CHARACTERIZATION OF ANTIOXIDANT ACTIVITIES OF SOYBEANS AND ASSESSMENT OF THEIR BIOACCESSIBILITY AFTER *IN VITRO* DIGESTION

Hyun Chung

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Sean F. O'Keefe, Chair

Kequan Zhou

Susan E. Duncan

Dongmin Liu

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ABSTRACT

Nine Virginia soybeans grown in a single location were compared for their antioxidant properties and isoflavone profiles. The extracts were evaluated for their total phenolic contents (TPC), Oxygen Radical Absorbance Capacity (ORAC), and DPPH$^{\bullet}$ radical scavenging activities. In order to evaluate efficient preparation methods for soybean antioxidants, three Virginia-grown soybeans were extracted using different extraction strategies. The extraction techniques included soxhlet extraction, conventional solvent extraction, and ultrasonic-assisted extraction (UAE) with 5 different common solvent systems including 50% and 80% aqueous acetone, 50 and 70% aqueous ethanol, and 80% aqueous methanol. The TPC in the soybean extracts and isoflavone compositions were significantly different among cultivars. Malonylgenistin was the major isoflavone in all soybean seeds, accounting for 75-83% of the total measured isoflavones. The V01-4937 variety had the highest total isoflavone and malonylgenistin contents, followed by V03-5794. The antioxidant activities of the soybean extracts were also significantly different. Overall, the V01-4937 soybean was the variety that stood out from the other tested Virginia soybeans because it had the highest TPC, ORAC value, and isoflavone contents as well as the second highest DPPH$^{\bullet}$ scavenging activity. Ultrasonic treatment improved the extraction of soybean phenolics by more than 50% compared to solvent alone. The UAE with 50% aqueous acetone was the most efficient for extraction of phenolic compounds in the soybean seeds. The conventional and UAE with 70% aqueous ethanol extracts had the highest ORAC values, while the soxhlet methanol extracts had the highest DPPH$^{\bullet}$ radical scavenging activities. Our results suggest that different extraction technologies have a remarkable effect on soybean antioxidant estimation and the UAE is more appropriate for soybean phenolic extraction because it is less time and solvent consuming than the conventional solvent and soxhlet extractions. The V01-4937 soybean with the highest TPC was evaluated for its antioxidant activity and isoflavone

contents in an *in vitro* digestive system. After gastrointestinal digestion, soybean extracts contained higher TPC and ORAC values than cooked soybean (before digestion) but they were relatively low in DPPH$^{\bullet}$ radical scavenging capacity. The glucosides, daidzin, genistin, and malonylgenistin showed stability during simulated digestion with 83.3 %, 59.4 %, and 10.7 % recovery, respectively. Aglycones, including daidzein and genistein, were recovered at 37 % and 73.7 %, respectively, after *in vitro* digestion. In this study, daidzin was the most stable and bioaccessible isoflavone determined using the *in vitro* digestive system. Among the aglycones, genistein was more stable and bioaccessible than daidzein after digestion. In conclusion, soybean antioxidant activities were different among cultivars and efficient extraction for TPC was found using UAE with 50% aqueous acetone. Furthermore, antioxidant activities were stable during digestion and genistein, within aglycones tested, was the most stable and bioaccessible compound following *in vitro* digestion. This information may provide manufacturers or researchers information required to develop food or nutraceutical products processed for better bioaccessibility of soybean bioactive components.

KEYWORDS: soybean antioxidant; isoflavones; TPC; ORAC; DPPH$^{\bullet}$; solvent extraction; ultrasonic-assisted extraction, bioaccessible soy extracts, *in vitro* digestive system, antioxidant activity

In memory of my father,

GyuTaek Chung

He always loved, was fully supportive and would be very proud of me.

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CHAPTER 1

INTRODUCTION

Reactive oxygen radical species (ROS) produced in normal cellular metabolism are well established in their roles as being both beneficial and harmful to living systems (*1*). ROS at low or moderate levels play beneficial roles in living systems, such as the defensive responses to infections and the functions in cellular signaling pathways. However, when ROS are produced excessively in biological systems, an imbalance results between ROS and the activity of enzymatic and non-enzymatic antioxidants in the defense system. This imbalance, so called oxidative stress, causes biological damage to cellular lipids, proteins, or DNA, which negatively affects their functions in the human body (*2, 3*). Because of this, oxidative stress has long been considered to be involved in the pathogenesis of human diseases such as atherosclerosis, inflammation, cancer, diabetes, central nervous system disorders as well as cardiovascular diseases (*4*).

Antioxidants are able to stabilize or deactivate free radicals before they attack cells. ROS can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, and catalase. Non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants. However, under oxidative stress conditions, enzymatic antioxidants may not be sufficient, and non enzymatic antioxidants (dietary antioxidants) may be required to maintain optimal cellular functions (*4-7*). Antioxidants may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress. There has been a great deal of recent interest in antioxidant effects of polyphenols.

It is generally recognized that antioxidants in fruits, vegetables, tea and red wine play a crucial role in functioning to reduce the risk of chronic diseases such as coronary heart disease, cancer, and diabetes (*8-13*). The consumption of fruits and vegetables rich in vitamins and antioxidants as well as red wine may increase the overall antioxidant status in the body (*14, 15*). Polyphenols are commonly found in plant foods and are major antioxidants present in the diet. The main dietary sources of polyphenols are fruits and beverages. There is increasing interest in

polyphenols' antioxidant properties, their abundance in the diet, and their possible role in the prevention of various diseases associated with oxidative stress including cancer, cardiovascular, and neurodegenerative diseases (*13*). However, not all nutrients are absorbed with equal efficacy after digestion in human body because components available to be absorbed and to be utilized in the body may vary quantitatively and qualitatively due to the physical properties of the food matrix (*16*). Moreover, the bioavailability of compounds in plant products (fruits and vegetables) may differ because of interactions between their chemical structures and macromolecules within the food matrix, as well as their uptake rates into the body. Bioaccessibility is defined as the amount of a food component released from a solid food matrix that is present in the gut and available for intestinal absorption (*17*). Bioavailability is described as the amount of food constituent ingested in the gut, available to be utilized and stored under normal physiological conditions in the human body (*18*).

Soybean intake has received increasing interest due to its health benefits, such as lowering the risk of chronic diseases such as heart diseases and cancers, especially breast and prostate cancers, osteoporosis, and diabetes (*19-25*). Soy antioxidant activities and the role of soy antioxidants such as isoflavones have received increasing interest since it has been recognized that soybeans may have therapeutic activities in addition to health promotion. The relationship between soybean consumption and health benefits has long been studied and the consumption of soy products rich in antioxidants, especially in isoflavones, has been shown to be associated with the prevention of cancers including breast and prostate cancers, cardiovascular diseases, bone health, and diabetes (*22, 24, 26-28*). The antioxidant activities of the extracts from food materials or food can be affected by various factors such as cultivar growth environment (climate, soil, and irrigation), methods of food manufacturing, and even the efficiency of extraction methodology (*29-31*).

Many studies have investigated in the bioavailability of soy antioxidants (isoflavones) using *in vitro* and *in vivo* models, but to date bioaccessibility of all soy antioxidants has not been examined. Most studies have focused on the bioavailability of soy isoflavone aglycone forms such as genistein and daidzein in human subjects, since these two aglycones are found at relatively high concentrations in plasma (*32-35*). These studies have suggested that the absorption of soy isoflavone aglycones is faster and at higher efficiency than their glycoside forms. When comparing aglycones, the concentration of genistein is higher than daidzein in

plasma. The isoflavone aglycone forms are generated by large intestine microbes and then absorbed (*36*), but recent reports have suggested the capability of the small intestine in the conversion of isoflavone glycosides to aglycones in rat small intestinal tissue (*37*). However, there is limited information of the bioaccessibility of soybean antioxidants. The isoflavones in soy bread were stable during *in vitro* gastrointestinal digestion (*38*). The information of the stability and bioaccessibility of total soy antioxidants including isoflavones is still not conclusive. Therefore the study of the bioaccessibility of soy antioxidants including isoflavones is required for better understanding the stability of antioxidant activity during the digestion in the gut.

There is lack of information on the antioxidant activities in Virginia-grown soy cultivars and more work is needed to clarify efficient extraction techniques. Furthermore, there is limited information on whether isoflavones and antioxidant capacities of soybeans are stable during digestion. Therefore, it was hypothesized that antioxidant activities of soybean can be affected by a variety of cultivars and extraction techniques, and are stable during *in vitro* digestion.

OBJECTIVES

The overall goal of this study was to investigate the antioxidant activities of soybean affected by different cultivars and extraction methods, and to determine the bioaccessibility of antioxidant extracts from soybean during *in vitro* digestion. Below are specific objectives.

1. Determine and compare the antioxidant properties and isoflavone contents of Virginia-grown soybeans
2. Compare the effect of extraction methodologies for assessing antioxidant capacity of selected Virginia-grown soybeans
3. Evaluate the antioxidant properties of bioaccessible soybean extracts and determine the bioaccessibility of isoflavones during *in vitro* digestion.

SIGNIFICANCE

Soybean isoflavones (a subclass of polyphenols) have been thought to promote health, by reducing the risk of cardiovascular diseases, cancers, osteoporosis, diabetes and obesity (*19-21,*

24, 25). Besides isoflavones, soybeans have also been investigated as a food source of polyphenols including tannins, proanthocyanidins, anthocyanins, flavonoids (majority of isoflavone), and phenolic compounds such as chlorogenic, caffeic, ferulic, and *p*-coumaric acids (*39-42*). Several *in vivo* and *in vitro* studies have shown that antioxidant extracts from soybean seed and soy products may increase the expression or activation of antioxidant enzymes including superoxide dismutase and catalase (*43*) and reduce LDL oxidation (*28, 44*). Soybean antioxidants, including isoflavones, are present in different quantities in various soy foods and concentrations also can be influenced by cultivars, extraction methods, and food processing methods (*41, 45-48*). Although soybeans have relatively high antioxidant activities, the antioxidants should be retained in the gut after release from food following digestion, and should be able to act as antioxidants in the body after absorption. The amount of bioactive compounds released from a food matrix before absorption through the intestinal wall refers to bioaccessibility. Bioavailability implies the bioaccessible compounds are used for their bioactivity in target cells after absorption. Most studies have investigated the bioavailability of isoflavones in soy food, supplements or pure compounds using both *in vivo* and *in vitro* methodologies (*33, 34, 37*). The bioaccessibility and bioavailability of isoflavones are affected by food processing methods (*49, 50*). However, there is limited information about the effect of food processing on bioaccessibility and bioavailability of soy total antioxidants. Few studies have evaluated the bioaccessibility of isoflavones from soy isoflavone-enriched food products (*38, 51*), and these studies did not account for soy total antioxidants. It is important to understand the bioaccessibility of soybean antioxidants ingested in the diet and their bioavailability to function in biological roles in the human body. Although not all bioaccessible antioxidants can be absorbed and act in a biological role in specific tissue sites, the information from this current study can help develop efficient food processing or nutraceutical products with better bioaccessibility of soybean bioactive components in the human body.

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CHAPTER 2

LITERATURE REVIEW

Oxidative stress, human diseases, and antioxidants

Free radicals, or reactive oxygen species (ROS), are constantly generated in the human body and include free radicals such as superoxide ($\cdot O_2^-$) and hydroxyl ($\cdot OH$), nitric oxide ($NO\cdot^-$) and non radicals such as hydrogen peroxide (H_2O_2) (*1*). ROS are produced in normal cellular metabolism and are well established in their roles as being both beneficial and harmful to living systems (*2*). ROS at low or moderate levels play beneficial roles in living systems, such as the defensive responses to infections and the functions in cellular signaling pathways. This balance between beneficial and harmful effects of ROS, which is controlled by redox regulation, has great importance to living organisms. Living organisms can be protected from various oxidative stresses by the process of redox regulation. Redox homeostasis is controlled by regulation of redox status *in vivo* (*3*). However, when ROS are produced excessively in biological systems, there results an imbalance between ROS and the activity of enzymatic and non-enzymatic antioxidants in the defense system. This imbalance, so called oxidative stress, causes potential biological damage to cellular lipids, proteins, or DNA, which negatively affects their functions in the human body (*4, 5*). Under mild oxidative stress, cells can be protected by the defensive systems, but under severe oxidative stress, cells are damaged and lead to death by apoptotic or further necrotic mechanisms (*2*). Because of this, oxidative stress has long been considered to be involved in the pathogenesis of human diseases such as atherosclerosis, inflammation, cancer, diabetes, central nervous system disorders as well as cardiovascular diseases (*1*).

- Reactive oxygen species (ROS) and human diseases

ROS can be generated both endogenously and exogenously. ROS, present in the atmosphere as pollutants, are generated by exogenous sources such as radiations (X-rays, γ-rays, and UV light irradiation), xenobiotics, metals, ions, chlorinated compounds, and environmental agents. In the human body, ROS are endogenously produced by catalyzed reactions and various other mechanisms in mitochondria as well as by neutrophils and macrophages during inflammatory cell activation (*2, 6*). As mentioned above, ROS include both free radicals and

non-free radicals. Superoxide, hydroxyl, and nitric oxide are major free radicals and hydrogen peroxide is a major non-free radical.

ROS produced endogenously will be examined and explained briefly. Superoxide ($\cdot O_2^-$) is reactive radical and produced mostly in cell mitochondria by leakage of a small number of electrons during energy transduction (*7*). The hydroxyl ($\cdot OH$) radical is the neutral form of the hydroxide ion, and $\cdot OH$ reacts with all components of the DNA molecule. This radical is highly reactive and making it a very dangerous radical (*8*). Nitric oxide ($NO\cdot^-$) acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including blood pressure regulation, defense mechanisms, smooth muscle relaxation and immune regulation (*9*). Hydrogen peroxide (H_2O_2), produced in mitochondria, is not a free radical but acts as a ROS precursor to superoxide. Even though H_2O_2 in low concentration may be poorly reactive, high concentration of H_2O_2 can attack several cellular energy-producing systems and form $\cdot OH$ in the presence of transition metal ions; $\cdot O_2^-$ facilitates this reaction (*1, 10*).

Highly concentrated ROS can play an important role as mediators of cell structure, and cause nucleic acids, lipids, and protein damage (*2*). Consequently, tissue injury itself can cause ROS generation, which may contribute to worsening of the injury (*11*). These oxidative damages lead to permanent modifications of genetic material and may cause mutagenesis, carcinogenesis, and aging (*12*). Furthermore, metal-induced generation of ROS also causes an attack on DNA, as well as on the other cellular components including the polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (*13*). Oxidative stress (imbalanced production of ROS) has been implicated in various pathogeneses involving cardiovascular disease, cancer, neurodegenerative disorders, diabetes, ischemia/reperfusion damage, aging as well as male infertility (*14-17*). These diseases are divided into two groups: 1) mitochondrial oxidative stress conditions causing cancer and 2) diabetes mellitus and inflammatory oxidative conditions causing atherosclerosis and chronic inflammation, ischemia, and reperfusion injury. The process of aging is caused by the damaging consequences of free radical action, which results in lipid peroxidation, DNA damage, and protein oxidation. Inflammatory cells may also increase DNA damage by activating pro-carcinogens to DNA-damaging species. Cancer can be considered a degenerative disease of old age, related to the effects of continuous damage over a life span by toxic oxygen (*14-17*).

- **Roles of antioxidants against oxidative stress**

When free radicals from various sources are exposed, organisms develop their own series of defense mechanisms. Defense mechanisms against free radical-induced oxidative stress include preventative mechanisms, repair mechanisms, physical defenses, and antioxidant defenses. Potentially damaging ROS are dealt with by enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, and catalase. Non-enzymatic antioxidants include ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione, carotenoids, flavonoids, and other antioxidants. Antioxidants are able to stabilize or deactivate free radicals before they attack the cells. ROS can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is very important to organisms' health and survival. However, under oxidative stress conditions, enzymatic antioxidants may not be sufficient, and non enzymatic antioxidants (dietary antioxidants) may be required to maintain optimal cellular functions. (*1, 18-20*).

<u>Enzymatic antioxidants</u>

Enzymatic antioxidants are endogenously produced in the human body. These endogenous antioxidants play a critical role in maintaining optimal cellular functions. The most efficient enzymatic antioxidants in endogenous antioxidants involve glutathione peroxidase, catalase, and superoxide dismutase (*18*).

- Glutathione peroxidase

Glutathione peroxidase is present in the mitochondria and cytosol. Glutathione peroxidase is one of the most important antioxidant defense mechanisms present in the cells. It is generally thought to be more important than catalase in the removal of hydrogen peroxide (H_2O_2) in humans. This enzyme associates with glutathione, which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxide (H_2O_2) or organic peroxide to water or alcohol. Glutathione peroxidase has a competitive reaction with catalase for hydrogen peroxide as a substrate, and is the major source of protection under the low levels of oxidative stress (*10, 18*).

- Catalase

Unlike glutathione perxidase and superoxide dismutase, catalase is located in peroxisome of aerobic cells and is very efficient at converting hydrogen peroxide (H_2O_2) to water and molecular oxygen. In animals, catalase and glutathione peroxidase detoxify hydrogen peroxide. Cells are protected by catalase from hydrogen peroxide generated within the cells (*18*).

