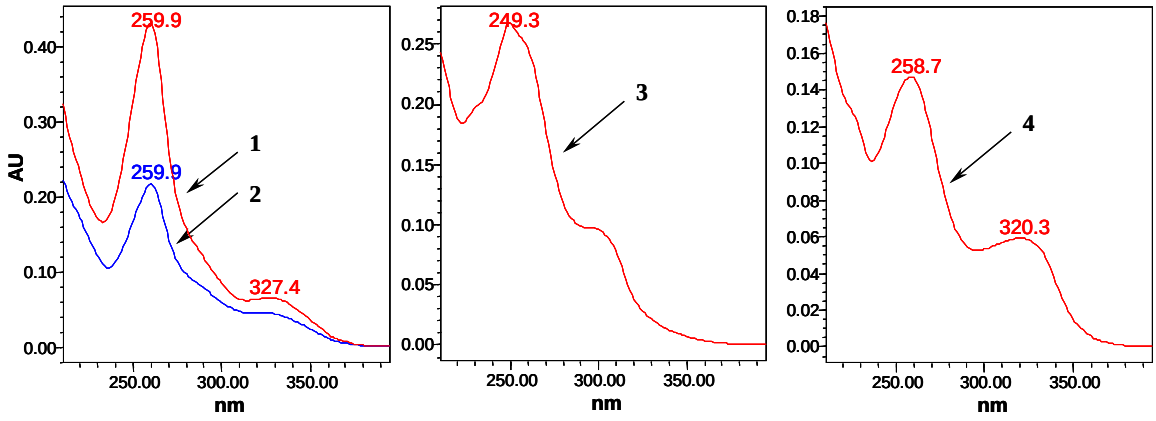


Figure 4.2: Spectra of different isoflavone isomers and genistein related isoflavone.
Legend: 1) Genistin, 2) Genistein related, 3) Daidzin, and 4) Glycitin.

Among all isoflavones, genistein derivatives were present in highest proportion (44.4% to 61.0%) in most of the cultivars followed by daidzein (27.8% to 47.1%) and glycitein (2.9% to 15.8%) derivatives. Only the early maturing food cultivars (Black Jet, Butterbean and Envy), and 1 Roundup ready experimental line (MD-99-0687-3) presented higher proportions of daidzin derivatives (50.0% to 56.4%) than genistin derivatives (27.9% to 47.4%).

Malonyl-isoflavone forms were found in higher amounts than other isoflavone isomers, with 6”-*O*-malonylgenistin and 6*”*-*O*-malonyldaidzin the most abundant. This corroborates previous reports (Liu 1997) that malonyl forms are predominant in soybeans.

4.2 Stability of Soybean Isoflavones During Storage

A study of isoflavone stability during storage was conducted. The purpose of this study was to determine the appropriate sample storage time during the study. Sample evaluation is very time consuming, and even when replications are needed it may not be feasible to analyze them at the same time. Therefore, we wanted to evaluate the changes in isoflavone content and profile during the storage of samples in the form of seed, flour and extracts.

There was a clear increase in the level of β−glucoside forms in the extracts stored at refrigeration temperatures compared to the extracts stored at freezing temperatures (Figures 4.3a and 4.3b) that increased in relative low proportions. The only glucoside that showed a clear increase during freezing was glycitin, being possibly more labile to storage conditions. In contrast, there was a clear reduction in the level of malonyl forms

in the extracts stored at refrigeration temperatures compared to the extracts stored at freezing temperatures (Figure 4.3c) that decreased in relative low proportions.

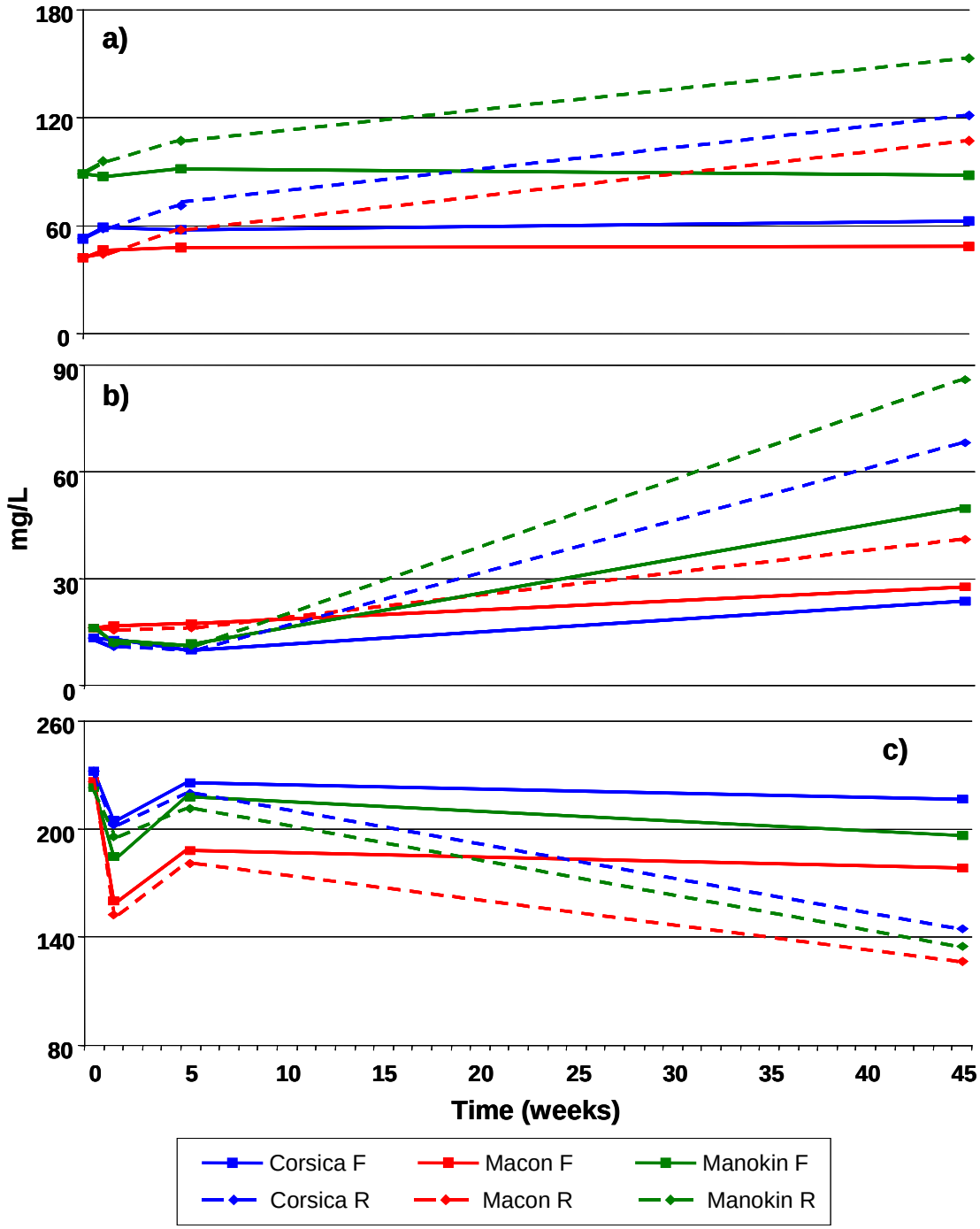


Figure 4.3: Stability of different isoflavone isomers under different storage conditions. In β−glycosides: a) daidzin, b) glycitin; and in malonyl forms: c) malonyl daidzin

According to these results, isoflavone extracts stored under refrigeration conditions followed a rapid process of degradation. This results goes in agreement with the results obtained by Wang and Murphy (1994) and Barnes and others (1994) where malonyl forms, thermally unstable, are degraded to β-glucoside forms due to the effect of the temperature.