- Superoxide dismutase

Similar to glutathione peroxidase, peroxide dismutase is distributed in the mitochondria and cytosol. This enzyme is one of the most effective intracellular enzymatic antioxidants and converts O_2- to hydrogen peroxide (H_2O_2) and then hydrogen peroxide (H_2O_2) to water either by catalase in the lysosomes or by glutathione peroxidase in the mitochondria. Under normal conditions, the high levels of SOD keep superoxide concentrations at low levels and prevent formation of peroxynitrite. SOD neutralizes superoxide ions during the process of successive oxidative and reductive cycles (*20, 21*).

Non-enzymatic antioxidants

As mentioned above, under the oxidative stress condition, enzymatic antioxidants may not be sufficient and, therefore, non enzymatic antioxidant (dietary antioxidants) may be required to maintain optimal cellular functions. Even though some dietary compounds do not contribute to neutralize free radicals, antioxidants may enhance the endogenous antioxidant activities (*10*). It is well known that fruits and vegetables are good sources of many antioxidants. It has also been reported that diets rich in fruit and vegetables are associated with reduced risks of chronic diseases such as cancer and heart diseases. Therefore, a healthy diet may maintain non-enzymatic antioxidants as well as exogenous antioxidants at or near optimal level, thus lowering the risk of tissue damage (*20*). In general, non enzymatic antioxidants involve vitamins E, C, glutathione, carotenoids, and flavonoids.

- Vitamin E and vitamin C

Vitamin E is fat-soluble and includes 8 different forms. The main function of vitamin E is to protect against lipid peroxidation. It inhibits lipid peroxidation by effectively scavenging the peroxyl radical in cell membranes (*22*). Among 8 different forms of vitamin E, α- tocopherol is the most active form *in vivo* and the major membrane bound antioxidant employed by the cell in human body (*23*). Additionally, α-tocopherol and vitamin C (ascorbic acid) function together during the antioxidant reactions. Vitamin C acts to regenerate α-tocopherol from α-tocopherol radicals in membranes and lipoproteins, and increases intracellular glutathione levels, thus playing an important role in protein thiol group protection against oxidation (*24*). Vitamin C is water-soluble and an important and powerful antioxidant working in aqueous environments of the body. As mentioned above, this vitamin is a partner with Vitamin E in scavenging radicals. In addition to work with vitamin E, it cooperates with carotenoids as well as with the antioxidant enzymes (*22, 25*).

- Glutathione

Glutathione is a major thiol antioxidant and has multiple functions as an intracellular antioxidant. It is the major water soluble antioxidant in these cell compartments and is present at high levels in the cytosol, nuclei, and mitochondria. The main protective roles against oxidative stress are to act as a co-factor for several detoxifying enzymes, to scavenge hydroxyl radical and singlet oxygen directly, and to regenerate vitamin C and E to their original active forms (*26*).

- Carotenoids

Carotenoids, present in plants and microorganisms, are mainly color pigments and contain conjugated double bonds. Their antioxidant activity arises due to the ability to delocalize unpaired electrons with resonant stabilization (*27*). Carotenoids can quench singlet oxygen and react with free radicals. They can prevent damage in lipophilic compartments by scavenging peroxy radicals. Even though it has been reported that β-carotenoids in high concentration can cause an increase in lipid peroxidation due to the adverse role as a pro-oxidants (*28*), many studies have epidemiologically revealed that the consumption of diets rich in carotenoids is correlated with a lower risk of age-related diseases (*29*).

- Flavonoids

Flavonoids are a large group of polyphenols which include phenolic acids and flavonoids. Over 4000 flavonoids have been identified and are divided into several groups such as flavonols (quercetin and kaempherol), flavanols (catechin), anthocyanidins, and isoflavones (daidzein and genistein) according to their chemical structures (*30*). Flavonoids, present in food mainly as glycosides and polymers, contain a substantial fraction of dietary flavonoids. They are a broad class of low molecular weight, ubiquitous plant metabolites, and are integral parts of the human diet (*31*). There are factors that determine whether a flavonoid will act as an antioxidant or as a modulator of enzyme activity; these biological properties include the nature and position of the substituents and the number of hydroxyl groups on the flavonoid. Flavonoids are mostly reported as being antioxidants that protect against oxidative stress due to their abilities to scavenge peroxyl radicals, effectively inhibiting lipid peroxidation, and by chelating redox-active metals, preventing catalytic breakdown of hydrogen peroxide. However, similar to carotenoids, flavonoids at certain concentrations can act as pro-oxidants (*31, 32*).

Dietary antioxidants and health benefit

Many studies with *in vitro* models have shown that dietary antioxidants, such as vitamin C, vitamin E, β-carotene (carotenoid), and flavonoids, act as antioxidants in biological systems including plasma, lipoproteins, and cultured cells. For example, vitamin C effectively inhibits lipid and protein oxidation in human plasma exposed to various physiologically relevant types of oxidative stress. Vitamin E, the most abundant lipid-soluble antioxidant in human lipoproteins and tissues, acts as an antioxidant against lipid peroxidation. The carotenoids β-carotene, lycopene, and lutein efficiently quench singlet oxygen and, thus, may protect the eye and skin against oxidative damage induced by UV light. These dietary antioxidants act together in an antioxidant defense system along with antioxidant enzymes and metal binding proteins that exist in cells and extracellular fluids (*33-35*). In addition to *in vitro* studies, numerous *in vivo* studies of dietary antioxidants with animal and human subjects have reported protection against oxidative damage; dietary antioxidants protect against oxidative damage and contribute to positive health benefits. For instance, the carotenoids lutein and zeaxanthin participate in antioxidant activities that have been shown to increase macular pigment concentration in the eye

(*36*). Flavonoids reportedly lower the risk of various degenerative diseases associated with aging, such as cancer, cardiovascular diseases, osteoporosis, and neurodegenerative diseases (*37, 38*). Furthermore, antioxidant nutrients have shown to prevent and treat type 2 diabetes (*39*). Natural antioxidants in grapes, cocoa, blueberries and teas have been shown to have beneficial effects on cardiovascular health, Alzheimer's disease, and even a reduction of the risk of some cancers (*40-43*) . Even though there is agreement on the health benefits of plant foods, it is still unclear which components of plant-based food are protective and what their mechanisms of action are. Many researchers are focusing on the effects of dietary antioxidants on prevention of chronic diseases related to aging.

Dietary antioxidants and diseases

Antioxidants may protect cell constituents against oxidative damage and, therefore, limit the risk of various degenerative diseases associated with oxidative stress. There has been a great deal of recent interest in antioxidant affects of polyphenols, which are largely explained by the complexity of their chemical structures. Numerous studies on animal models have shown that they limit the development of cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, and osteoporosis (*37*).

- Cardiovascular disease (coronary heart diseases)

Alpha-tocopherol has shown to protect low density lipoproteins (LDL) at a concentration of about 6 mol/ mol LDL. Gamma tocopherol, β-carotene, lycopene, α-carotene, β-cryptoxanthin, lutein, zeaxanthin, cantaxanthan, and phytofluene have also been shown to minimize the oxidation of LDL after the exposure of LDL to oxidant stress (*44, 45*). In addition, dietary flavonoid intake from tea was shown to significantly lower deaths from heart disease (*46, 47*). Epidemiological study consistently shows a protective association between β-carotene or foods in rich carotenoid and cardiovascular disease (*43*). In prospective studies of non-diabetic individuals, vitamin E supplementation was associated with protection against coronary heart diseases. Non-insulin dependent diabetes mellitus (NIDDM) in human and animal models have shown that vitamin E reduced vascular oxidative stress and preserved endothelial function and,

thus, inhibit the development of atherosclerosis (*48*). Art et al. have also shown that catechin intake is related to lower risk of coronary deaths (*47*).

- Cancer

Anticarcinogenic effects of polyphenols which include phenolic acids and flavonoids are well verified in animals such as rat or mice. Polyphenols have been shown to inhibit the formation and growth of tumors from initiated cells and cell proliferation *in vitro* (*49*). Particularly, flavonoids, including isoflavones, catechins, red wine polyphenols, and resveratrol, have been observed to have protective and inductive effects by lowering the number of tumors or of their growth. These effects have shown at a variety of sites including mouth, stomach, duodenum, colon, liver, lung, mammary, and skin (*37, 50*).

- Neurodegenerative disease

Oxidative stress can specifically influence brain tissues, and antioxidants possibly participate in preventing neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (*51*). Dietary polyphenols have been shown to limit the development of neurodegenerative diseases (*37*). Joseph et al. reported that aging rats fed in a diet supplemented with aqueous extracts of spinach, strawberry, or blueberry rich in polyphenols improved their cognitive functions, and blueberries rich in anthocyanins were efficient, in particular. Since insufficient vitamins E and C in the brain was not observed, it is suspected that polyphenols as antioxidants act directly in these functions (*52, 53*).

- Diabetes

Dietary supplementation with antioxidants in human and rodents studies is associated with decreased risk of non-insulin dependent diabetes mellitus (NIDDM) (*54*). Prospective epidemiological studies demonstrated that high levels of α-tocopherol (vitamin E) in serum is associated with lowering the risk of NIDDM (*55*). Polyphenols in plants, which have been used for treating diabetes traditionally, may contribute their therapeutic activity, and many studies on polyphenols and treatment of diabetes have been conducted *in vitro* and *in vivo* (*56*). Anthocyanin-rich extracts also showed hypoglycemic effects (*57*). Several *in vitro* studies on

cultured cells have shown that polyphenols such as caffeic acid, ECGC, and isoferulic acids may increase glucose uptake by peripheral tissues (*58*). There have also been reports that acute or chronic administration of polyphenols, including caffeic and isoferulic acids, to experimental animals reduces fasting glycemia and lessens the increase of plasma glucose (*59, 60*). However, adverse effects of quercetin and genistein were also reported, which both of which inhibited glucose uptake in rat adipocytes (*61, 62*).

Sources of dietary antioxidants (polyphenols)

It is generally recognized that antioxidants in fruits, vegetables, tea and red wine play a crucial role in functioning to reduce the risk of chronic diseases such as coronary heart disease, cancer, and diabetes (*25, 37, 63-66*). The consumption of fruits and vegetables, rich in vitamin and antioxidants, and red wine may increase the overall antioxidant status in the body (*67, 68*). In experimental and epidemiological studies, consumption of grains, fruits and vegetables rich in antioxidants provided health benefits by lowering the incidence of aging diseases related to oxidative stress. There is increasing interest in polyphenols' antioxidant properties, their abundance in the diet, and their possible role in the prevention of various diseases associated with oxidative stress including cancer, cardiovascular, and neurodegenerative diseases (*37*). In the most case, polyphenols are found in foods as complex, poorly characterized mixtures. Food sources with high polyphenol contents will next be discussed.

Polyphenols are commonly found in foods of plant origin and are major antioxidants present in the diet. The main dietary sources of polyphenols are fruits and beverages. Fruits, such as apples, grapes, pears, cherries, and various berries, contain up to 200 – 300 mg polyphenols/ 100g of fresh weight. A glass of red wine or a cup of green tea or coffee also contains about 100 mg of polyphenols. Cereals, chocolate, and dry legumes are other polyphenol sources. The total dietary polyphenols intake (about 1g/day) is much higher than that of all other known dietary antioxidants, and is about 10 times higher than of vitamin C and 100 times higher than of vitamin E and carotenoids (*69, 70*). The most common flavonoids include quercetin, catechin, anthocyanins, daidzein, proanthocyanidins, and caffeic acid. Quercetin is abundant in onion, tea, and apples. Catechin is found in tea and several fruits. Anthocyanin provides the color to many red fruits such as grape, blackcurrant, raspberry, and strawberry. Daidzein, a type of isoflavone, is found mainly in soybean but is found in other products such as kudzu. Proanthocyanidins are

common in many fruits including apple, grape, and cocoa. Caffeic acid, one of the most common phenolic acids, is present in many fruits and vegetables (*37*).

Various fruit have been investigated for their polyphenol contents. Manach et al. have summarized the dietary polyphenol contents in various food sources (*70*). Specifically, berries have been shown to contain high levels of polyphenols. Blackberries, raspberries, and blackcurrents contain 8 – 27, 6 – 10, and 4 – 13 mg of phenolic acids, such as hydroxybenzoic acids, per serving (100 g), respectively. Strawberry contains 4 – 18 mg of hydroxybenzoic acids per 200g serving. Hydroxycinnamic acids, another type of phenolic acid, are found in blueberry at 200 – 220 mg/ 100g serving. Blackberries, blueberries, and black currents contain 100 –400, 25 – 500, and 130 - 400 mg of anthocyanins per 100g serving,respectively. Thirty to one hundred milligrams of anthocyanin is contained in a 200g serving of strawberries. Blackberries also contain catechin, around 13 mg per 100g serving (*70*). Red wine has an anthocyanin content around 20 – 35 mg/ 100 mL. In grapes, a 200g serving contains 60 – 1500 mg of anthocyanins. One hundred milliliters of red wine and 200g of black grapes contain 4 – 7 and 3 – 8 mg of quercetin, respectively. Catechin is found at 6 – 35 and 8 – 30 mg in 200g of grape and 100 mL of red wine, respectively (*70*). Chicory and potato contain 40 – 100 and 20 – 38 mg of phenolic acids per 200g serving. Red cabbage reportedly contains 50 mg of anthocyanin per 200g. Yellow onion contains quercetin around 35 – 120 mg/100g. Green or white beans (200g) contain 2 – 10 mg of quercetin. Catechin is found at levels around 70 – 110 mg per 200g of beans.

Isoflavones are found in most leguminous plants. Soybean and soybean products are a major source of isoflavones, one of the subclasses of flavonoids in human diet. The isoflavone contents of soybeans and its manufactured products vary greatly, depending on their growing zone, growing conditions, variety of soybeans, and processing. Boiled soybeans (200g), soy flour (75g), and soymilk (1L) contain 40 – 180, 60 – 135, and 30 - 175 mg of isoflavones per serving (*70, 71*).

Bioaccessibility and bioavailability of antioxidants (polyphenols)

As mentioned above, the polyphenol daily intake in the US is recognized as being around 1g/ day. In the Spanish diet, the mean daily intake of polyphenols was estimated to be between 2.59 and 3.02 g/day (*72*). However, not all nutrients are absorbed with equal efficacy after digestion in human body, because components available to be absorbed and to be utilized in the

body may vary quantitatively and qualitatively due to the physical properties of food matrix (*73*). Moreover, the bioavailability of compounds in plant products (fruits and vegetables) may differ because of interactions between their chemical structures and macromolecules within the food matrix, as well as their uptake rates into the body. For example, carotenoids in vegetables showed remarkably low rates of absorption both in animals and humans due to their chemical structures (*74*). Carotenoids in raw carrots and tomato products were examined for their bioaccessibility using an *in vitro* digestion model, and only 1–3% of the β-carotene in raw carrots and 1% of lycopene in canned and fresh tomatoes were accessible for absorption (*75*). Furthermore, more than 70% of the original carotenoids from fruit and vegetables remained in the final digesta (*76*).

Polyphenols released from the food matrix by the action of digestive enzymes from small intestine and bacterial microflora from large intestine are bioaccessible in the gut and, thus, potentially bioavailable. Bioaccessibility is defined as the amount of a food fraction released from solid food matrix that is present in the gut and available for intestinal absorption (*77*). Bioavailability is described as the amount of food constituent ingested in the gut available to be utilized and stored under normal physiological conditions in the human body (*78*). Knowledge of the dietary intake of polyphenols and their bioaccessibility in the human digestive system are key factors in assessing their significance in human health. In addition to bioaccessibility, knowing the bioavailability and metabolism of the various polyphenols is necessary to evaluate the biological roles of polyphenols from foods to the human body. *In vitro* and *in vivo* methodologies have been established to assess the bioaccessibility and bioavailability of antioxidants from food matrices or supplements.

- Determining bioaccessibility of antioxidants by *in vitro* digestion

Simulating gastric and intestinal digestion for the approach of in vitro digestion of food and pure components has been used to investigate digestion of proteins, starch, lipids, polyphenols, and carotenoids from various matrices (*72, 75, 79-81*). Digestive enzymes including pepsin and pancreatin along with bile salts, time, pH, and temperature can be controlled for a simulated digestion procedure (*78*). These simulated digestion procedures contribute information about the stability of compounds during gastrointestinal digestion. An *in vitro* gastrointestinal system has been used to assess the bioaccessibility of polyphenols and

carotenoids in different food sources such as grape seed and peel (*82*), green tea (*83*), carrot, tomatoes(*75*), and leafy vegetables (*84*). For example, Green et al. have reported that green tea beverage formulations altered the recovery of tea catechins in human using an *in vitro* digestion model (*83*). Leafy vegetables were examined for bioaccessibility using an *in vitro* digestion system, and vegetables cooked with oil showed 2 – 5 times more bioaccessible all *trans* β – carotene than vegetables cooked without oil (*84*). The stability of carotenoids and chlorophyll was also examined during the gastric and small intestinal digestion processes using an *in vitro* digestive system by Ferruzzi et al. (*85*). The bioaccessibility of soy isoflavones from soy bread was examined by Walsh et al. They revealed that the isoflavones were stable during simulated digestion and bile salt significantly improved partitioning of soy isoflavones into the aqueous fraction (becoming more bioaccessible) (*86*).

- Determination of activities of bioaccessible antioxidants (polyphenols)

As mentioned previously, the first step of bioavailability can be considered the fraction of the nutrient released from the food during the gastrointestinal digestion, which is available for absorption and for biological activity. To estimate the bioaccessibility of antioxidants released from food or food materials during in vitro digestion, HPLC analysis and total antioxidant activity assays have been utilized (*76, 83, 86-90*). The methods used to evaluate the antioxidant release from foods during the digestion usually employ the total phenolic content (TPC), the ferric reducing antioxidant capacity (FRAP), Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorption capacity (ORAC), and the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). Several studies have reported the effect of *in vitro* gastrointestinal digestion on the stability and bioaccessibility of antioxidant compounds from food. Various methods are used for evaluating the antioxidant capacities of food because antioxidant compounds may act *in vivo* through different mechanisms. Pomegranate was shown to be an important source of anthocyanins, other phenolic compounds, and ascorbic acid (vitamin C). The release of anthocyanins, vitamin C, and total phenols from the pomegranate juice during gastrointestinal digestion was evaluated using HPLC analysis and TPC assay by Perez -Vicente et al (*87*). This study showed that pomergranate phenolic compounds were accessible during the *in vitro* digestion whereas anthocyanins and vitamin C were degraded during small intestinal digestion, presumably via pH effects. Nagah and Seal assessed the antioxidant activity of the

fractions released from whole grain foods (uncooked and cooked) during *in vitro* gastrointestinal digestion (*88*). The antioxidant activity of *in vitro* food digestion was determined by FRAP, TEAC, and ORAC. These methods provided different trends and ranges of antioxidant values within the *in vitro* digestion foods. Noguer et al. reported the antioxidant activity of red wine fractions tested using an *in vitro* digestive system and assessed by ORAC and FRAP assays (*89*). The antioxidant activity of red wine fractions showed 100 to 1000 times higher antioxidant activity after than before gastrointestinal digestion. This increase in antioxidant activity of red wine fractions after digestion can be partly explained by a release of phenolic compounds with small molecular weights from polymeric factions of red wine during digestion (*89*). The antioxidant activity of fruit beverages with an *in vitro* gastrointestinal digestion model was studied using TEAC and ORAC assays by Perales et al. (*91*). The total antioxidant activity of the bioaccessible fractions of the beverages were lower than the original beverages (*91*). The antioxidant activity of wheat bread after *in vitro* digestion was reported by Gawlik-Dzidi et al. They determined the antioxidant activities of the bioaccessible fractions of wheat bread after gastrointestinal digestion by TPC and $DPPH^{\bullet}$ assays. The antioxidant activities of the wheat bread fractions were compared at different digestion stages such as mouth, gastric, and small intestinal digestions. The TPC of the fractions after intestinal digestion revealed that the highest antioxidant activity after gastrointestinal digestion (*90*). Saura-Calixto et al. have investigated the bioaccessibility of polyphenols in plant foods including cereals, vegetables, legumes, fruits, nuts, beverages, and oils during *in vitro* gastrointestinal digestion using a TPC assay (*72*). This study reported that 48% and 42% of dietary polyphenols are bioaccessible in small intestine and in large intestine, respectively, while 10% of the dietary polyphenols remained in the food matrix after digestion.

- Bioavailability of antioxidants

To estimate bioavailability of nutrients, antioxidants or antioxidant capacity of human plasma can be measured during *in vivo* assessment of absorption processes (*92*), and *in vitro* model studies can be conducted using Caco-2 cells, which are intestinal cells and can be used to evaluate absorption (*93*). The bioavailability of components such as antioxidants that exist in fruits and vegetables is currently a very important area of food and nutrition study. These studies can help to determine the real contribution of foods as antioxidant sources and to establish

processing condition that may be able to increase possible health benefits. Food processing including grinding, fermentation, and/or heating may enhance the bioavailability of antioxidants due to disruption of the plant tissue cell walls, may cause the dissociation of antioxidant-matrix complexes, or may result in a transformation into more active molecular structures (*78*). Numerous researchers have examined bioavailability of carotenoids and polyphenols, including flavonoids such as anthocyanins, in various food sources by using *in vitro* and *in vivo* digestion methods (*70, 94-97*). Ferruzzi et al. demonstrated that the uptake of carotenoids and chlorophyll derivatives absorbed from spinach puree by human Caco-2 cells was 20-40% and 5-10% for carotenoids and chlorophyll derivatives, respectively (*85*). The bioavailability of carotenoids transferred from baby foods containing carrots, spinach, and a meat, and tomato paste were determined to be 28-46 % for carotenoids in Caco-2 human intestinal cells (*98*). Numerous studies on the bioavailability of polyphenols have established that most classes of polyphenols are sufficiently absorbed to exert biological effects. For instance, quercetin from onions, catechins from red wine, and isoflavones from soy consumption attained micromolar concentration in the blood samples (*97, 99, 100*). Anthocyanin bioavailability in black raspberry was reported by He et al (*101*). This study showed that 7.5% of anthocyanins are transported into the small intestinal tissue in the rat (*101*). The bioavailability of soy isoflavones including daidzein and genistein in American women ingesting the aglycone or glucoside form of isoflavones was investigated by the assessment of blood plasma from collected blood samples (*102*). Zubik and Meydani concluded that there was no difference between daidzein and genistein bioavailability when glucoside and aglycone forms of isoflavones were consumed (*102*). The concentrations of polyphenols needed to test the effectiveness using *in vitro* models ranges from <0.1 mol/L to >100 mol/L, even though most polyphenols can be absorbed. Furthermore, the absorption of polyphenols is associated with their conjugation and metabolism, and the forms of polyphenols in blood samples may be different from the forms in food (*99, 103*).

Soybean as an antioxidant source

The consumption of soybean and soy-based products has been dramatically increasing with a growth in US soy food sales from $300 million to $4 billion from 1992 to 2008 (*67*). Soybean is mainly composed of proteins, carbohydrates, and lipids as macronutrients. Soybeans

usually contain about 40% of protein, 30% of carbohydrate, 18% of oil, and 14% of moisture, ash, and other materials such as hull (*104-106*). Protein contents of soybeans differ somewhat between cultivars, but about 80% of the total protein contents are composed of heat-stable storage proteins including 7S globulin (β-conglycinin) and 11S globulin (glycinin) (*107*). Some studies have shown that β-conglycinin can improve serum lipid profiles in mice and humans, and that this was not related to the effects of isoflavones (*108, 109*). Carbohydrates include 15% of soluble carbohydrates such as sucrose, raffinose, and stachyose as well as 15% of insoluble carbohydrates such as dietary fiber (*106*). Soybean also contains micronutrients including isoflavones, phytic acid (phytate), saponins, plant sterols (phytosterols), vitamins and minerals. The bioactivities of soy protein, peptides, saponins and phytosterols have been shown to promote human health (*105, 110, 111*). Moreover, soy antioxidant activities and the role of soy antioxidants such as isoflavones have received increasing interest since it has been recognized that soybeans have potential properties to have therapeutic activities as well as to promote health. The consumption of soy products rich in antioxidants, especially in isoflavones, has beneficial effects such as prevention of cancers including breast and prostate cancers, cardiovascular diseases, diabetes, and may improve bone health (*112-116*).

Soybean antioxidants

Antioxidant properties of soy have been associated with isoflavones, their interaction with soy protein, and antioxidant peptides obtained from soy protein fractions, soybean seeds, and soy products (*117-121*). Studies have recently shown that soybeans and soybean products exhibit significant antioxidant activities (*122-126*). These reports indicate that along with isoflavones, soybeans antioxidants have been received increasing interest and further investigation as a potential natural antioxidant source for disease prevention and health promotion.

- Isoflavones

It has been known that soybean and soy products are the best sources of isoflavones in foods. Soybean contains significant amounts of isoflavones which are a subclass of flavonoids. Soybean isoflavones include four main forms such as β-glucosides, their aglycones,

acetylglucosides, and malonylglucosides (*102, 127*). Beta-glucosides include genistin, daidzin, glycitin which are aglycones conjugated with sugar (Fig 1). Aglycones include genistein, daidzein, and glycitein (Fig 1). Malonylglucosides are major forms present in soybean seeds and unfermented soy foods (*128, 129*). After consumption of soybean, only aglycones (genistein and daidzein) hydrolyzed from glycosides by bacterial β-glucosidases are absorbed by the intestinal tract and have biological activity in human body. Genistein and daidzein are predominant isoflavones detected in the blood and urine of humans and animals (*130, 131*). Furthermore, fermented soy foods including miso, tempeh, and chungkookjang which are predominantly consumed in Asia, contain high levels of aglycones forms, hydrolyzed from glycosides by bacterial β-glucosidases during fermentation (*132-135*). Studies have shown that total isoflavone contents and compositions in soybeans and soy products differ from a variety of cultivars, growth in environmental condition, and food processing methods (*117, 126, 128, 136-138*). Recently, studies have reported that the isoflavone aglycones were considerably increased by pretreatment of soybean, and their composition was changed during heat treatments such as cooking, roasting, boiling, steaming, and pressure steaming processing (*139-141*). Furthermore, as a group of natural flavonoids, soy isoflavones in soybean and soy products have also exhibited antioxidant activities *in vitro* and *in vivo* by inhibiting lipid peroxidation (*120, 142*), LDL oxidation (*143-145*), scavenging free radicals (*118, 145-147*), and promoting the expression of antioxidant enzymes (*118, 148*). Consumption of soy isoflavone-rich food has been shown to have preventive properties against cardiovascular diseases (*149, 150*), diabetes and obesity (*116, 151*). In addition to antioxidant capacities and preventive effects of isoflavones on chronic diseases, soy isoflavones have similar structures to estrogens, and, therefore, have exerted beneficial effects on hormone-related cancers such as breast and prostate cancers and bone health in postmenopausal women (*114, 152, 153*).

- Total soybean antioxidants

Unlike the investigation of isoflavones in soybean, few studies have reported the composition of soybean antioxidants and their antioxidant activities (*119, 124-126*). Plant foods such as soybeans, have been known to contain a large variety of phenolic compounds, including simple phenols, benzoic acid derivatives, flavonoids, tannins, and lignans (*154*). Soybeans have been investigated as a food source of polyphenols including tannins, proanthocyanidins,

anthocyanin, flavonoids (majority of isoflavone), and phenolic compounds such as chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid (*124, 155-157*). Similar to isoflavone contents in soybean, the content of soy antioxidants is generally influenced by environmental factors such as location, weather, harvested year and cultivar (*156*). The contents and compositions of both phenolic compounds and isoflavones are also affected by storage time, germination of seed, and processing methods (*123, 158, 159*). Recently, Xu and Chang compared the antioxidant activities of soybeans with different extraction solvents and thermal processing (*141, 160*). For instance, among the solvents tested, 50% aqueous acetone yielded significantly higher total phenolic contents in soybean extracts than other solvents examined. Moreover, pressure steaming provided significant increases in measured total phenolic contents, condensed tannin content, ORAC, FRAP, and DPPH$^{\bullet}$ values in soybeans.

Several *in vivo* and *in vitro* studies have shown that antioxidant extracts from soybean seed and soy products increased the activation of antioxidant enzymes including superoxide dismutase and catalase (*122*) and reduced LDL oxidation (*116, 145*). Total phenolic contents are generally evaluated in most studies of the antioxidant activities in soybean extracts. Furthermore, soybean extracts have shown to exert oxygen radical absorbance capacity (ORAC), ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activities (*123, 124, 126, 161*). Ekor et al. reported that soybean polyphenolic extracts protect the kidney against gentamicin-induced nephropathy using an *in vivo* rat model (*162*). These studies indicate that other natural antioxidants beside isoflavones may be involved in soy-based disease prevention and health promotion.

Bioaccessibility and bioavailability of soybean antioxidants

Three main factors are important when considering potential antioxidant activities of dietary bioactive compounds. The first concern is that after food consumption the bioactive compounds can be released from the food matrix and become bioaccessible and then are absorbed by intestinal cells. The second concern is that antioxidant activities of compounds are stable during the digestion process and persist in plasma after being absorbed into the body. The third concern is that the derivatives of the compounds after metabolic pathways are still bioactive (still have an antioxidant capacity).

There are numerous studies that have investigated the bioavailability of polyphenols using *in vitro* and *in vivo* models (*78, 97, 99*), but few reports have examined the bioaccessibility of polyphenols. Recently, the bioaccessibility and potential antioxidant activities of polyphenols from various foods such as olive oil (*163*), red wine (*89*), pomegranate juice (*87*), black raspberry (*101*), grape (*82*), soy bread (*86*), and wholegrain foods (*88*) have been reported. Similarly, many studies have examined bioavailability of soy antioxidants (isoflavones) *in vitro* and *in vivo*, but bioaccessibility of soy antioxidants has not been widely studied. Most studies have focused on the bioavailability of soy isoflavone aglycone forms such as genistein and daidzein in human subjects, since these two aglycones are found in relatively high concentration in plasma (*99, 164-166*). These studies have supported the idea that the absorption of soy isoflavone aglycones is faster than their glycoside forms. When comparing aglycones, the plasma concentration of genistein is higher than daidzein after soy intake. For instance, Izumi et al. found that the plasma concentration of aglycones was more than 100% higher than of glycosides after soy product ingestion. Within the aglycones, the concentration of genistein was also 5 times higher than daidzein in plasma at similar levels of intake (*167*). Cassidy et al. supported Izumi et al. when they reported the concentration of genistein in serum was greater than daidzein (*168*). They also reported that the bioavailability of soy isoflavones in humans was influenced by factors such as physiological relevant intakes, gender-, and age-related differences. The absorption of daidzein was faster in women than men, and the absorption of daidzein and genistein from soy milk was faster than from tempeh or textured vegetable protein (*168*). The concentrations of isoflavones in plasma are generally less than 10 μmol/L (*99*). The isoflavone aglycone forms are generated by microbial activity in the large intestinal where the aglycones are absorbed (*169*). Recent reports have suggested that the small intestine may be involved in the conversion of isoflavone glycosides to aglycones in rat small intestinal tissue (*170*). However, there is limited information of the bioaccessibility of soybean antioxidants.

The isoflavones from a soy-based bread were stable during *in vitro* gastrointestinal digestion. Walsh et al reported that the higher solubility of daidzein made more accessible than genistein for potential absorption from the aqueous fraction of digesta, which is bioaccessible in the small intestine (*86*). Information of the stability and bioaccessibility of soy antioxidants including isoflavones is still not complete.

Soybean and health benefits

Soybean intake has been receiving increasing interest due to potential health benefits, such as lowering the risk of chronic diseases such as heart diseases and cancers, especially breast and prostate cancers, osteoporosis, and diabetes (*114, 115, 171-175*). There is the shift in focus from examining the biological activity of soybean protein to specific phenolic compounds of soybean, such as isoflavones, since health benefits have been more closely linked to the biological activities of isoflavones (*149*).

- Cardiovascular diseases

Dietary soybean appears to protected against coronary heart disease by reducing blood lipids, oxidized LDL, and blood pressure (*143*). Soy consumption has been linked to a reduced risk of heart disease, according to numerous epidemiological studies. In 1999, the FDA approved a health claim where soy protein may attenuate coronary heart disease risk because of the hypocholesterolemic effect with soy protein (*69*). In addition, soybean isoflavones are believed to be largely responsible for the health benefits assigned to soy protein because soy protein isolates typically contain isoflavones (*176*). Taku et al. reported that soy isoflavone significantly decreased serum total and LDL cholesterol without changing HDL cholesterol or triacylglycerol (*171*). Numerous studies have demonstrated the cardio-protective activities of soybean even though it is unclear whether the mechanisms for the hypocholesterolemic effects of soy products result from soy protein itself, bioactive components of soy, or the combination of the two (*113, 149, 150, 177*). Some studies have suggested that soy protein as well as other components (without isoflavones) may lower serum cholesterol (*150, 177*), while other studies reported that the improvement of serum lipid profiles following chronic intake of soy may be due to the isoflavone content of soy products consumed (*149*). In a study on the effect of soy protein containing enriched or depleted isoflavones on the lipid profiles, it was reported that both soy proteins with or without isoflavones significantly lowered LDL cholesterol, and soy protein containing isoflavones increased HDL cholesterol (*171*). In spite of recent results from meta-analysis indicating more modest effects than initial reports on the effects of soybean on heart diseases, Menissa and Lane concluded that soy foods can help decrease the mean serum

cholesterol levels of populations and may reduce vascular reactivity because of isoflavones (*172*).

- Cancer

The consumption of whole-grain cereals has been linked to a reduced risk of various cancers (*178*), and the consumption of soybean, a dietary source of phytoestrogens (isoflavones), has also been associated with lower cancer risks (*37*). Epidemiological studies have shown that soy products, rich in isoflavones, have a protective role against hormone-related cancers such as breast and prostate cancers (*112, 114*). In the past 20 years the high soy consumption has been associated with the lowered incidence of breast cancer. Epidemiological studies indicate that soy consumption by Asians is associated with a lowered risk of breast cancer (*112*). Yamamoto et al. also reported that the consumption of miso, which is a fermented soybean paste rich in isoflavones, was associated with a reduced risk of breast cancer in Japan (*179*). Indeed, many studies show that the intake of soybean products is associated to a decreased risk of breast cancer, and suggests a potential beneficial effect of soybean products in the prevention of breast cancer (*180-182*). However, Suzuki et al. reported that a reduced risk was observed only in patients with specific tumor lines, and indicated that the protective role of soy against breast cancer risk depends on the behavior of breast cancer with different estrogen receptor status (*152*).