Our results were in agreement with these reported stabilities of the different isoflavone forms (Wiseman and others 2002). Higher stability of the glucosides as compared with the malonylated forms was observed in this study (Figures 4.3a, 4.3b, 4.3c). The aglycone form is more stable than the β-glucosides, and these are more stable than the acetyl glucosides. The least stable of all are the malonyl forms (Wiseman and others 2002).

Freezing of isoflavone extracts seemed to be suitable for storage of samples with minimal compound degradation over a limited storage time, with 5 weeks being an acceptable period of storage time for the right preservation of the different forms of isoflavones (less than 10% interconversion from malonyl to glucoside forms). Meanwhile, long refrigeration periods should be avoided for the storage of isoflavone extracts since significant changes in content and profile are observed after a few weeks of storage. For our study refrigerated samples were analyzed before a week of storage, usually within 3 days. Samples were frozen to be used only if an additional replication was needed. Those frozen samples were analyzed within a couple of weeks.

4.3 Cultivar Comparison

Large differences were observed for total isoflavone content among cultivars. When comparing 40 cultivars planted at the Wye location, the isoflavone content ranged from 212 mg/Kg for Black Jet cultivar to ~2504 mg/Kg for MD-99-0687-3 cultivar (Figure 4.4, and Tables 4.1 and 4.2). Even higher isoflavone concentrations were found when cultivars were evaluated at a different location and planting time. Up to 3056 mg/Kg total isoflavones were obtained from Stressland planted at Poplar Hill in a double crop system.

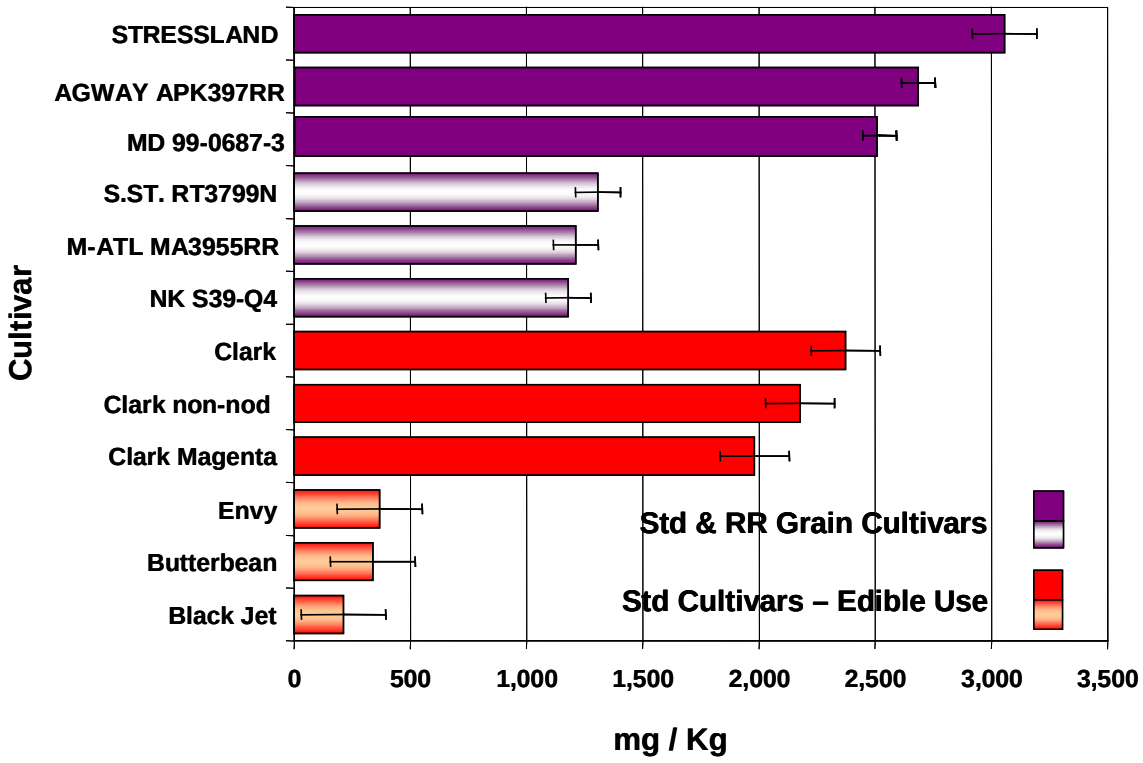


Figure 4.4: Comparison of total isoflavone content in selected cultivars with highest and lowest total isoflavone concentrations

In general, the range of total isoflavones found in our experiment (212 to 3056 mg/Kg) goes in agreement with the range (199 to 3510 mg/Kg) of total isoflavone content in whole seed of American and Japanese soybean cultivars found by Tsukamoto and others (1995).

There were remarkable differences on the total isoflavone content among the different standard cultivars, with the grain cultivars having higher concentrations of total isoflavones compared to the food cultivars. Harosoy and Harosoy Magenta grain cultivar were the exception (Table 4.1), showing concentrations comparable to those found for the food type cultivars (1159 and 1027 mg/Kg respectively).

Table 4.1: Differences of Total Isoflavone Content (mg/Kg seed) for 16 Soybean Standard Cultivars According to Type of Grain

SEED TYPE	CULTIVAR	MATURITY GROUP	TOTAL ISOFLAVONES* (mg / Kg seed)	SD
Grain	**Harosoy**	II	1,159	4.5
	Harosoy Magenta	II	1,027	8.4
	Kunitz	III	1,597	110.7
	Clark	IV	2,372	239.2
	Clark Magenta	IV	1,981	104
	Clark non-nod	IV	2,176	122.8
	Corsica Black	IV	1,497	95.7
	Corsica Breeders	IV	1,535	26.7
	Corsica Brown	IV	1,564	84.9
Green	**Verde**	III	1,449	26.5
	Emerald	IV	1,093	40.6
Food	**Black Jet**	000	212	7
	Envy	0	367	20.9
	Butterbean	I	337	53.5
	Jack	II	866	106
	Japan L1L2L3	N.A.**	1,167	238

* Values are average of two replications

** N.A.: not available

Table 4.2: Variation in the Total Isoflavone Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	TOTAL ISOFLAVONES * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	1,928	2,597	1,689	N.A.
	IA-3010		1,804	2,649	1,521	N.A.
	MACON		2,004	2,636	1,810	N.A.
	LS-93-0375	IV	2,185	2,515	1,803	N.A.
	STRESSLAND		2,349	3,056	1,946	N.A.
	KS-4694	IV-S	1,733	2,224	1,540	N.A.
	MANOKIN		2,089	1,743	1,828	N.A.
	MD-97-6491		1,709	2,012	1,440	N.A.
	CLIFFORD	V	1,955	1,803	1,854	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	2,682	2,418	1,241	2,206
	ASGROW-AG3902		2,184	2,182	1,344	1,970
	DEKALB-DKB38-51		2,590	2,516	1,319	2,294
	M-ATL-MA3955RR		2,405	2,496	1,211	2,201
	NK-S39-Q4		2,259	2,146	1,179	1,985
	S.ST.-RT3799N		1,837	2,197	1,306	2,067
	ASGROW-AG4403	IV	1,869	2,294	1,349	2,036
	DEKALB-DKB44-51		1,998	2,307	1,531	2,072
	M-ATL-MA4355RR		2,175	2,586	1,499	2,233
	MD-99-0198-3		2,477	2,322	2,062	2,380
	DPL-DP4690RR	IV-S	2,072	1,992	1,584	1,871
	USG-7489RR		2,022	2,074	1,742	1,767
	VIGORO-V742NRR		2,531	2,522	2,178	2,171
	MD-99-0687-3	V	2,745	2,164	2,369	2,640
	USG-7509nRR		2,207	2,108	2,032	2,078

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Food cultivars showed variability from 212 to 1167 mg/Kg (lipoxygenase-free cultivar Japan L1 L2 L3). It is noticeable that 3 of the 5 cultivars had very low isoflavone concentrations: Black Jet, Envy and Butterbean, with isoflavone concentrations of 367 mg/Kg or below (Table 4.1 and Figure 4.5). These were early maturing cultivars with maturity groups 000, 0 and I.