The relation between soy consumption and prostate cancer has been investigated by many researchers. Unlike the effect of soy consumption on the risk of breast cancer, an initial study on the consumption of soy products rich in genistein, an isoflavone aglycone, showed a slight protective effect on prostate cancer, but this was not statistically significant (*183*). However, Yan and Spitznagel recently conducted a meta-analysis and concluded that soy food consumption is associated with a reduced risk of prostate cancer in men. They also suggested that the quality and quantity of soy food intake may be related to protect effects against prostate cancer (*114*). However, epidemiological studies on the association between the soybean intake and incidence of cancer are inconclusive (*112, 184*).

- Bone health

Soy foods and isoflavones have been shown to have a potential role in bone health, particularly in postmenopausal women (*115, 185*). Numerous epidemiological studies have demonstrated a relationship between the incidence of osteoporosis and chronic intake of soy product or isoflavones. The effect of isoflavones or isoflavone-rich soy protein on bone mineral density was evaluated, and suggested that soy intake for more than 6 months exerted beneficial effects by attenuating bone loss in postmenopausal women (*186, 187*). Ye et al. indicated that daily supplementation of isoflavones for 6 months decreased bone loss in postmenopausal women with a dose dependent effect (*188*). Recent studies have focused on the effect of soy product consumption on bone health in menopausal or young women. Daily consumption of isoflavones (>90mg) for 6 months may be enough to provide beneficial effects on spine bones in menopausal women (*189*). Soybean and isoflavone consumption also have a positive effect on bone mass density in young women (*190*). However, not all studies confirmed the beneficial effect of isoflavone on bone health (*185, 191*) because the beneficial effects of soy isoflavones on bone may be life-stage specific and dependent on the estrogen receptor numbers in individuals. Further studies in humans are required to support the beneficial effect of soy, soy products, or soy isoflavone consumption on bone health in women (*185*).

- Diabetes

Studies in humans and rodent models have supported the potential benefits of soy or soy isoflavones in diabetes (*116, 151, 192-195*). Villegas et al. established the relationship between the soy food or soy protein consumption and the incidence of type 2 diabetes in women. In this study, they suggested that soy product intake was associated with reducing the risk of type 2 diabetes in women (*175*). Furthermore, dietary supplementation with soy protein and soy isoflavones positively affected insulin resistance, glycemic control, and serum lipoproteins in postmenopausal women with type 2 diabetes (*116*). However, the protective mechanisms are still unclear and the long term effects of soy isoflavones on diabetes in human should be the subject of further investigation (*151*).

Extraction methodologies for antioxidants from plant foods

Antioxidants should be first extracted from food materials before their commercial uses in food or nutraceutical industries. The antioxidant activities of the extracts from food materials or food can be affected by various factors such as cultivars growth environment (climate, soil, and irrigation), methods of food manufacturing, and even the efficiency of extraction methodology (*160, 196, 197*). A variety of extraction process have been employed for antioxidant extraction. Food antioxidant extraction methods have employed classical methods such as soxhlet (*198*) and aqueous solvent extractions (*160*) to novel extraction methods such as ultrasonic-assisted extraction (UAE) (*196*) and microwave-assisted extraction (MAE) (*199*). These extraction methods have been compared based on extraction time, temperature, solvents and yield (*200, 201*).

Conventional extraction

- Soxhlet extraction

Soxhlet extraction is a traditional method for the extraction of bioactive compounds from plant or food materials. Different solvent systems yield different antioxidant extracts and extract compositions. Absolute ethanol, methanol, or mixtures with water have commonly been used for extracting antioxidants from plant foods (*198, 202, 203*). For example, absolute ethanol extraction with a soxhlet extractor was used to extract antioxidant from wheat bran (*198*). Absolute methanol was used for extracting isoflavones from spices (*202*). Various optimal extraction time periods have been reported for extracting food antioxidants, ranging from 2 to 12 hours (*198, 203, 204*). Soybean isoflavones were extracted using a soxhlet extractor for 3 hours (*204*). Although soxhlet extraction is cheap, very simple, and good for repeatedly using fresh solvent in contact with the food material, it has well known disadvantages such as large amounts of solvent usage and a long time of extraction (*201*).

- Aqueous solvent extraction

This solvent extraction has been used to extract antioxidants from fruit or plant foods. Similar to soxhlet extraction, solvent, temperature, and time are crucial factors and contribute to

extraction effectiveness. Different solvent systems have been used to extract antioxidants from plant food materials. Acetone, ethanol, and methanol combined with water are usually used to extract antioxidants in plant foods. Antioxidants in several fruits including mango, strawberry, and tomato have been extracted using 50% aqueous methanol or 70% aqueous acetone for 1 hour at ambient temperature (*205*). For phenolic compound extraction from legumes, 50% aqueous acetone, 70% aqueous ethanol, and 70% aqueous methanol have used to extract antioxidants for 15 hours at room temperature (*160*). Aqueous ethanol at 80% concentration was used for wheat antioxidant extraction at 4 °C for 16 hours (*206*). This solvent system method is commonly used for phytochemical extractions from plant foods because it is cheap, safe, and requires less solvent usage than soxhlet extraction. Many researchers are still interested in the optimization of solvent systems used to extract antioxidants from different plant foods.

Ultrasonic-assisted extraction

Ultrasonic-treatment has been applied to the extraction of antioxidants from foods due to its efficiency: short extraction time and higher yield of desired compounds. Generally, there are two types of ultrasonic assisted extractors including ultrasonic baths or closed extractors equipped with an ultrasonic probe. Ultrasound can be induced to penetrate from the solvent into the food materials. Biological cell walls of plant foods dissolved in solvent systems can be disrupted by ultrasound during the extraction and, therefore, desired components can be released from cells and transferred into the solvent system (*207*). Solvent systems, extraction time, and temperature may be modified with UAE to improve antioxidant extraction from plant foods. Ultrasonic assisted extraction is reportedly effective at extracting antioxidants from plant foods such as *Rosemarinus officinalis* (*208*), citrus peel (*209*), wheat bran (*210, 211*), barley (*196*), bean (*212*), and soybean (*213*). Although contributing to an efficient extraction of antioxidants, ultrasound also produces heat, so extraction temperature and time should be controlled to avoid degradation of the extracts. Many researchers have studied the optimal conditions to extract antioxidants from plant food materials. Wang et al. reported that the optimal conditions for antioxidant extraction from wheat bran were 64% of aqueous ethanol at 60 °C for 25 minutes (*211*). Hromadkova et al. suggested that a shorter time of extraction, up to 10 minutes, was optimal for extracting antioxidants from wheat bran (*210*). UAE is a relatively simple and inexpensive as well as efficient alternative method to conventional extraction. However,

characteristics of plant materials such as phase dispersion can influence the extraction capacity of UAE.

Microwave-assisted extraction

Microwave-assisted extraction (MAE) provides a rapid transport of energy to a total volume of solvent and solid plant food matrix while heating the solvent and food matrix, efficiently and homogeneously (*201*). Microwaves can penetrate food materials (solid) and interact with polar molecule such as water to generate heat. Microwaves, consequently, can heat a whole material and desired molecules can be released from the food matrix into the extraction solvent. MAE can be operated under controlled extraction time and temperature to avoid degradation of compounds of interest. In order to obtain better yield of antioxidants, selection of proper solvents is also important for extraction of antioxidants from plant foods (*201*). Recent studies have shown that the MAE was efficient for extracting antioxidants from plant foods such as asparagus, wheat bran, and common beans. For example, Sun et al. reported that microwave sterilization increased the antioxidant activities of asparagus (*214*). MAE with absolute methanol for 20 minutes significantly increased the antioxidant activities of wheat bran (*199*). Sutivisedsak et al. reported that MAE with 50% aqueous ethanol for 15 minutes effectively extracted antioxidants from common beans, *Phaseolus rulgaris* L. (*215*). Furthermore, absolute ethanol MAE for 20 minutes yielded maximum antioxidant capacities of *Hippophae rhamnoides* seed extracts (*216*). Like UAE, cost and time efficiencies of MAE provide advantages for extracting nutraceuticals from plants or foods. In addition to economical aspects, MAE can be a strong novel extraction method for the nutraceutical extraction.

Extraction of soybean antioxidants

Antioxidants of soybean or soy based food can be extracted by aqueous solvent extraction or with USE with aqueous solvents. Various concentrations of aqueous solvent systems have been used for soy antioxidant extraction. In addition to a variety of solvent systems, time, temperature, and physical conditions have been applied for extraction of soybean antioxidants. Xu and Chang recently compared several concentrations of solvent solutions including 50%, 80%, and 70% acidified aqueous acetone, 80% aqueous methanol, and 70% and

absolute ethanol (*124*). After vigorous shaking a mixture of aqueous solvent and finely ground soybean, antioxidants were further extracted at room temperature for additional 12 hours. In this study, they found that 50% aqueous acetone provided the highest yield of total phenolics from the soybean extracts. With this information, current studies have been using 50% aqueous acetone extraction method for antioxidant extraction from soybean seeds (*126, 141, 217*). In addition to antioxidant extraction from soybean seeds, phenolic compounds from fermented soybean were extracted using different temperatures and ratios of a mixture of ethanol and water. Wardhani et al. found that 74.2 % aqueous ethanol at 65.3C provided the optimal extraction system for fermented soybean antioxidants, recovering 56.2mg of GAE/g (*218*). Besides simple aqueous solvent extraction methods, UAE with different solvent systems has also been used for extraction of soybean antioxidants as well as a pretreatment procedure for antioxidant extraction. Takahashi et al. extracted soybean antioxidants with an acidified methanol solution for 24 hours at 4°C after a pretreatment for 20 min using UAE (*213*). An ultrasonic bath was also used to extract soy polyphenols with 70% aqueous acetone for 20 minutes (*155, 219*). However, there is little information on the comparison of different extraction methods for soybean antioxidants.

Isoflavones, major antioxidants in soybean, have been extracted using similar procedures such as aqueous solvent extraction or UAE. For aqueous solvent extraction methods, different concentrations of ethanol and acidified aqueous acetonitrile are generally used to extract isoflavones from soybean. When comparing acetonitrile extraction and other solvents, an aqueous acetonitrile extraction was shown to increase the yield of isoflavone from soybean (*220, 221*). Aqueous acetonitrile-HCl at room temperature are often used for extraction of isoflavones (*156, 221*). Sometimes, the extraction can be conducted by boiling (*159*). Extraction time ranges from 2 to 10 hours (*220-222*). Isoflavone extraction has also been conducted by using aqueous ethanolic, methanolic, and acetonitrile with or without UAE with varying time and temperature. Lee and Row investigated extraction time, solvent concentration, and temperature effects on the yield of isoflavones, and found that extraction with 60% aqueous ethanol for 10 min at 40C with a frequency of 20 KHz provided improvements in total glycoside and aglycone isoflavone extraction from soybean (*223*). As a sole mean of isoflavone extraction, 20 min sonication with 50% aqueous ethanol at 60°C enhanced total isoflavone extraction from soybean (*222*). Solvent extractions and UAE methods have been compared to enhance the isoflavone extraction. Several solvent compositions (i.e., 80% aqueous acetonitrile- 0.1N HCl, 80%

aqueous methanol, and 80% aqueous ethanol) were used for solvent extraction. A remarkably improved extraction of total isoflavones of soybean was obtained by using 80% aqueous acetonitrile-0.1N HCl for 10 hours extraction after 15 minutes sonication (*220*).

Measurement of *in vitro* antioxidant capacities in food materials

In the past years, antioxidants protective role in the pathogenesis of several human diseases including cardiovascular diseases, cancers, and neurodegenerative diseases generated from reactive oxygen species (ROS) has been studied (*30, 224*). Researchers require a rapid means to determine the antioxidant activities of compounds extracted from foods, fruits, vegetables, medicinal plants, or biological samples (*225*). There are various types of assays for the determination of antioxidant capacities. These assays can be divided into two main types, scavenging capacity assays against specific ROS and scavenging capacity assays against stable, non-biological radicals. In order to determine the antioxidant activities of fruit, vegetables, and food materials, there are several assays that are often used: total phenolic contents assay (TPC), oxygen radical absorbance capacity assay (ORAC), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging assay, and the ferric reducing antioxidant power (FRAP) assay. Of those assays, TPC and ORAC are most likely used together to evaluate the antioxidant activity of foods. In addition to TPC and ORAC, other specific assays are usually used to comprehensively study different antioxidants (*226*).

Total phenolic contents (TPC) assay

This is also called as Folin-Ciocalteu (FC) reducing capacity assay or TPC assay. This assay is most commonly used to quantify the total phenolic contents of food extracts. When existing phenolic compounds react, they are oxidized and the Folin-Ciocalteu (FC) reagent is reduced in the alkaline (base) solution and then a blue color is formed. The absorbance of the blue colored solution is detected spectrophotometrically at 750-765 mm. The quantification of phenolic contents is expressed as gallic acid equivalents (mg/L or g), using gallic acid as a reference. The FC reagent is known to be non specific to phenolic compounds because other non-phenolic compounds such as aromatic amines can also reduce the FC reagent (*227*). Therefore, the TPC assay is useful to quantify the total reducing capacity of antioxidants (*226*).

TPC assay is usually performed with other assays including DPPH•, TEAC, and ORAC assays to assess the antioxidant activities of samples. It has been reported that the TPC assay is highly correlated with the DPPH• assay in the measurement of antioxidant activities of beverages including red wine, tea infusion, fruit juices, and beer (*228*). Furthermore, a good correlation between the FC assay and ORAC was reported (*229*). Therefore, the TPC assay has been used to assess the antioxidant capacity of a variety of food samples including fruit and vegetables (*230, 231*), legumes including soybeans (*126, 157, 160*), and grains (*199, 211*). Although this assay is for hydrophilic compounds because it is conducted in aqueous solution, it is used for assessment of dietary antioxidant activity due to its simplicity and reproducibility.

Oxygen radical absorbance capacity ($ORAC_{FL}$)

This assay is an *in vitro* method for determination of peroxyl radical scavenging of sample extracts. Water soluble 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH), as a radical generator, causes the oxidation and generates peroxyl radical (ROO·). Peroxyl radical is scavenged by hydrogen atom transfer reactions of an antioxidant in sample extracts. In order to measure the ability of antioxidants to scavenge peroxyl radicals, the intensity of fluorescence decrease of AAPH is determined fluorometrically within 1-2 hours under reproducible and constant flux of peroxyl radicals at 37 °C (*232*). Recently, a high-throughput assay has been used with a microplate fluorescence reader using 96-well plates. It is necessary to control the temperature of buffer and plate at 37 °C since this assay is temperature sensitive (*225, 232*). The measurement of fluorescence decay during the reaction can be quantified based on net area under the curve (AUC) expressed as Trolox equivalents (μM/L or g). This AUC method to quantify antioxidant radical scavenging activity is applicable for antioxidants and sample extracts exhibiting both distinct lag phases and no lag phases. As mentioned above, the ORAC assay is mostly used to determine antioxidant activities in conjunction with the TPC assay. The ORAC assay has been used for evaluation of radical scavenging activity of antioxidants in various food extracts (*126, 136, 233-236*). It can be used for measuring the activity of both hydrophilic and lipophilic antioxidants (*232*).

DPPH• scavenging activity assay

This assay is one of the *in vitro* scavenging capacity assays using stable, non-biological radicals for the evaluation of total reducing capacity. The purple-colored radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is reduced by antioxidant compounds and consequently the solution color fades. During this reaction, the absorbance decrease is monitored spectrophotometrically at 515 – 528 nm for 30 min or until the absorbance is stable. The absorbance decrease can be expressed as % DPPH• remaining; Trolox equivalents (μM/L or g) can also be measured. In general, this assay is performed with a mixture of DPPH• solution and antioxidant extracts in methanol or ethanol. Recently, like ORAC, a high throughput method for DPPH• assay using a microplate spectrometric reader with 96-well plates was developed by Cheng et al. (*237*). This assay uses the relative % DPPH• scavenging capacity instead of % DPPH• remaining. The absorbance decrease is estimated based on AUC of % DPPH• quenched, and AUC is expressed as Trolox equivalents (mM/g) (*238*). The scavenging capacity against DPPH• radical is strongly influenced by the solvent and pH (*228*). In addition, the spectrometric measurements of the absorbance decrease can be affected by compound characteristics. For example, carotenoid absorbs at the wavelength of determination. Turbidity of some sample can also affect the measurements. Despite the drawbacks, the DPPH• radical method is widely used for screening and measuring antioxidant capacity of food materials (*126, 206, 235, 239-241*) since this assay is stable, rather simple and easy to perform (*225*).