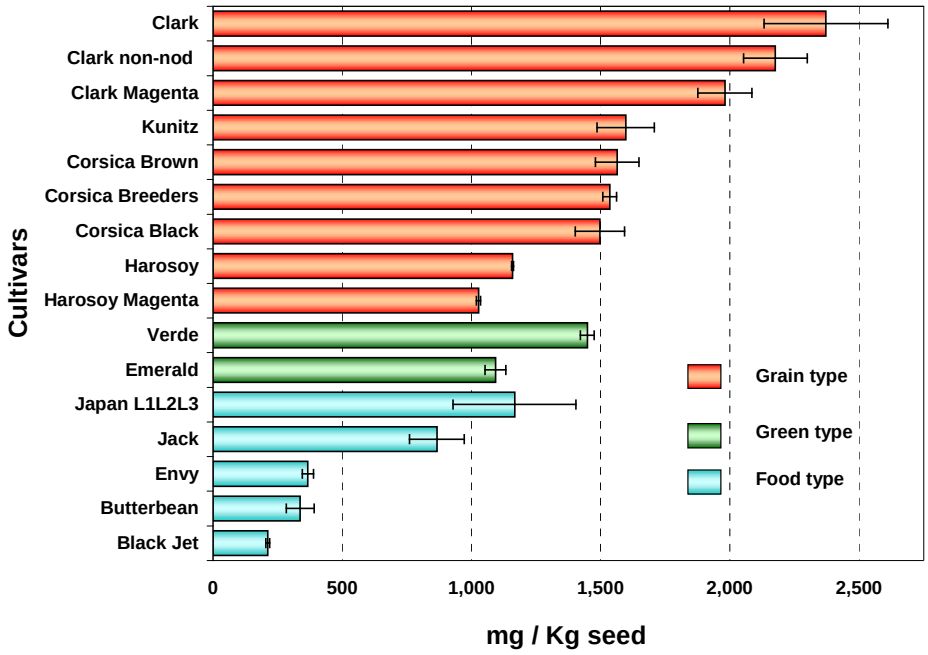


Figure 4.5: Differences in the total isoflavone content (mg/Kg seed) in 16 soybean standard cultivars according to type of grain

It is also noticeable that in the case of the variants (single gene difference) of the grain cultivars Clark (Clark non-nod and Clark Magenta), and Harosoy (Harosoy Magenta) cultivars, the level of total isoflavone content was lower compared to their respective parent cultivar.

The characteristic of the Magenta variant is that has reduced flavonol glycoside content in the leaves and also lower photosynthetic rate (Buzzell and others 1977). Then, it is possible that, as well as there is less production of flavonol glycosides in the leaves, could be also less isoflavone synthesis in the seeds.

According to different studies, undesirable flavors in soybeans and soy products can be related to the presence of soy isoflavones and other compounds (Huang and others 1981, Matsuura and others 1989, Kudou and others 1991, and Okubo and others 1992), hence, isoflavones may influence the consumers' acceptation of soybean and soybean products. The low isoflavone content found in the food cultivars studied, make them more appropriate for direct consumption and also for the preparation of soybean-based foods, like soymilk, tofu, etc.; consequently, obtaining products with more attractive flavor than products prepared with cultivars rich in isoflavone content.

A comparison of individual isoflavones was also performed for standard (edible) cultivars, and the data is presented in Appendixes 10 to 12.

Standard and RR grain cultivars showed variability ranging from 1179 (i.e. NK-S39-Q4, M-ATL-MA3955RR, S.ST.-RT3799N) to 3056 mg/Kg (i.e. Stressland, MD-99-0687-3, AGWAY-APK397RR) of total isoflavones (Table 4.2 and Figure 4.4).

It is noticeable that cultivars AGWAY-APK397RR, IA-3010, DEKALB-DKB38-51, M-ATL-MA4355RR and M-ATL-MA3955RR showed a wide variability on their isoflavone content. All these grain cultivars were found as having high and low isoflavone content, depending on the specific growing location and planting time. This is maybe due to the different environmental condition during the growth of the plants. The

potential effects of environmental factors like environment temperature and precipitation are discussed later.

Also, individual isoflavones were compared for all grain cultivars, and the data is presented in Appendixes 1 to 9.

4.4 Evaluation of Location and Planting Time on the Isoflavone Content for Grain Cultivars

Two locations in the eastern shore of Maryland belonging to the Maryland Cooperative Extension Project were selected for this study, Poplar Hill Facility (Quantico) and Wye Farm (Queenstown); both of them located very close to the Chesapeake Bay. The Wye Farm location has a latitude/longitude of 38°55'N / 76°08'W and is geographically positioned to the northwest of the Poplar Hill location (38°20'N / 75°31'W).

Despite their relative closeness, both locations had different climatic characteristics of temperature and level of precipitation. Another important factor to mention is that Wye Farm location is adjacent to the Wye River; however, Poplar Hill location is more surrounded by land.

Two different planting times were chosen for both locations: an early planting for growth during the full season period (FS), and a late planting to allow for two different harvest during one whole season, being soybeans the second crop (double crop, or also know as after barley). The different locations and planting time combinations allowed different environmental conditions for growing of the plants, and hence, different

conditions for the isoflavone formation. No data were available for standard soybeans planted at Wye farm in a double-crop system.

4.4.1 Effect of Location

The effect of location for the 24 different cultivars evaluated in this study is illustrated in Table 4.2. The location where soybeans were grown affected the total isoflavone content, with soybeans grown at the Poplar Hill location exhibiting higher concentrations of isoflavones than the same cultivars planted at the Wye location.

The BLUPS analysis showed that the level of correlation was high among standard (non Roundup Ready tolerant) cultivars at the different locations (ρ=0.80, p=0.01), being 63.8% of the observations correlated in the locations. This indicates that the differences observed in the isoflavone content may be explained by location, and not due to randomness.

4.4.1.1 Environmental Factors in Planting Location

According to Tang and others (1995), plant stress can be broadly defined as any external condition unfavorable to its growth and survival. In nature, plant stress may be caused by many biotic and abiotic factors, including water, temperature, radiation, mechanical force, pollutants, pesticides, ozone, heavy metals allelochemicals, herbivores and pathogens.

Stress factors like drought and temperature affect the chemical composition of soybeans (Dornbos and Mullen 1992). They reported that severe drought increased the

protein content, whereas the oil content decreased. As drought stress increased, as measured by accumulating stress degree-days, protein content increased linearly and oil content decreased linearly at each air temperature.

In addition, different climate conditions might be the attributing factor to variation in isoflavone content (Wang and Murphy 1994). It is possible that different temperatures during the plant growth could induce stress in the plant, causing the increase of phenolic compounds like isoflavones (Tang and others 1995).

The results illustrated in Table 4.2 showed that for all the cultivars, the isoflavone content found at Poplar Hill location (PH) was higher in comparison to the one found at Wye Farm location. Environmental differences between the locations may have contributed to the effect of stress on the isoflavone formation.