Ferric reducing antioxidant power (FRAP) assay

FRAP assay measures spectrophotometrically the absorbance of a blue color generated by a reduction from the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe(III)-(TPTZ)_2]^{3+}$ to ferrous complex $[Fe(II)-(TPTZ)_2]^{2+}$ by antioxidants in acidic solution at 593 nm. In the FRAP method, the antioxidant activity is quantified using a ferrous ion standard or ascorbic acid as a reference (*225*). This method has also been adapted to a 96-well micro plate spectrophotometric reader, providing better reproducibility and higher sample throughput (*242*). The reaction time may depend on the antioxidants, since not all antioxidants reduce Fe(III) in the same time frame. For instance, Pulido et al. reported that dietary polyphenols need longer reaction times, perhaps as much as 30 minutes for total quantification (*243*). In this study, polyphenols with such behavior

included caffeic acid, ferulic acid, quercetin, and tannic acid. The FRAP data should be compared with the results obtained by radical scavenging methods such as ORAC because a strong correlation between FRAP and ORAC values is not always observed (*244, 245*). The FRAP method is inexpensive, simple, and useful to determine a putative index of antioxidant capacity. For these reasons, the FRAP assay is still used to evaluate the antioxidant activity of food samples (*205, 233, 240, 242*). (*58*)

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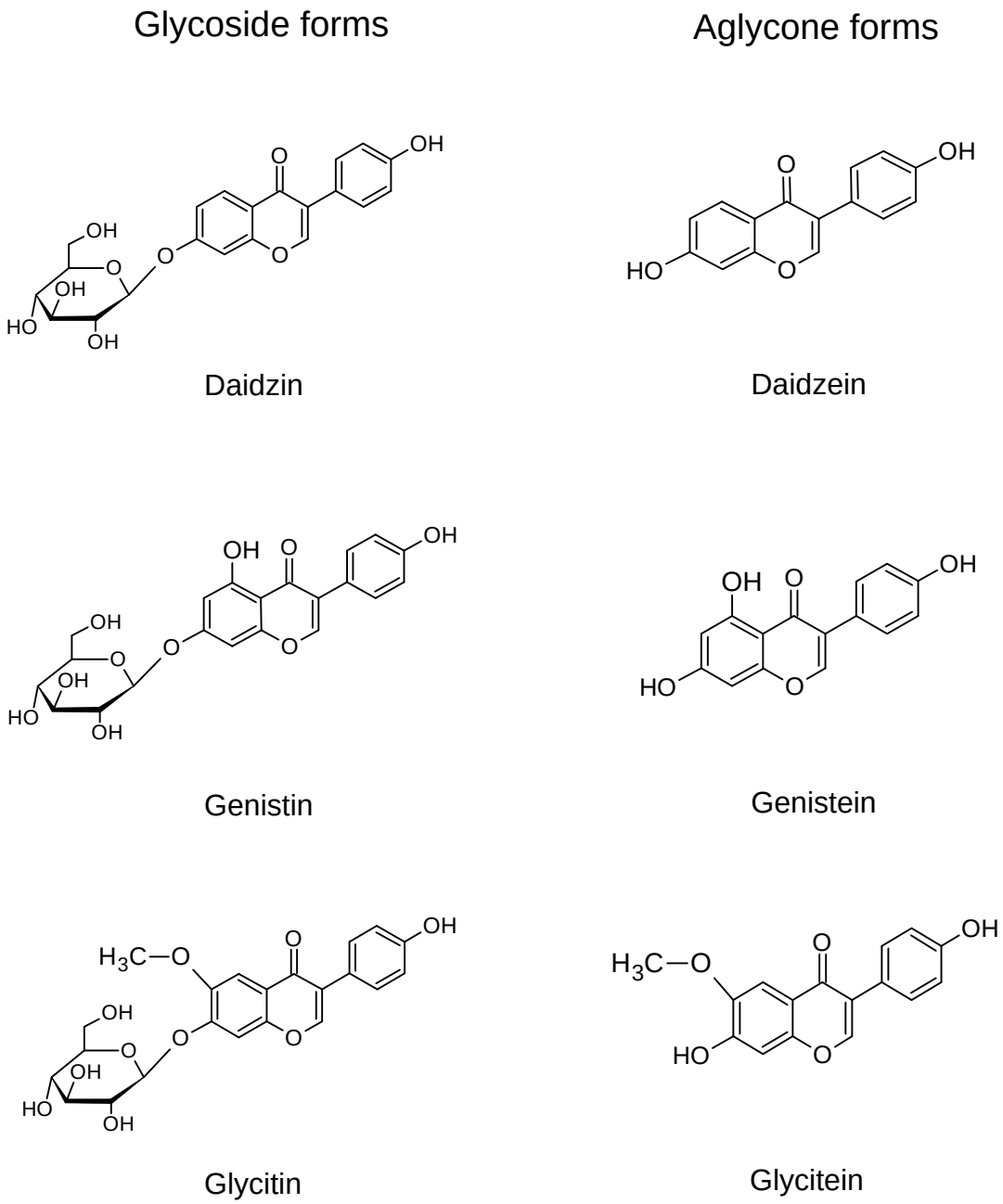

Figure 2.1. Structures of glycoside and aglycone forms of soy isoflavones.

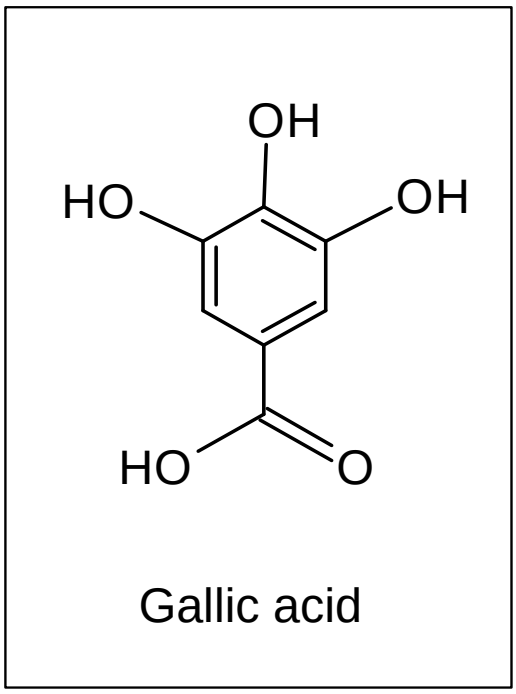

Figure 2.2. Structure of gallic acid as a standard for total phenolic contents assay.

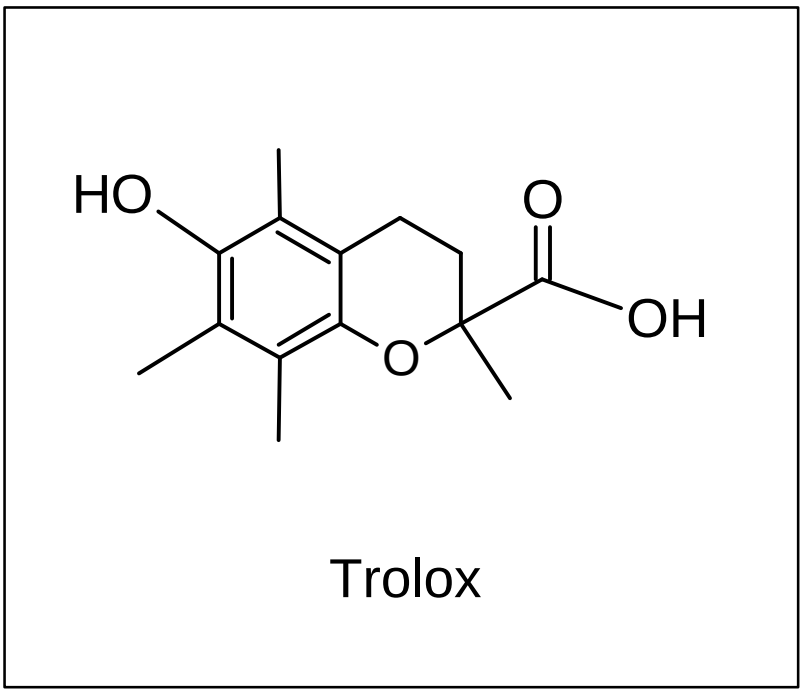

Figure 2.3. Structure of Trolox as a standard for ORAC and DPPH$^{\bullet}$ assays.

CHAPTER 3

Characterization and Comparison of Antioxidant Properties and Bioactive Components of Virginia Soybeans

ABSTRACT

Nine Virginia soybeans grown in a single location were evaluated and compared for their antioxidant properties and isoflavone profile. The total phenolic content (TPC) in the soybean extracts was significantly different among different genotypes. The V01-4937, V03-1144, and MFS-511 soybeans had the highest TPC values of 3.89, 3.63, and 3.53 mg gallic acid equivalents per gram seeds, respectively. The isoflavone composition was also different among the different soybean varieties. Malonylgenistin was the major isoflavone in all soybean seeds accounting for 75-83% of the total measured isoflavones. The V01-4937 variety had the highest total isoflavones and malonylgenistin content followed by the V03-5794. The antioxidant activities of the soybean extracts were also significantly different. The V01-4937 and Teejay showed the strongest ORAC values which were 70% higher than that of the V00-3493 soybean with the lowest ORAC value (115.7 μmoles Trolox equivalents/g seeds). However, their ORAC values were neither correlated with total phenolic content nor total isoflavone content. The MFS-511, V01-4937, and Teejay soybeans had the highest DPPH$^{\bullet}$ radical scavenging activity of 4.94, 4.78, and 4.64 mmoles Trolox equivalents/g seeds. Overall, the V01-4937 soybean was the variety which stood out the tested Virginia soybeans in regards to having the highest TPC, ORAC value, and isoflavone content as well as the second highest DPPH$^{\bullet}$ scavenging activity.

KEYWORDS: soybean antioxidant; isoflavones; TPC; ORAC

INTRODUCTION

Human consumption of soybean and soy-based products has been soaring recently, with a growth in sales from $300 million to $3.9 billion from 1992 to 2006 (*1*). This trend has been in line with the increasing volume of research linking soybean consumption with lower serum total and LDL cholesterol in humans (*2*), the reduced risk of certain types of cancers particularly prostate and breast cancer (*3*, *4*). The diverse potential health benefits of soybean consumption have prompted scientists to further investigate specific bioactive ingredients in the soybean. Soybean isoflavones have become one of the most investigated food functional ingredients with a wide variety of beneficial activities being revealed in *in vitro* and in clinical studies. Along with isoflavones, soybean antioxidants have also been receiving increased attention.

Soybean and soy products contain significant amount of isoflavones known as aglycones as genistein, glycitein, and daidzein and their glycosides as β-glucosides, 6″-O-acetyl- β-glucosides, and 6″-O-malonyl-β-glucosides. It has been postulated that the purported health benefits of soy products are in part due to isoflavone estrogenic activity or antioxidant activity (*5*). The structural similarities of soy isoflavones to estrogens make these bioactive compounds a unique group of phytoestrogens which may protect hormone-dependent cancers and produce immune effects by modulating activity of estrogen (*6*). Furthermore, as a group of natural flavonoids, soy isoflavones were also shown significant antioxidant activities by inhibiting lipid oxidation (*7*), scavenging free radicals, and promoting the expression of antioxidative enzymes (*8*). In addition to isoflavones, soybeans contain a number of other natural antioxidants such caffeic acid, chlorogenic acid, and ferulic acid, tannins, and proanthocyanidins (*9*). Soy antioxidant extracts were shown to reduce low density lipoprotein (LDL) oxidation and exert oxygen radical absorbance capacity (ORAC), ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$) free radical scavenging activity (*10*).

It was known that both soybean isoflavones and antioxidant properties can be significantly affected by their genotypes (*11, 12*). For instance, 14 Brazilian soybeans had more than three times variation on their isoflavone content and more than twice the difference on their total phenolics content (*13*). These findings suggested the possibility of identifying and developing premium soybean varieties rich isoflavones and natural antioxidants for human consumption with enhanced health benefits. Soybeans are Virginia's largest row crop with production of 15 million bushels in 2005, which contributed to over 85 million dollars to the

local agricultural economy. However, little is known about isoflavone composition and other beneficial components in Virginia soybeans as well as their associated antioxidant properties. The present study was undertaken to characterize isoflavone and phenolic content of 9 soybean varieties grown in Virginia and to provide information related to their antioxidant characteristics. This project is our continual effort towards the development of Virginia soybean cultivars with increased levels of isoflavones and/or natural antioxidants, which may potentially benefit Virginia soybean growers and local agricultural economy.

MATERIALS AND METHODS

Materials. The nine soybean varieties used in this experiment were grown in Warsaw, VA, by a soybean breeding project at Virginia Polytechnic Institute and State University and harvested in 2006. The MFS-511, V00-3493, and V01-4397 were small seeded varieties that could be used for food grade breeding. SS-516 is a large seeded food grade variety. Teejay and Hutcheson were varieties that have been used for more conventional soybean uses such as meal and oil. They are not considered food grade. Folin-Ciocalteu reagent, fluorescein (*14*), 2,2'-bipyridyl, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and phenolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO), and 2,2'-azobis(2-amino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals U.S.A. (Richmond, VA). Isoflavone standards (daidzin, genistin, malonylgenistin, daidzein, glycitein, genistein) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical or HPLC-grade.

Sample Preparation. Five grams of each soybean sample was ground to a fine powder using a micro-mill and extracted with 50 mL of 50% acetone for 15 hours by shaking at ambient temperature (*10*). The extracts were filtered with a 1.5µm filter paper and kept in the dark until analyses. The soybean extracts were then subjected to a variety of antioxidant evaluations. To determine the isoflavone composition, the milled soybean seeds were extracted by the mixture of 0.1 normal hydrochloric acid, acetonitrile and distilled water (2/7/3, v/v/v) (*15*). After the extraction, the solution was centrifuged and the supernatant was filtered and collected. The

filtration was then evaporated under the nitrogen gas. The residue was reconstituted in 1ml methanol and filtered with a 0.45μm disk filter prior to HPLC analysis.

Total Phenolic Content (TPC). The TPC of soybean extracts were determined using Folin-Ciocalteu reagent with gallic acid as phenolic standard (*16*). In brief, the appropriate dilutions of extracts were mixed with Folin-Ciocalteu reagent and 20% sodium carbonate (Na_2CO_3) at ambient temperature. After reaction incubation for 2 hours, the blue color developed in each assay mixture and the absorbance was recorded at 760 nm (Thermo Electron Corporation, Genesys 10-UV scanning, Madison, USA). The TPC value of the soybean extracts was expressed in micrograms of gallic acid equivalent (GAE) per gram of soybean (mg/g).

Oxygen Radical Absorbance Activity (ORAC). The $ORAC_{FL}$ assay was conducted to measure the peroxyl radical scavenging activity of soybean samples with Trolox as an antioxidant standard according to the method reported previously (*17*). In brief, a fluorescein stock solution (100 μM) in phosphate buffer (75mM, pH 7.4) was prepared and kept at 4° C in the dark. A fresh working fluorescein solution (100 nM) was prepared daily by diluting the stock solution in phosphate buffer. Two hundred microliter of the working fluorescein solution was added to each 40μL of sample or Trolox standard (a water soluble analogue of vitamin E) prepared in the phosphate buffer (20, 40, 80,100, and 200 μM) in a black 96-well plate and incubated for 20 minutes at 37°C. The assay was initiated by adding the peroxyl radical generator prepared in the phosphate buffer. Specifically, 35μL of 0.36 M 2,2'-Azobis-2-amidinopropane (AAPH) was added and the fluorescence was measured (λ_{ex} = 485 nm and λ_{em} = 535 nm) every minute using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) maintained at 37° C until the reading had declined to less than 5% of the initial reading. Standards and samples were run in triplicate. Results for ORAC were determined by using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The $ORAC_{FL}$ value of each soybean extract was expressed in micromoles of Trolox equivalents per gram sample (μmoles/g).

$DPPH^{\bullet}$ Scavenging Activity. Antioxidant activity of soybean samples was tested using the radical 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay with slight modification. This high-throughput assay, based on the reduction of the free radical $DPPH^{\bullet}$, was carried out using a

Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). To begin, the reaction mixture contained 100 μL of antioxidant soybean extracts and 100 μL of 0.2 mM DPPH$^{\bullet}$ solution. The absorption at 515 nm was determined immediately when the reaction was initiated by gentle shaking. Each plate was read once every minute for 1.5 hours. The relative DPPH$^{\bullet}$ scavenging capacities were expressed as micromoles of Trolox equivalents (TE) per gram of sample (mmoles/g).

HPLC Analysis of Isoflavone Composition. Isoflavone profile in the soybean extracts was performed on an Agilent 1200 quaternary HPLC system (Agilent Technologies, CA) equipped with a photodiode array detector. The isoflavone standards were separated on a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size 5μm) using a linear gradient elution program with a mobile phase containing solvent A (0.1% glacial acetic acid in H_2O) and solvent B (0.1% glacial acetic acid in acetonitrile) (*18*). The solvent gradient was linear programmed from 15 to 35% solvent B in 50 minutes with a flow rate of 1.0 mL/min. Identification of isoflavones in each soybean sample was accomplished by comparing the retention time and absorption spectra of peaks in the extracts to that of the standard compounds. Quantification of individual isoflavone was conducted using total area under each peak with external standards.

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations. The mean values within each test were compared by a two-sample *t*-test. Data is presented as mean ± SD (Standard Deviation). Significance of variety differences was determined by analysis of variance. Difference was considered statistically significant when the P value was <0.05. A two-tailed Pearson's correlation test was conducted to determine the correlations among means.

RESULTS AND DISCUSSION

Total Phenolic Content. Natural phenolic compounds have been receiving increased attention due largely to their notable antioxidant activities. The unique structures make the phenolic compounds inherently excellent electron or hydrogen donors, which enable them to readily stabilize some reactive oxygen species (ROS) (*19*). In fact, phenolic compounds have been shown to effectively inhibit lipid oxidation of low density lipoprotein (LDL), liposome, and

food model systems by interacting with transitional metals and free radicals such as peroxyl, hydroxyl, and superoxide radicals (*20, 21*).

Total phenolic content (TPC) of the extracts from selected soybeans was presented in Fig 3.1. The tested soybeans had a TPC range of 2.9-3.9 mg gallic acid equivalents (GAE)/g fresh weight with most falling between the range of 3.2-3.6 mg GAE/g. The TPC values were significantly different among different soybean varieties. In particular, the V01-4937 soybean had the highest TPC of 3.9 mg GAE/g, while the Hutcheson was the lowest with 2.9 mg GAE/g. This distinct difference may be attributable to their genetic variation as all the soybean samples were collected from a single growing location which minimized the environmental influence. Among the tested Virginia soybeans, the Hutcheson, V03-5794, and SS-516 varieties had the lowest TPC values that ranged between 2.9- 3.0 mg GAE/g. The V01-4937 had a exceptionally higher TPC than other eight varieties, indicating that this specific soybean may have unique genetic characteristics in favor of producing phenolic compounds. Overall, the TPC range of the tested Virginia soybeans was comparable to previously reported values determined in the seeds of 20 soybean hybrid (2.7-4.9 mg/g) (*9*), and in 6 yellow soybean seeds (3.0-4.5 mg/g) (*22*). Lin et al also reported that the black soybeans had significantly higher amount of total phenolic content than the yellow soybeans did (*22*).

Oxygen Radical Absorbance Activity (ORAC). ORAC measures the ability of the soybean extracts to scavenge peroxyl radicals generated in an aqueous solution. The ORAC values of the selected soybeans were expressed as μmoles Trolox equivalents (TE)/g (Fig 3.2). Different soybeans showed significantly different ORAC values which varied from 115.7-228.6 μmoles TE /g. The V01-4937 soybean had the highest value at 228.6 μmoles TE /g, the only soybean variety in our study higher than 200 μmoles TE/g. This exceptionally high ORAC value of the V01-4937 may be associated with its highest TPC among the tested varieties. However, there was no significant correlation between the ORAC and TPC of the soybeans in our experiments. The V00-3493 soybean had the lowest ORAC value (115.7 μmoles TE /g), less than half of that of the V01-4937. Other soybeans with significantly lower ORAC values were the V03-1144, SS-516, NC Roy, and Hutcheson varieties in 136.7, 137.3, 142.5, and 149.0 μmoles TE/g, respectively. The difference of ORAC values strongly suggests that soybean varieties may significantly affect their antioxidant activities against peroxyl radicals. Current reports on the ORAC data of soybean seeds are scarce. Xu and Chang recently reported a range

of 40.81 to 86.84 μmol TE/g in the various extracts of a yellow soybean (*23*). However, our ORAC results for the Virginia soybeans were considerably higher; this may be due to the different sample preparations as well as the effect of soybean varieties and growing environment.

DPPH• Scavenging Activity. Other than ORAC which measures antioxidant activity based on hydrogen transfer mechanisms, DPPH• assay involves electron transfer mechanisms (*24*). This explains that the higher ORAC values of the samples did not necessarily suggest the stronger DPPH• scavenging activity. Soybean extracts and derived compounds have been shown to be effective scavengers of DPPH• radicals (*5, 9*). For instance, Takahashi et al reported that the IC50 of soybean polyphenols for DPPH• radicals were 39 and 34 μg/g for yellow soybean and black soybean, respectively (*25*). The DPPH• results of our selected soybeans were expressed as mmoles Trolox equivalents (TE)/g (Fig 3.3). The highest DPPH• scavenging activity was observed on the MFS-511 (4.9 mmoles TE /g), followed by the V01-4937 (4.8 mmoles TE /g), and the Teejay (4.6 mmoles TE /g). Other soybeans were in the range of 4.0-4.4 mmoles TE /g except for two varieties: NC Roy and V03-1144, which had remarkably lower DPPH• values of 2.9 and 3.0 mmoles TE /g, respectively. This range was higher than that of the yellow soybean extracts (0.6- 2.0 mmoles TE /g) and significantly lower than that of the black soybean extracts (7.1-17.9 mmoles TE /g) (*10*). The DPPH•scavenging activities of the soybeans were neither significantly corrected with ORAC nor with TPC values even though the V01-4937, MFS-511, and Teejay were the top three varieties identified in both ORAC and DPPH• experiments. These results suggest that the V01-4937, MFS-511, and Teejay varieties may potentially provide enhanced health benefits as a result of their stronger antioxidant activities as compared to the other Virginia soybean varieties. Overall, further investigation into the individual soybean antioxidants and their bioactivities is warranted.

HPLC Analysis of Isoflavone Composition. Isoflavones belong to a class of plant compounds called phytoestrogens, which exhibited both estrogenic and antiestrogenic properties in both cell and animal models (*26, 27*). Isoflavones are also a group of flavonoids that showed potent antioxidant properties. The unique chemical and functional properties of isoflavones have widely stimulated the research on their potential health benefits. In fact, isoflavones have been associated with the protection of a wide variety of chronic diseases and hormone related complications such as atherosclerosis (*28*), breast cancer (*29*), osteoporosis, and menopausal

symptoms (*30*). Isoflavones are being marketed as dietary supplements and the main dietary sources of isoflavones are soybeans and soy based products. However, there is a large variability in isoflavone concentration and profile among the soybeans depending on factors such as their genotypes and environmental conditions.

The isoflavone compositions of the selected soybeans are presented in Table 3. 1. The total measured isoflavones were in the range of 2495.8 – 3205.1 μg/g which is comparable to that of 1563 – 3309 μg/g reported in 8 American and 3 Japanese soybeans (*31*), and to that of 1443.1 - 3803.6 μg/g detected in 17 Ohio soybeans (*5*). The soybean V01-4937 was found to have the highest total isoflavone content followed by V03-5794 (3127.4 μg/g) and V03-1144 (2931.7μg/g), whereas the lowest isoflavone content was observed from the soybeans SS-516 (2495.8 μg/g), Teejay (2570.1 μg/g), and Hutcheson (2566.3 μg/g). The isoflavones variation among 11 soybean varieties was relatively smaller than the findings of Kirakosyan et al who reported that total isoflavones in 2 American varieties (Cisne and Ripley) and 3 from China varied from 425-6115 μg/g (*32*). Both results suggest that soybean genotypes played an important role on the total isoflavone content in the seeds. The isoflavone profile was also different among the different soybeans. Malonylgenistin was the major isoflavone determined in all the soybean samples, representing 75-84% of the total isoflavones, followed by genistin (6-13%) and daidzin (5-10%), respectively. Daidzein and genistein were detected in very low amounts. This finding indicated that most soy isoflavones exist in the seeds in the form of glucosides instead of aglycones (*33*). These results were in agreement with previous reports that malonylgenistin was the predominant isoflavone in soybean seeds (*11, 12, 31*). Similar to the total isoflavones, the amounts of the individual isoflavones were also found to be significantly different among the soybeans. For instance, the V01-4937 soybean contained the highest malonylgenistin content (2539.8 μg/g), which was 27% and 23% higher than the NC Roy and SS-516, respectively. Such significant difference was also reflected in the amounts of genistin and daidzin. The malonylgenistin content in the tested Virginia soybeans was significantly higher than that of 11 soybeans grown in the state of Iowa (290-958 μg/g) (*31*), but comparable to 17 Ohio soybeans (1213.1-3048.4) (*5*). These differences may be attributable to the soybean varietal difference, growing conditions or even sample treatment procedures. In brief, our results suggested that the V01-4937 and V03-5794 soybean could be recommended as potential Virginia varieties with significantly higher isoflavone content.

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Table 3.1. Isoflaovone composition of Virginia soybean samples

Variety	Daidzin (μg/g)	Genistin (μg/g)	Malonylgenistin (μg/g)	Daidzein (μg/g)	Genistein (μg/g)	Total (μg/g)	Malonygenistin/total (%)
NC Roy	275.2e ± 5.5	301.1e ± 2.4	1998.7a ± 12.8	33.4d ± 0.9	27.0f ± 0.5	2635.4	75.8
V03-1144	268.5e ± 12.8	376.2g ± 9.2	2225.8c ± 88.0	32.1c,d ± 0.6	29.1g ± 0.0	2931.7	75.9
MFS-511	231.9e ± 2.2	179.8a ± 0.7	2317.8d ± 0.0	42.7e ± 1.7	20.4d ± 1.4	2792.6	83.0
SS-516	212.4d ± 2.3	179.5a ± 8.6	2064.6a,b ±4.5	26.6a,b ± 0.8	12.7a ± 1.6	2495.8	82.7
V00-3493	194.4c ± 1.1	198.6b ± 9.6	2244.9c ± 36.8	33.9d ± 2.1	17.8b ± 0.2	2689.6	83.5
V01-4937	251f ± 2.4	340.8f ± 2.8	2539.8e ± 94.2	43.1e ± 0.6	30.4h ± 2.9	3205.1	79.2
Teejay	176.9b± 1.4	247.7c ± 26.5	2102.4b ± 98.7	26.5a,b ± 0.2	16.6b ±2.4	2570.1	81.8
Hutcheson	152.9a ± 6.9	269.3d ± 10.1	2093a,b ± 144.739	27.5b,c ± 3.5	23.6e ± 0.5	2566.3	81.6
V03-5794	275.8e ± 2.7	408.4f ± 2.5	2354.9d ± 50.1	50.2f ± 1.8	38.1i ± 4.0	3127.4	75.3

Entries in a column marked by the same letter are not significantly different ($P < 0.05$).

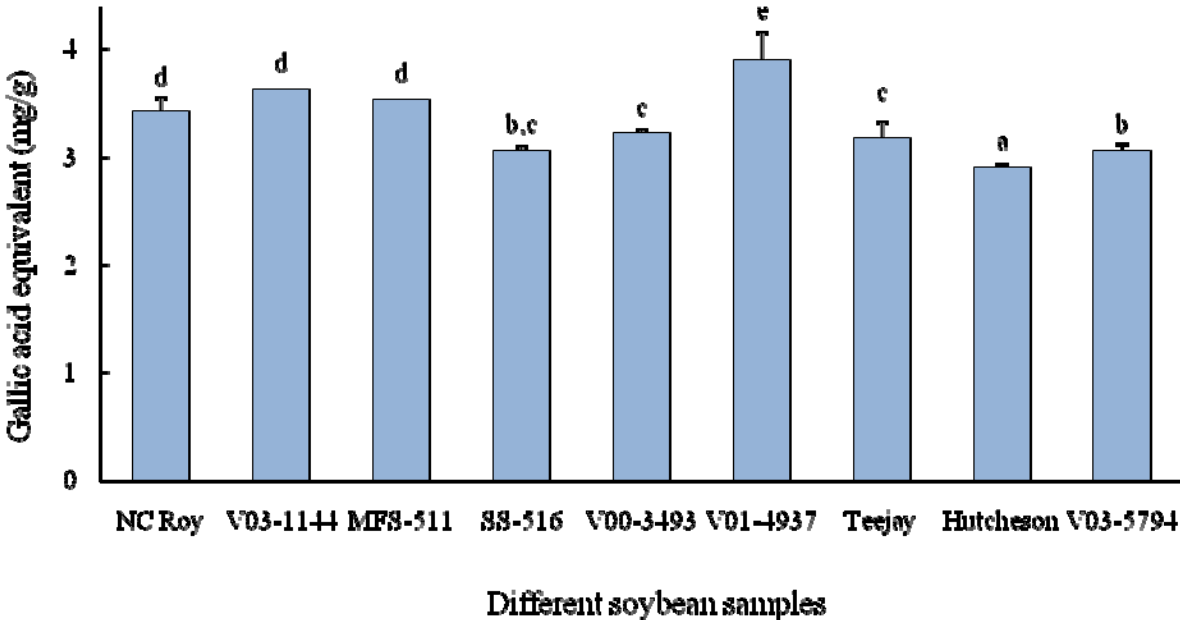

Figure 3.1. Total phenolic content (TPC) of Virginia soybean samples. Results are expressed as milligrams of gallic acid equivalents (GAE) per gram of soybean seed (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$)

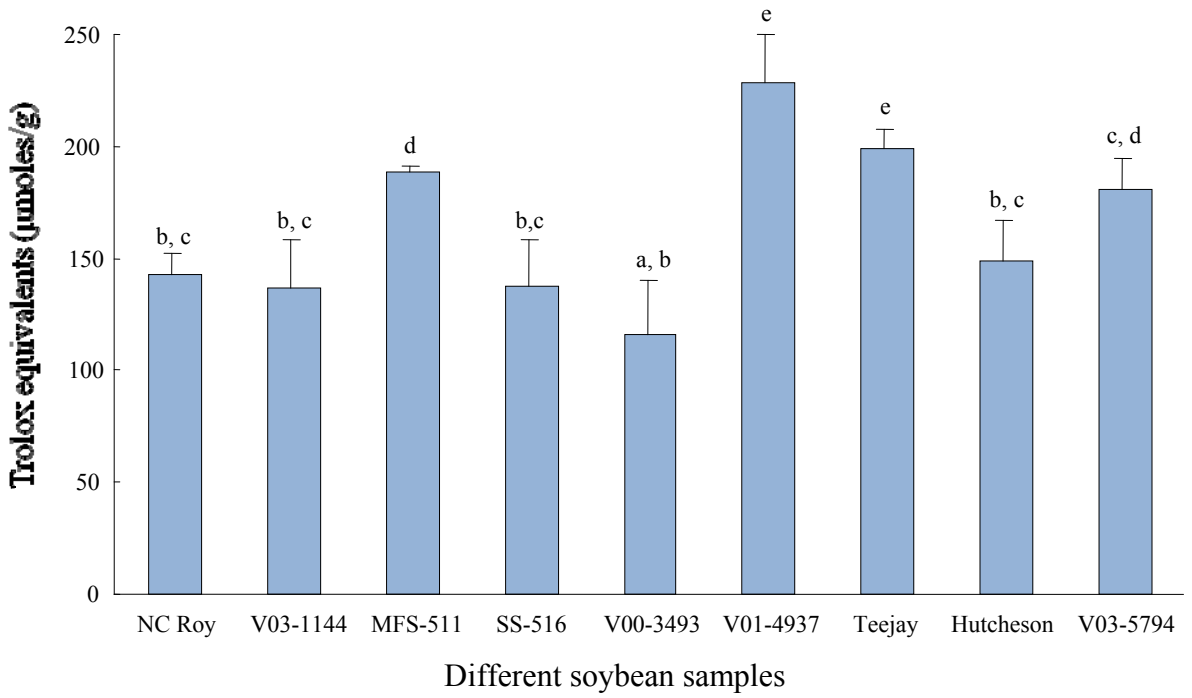

Figure 3.2. Oxygen radical absorbance capacity (ORAC) of Virginia soybean samples. Results are expressed as micromoles of Trolox equivalents (TE) per gram of soybean seed (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$)

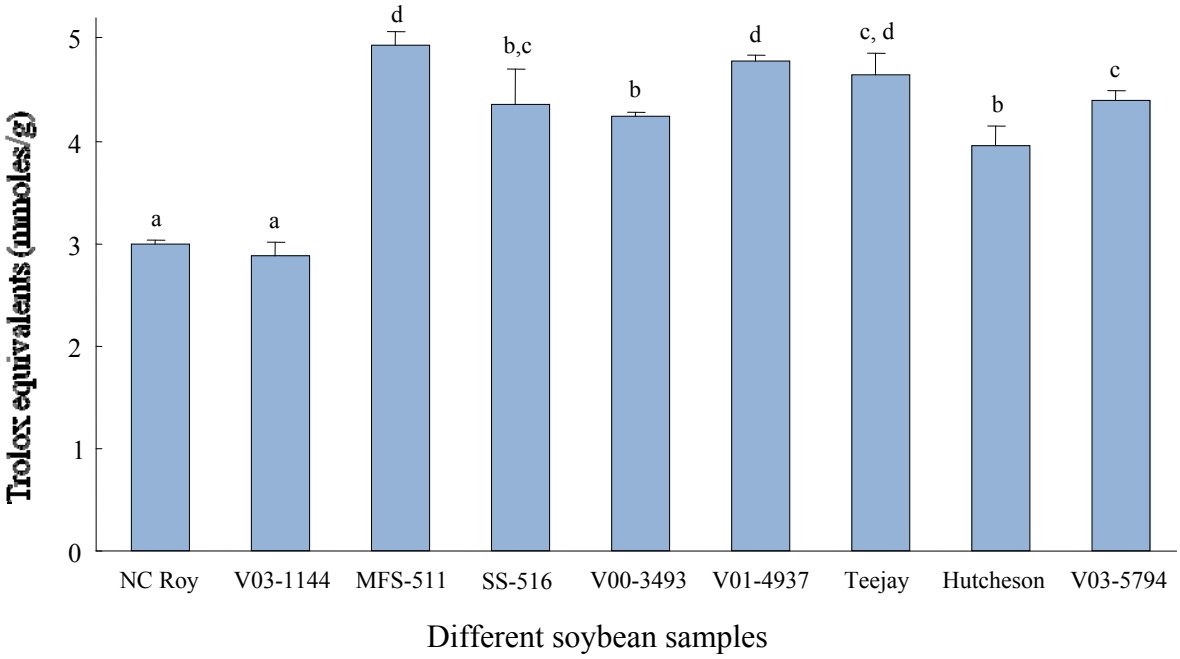

Figure 3.3. DPPH$^{\bullet}$ radical scavenging activity of Virginia soybean samples. Results are expressed as mmoles of TE per gram of soybean seed (mean ± SD, $n = 3$). Bars marked by the same letter are not significantly different ($P < 0.05$)

CHAPTER 4

Comparison of Different Extraction Strategies for Soybean Antioxidants

ABSTRACT

Different extraction strategies were investigated in search for efficient preparation methods for soybean antioxidants. The extraction techniques examined included soxhlet extraction, conventional solvent extraction, and ultrasonic-assisted extraction (UAE) with five common solvent systems including 50% and 80% aqueous acetone, 50 and 70% aqueous ethanol, and 80% aqueous methanol. Extracts were evaluated for their total phenolic contents (TPC), Oxygen Radical Absorbance Capacity (ORAC), and DPPH$^{\bullet}$ radical scavenging activities. The ultrasonic treatment improved the extraction of soybean phenolics by more than 50%. The UAE with 50% aqueous acetone was the most efficient solvent for extraction of phenolic compounds in the soybean seeds. However, the antioxidant activities of the soybean extracts were not correlated with their TPC. The conventional and UAE 70% aqueous ethanol extracts had the highest ORAC values, while the soxhlet methanol extracts had the highest DPPH$^{\bullet}$ radical scavenging activities. Our results suggest that different extraction technologies had a remarkable effect on soybean antioxidant estimation and that UAE is more appropriate for soybean phenolic extraction because it is less time – and solvent – consuming than convention solvent and soxhlet extractions.