Figure 4.6 shows that the Poplar Hill facility was subject to more extreme temperatures (maximum and minimum) than Wye Farm, reaching lower temperatures than Wye Farm during the seed filling stage. It is important to indicate that the pod development and seed filling stage occurred during the fall season where the air temperature is starting to drop. According to this analysis, it seems that the isoflavone formation was triggered in a high proportion at Poplar Hill location, being induced by the stress caused by low temperatures (Tsukamoto and others 1995).

Another form of stress, and also the most common stress encountered by plants is probably water stress. In general, water deficit increases the concentrations of secondary metabolites. Among them, phenolics and terpenes were most commonly studied (Tang and others 1995).

According to NCDC (2002), the year 2001 was a year of low precipitation, where the fluctuation of Maryland's precipitation during the seed filling stage was from below normal during September to much below normal for October and November.

The Palmer Hydrological Drought Index for the same period varied from mid-range in September to moderate drought in October and November (NCDC 2002). As a result, the lack of precipitation could have induced stress in the plants, affecting the metabolism, and the rate of formation of isoflavones in the seeds.

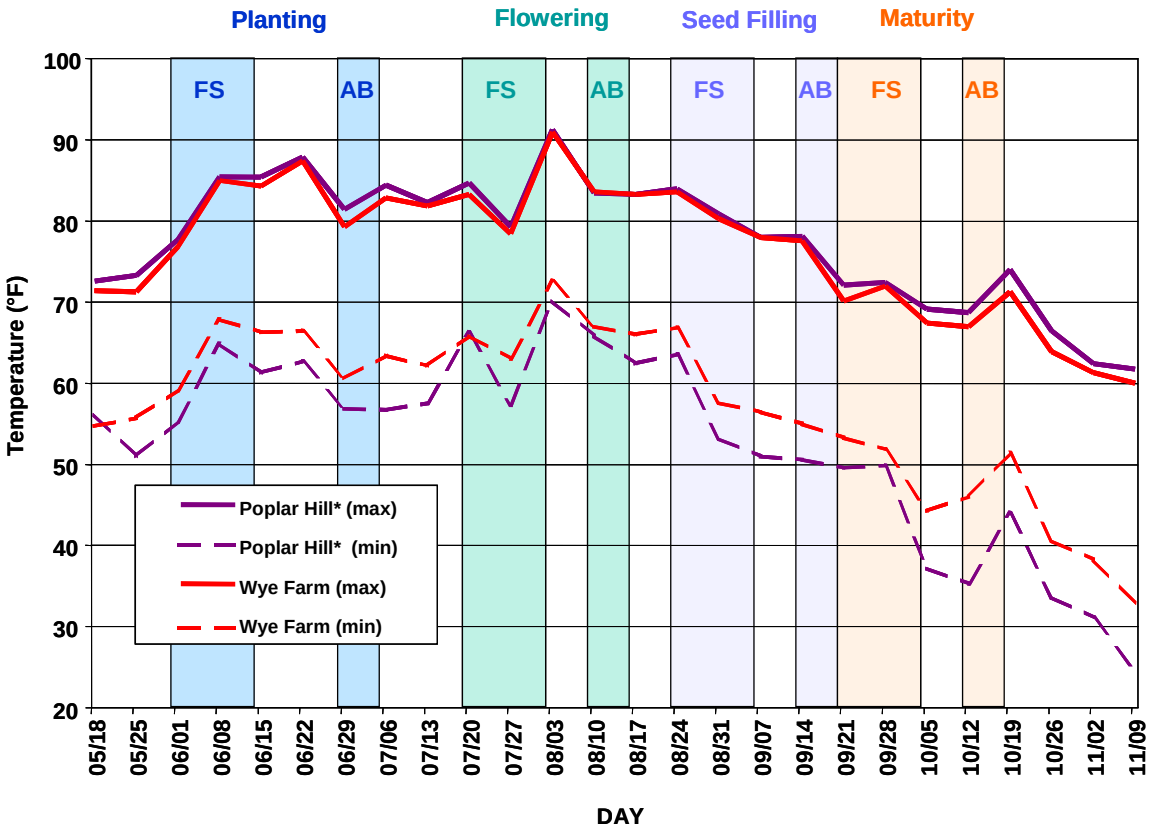


Figure 4.6: Weekly temperature (°F) during growing season at different test locations. (* Taken at Salisbury airport). Source: NCDC (2002)

Shaded areas correspond to the beginning of the respective period

According to Figure 4.7, Wye Farm facility received more precipitation than Poplar Hill. These results indicate that possibly Poplar Hill was subject of water stress in a high level compared to Wye farm, being the formation of isoflavones more feasible at this location due to the higher water stress. Our results showed that more isoflavones were found in cultivars grown at Poplar Hill, the location subject to a higher water stress.

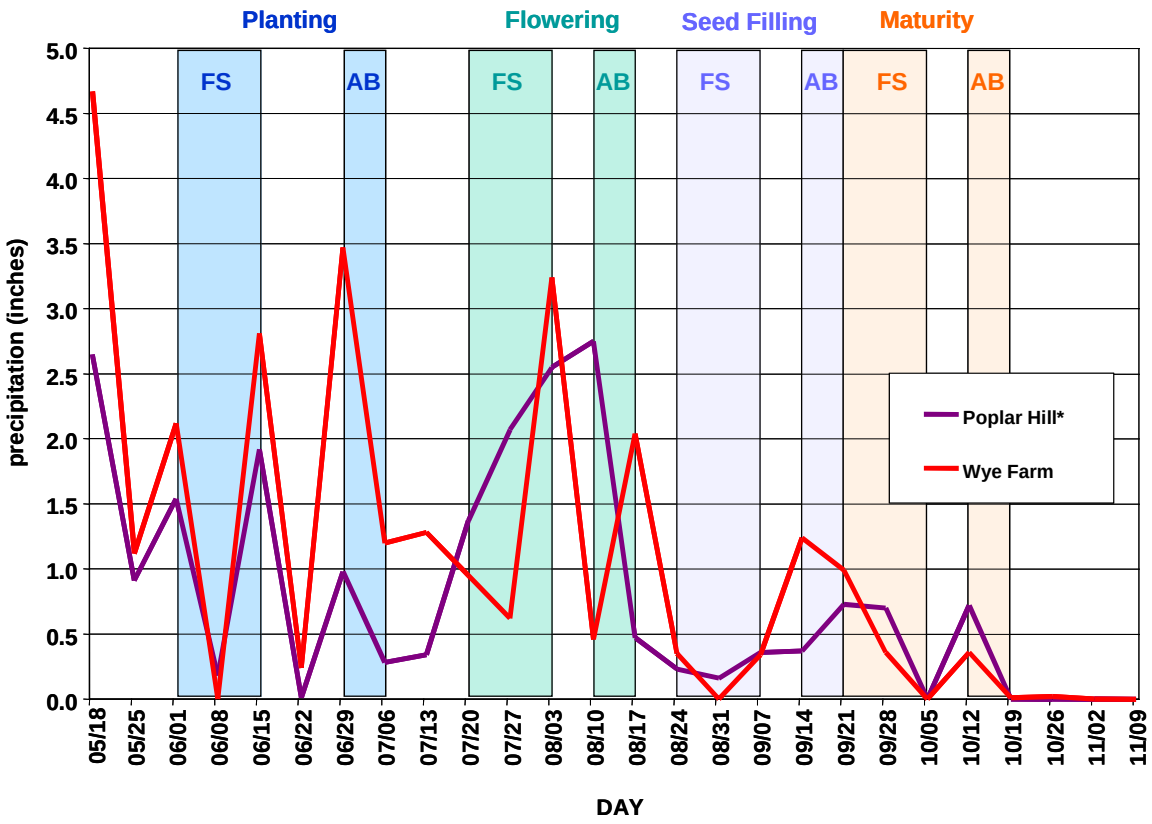


Figure 4.7: Weekly precipitation (inches) during growing season at different test locations. (* Taken at Salisbury Airport). Source: NCDC (2002)

Shaded areas correspond to the beginning of the respective period

4.4.2 Effect of Planting Time

The effect of planting time is presented in Table 4.2. In the case of standard grain cultivars (non Roundup Ready), most of the cultivars planted at Poplar Hill location (PH) during the double crop period (AB), showed more isoflavone content than cultivars planted during the full season (FS). The only exceptions were Manokin and Clifford cultivars. This behavior maybe explained with the observations from the report of Kenworthy and Wood (2002), that a frost affected some standard cultivars grown at Poplar Hill location in the double crop system (AB) before they matured, and did not complete the formation of isoflavones as compared to cultivars that matured normally. The cultivars affected were Manokin (IV-S) and Clifford (V), the same cultivars that showed the opposite behavior.

There was a high correlation level among standard grain cultivars at the different planting times ($r=0.80$, $p=0.01$), being 63.8% of the observations correlated in the different treatments. This indicates that the differences observed in the isoflavone content could be explained by planting time, and not due to randomness.

Meanwhile, the correlation level among Roundup Ready tolerant cultivars (RR) at the different locations varied according to the planting time. Table 4.2 illustrates the behavior of Roundup Ready tolerant (RR) grain cultivars planted at Wye location in a double crop system (AB). They showed a noticeable increase in isoflavone content compared to the ones planted during the full season (FS). The results obtained showed similarity to the ones obtained by Kitamura and others (1991), who studied soybeans grown under two different culture conditions (standard and late culture conditions). They found that soybeans growth under standard culture conditions (early planting) produced

seeds with a stable low concentration of isoflavones compared to the late culture conditions.

In the case of Roundup Ready cultivars planted during the double crop system (AB), a medium correlation was observed among the cultivars (ρ=0.55, p=0.03), being 30.2% of the observations correlated.

Different results were observed during the full season (FS), where Roundup Ready cultivars showed a very low correlation (ρ=0.26, p=0.36), indicating that the differences in the isoflavone content among the cultivars could be due to random effect.

The same type of grain cultivars (Roundup Ready tolerant) did not exhibit a clear effect of planting time when seeded at the Poplar Hill location. This behavior maybe explained with the observations reported by Kenworthy and Wood (2002). According to their report, a frost affected some Roundup Ready cultivars grown at Poplar Hill and Wye Farm during the double crop system (AB), causing that the cultivars did not reach the maturity. The cultivars affected at Poplar Hill were the ones with maturity group IV-S (USG-7489RR, DPL-DP4690RR) and the ones with maturity group V (USG-7509nRR, MD-99-0687-3). The cultivars affected at Wye Farm were the ones with maturity group V (USG-7509nRR, MD-99-0687-3).

According to Kudou and others (1991), the accumulation of isoflavones occurs during seed filling. Since the frost affected the maturity of the seeds, and they never finished the seed filling, it is likely that the isoflavone formation was truncated. With this observation and with our results, we can infer that due to the frost and not reaching the maturity level, these cultivars were not able to complete the seed filling and hence, not completing the formation of isoflavones as in the seeds that reached the complete

maturity. Our results showed that the cultivars affected by the frost at Poplar Hill (in a double crop system) showed less isoflavone formation that the ones growth during the full season at the same location. Most of the other cultivars grown during the double crop system showed higher isoflavone content that the ones grown during the full season at the same location.

After excluding the observations of samples affected by the frost, another BLUPS analysis was conducted. The results obtained by this analysis showed an increase in the correlation level for all the treatments. The standard cultivars at the different locations showed an increase in the correlation level (ρ=0.87, p=0.0026), being 75.7% of the observations correlated in the locations. Among Roundup Ready tolerant cultivars (RR) at the different locations, the correlation level varied according to the planting time. During the double crop system (AB), there was now a high correlation (ρ=0.87, p=0.0005), being 75.7% of the observations correlated. During the full season (FS) the correlation also increased, but there was still low (ρ=0.26, p=0.198). This indicates that the differences observed in the isoflavone content could not be explained by planting time or other effect, but due to randomness.

4.4.2.1 Environmental Factors in Planting Time

According to our results, cultivars grown under a double crop system (AB) presented higher levels of isoflavones that the ones grown during the full season (FS).

Another important fact is that cultivars planted in a double crop system (AB) are subject to lower temperatures during isoflavone formation (seed filling stage), than the

cultivars planted during the full season (FS), therefore, it seems that the isoflavone formation was induced due to a stress caused by low temperatures.

Our results are in agreement with the results of Tsukamoto and others (1995). Their results suggested that temperature during the seed filling stage was related as a major factor in determining the levels of isoflavones in seeds, and, according to their results, the isoflavone contents of the soybeans harvested in an earlier harvest period were significantly lower than those of the group harvested in a later period. Soybeans grown under late culture conditions (double crop) causes the plants to be exposed to lower temperatures during seed fill. The low temperatures and mid to moderate drought conditions that our seeds were exposed to during the seed filling could explain the fact that the cultivars planted in a double crop system (AB) produced more isoflavones that the cultivars grown during the full season.

4.5 Isoflavone Production from Soybean

The seed yield of the soybean cultivars was influenced by different factors like the growing location, the planting time and also by the genotype effect.

With the purpose of selecting seeds with high isoflavone content we evaluated first the isoflavone yield per seed weight (mg isoflavones / Kg seed), finding that Poplar Hill location under a double crop system showed the higher values of isoflavone content compared to isoflavone concentrations found in other locations. Standard cultivars that showed the highest values (~ 2,600 mg/Kg seed or higher) were Stressland, Macon, IA-3010 and General cultivars, planted at these conditions (Table 4.2). Roundup Ready

cultivars with the highest isoflavone content (~2500 mg/Kg seed or up) were M-ATL-MA-355RR, Vigoro-V742NRR, Dekalb-DKB38-51.

However, stress conditions that may favor isoflavone synthesis, may as well decrease the overall seed production. Therefore, with the purpose of investigating the yield of isoflavones in the field production (Kg isoflavones / Ha) we combined the information of isoflavone yield in the seeds (mg isoflavones / Kg seed) with the information of soybean yield (Kg soybeans / Ha) obtained from the Information bulletin of the 2001 Maryland soybean cultivar tests, published by the Maryland Cooperative Extension (Kenworthy and Woods 2002). This information is shown in Table 4.3, and could help to determine which cultivars, planting time and location offer more possibilities for high soybean isoflavones production in a given area.

Results from Table 4.3 illustrates that seed yields obtained by soybeans planted in a double crop system were lower than seed yields of soybeans planted during the full season. These results go in agreement with Wesley (1999), where soybeans yields obtained of monocrop systems (full season) were higher than soybeans yields obtained by double crop systems. From the same table is also noticeable that most of the cultivars obtained higher yields at Poplar Hill location compared to Wye Farm where yields were lower.

Other observation is that higher seed yields (Kg seeds / Ha) were obtained for the Roundup Ready cultivars, having the standard cultivars lower yields (Table 4.3).