KEYWORDS: Soybean; antioxidant activity; solvent extraction; ultrasonic-assisted extraction; TPC; ORAC; DPPH$^{\bullet}$

INTRODUCTION

High intake of soybean and soy-based products has been associated with lower levels of serum total and low density lipoprotein (LDL) cholesterol in humans and a reduced risk of certain types of cancers, particularly breast and prostate cancer (*1-3*). The potential health benefits of soybean consumption have prompted researchers to further investigate specific bioactive ingredients in the soybean, and many believe that natural antioxidants in soybean make significant health-promoting contributions. Indeed, soybean antioxidants have received increased attention due to a wide variety of beneficial activities demonstrated in both *in vitro* and *in vivo* studies, such as protection against oxidative modification of LDL (*4*), inhibition of lipid oxidation (*5, 6*), scavenging free radicals (*7, 8*), and promoting the expression of antioxidative enzymes in cells (*9*).

Soybeans contain a diverse number of natural antioxidants including isoflavones, also known as phytoestrogens, proanthocyanidins, and phenolic acids such as caffeic, chlorogenic, and ferulic acids (*10*). An appropriate extraction strategy is required in order to maximize extraction efficiency of natural antioxidants in soybeans. An efficient extraction method can be used by food and nutraceutical industries to extract antioxidants, or by researchers to further investigate antioxidants. The methods commonly used for antioxidant extraction in natural products include extraction with aqueous mixtures of different organic solvents (conventional extraction). For instance, 50% aqueous ethanol, 70% and 80% aqueous methanol, 50% and 80% aqueous acetone are frequently used for extraction of natural antioxidants in vegetables, fruits, cereals, and other food products (*11-16*). For soybean or legume antioxidant extraction, Xu and Chang have compared 6 different solvent mixtures and found that 50% acetone was the most efficient to extract phenolic compounds in soybeans (*7*). Other solvents used for soybean antioxidant extraction include 70% aqueous ethanol (*17*), 80% aqueous ethanol (*8*), acidified aqueous methanol (*6*), and 70% acidified aqueous acetonitrile (*18*).

In addition to conventional solvent extractions, some instrument-assisted techniques such as ultrasonic-assisted extraction (UAE) (*19, 20*) and microwave-assisted extraction (MAE) (*21, 22*) have been used for the extraction of natural antioxidants. UAE has now become a popular method for extracting antioxidants in food materials, herbs, and other natural products. For instance, aqueous ethanol extractions with ultrasonic treatment have widely been applied for the

antioxidant extraction from fruits, wheat bran, and plants (*14, 23-28*). Several studies have also reported the use of ultrasonic-assisted systems with different solvent mixtures such as 70% aqueous acetone, 40% and 60% aqueous ethanol for soybean antioxidants or isoflavones extraction (*6, 19, 20, 29, 30*).

Despite the increased application of UAE for soybean antioxidants, it is still not known whether UAE (with an appropriate solvent system) is more efficient than commonly used conventional solvent or soxhlet extractions. The ideal method would be the one that can maximize antioxidant extraction in soybeans with shortened time and reduced solvent consumption, while avoiding toxic solvents. The extraction methods play a crucial role in analysis of soybean antioxidants and their activities. Although several studies have compared different extraction strategies for soybean antioxidants or isoflavones, they have focused on solvent selection for soybean total antioxidants, but have not compared the extraction methods for all antioxidants (*7, 31, 32*). There is limited information on different extraction methods using the same solvent for the specific assay of soybean antioxidant activity. Therefore, the objective of this study was to evaluate the extraction efficiency of three different extraction strategies: conventional, ultrasonic-assisted, and soxhlet extraction with different solvent systems for the extraction of the soybean antioxidants. This study may lead to the development of an efficient extraction and preparation method for soybean antioxidants that may lay groundwork for further soybean nutraceutical development.

MATERIALS AND METHODS

Materials. Three soybean varieties, NC Roy, V00-3493, and V00-3636, were used in this experiment and were grown in Warsaw, VA, by a soybean breeding project at Virginia Polytechnic Institute and State University and harvested in 2006. Folin-Ciocalteu reagent, fluorescein (FL), 2,2'-bipyridyl, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and phenolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO), and 2,2'-azobis(2-amino-propane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. *Conventional solvent extraction.* Soybean seeds were ground to a fine powder using a Bel-Art micro-mill grinder. Each ground soybean sample (0.5g) was extracted twice with a constant volume (10 mL) using five different solvent mixtures: 50% or 80% aqueous acetone, 50% or 70% aqueous ethanol, and 80% aqueous methanol (v/v). The extraction was conducted with shaking for 15 hours at ambient temperature (*7*). After filtration using Whatman No. 2 filter paper, the extracts were centrifuged using an Optima L-90K Ultracentrifuge (Beckman Coulter Lid, CA, USA) at 1,500 x g and 4 °C for 10min. The supernatant was collected and further filtered with a 0.45μm syringe filter (Acrodisc, Gelmen Science). The clear extract was kept in the dark room at 4 ° C for further antioxidant analysis.

Soxhlet extraction (SOX). One gram of finely ground soybean sample was extracted in 50 mL of absolute methanol and ethanol using a Soxhlet extractor. Soxhlet extraction was conducted for 4 hours. After cooling, the antioxidant extracts were filtered and stored in the dark at 4°C until further antioxidant analysis.

Ultrasonic-assisted extraction (UAE). A slight modification of a previous method (*10*) was used. *G*round soybean (0.1g) was extracted using 5 ml of five separate extraction solvents: 50% or 80% aqueous acetone, 50% or 70% aqueous ethanol, or 80% aqueous methanol using an ultrasound liquid processor (Sonicator 3000, Misonix, NY, USA). The actual power delivered into the extraction system was 40 W (at 20% amplitude) for 3 min (1 min at a time to control temperature). An ultrasonic probe with a tip diameter of 7 mm was fitted into the flask and the tip was inserted at half height of the extraction solvent. The mixture was then centrifuged and filtered. The extracts were kept in the dark at 4°C for further analysis.

Total Phenolic Content (TPC). The total phenolic content (TPC) of soybean extracts were determined using the Folin-Ciocalteu reagent with gallic acid as the phenolic standard (*33*). In brief, the appropriate dilutions of extracts were mixed with Folin-Ciocalteu reagent and 20% sodium carbonate (Na_2CO_3) at ambient temperature. After incubation for 2 hours at room temperature, the absorbance was recorded at 760 nm (Thermo Electron Corporation, Genesys 10-UV scanning, Madison, USA). The TPC value of the soybean extracts was expressed in micrograms of gallic acid equivalent (GAE) per gram of soybean (dry basis).

Oxygen Radical Absorbance Activity. The $ORAC_{FL}$ assay was performed to measure the peroxyl radical scavenging activity of soybean samples with Trolox as an antioxidant standard according to the method reported previously (*34*). In brief, a fluorescein stock solution (100 μM) in phosphate buffer (75mM, pH 7.4) was prepared and kept at 4° C protected from light. A fresh working fluorescein solution (100 nM) was prepared daily by diluting the stock solution in phosphate buffer (75mM, pH 7.4). Two hundred microliters of the working fluorescein solution was added to 40μL of sample or Trolox standard prepared in the phosphate buffer (10, 20, 40, 80,100, and 200 μM) in a black 96-well plate and incubated for 20 minutes at 37°C. The assay was initiated by adding the peroxyl radical generator prepared in the phosphate buffer. Specifically, 35μL of 0.36 M 2,2'-Azobis-2-amidinopropane (AAPH) was added and the fluorescence was measured (λ_{ex} = 485 nm and λ_{em} = 535 nm) every minute using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) maintained at 37° C until the reading had declined to less than 5% of the initial reading. Standards and samples were run in triplicate. Results for ORAC were determined by using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve (AUC). The $ORAC_{FL}$ value of each soybean extract was expressed as micromoles of Trolox equivalents per gram sample (μmoles/g).

DPPH$^{\bullet}$ Scavenging Activity. This high-throughput DPPH$^{\bullet}$ assay, based on the reduction of the free radical DPPH$^{\bullet}$, was slightly modified from a previous report (*35*) and carried out using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Briefly, the reaction mixture contained 100 μL of soybean extracts and 100 μL of 0.208 mM DPPH$^{\bullet}$ solution. The absorption at 515 nm was determined immediately when the reaction was initiated by gentle shaking. Plates were read every minute for 30 minutes. The relative DPPH$^{\bullet}$ scavenging capacities were expressed as millimoles of Trolox equivalents (TE) per gram of soybean dry mass (mmoles TE/g), calculated using the net area under the kinetic curve (AUC).

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations. All results were reported as a dry weight basis. The mean values within each test were compared by using a two-sample Student's *t*-test. Data are presented as mean ± SD (Standard Deviation). Significance of variety differences was determined by analysis of variance. Difference was

considered statistically significant when the P value was <0.05. A two-tailed Pearson's correlation test was conducted to determine the correlations among means.

RESULTS AND DISCUSSION

Total Phenolic Content. Total phenolic contents (TPC) of the selected soybean samples are shown in Table 1. For conventional solvent extractions, the TPC of soybean extracted by 5 different solvent mixtures were significant different, and ranged from 3.13 - 3.42 mg gallic acid equivalents (GAE)/g for NC Roy, 2.75 - 3.22 mg GAE/g for V00-3493, and 2.50 - 3.45 mg GAE/g for V00-3636. The TPC of the Virginia soybeans under our experimental conditions were comparable to previous studies. Xu and Chang reported a TPC of 2.67 mg GAE/g in soybean using a 50% aqueous acetone extraction, while 80% aqueous methanol extract of soybean seeds by Lin and Lai reportedly yielded 4.5 mg GAE/g (*7, 16, 36*). It should be noted that the comparison includes different soybean varieties, which may have a significant impact on their total phenolic contents (*37*). Therefore, we randomly selected three Virginia-grown soybean varieties in our investigation in order to comprehensively examine the effect of extraction solvents on different soybean seeds. For both NC Roy and V00-3493 soybean seeds, the 50% aqueous ethanol and 50% aqueous acetone extraction yielded the highest TPC, followed by aqueous mixtures of 70% ethanol, 80% methanol, and 80% acetone. For V00-3636 soybean seeds, the 50% aqueous acetone extract showed significantly higher TPC than other solvent extracts ($P < 0.01$). The results suggested that 50% aqueous acetone is a better solvent mixture than the others for the extraction of phenolic compounds in soybean seeds under conventional extraction conditions, and this data confirmed the result reported by Xu and Chang (*7*).

For UAE, the same 5 different solvent mixtures were examined for their extraction efficiency. The 50% ethanol extract was too turbid to obtain clear solutions even after 0.45 μm filtration. Therefore, this turbid extract was not included in this investigation. The results showed that 50% aqueous acetone extraction had the highest TPC values (6.93-7.80 mg GAE/g dry soybean seeds) for all soybean varieties. These TPCs were higher than the other three solvent mixtures, which ranged in TPC from 4.49 - 6.34 mg GAE/g. On average, the 70% aqueous ethanol extracts had the second highest TPC values while the 80% aqueous methanol and 80% aqueous acetone extracts had the lowest TPC values. A previous study reported that

UAE with 70% aqueous acetone had a TPC of 4.9 mg GAE/g in soybean seeds (*10*). The UAE also had approximately 54-139 % higher TPC values than the corresponding conventional solvent extraction, suggesting the potential application of ultrasonication to improve the solvent extraction efficiency for soybean phenolic compounds.

The soxhlet extractions of soybean seeds with absolute methanol and ethanol had TPC values of 2.11- 4.16 mg GAE/g, which are comparable to the conventional solvent extractions. For all the three soybean seeds, the soxhlet extraction with ethanol was more efficient than the one with methanol for soy phenolics ($P < 0.05$).

Overall, the results from the conventional extractions and UAE suggest that 50% aqueous acetone is the best solvent mixture for soybean phenolic extraction. However, even though the sample mass/solvent ratio was much lower than conventional extraction, UAE showed more than 50% higher efficiency than the corresponding conventional solvent extractions for extracting soy phenolics. This result clearly shows that ultrasonic treatment provides more effective mass transfer from the soy into the solvent compare to conventional solvent extraction (*14*). The ultrasonic extraction can be completed within 30 min (*20*), or as little as 3 min in our study, and is much less time-consuming than the conventional solvent extraction when compared to around 15h conventional extraction time used by many researchers (*7, 37-39*). Therefore, the UAE with 50% aqueous acetone could be a more appropriate extraction method for laboratory or the food and nutraceutical industries for the investigation of soybean phenolics.

Oxygen Radical Absorbance Ability. The ORAC values of soybean extracts from selected solvent mixtures and methods are presented in Table 2. For the conventional extractions, the ORAC of the extracts were significantly different, ranging from 248.5 to 427.2 μmol Trolox equivalents (TE)/g for NC Roy, 208.4 - 457.2 μmol TE/g for V00-3493, and 180.4 - 319.1 for μmol TE/g V00-3636. Our ORAC values were higher than most of the reported ORAC values in soybean extracts, which are between 38.7 and 228.6 μmol TE /g (*37-40*). For all three of the soybean cultivars, the 70% ethanol extracts showed the highest ORAC values (319.1-457.2 μmol TE/g), which were higher than the extracts of other solvents ($P < 0.01$). However, the 80% methanol soybean extracts showed the lowest ORAC values, between 180.4 and 274.8 μmol TE/g. The ORAC values of the soybean extracts were not correlated with their TPC values. We expected that the soybean extracts with 50% acetone or 50% ethanol would

have had higher ORAC values because they had significantly higher TPC, which measures phenolics, the antioxidants in soybean seeds (*20*). Other research has shown that the antioxidant activity of soybean seeds was significantly correlated with their TPC values (*38*). We speculate that the phenolic profiles of the soy extracts using different solvents are different. According to Xu and Chang, total flavonoids and condensed tannin contents of soybean seeds were affected by different solvent systems (*7*). The soybean phenolics extracted with 70% aqueous ethanol appear to be more effective against peroxyl radicals because they exerted significantly higher ORAC values than the extracts from other solvents.