Table 4.3: Variation in the Seed Yield (Kg/Ha) and Total Isoflavone Yield ((Kg/Ha) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

Seed Type	Brand-Cultivar	Maturity Group	SEED YIELD (Kg/Ha) * *				ISOFLAVONE YIELD (Kg / Ha)			
			PH - FS	PH - AB	Wye - FS	Wye - AB	PH - FS	PH - AB	Wye - FS	Wye-AB
STANDARD (non Roundup Ready)	GENERAL	III	3847	4021	3934	N.A.	7.41	10.44	6.64	N.A.
	IA-3010		3685	4176	3840	N.A.	6.65	11.06	5.84	N.A.
	MACON		3833	3988	3941	N.A.	7.68	10.51	7.13	N.A.
	LS-93-0375	IV	4425	3732	4008	N.A.	9.67	9.39	7.23	N.A.
	STRESSLAND		3947	3840	3927	N.A.	9.27	11.73	7.64	N.A.
	KS-4694	IV-S	4129	3726	3598	N.A.	7.16	8.29	5.54	N.A.
	MANOKIN		3288	2831	3174	N.A.	6.87	4.93*	5.8	N.A.
	MD-97-6491		3961	3403	4075	N.A.	6.77	6.85	5.87	N.A.
	CLIFFORD	V	3934	3006	3396	N.A.	7.69	5.42*	6.3	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	5010	3833	3974	3564	13.44	9.27	4.93	7.86
	ASGROW-AG3902		5212	3806	3820	3759	11.38	8.3	5.14	7.4
	DEKALB-DKB38-51		4876	3712	3914	3181	12.63	9.34	5.16	7.3
	M-ATL-MA3955RR		4775	3578	3941	3712	11.48	8.93	4.77	8.17
	NK-S39-Q4		5266	3645	4371	3786	11.9	7.82	5.15	7.52
	S.ST.-RT3799N		4855	3968	4069	3524	8.92	8.72	5.31	7.28
	ASGROW-AG4403	IV	4869	3699	4418	3887	9.1	8.49	5.96	7.92
	DEKALB-DKB44-51		4990	3726	4297	3840	9.97	8.6	6.58	7.96
	M-ATL-MA4355RR		5138	3726	4358	3813	11.17	9.63	6.53	8.52
	MD-99-0198-3		4997	3880	4284	3544	12.37	9.01	8.83	8.43
	DPL-DP4690RR	IV-S	5252	3410	4707	3591	10.88	6.79*	7.46	6.72
	USG-7489RR		5286	3631	4694	3591	10.69	7.53*	8.18	6.35
	VIGORO-V742NRR		5064	3490	4317	3477	12.82	8.8	9.4	7.55
	MD-99-0687-3	V	4714	3336	3537	3087	12.94	7.22*	8.38	8.15*
	USG-7509nRR		4788	3315	4506	3060	10.57	6.99*	9.16	6.36*

N.A.: not available. Tests not performed at that location. * Values affected by frost * * Source: Kenworthy and Wood (2002)

By combining isoflavone production per weight of seed with the information on seed yields we found that (Table 4.3) for the Roundup Ready cultivars, the highest isoflavone yields (Kg isoflavone / Ha) were obtained at the Poplar Hill location during the full season period; meanwhile, for the standard cultivars the highest yields were obtained at the same location but during the double crop system.

In general, Roundup Ready cultivars showed higher isoflavone yields (Kg / Ha) compared to standard cultivars, maybe these results were influenced by the higher production yields (Kg seeds / Ha).

Roundup Ready cultivars that showed high yields in isoflavones (Kg / Ha) were MD 99-0198-3, Dekalb DKB38-51, Vigoro V742NRR, MD 99-0687-3 and Agway APK397RR, ranging from 12.4 to 13.4 Kg of total isoflavones per Hectare. Standard cultivars with high total isoflavone yield (Kg / Ha) were General, Macon, IA 3010 and Stressland. In these cultivars the total isoflavone yields fluctuated from 10.4 to 11.7 Kg of total isoflavones per Hectare. It is noticeable that the same standard cultivars that showed high seed isoflavone yield (mg isoflavones / Kg seed), showed the highest isoflavone yields (Kg isoflavones / Ha). These cultivars could be used to obtain high isoflavones yields (Kg / Ha), as required in industrial level, where higher volumes are demanded for nutraceutical purposes.

CHAPTER 5: CONCLUSIONS

Drastic differences among cultivars were observed for total isoflavone content (from 212 mg/Kg to 3056 mg/Kg). Standard and Roundup Ready tolerant grain cultivars showed higher total isoflavone concentrations than the food cultivars. Roundup Ready cultivars, showed higher isoflavone yields in the field (Kg / Ha) compared to standard cultivars probably due to the higher seed production yields (Kg seeds / Ha).

Planting time and location had an impact on soybean recoveries and profiles. Selection of planting time and location affected the concentration of isoflavones in the seeds. Our results suggest that for most cultivars higher isoflavone content in the seeds is obtained using double cropping practices (barley or similar followed by soybeans), caused maybe by stronger stress conditions (lower temperatures and less water availability) characteristic of this period. However, comparable total isoflavone yields per Ha can be obtained at the different planting times after accounting for the reduced seed yields obtained with the double crop system.

Stress conditions caused by low temperatures and low precipitation during seed formation may be responsible for the higher isoflavone yields obtained from seeds planted at Poplar Hill in a double crop system.

It may be possible to select for specific cultivars with higher isoflavone content and environmental conditions such as temperature and water supply that would favor the desirable isoflavone production: high isoflavone content for seeds used for nutraceutical purposes, or low isoflavone production for food product developments where bitter flavor associated with isoflavones may be undesirable.

CHAPTER 6: FUTURE RESEARCH

This study is the first step on what could be a larger study to understand the potential environmental factors that affect the isoflavone content and profile in soybeans.

It would be recommended that the comparison of isoflavone content and profile be replicated under the same experimental condition as the ones already evaluated, using the same cultivars one more time, to account for year to year variability.

A complete study on the effect of water availability and temperature during seed formation on the isoflavone synthesis should be performed. Growing chambers could be used with this purpose.

Factors that may be worth further investigation are the use of different stress conditions during the plant growth in the effect of the isoflavone content, such as ozone exposure, UV radiation, the use of herbicides, pesticides and fungicides, different soil conditions, presence of weeds, insects, diseases during growing conditions.

The identity of the genistein related peak should be elucidated. Mass spectroscopy could be used for this purpose.

APPENDICES

Appendix 1: Variation in the Malonyl Genistin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Malonyl genistin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	920	1,267	821	N.A.
	IA-3010		765	1,134	630	N.A.
	MACON		980	1,208	850	N.A.
	LS-93-0375	IV	990	1,093	826	N.A.
	STRESSLAND		1,010	1,300	846	N.A.
	KS-4694	IV-S	828	1,110	744	N.A.
	MANOKIN		927	803	808	N.A.
	MD-97-6491		786	1,037	671	N.A.
	CLIFFORD	V	971	926	914	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	1,084	1,098	557	979
	ASGROW-AG3902		961	1,028	614	952
	DEKALB-DKB38-51		996	1,059	556	987
	M-ATL-MA3955RR		997	1,086	542	969
	NK-S39-Q4		977	995	556	896
	S.ST.-RT3799N		812	972	591	919
	ASGROW-AG4403	IV	884	1,032	639	942
	DEKALB-DKB44-51		920	1,039	709	945
	M-ATL-MA4355RR		986	1,165	693	990
	MD-99-0198-3		1,006	957	852	985
	DPL-DP4690RR	IV-S	1,034	945	826	965
	USG-7489RR		1,046	981	900	914
	VIGORO-V742NRR		1,037	1,041	953	997
	MD-99-0687-3	V	1,105	861	987	1,015
	USG-7509nRR		1,037	971	974	995

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 2: Variation in the Malonyl Daidzin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Malonyl daidzin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	630	812	522	N.A.
	IA-3010		729	1,072	612	N.A.
	MACON		598	865	545	N.A.
	LS-93-0375	IV	765	946	633	N.A.
	STRESSLAND		870	1,114	703	N.A.
	KS-4694	IV-S	551	664	476	N.A.
	MANOKIN		804	654	720	N.A.
	MD-97-6491		563	595	476	N.A.
	CLIFFORD	V	603	544	591	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	1,163	922	421	830
	ASGROW-AG3902		835	781	436	678
	DEKALB-DKB38-51		1,170	1,035	511	913
	M-ATL-MA3955RR		1,020	979	445	861
	NK-S39-Q4		951	850	427	785
	S.ST.-RT3799N		666	755	415	712
	ASGROW-AG4403	IV	627	763	423	681
	DEKALB-DKB44-51		687	780	487	702
	M-ATL-MA4355RR		774	750	480	790
	MD-99-0198-3		988	893	785	945
	DPL-DP4690RR	IV-S	623	610	459	558
	USG-7489RR		573	641	517	518
	VIGORO-V742NRR		980	922	802	763
	MD-99-0687-3	V	1,203	966	979	1,258
	USG-7509nRR		752	742	671	733

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 3: Variation in the Malonyl Glycitin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Malonyl glycitin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	77	106	113	N.A.
	IA-3010		57	85	79	N.A.
	MACON		135	130	122	N.A.
	LS-93-0375	IV	127	117	119	N.A.
	STRESSLAND		124	150	147	N.A.
	KS-4694	IV-S	116	116	113	N.A.
	MANOKIN		55	59	51	N.A.
	MD-97-6491		100	62	102	N.A.
	CLIFFORD	V	97	101	98	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	64	79	73	90
	ASGROW-AG3902		68	108	85	99
	DEKALB-DKB38-51		64	76	59	94
	M-ATL-MA3955RR		54	68	55	76
	NK-S39-Q4		60	50	39	67
	S.ST.-RT3799N		68	125	87	117
	ASGROW-AG4403	IV	100	136	113	134
	DEKALB-DKB44-51		110	132	115	135
	M-ATL-MA4355RR		110	282	135	136
	MD-99-0198-3		76	112	114	115
	DPL-DP4690RR	IV-S	82	118	89	91
	USG-7489RR		81	124	101	106
	VIGORO-V742NRR		96	129	129	87
	MD-99-0687-3	V	65	79	101	59
	USG-7509nRR		89	120	128	90

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 4: Variation in the Genistin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Genistin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	150	236	105	N.A.
	IA-3010		113	183	89	N.A.
	MACON		153	242	136	N.A.
	LS-93-0375	IV	144	183	92	N.A.
	STRESSLAND		154	251	100	N.A.
	KS-4694	IV-S	127	186	93	N.A.
	MANOKIN		158	112	121	N.A.
	MD-97-6491		126	176	79	N.A.
	CLIFFORD	V	161	124	124	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	172	169	82	149
	ASGROW-AG3902		157	147	96	123
	DEKALB-DKB38-51		152	174	78	142
	M-ATL-MA3955RR		153	190	69	143
	NK-S39-Q4		128	127	68	112
	S.ST.-RT3799N		141	182	94	156
	ASGROW-AG4403	IV	125	191	72	137
	DEKALB-DKB44-51		134	189	92	136
	M-ATL-MA4355RR		147	199	80	154
	MD-99-0198-3		194	183	127	147
	DPL-DP4690RR	IV-S	185	177	103	141
	USG-7489RR		187	183	119	128
	VIGORO-V742NRR		211	233	135	178
	MD-99-0687-3	V	179	117	137	136
	USG-7509nRR		177	142	128	138

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 5: Variation in the Daidzin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Daidzin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	117	139	93	N.A.
	IA-3010		117	156	101	N.A.
	MACON		93	149	107	N.A.
	LS-93-0375	IV	113	138	98	N.A.
	STRESSLAND		138	193	107	N.A.
	KS-4694	IV-S	75	113	82	N.A.
	MANOKIN		129	96	111	N.A.
	MD-97-6491		101	112	84	N.A.
	CLIFFORD	V	91	80	96	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	175	129	87	136
	ASGROW-AG3902		138	91	88	94
	DEKALB-DKB38-51		185	150	95	138
	M-ATL-MA3955RR		162	154	82	134
	NK-S39-Q4		129	111	74	110
	S.ST.-RT3799N		120	119	84	128
	ASGROW-AG4403	IV	98	126	65	104
	DEKALB-DKB44-51		107	126	84	115
	M-ATL-MA4355RR		115	134	71	125
	MD-99-0198-3		180	142	147	155
	DPL-DP4690RR	IV-S	116	102	74	84
	USG-7489RR		100	103	71	74
	VIGORO-V742NRR		170	157	125	115
	MD-99-0687-3	V	169	119	144	155
	USG-7509nRR		120	103	98	94

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 6: Variation in the Glycitin Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Glycitin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	33	37	34	N.A.
	IA-3010		24	19	10	N.A.
	MACON		45	42	49	N.A.
	LS-93-0375	IV	45	39	35	N.A.
	STRESSLAND		52	48	42	N.A.
	KS-4694	IV-S	37	35	31	N.A.
	MANOKIN		17	18	16	N.A.
	MD-97-6491		33	28	29	N.A.
	CLIFFORD	V	32	30	31	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	23	21	23	22
	ASGROW-AG3902		25	26	25	22
	DEKALB-DKB38-51		22	22	20	20
	M-ATL-MA3955RR		19	19	17	17
	NK-S39-Q4		14	14	15	14
	S.ST.-RT3799N		30	43	34	35
	ASGROW-AG4403	IV	36	46	38	39
	DEKALB-DKB44-51		39	41	43	39
	M-ATL-MA4355RR		43	56	40	38
	MD-99-0198-3		33	36	37	32
	DPL-DP4690RR	IV-S	33	40	33	32
	USG-7489RR		34	42	35	28
	VIGORO-V742NRR		36	40	35	31
	MD-99-0687-3	V	24	21	21	17
	USG-7509nRR		32	31	33	29

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications

Appendix 7: Variation in the Acetyl Genistin[a] Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Acetyl genistin * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	14.3	10.0	12.5	N.A.
	IA-3010		10.8	6.8	10.5	N.A.
	MACON		9.3	9.3	10.8	N.A.
	LS-93-0375	IV	8.3	10.0	9.0	N.A.
	STRESSLAND		12.5	12.0	10.8	N.A.
	KS-4694	IV-S	6.8	5.0	8.0	N.A.
	MANOKIN		5.0	6.8	9.3	N.A.
	MD-97-6491		9.3	13.5	8.8	N.A.
	CLIFFORD	V	7.5	5.8	7.5	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	11.8	7.8	11.3	10.8
	ASGROW-AG3902		9.0	7.0	7.5	9.0
	DEKALB-DKB38-51		11.5	7.0	8.3	11.8
	M-ATL-MA3955RR		12.8	8.0	8.5	10.0
	NK-S39-Q4		12.0	5.3	8.3	9.3
	S.ST.-RT3799N		13.0	6.8	7.3	9.3
	ASGROW-AG4403	IV	11.0	6.0	6.3	10.3
	DEKALB-DKB44-51		11.0	7.3	7.3	9.8
	M-ATL-MA4355RR		11.5	11.0	7.0	9.8
	MD-99-0198-3		13.8	6.3	9.0	11.3
	DPL-DP4690RR	IV-S	14.8	5.8	9.0	9.5
	USG-7489RR		13.0	6.0	9.3	10.3
	VIGORO-V742NRR		14.3	6.5	10.3	10.3
	MD-99-0687-3	V	13.3	5.3	9.5	6.0
	USG-7509nRR		14.3	6.0	9.5	7.8

N.A.: not available. Tests were not performed at that location.

* Values are average of two replications.

[a] Acetyl daidzin and acetyl glycitin were not detected in the samples.

Appendix 8: Variation in the Genistein[a] Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Genistein * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	9.0	0.5	5.8	N.A.
	IA-3010		8.0	0.5	10.3	N.A.
	MACON		N.D.	N.D.	N.D.	N.A.
	LS-93-0375	IV	5.5	N.D.	N.D.	N.A.
	STRESSLAND		6.5	8.3	N.D.	N.A.
	KS-4694	IV-S	9.3	3.3	N.D.	N.A.
	MANOKIN		N.D.	7.5	N.D.	N.A.
	MD-97-6491		9.5	7.8	N.D.	N.A.
	CLIFFORD	V	N.D.	6.0	N.D.	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	7.0	N.D.	N.D.	N.D.
	ASGROW-AG3902		7.0	0.8	N.D.	N.D.
	DEKALB-DKB38-51		8.5	2.3	N.D.	9.8
	M-ATL-MA3955RR		6.8	2.3	N.D.	7.8
	NK-S39-Q4		6.8	N.D.	N.D.	6.0
	S.ST.-RT3799N		7.8	N.D.	N.D.	11.0
	ASGROW-AG4403	IV	5.3	0.0	N.D.	N.D.
	DEKALB-DKB44-51		6.0	N.D.	N.D.	N.D.
	M-ATL-MA4355RR		6.0	0.8	N.D.	7.0
	MD-99-0198-3		8.0	N.D.	N.D.	7.0
	DPL-DP4690RR	IV-S	10.8	N.D.	N.D.	3.5
	USG-7489RR		9.0	N.D.	N.D.	N.D.
	VIGORO-V742NRR		12.0	N.D.	N.D.	4.8
	MD-99-0687-3	V	9.8	1.0	N.D.	5.0
	USG-7509nRR		9.3	N.D.	N.D.	4.0

N.A.: not available. Tests were not performed at that location.
N.D.: not detected.
* Values are average of two replications
[a] Glycitein was not detected in the samples.

Appendix 9: Variation in the Daidzein[a] Content (mg/Kg seed) for 24 Soybean Grain Cultivars Growth at Two Different Locations and Two Different Planting Times

SEED TYPE	BRAND-CULTIVAR	MATURITY GROUP	Daidzein * (mg / Kg seed)			
			POPLAR HILL		WYE FARM	
			Full Season	Double Crop	Full Season	Double Crop
STANDARD (non Roundup Ready tolerant)	GENERAL	III	6.0	N.D.	3.5	N.A.
	IA-3010		8.0	N.D.	7.5	N.A.
	MACON		N.D.	N.D.	3.5	N.A.
	LS-93-0375	IV	N.D.	N.D.	2.3	N.A.
	STRESSLAND		3.8	8.8	2.0	N.A.
	KS-4694	IV-S	N.D.	N.D.	2.5	N.A.
	MANOKIN		N.D.	7.0	2.8	N.A.
	MD-97-6491		N.D.	4.5	2.8	N.A.
	CLIFFORD	V	N.D.	N.D.	2.5	N.A.
ROUNDUP READY	AGWAY-APK397RR	III	6.0	N.D.	3.0	4.3
	ASGROW-AG3902		5.5	N.D.	4.0	3.0
	DEKALB-DKB38-51		9.0	N.D.	3.8	4.8
	M-ATL-MA3955RR		6.8	N.D.	2.8	3.8
	NK-S39-Q4		N.D.	N.D.	3.0	3.3
	S.ST.-RT3799N		6.5	N.D.	4.5	5.3
	ASGROW-AG4403	IV	6.0	N.D.	2.5	3.0
	DEKALB-DKB44-51		4.5	0.8	3.0	3.0
	M-ATL-MA4355RR		4.3	N.D.	3.0	3.5
	MD-99-0198-3		5.3	0.5	3.8	3.8
	DPL-DP4690RR	IV-S	6.8	N.D.	3.3	3.0
	USG-7489RR		4.5	N.D.	2.0	3.8
	VIGORO-V742NRR		9.3	N.D.	3.8	4.0
	MD-99-0687-3	V	7.0	N.D.	4.0	5.3
	USG-7509nRR		5.5	N.D.	3.3	3.8

N.A.: not available. Tests were not performed at that location.
N.D.: not detected.
* Values are average of two replications
[a] Glycitein was not detected in the samples.

Appendix 10: Variation in the Malonyl Genistin, Malonyl Daidzin and Malonyl Glycitin Contents (mg/Kg seed) for 16 Soybean Standard Cultivars According to Type of Grain

SEED TYPE	CULTIVAR	MATURITY GROUP	ISOFLAVONES* (mg / Kg seed)		
			Malonyl genistin	Malonyl daidzin	Malonyl glycitin
Grain	**Harosoy**	II	529	399	79
	Harosoy Magenta	II	481	368	52
	Kunitz	III	739	508	111
	Clark	IV	1,147	734	93
	Clark Magenta	IV	993	639	92
	Clark non-nod	IV	1,005	564	114
	Corsica Black	IV	667	549	67
	Corsica Breeders	IV	660	585	76
	Corsica Brown	IV	697	580	68
Green	**Verde**	III	644	534	51
	Emerald	IV	525	367	62
Food	**Black Jet**	000	48	101	26
	Envy	0	105	170	28
	Butterbean	I	95	148	30
	Jack	II	447	209	71
	Japan L1L2L3	N.A.	541	351	102

* Values are average of two replications
N.A.: not available

Appendix 11: Variation in the Genistin, Daidzin and Glycitin Contents (mg/Kg seed) for 16 Soybean Standard Cultivars According to Type of Grain

SEED TYPE	CULTIVAR	MATURITY GROUP	ISOFLAVONES* (mg / Kg seed)		
			Genistin	Daidzin	Glycitin
Grain	**Harosoy**	II	69	60	23
	Harosoy Magenta	II	61	49	17
	Kunitz	III	112	89	39
	Clark	IV	218	142	38
	Clark Magenta	IV	132	92	33
	Clark non-nod	IV	252	176	66
	Corsica Black	IV	96	96	22
	Corsica Breeders	IV	98	91	24
	Corsica Brown	IV	101	94	23
Green	**Verde**	III	106	97	15
	Emerald	IV	68	52	18
Food	**Black Jet**	000	11	19	7
	Envy	0	20	28	16
	Butterbean	I	19	31	13
	Jack	II	77	31	31
	Japan L1L2L3	N.A.	78	54	42

* Values are average of two replications
N.A.: not available

Appendix 12: Variation in the Acetyl Genistin, Genistein and Daidzein[a] Contents (mg/Kg seed) for 16 Soybean Standard Cultivars According to Type of Grain

SEED TYPE	CULTIVAR	MATURITY GROUP	ISOFLAVONES* (mg / Kg seed)		
			Acetyl genistin	Genistein	Daidzein
Grain	**Harosoy**	II	5.8	11.3	N.D.
	Harosoy Magenta	II	N.D.	N.D.	N.D.
	Kunitz	III	9.3	13.1	N.D.
	Clark	IV	8.3	15.6	N.D.
	Clark Magenta	IV	7.3	16.3	N.D.
	Clark non-nod	IV	9.8	30.6	N.D.
	Corsica Black	IV	10.8	10.6	3.8
	Corsica Breeders	IV	8.5	11.3	N.D.
	Corsica Brown	IV	7.5	10	N.D.
Green	**Verde**	III	5.0	13.1	N.D.
	Emerald	IV	4.8	7.5	N.D.
Food	**Black Jet**	000	15.5	8.8	N.D.
	Envy	0	3.0	4.4	N.D.
	Butterbean	I	7.8	5.6	N.D.
	Jack	II	4.5	18.8	N.D.
	Japan L1L2L3	N.A.	3.5	5.6	N.D.

* Values are average of two replications
N.A.: not available
N.D.: not detected.
[a] Acetyl daidzin, acetyl glycitin and glycitein were not detected in the samples.

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