For the UAE, the ORAC were also significantly different ranging from 203.7-392.2 µmol TE/g for NC Roy, 208.9-438.6 µmol TE/g for V00-3493, and 144.7-393.5 µmol TE/g for V00-3636. These ORAC values were comparable to the conventional extracts, although the TPC of the UAE extracts were more than 50% higher than those of the conventional extracts. One other possible reason explaining these results could be that the ultrasonic extraction process may lower the antioxidant activity of soybean phenolics. For NC Roy, the UAE-80% methanol extract had significantly higher ORAC (392.2 µmol TE/g), followed by the UAE-70% ethanol (341.8 µmol TE/g), UAE-80% acetone extract (264.7 µmol TE/g), and UAE-50% acetone (203.7 µmol TE/g). For both V00-3493 and V00-3636, the UAE-70% ethanol extracts had the highest antioxidant activity, followed by UAE-80% methanol, UAE-50% acetone, and UAE-80% acetone extracts. There was no correlation between the ORAC and TPC values of the soybean UAE extracts. For the soxhlet extraction, the methanol extracts of all three soybeans had significantly higher ORAC (295.2-345.1 µmol TE/g) than the ethanol extracts (144.4-221.3 µmol TE/g), despite the lower TPC in the methanol extracts. On average, the conventional 70% and UAE-70% ethanol extracts had the highest ORAC values. However, the UAE did not significantly improve the ORAC value of the soybean extracts compared to the conventional and soxhlet extraction and no correlation was detected between the ORAC and TPC of the soybean extracts.

DPPH$^{\bullet}$ Scavenging Activity. DPPH$^{\bullet}$ scavenging activities of the soybean antioxidant extracts with different solvents systems and techniques is presented in Table 3. The 50% aqueous acetone and 50% aqueous ethanol extracts were not included for this comparison because the spectrophotometric measurement of the extract- DPPH$^{\bullet}$ mixture was affected by the turbidity of the mixture of sample and DPPH$^{\bullet}$solution in ethanol. Indeed, this is one of the

disadvantages of using the DPPH• assay (*41*). For the conventional solvent extractions, the DPPH• scavenging activities of the extracts were somewhat different, ranging from 0.25 – 1.22 mmol TE/g for NC Roy, from 0.09 – 1.27 mmol TE/g of soybean for V00-3493, and from 0.25 – 1.31 mmol TE/g for V00-3636. Antioxidant activities were considerably higher than what was reported in soybean using 50% aqueous acetone extraction (1.16 μmol TE /g) (*40*) and 70% aqueous ethanol extracts (2.1 μmol TE /g) (*7*). For NC Roy, the 80% aqueous methanol and 80% aqueous acetone extracts had comparable antioxidant activities (1.16 and 1.22 mmol TE/g, respectively), which were significantly higher than the 70% aqueous ethanol extracts (0.25 mmol TE/g). For V00-3493 and V00-3636, the 80% aqueous acetone extracts had the highest antioxidant activity, followed by 80% aqueous methanol and 70% aqueous ethanol extracts. These results differ from the report of Xu and Chang (*7*). These authors indicated that 70% aqueous ethanol extraction was the most efficient method for DPPH• assay. The DPPH• scavenging activities of soybean extracts using conventional aqueous solvent systems were not similar in pattern to their ORAC or TPC values, although the DPPH• values from 50% aqueous acetone extracts could not be compared to TPC values.

DPPH• scavenging activities of soybean extracts by UAE were also significantly different between cultivars, ranging from 1.97 – 3.11 mmol TE/g for NC Roy and 0.67 – 2.80 mmol TE/g for V00-3493, respectively while V00-3636 extracts had slightly different ranging 0.57 – 2.74 mmol TE/g of soybean. For all three soybean varieties, the UAE- 80% aqueous methanol extract exerted the highest antioxidant activity, followed by the UAE-70% aqueous ethanol and UAE-80% aqueous acetone extracts. The DPPH• scavenging activities of the NC Roy extracts (but not V00-3493 and V00-3636) were correlated with their ORAC values. For the soxhlet extractions, the methanol extracts showed significantly higher DPPH• scavenging activities than the ethanol extracts, which was similar to the ORAC data for the soxhlet extracts. Overall, the soxhlet methanol extracts had the highest DPPH• scavenging activities.

In conclusion, we have shown a significant effect of the extraction technologies on soybean antioxidant activities. The extractions were performed using commonly employed conditions with a sample mass/solvent volume ratio which has been commonly used for each method. The ultrasonication process improved the extraction efficiency of the soybean phenolic compounds by 54-139%, when compared to the corresponding conventional solvent extractions. The selection of solvent mixtures was an important factor affecting the extraction efficiency of

soybean phenolics. We have found that 50% aqueous acetone was the best solvent system for both the conventional and UAE for soybean phenolics. However, the antioxidant activities of the soybean extracts were not related with their TPC values. The conventional and UAE-70% aqueous ethanol extracts had the highest ORAC values, while the soxhlet methanol extracts showed the highest $DPPH^{\bullet}$ scavenging activities. Overall, the UAE is more appropriate for soybean phenolic extraction because it is less time – and solvent – consuming and appears to have a more efficient mass transfer of phenolic into the solvent compared to convention solvent and soxhlet extractions. Improved antioxidant extraction strategies can contribute to improvements in food or nutraceutical industry using soy antioxidants, and be of value to researchers investigating soybean antioxidants.

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Table 4.1. Total phenolic content of soybean seeds with different extraction methods.

Total phenolic content (mg gallic acid equivalents /g dry mass of soybean)			
	NCRoy	V00-3493	V00-3636
Conventional solvent extraction			
50% aqueous acetone	3.26e,f ± 0.45	3.15e ± 0.36	3.45d ± 0.05
80% aqueous acetone	2.90f ± 0.25	2.90e,f ± 0.10	2.50g ± 0.13
50% aqueous ethanol	3.42e ± 0.13	3.22e ± 0.17	2.97e ± 0.10
70% aqueous ethanol	3.22e,f ± 0.11	2.75f ± 0.16	2.69f ± 0.13
80% aqueous methanol	3.13e,f ± 0.16	2.88e,f ± 0.17	2.93e,f ± 0.25
Ultrasonic-assisted extraction			
50% aqueous acetone	7.80a ± 0	7.05a ± 0	6.93a ± 0
80% aqueous acetone	5.46d ± 0.04	4.49c ± 0.09	5.05b,c ± 0.62
70% aqueous ethanol	6.34b ± 0.04	5.40b ± 0.44	5.65b ± 0.04
80% aqueous methanol	6.05c ± 0.18	4.68c ± 0.18	4.62c ± 0
Soxhlet extraction			
Ethanol	2.41g ± 0.06	4.16d ± 0.01	3.02e ± 0.24
Methanol	2.11h ± 0.12	2.24g ± 0.25	2.22h ± 0.07

The data in each column marked by the same letter are not significantly different ($P < 0.05$).

Table 4.2. ORAC value of soybeans with different extraction methods.

ORAC value (μM Trolox equivalents /g dry mass of soybean)			
	NCRoy	V00-3493	V00-3636
Conventional solvent extraction			
50% aqueous acetone	270.92c,d ± 71.64	275.39e ± 3.95	276.70c,d ± 26.13
80% aqueous acetone	339.06b ± 9.64	295.21d,e ± 40.69	288.20c,d ± 47.96
50% aqueous ethanol	248.52d ± 49.24	307.68d ±22.39	240.35d ,e ± 27.96
70% aqueous ethanol	427.20a ± 21.92	457.21a ± 5.10	319.14b,c ± 33.09
80% aqueous methanol	274.82c ± 11.09	208.38f ± 0.40	180.41f ± 18.50
Ultrasonic-assisted extraction			
50% aqueous acetone	203.71d ± 42.08	217.00f ± 12.68	227.92e ± 14.26
80% aqueous acetone	264.69c,d ± 26.20	208.87f ± 13.84	144.67g ± 3.10
70% aqueous ethanol	341.85b,c ± 47.40	438.63b ± 7.22	393.47a ± 17.34
80% aqueous methanol	392.25a,b ± 17.75	298.44d ± 4.61	223.54e,f ± 34.84
Soxhlet extraction			
Ethanol	144.44e ± 58.04	221.33f ± 11.97	168.96f ± 10.16
Methanol	324.22b,c ± 17.54	345.10c ± 1.64	295.14b,c ± 16.96

The data in each column marked by the same letter are not significantly different ($P < 0.05$).

Table 4.3. DPPH• scavenging activity of soybeans with different extraction methods.

DPPH• scavanging activity (mM Trolox equivalents /g dry mass of soybean)			
	NCRoy	V00-3493	V00-3636
Conventional solvent extraction			
80% aqueous acetone	1.16b ± 0.02	1.27c ± 0.02	1.31a,b,c ± 0.11
70% aqueous ethanol	0.25a ± 0.00	0.09a ± 0.11	0.25a ± 0.04
80% aqueous methanol	1.22b ± 0.10	1.24b ± 0.07	1.02a,b ± 0.03
Ultrasonic-assisted extraction			
80% aqueous acetone	1.97c ± 0.04	0.67b ± 0.09	0.57a,b ± 0.07
70% aqueous ethanol	2.55d ± 0.04	1.73d ± 0.25	1.87b,c,d ± 0.40
80% aqueous methanol	3.11e ± 0.22	2.80e ± 0.01	2.74d ± 0.90
Soxhlet extraction			
Ethanol	1.78c ± 0.22	1.48c,d ± 0.05	2.66c,d ± 0.04
Methanol	5.22f ± 0.01	5.00f ± 0.04	5.74e ± 0.06

The data in each column marked by the same letter are not significantly different ($P < 0.05$).

CHAPTER 5

Antioxidant Properties of Bioaccessible Extracts and Bioaccessibility of Isoflavones from Soybean During *in vitro* Digestion

ABSTRACT

The antioxidant activity and isoflavone contents of a Virginia-grown soybean with a high total phenolic content (TPC) were evaluated using an *in vitro* digestion procedure. Samples were obtained after treatment with pepsin at pH 2 (gastric digestion) and after subsequent treatment with porcine pancreatin and bile salts at pH 8 (gastrointestinal digestion). Soluble (bioaccessible) and insoluble (indigestible) fractions were obtained after each digestion step and the experiment was repeated with heat denatured enzyme to discount nonspecific effects. Isoflavones were quantified by high performance liquid chromatography and the total phenolic contents (Folin-phenol), oxygen radical absorptive capacity (ORAC), and DPPH radical scavenging activity were determined on soluble and insoluble fractions. Raw and cooked soybean samples were also analyzed as controls. Heat inactivation of the enzymes resulted in lower TPC, ORAC and DPPH in soluble fractions ($p<.01$). After gastrointestinal digestion, soybean extracts had significantly higher TPC and ORAC ($p<.05$) but not DPPH than cooked soybean. Gastrointestinal digestion resulted in an increased TPC (12.2 mg/g gallic acid equivalents, GAE) compared to cooked soybean (4.9 mg/g GAE). Similarly, ORAC was higher at 143.1 μM/g Trolox equivalents (TE) in raw soybeans compared to 95 μM/g TE in cooked. Glycosides, including daidzin, genistin and malonylgenistin, appeared relatively stable with recoveries of 83.3 %, 59.4 %, and 10.7 %, respectively, in the soluble fraction after *in vitro* gastrointestinal digestion and the aglycones daidzein and genistein, were recovered at 37 % and 73.7 %, respectively. Genistein was the most stable and bioaccessible of the aglycones after *in vitro* digestion.

KEYWORDS: Bioaccessible soy extracts, *in vitro* digestive system, antioxidant activity, TPC, ORAC, $DPPH^{\bullet}$, isoflavones

INTRODUCTION

Soybean consumption has been associated with health benefits such as lowered risk of heart diseases, osteoporosis, diabetes, and cancers, especially breast and prostate (*1-5*). Soybeans have also been investigated as a food source of polyphenols, including tannins, proanthocyanidins, anthocyanin, flavonoids (mainly isoflavones), and phenolic acids such as chlorogenic, caffeic, ferulic, and *p*-coumaric acids (*6-9*). Antioxidants present in soybeans include isoflavones, a group of natural flavonoids from soybean and soy products which have exhibited the potential to inhibit lipid peroxidation (*10, 11*), LDL oxidation (*12-14*), scavenge free radicals (*14-17*), and promote the activation of antioxidative enzymes including superoxide dismutase and catalase (*17-19*). The total phenolic content (TPC) is generally evaluated in most studies on the antioxidant activities of soybean extracts. Soybean extracts have been shown to have significant antioxidant activity using a variety of assessment procedures including oxygen radical absorbance capacity (ORAC), ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$) free radical scavenging activity (*8, 20-22*). These studies indicate that soybeans are a good dietary source of antioxidants that may be important for disease prevention and health promotion.

The contents and compositions of soybean antioxidants are affected by storage time, seed germination, and processing methods (*22-24*). Recently, Xu and Chang compared the antioxidant activities of soybeans as affected by extraction solvents and thermal processing (*25, 26*). Isoflavone aglycones are considerably increased by soybean pretreatment and aglycone composition is changed during heat treatments such as cooking, roasting, boiling, steaming, and pressure steaming (*26-28*).

Information on the dietary intake of antioxidants and their bioaccessibility in the human digestive system is critical for the assessment of antioxidant significance in human health. In addition to bioaccessibility, the bioavailability of antioxidants is important to accurately evaluate the biological roles of antioxidants from foods in the human body. As shown in Figure 5.1, bioaccessibility is defined as the amount released (solubilized) from a solid food matrix. This soluble fraction, when present in the gut, is available for intestinal absorption (*29*). Bioavailability is described as the amount of a food constituent ingested, absorbed in the gut, and

available to be utilized or stored under normal physiological conditions in the human body (*30*). Several studies have reported the bioavailability of soy isoflavones using *in vitro* and *in vivo* model systems. Since these two aglycones are found at relatively high concentrations in the plasma and urine of humans and animals, many studies have focused on the bioavailability of soy isoflavone aglycones such as genistein and daidzein (*31-36*). In addition to the bioavailability of isoflavones, studies have shown that the isoflavones from soy bread were stable during *in vitro* gastrointestinal digestion (*37*). Walsh et al. reported that the higher solubility of daidzein might lead to it being more accessible than genistein in the aqueous fraction of digesta, and be available for potential absorption (be bioaccessible) in the small intestine (*37*). However, the stability and bioaccessibility of all soy antioxidants including isoflavones is still not clear. Therefore the purpose of this study is to examine the bioaccessibility of soy antioxidants including isoflavones to provide a better understanding the stability and the bioaccessibility of soybean antioxidant components during digestion in the gut.

In order to determine the bioaccessibility of antioxidants of food, one of the approaches is to use an *in vitro* digestion model to simulate gastrointestinal (gastric + small intestine) digestion. This approach has been used to investigate digestion of polyphenols and carotenoids from foods (*38, 39*). An *in vitro* gastrointestinal system has been used to assess the bioaccessibility of polyphenols and carotenoids in different foods such as grape seed and peel (*40*), green tea (*41*), carrot, tomatoes(*39*), and leafy vegetables (*42*). Digestive enzymes including pepsin and pancreatin along with bile salts, time, pH, and temperature can be controlled to provide a simulated digestion (*30*). Components that are solubilized under these conditions are bioaccessible. These simulated digestion procedures contribute important information about the stability of compounds during gastrointestinal digestion.

Various i*n vitro* assays, including the total phenolic content (TPC), oxygen radical absorptive capacity (ORAC), and ferric reducing antioxidant power (FRAP), have been established to assess the amounts of bioaccessible antioxidants in food products or supplements. The TPC assay was used to investigate the bioaccessibility of polyphenols in plant foods including cereals, vegetables, legumes, fruits, nuts, beverages, and oils during *in vitro* gastrointestinal digestion (*38*). The antioxidant activity of red wine fractions using an *in vitro* digestive system was estimated by using ORAC and FRAP assays (*43*). In a previous study for

assessing the stability and the bioaccessibility of soy isoflavones, HPLC analysis was used to determine amounts of isoflavones (*37*).

After food consumption, the potent antioxidants should be released from the food matrix and diffused in the digestible aqueous fraction in the gut. The antioxidants released from foods should have their biological activities in the gut, although these antioxidant compounds may be subject to absorption and become bioavailable in the intestines. Not all nutrients are absorbed with equal efficacy after digestion in the human body because components available to be absorbed and to be utilized may vary quantitatively and qualitatively due to the physical properties of the food matrix (*44*). Moreover, the bioavailability of compounds in plant products (fruits and vegetables) may differ because of interactions between their chemical structures and macromolecules within the food matrix, as well as their differential uptake rates into the body. Therefore, it is important to know whether the antioxidants are stable and bioaccessible, and available for absorption into the body where they may exert their potential bioactivities. The objectives of this study were to investigate antioxidant activities of bioaccessible extracts and characterize the distribution of bioaccessible isoflavones from soybeans during *in vitro* digestion.

MATERIALS AND METHODS

Antioxidant Extraction. Soybean seed was ground to a fine powder using a Bel-Art micro-mill grinder. One gram of ground soybean sample was then extracted with 10 mL of 50% aqueous acetone (w/v; mass/solvent ratio 1:10). The extraction was conducted under shaking for 15 hours at ambient temperature (*20, 25*). After filtration using filter paper (Whatman No. 2), the extracts were centrifuged using an Optima L-90K Ultracentrifuge (Beckman Coulter Lid, CA, USA) at 1,500 x g and 4 °C for 10min. The supernatant was collected and filtered using a 0.45μm syringe filter (Acrodisc, Gelmen Science). The extract was kept in the dark at 4 °C for further antioxidant analysis. Cooked soybean, boiled for 2 hours after soaking for 15 hours (*26*), was lyophilized and then ground to a fine powder using a Bel-Art micro-mill grinder. One gram of powdered, cooked soybean was extracted with 50% (w/v) aqueous acetone to extract antioxidants as described above. The clear extract was kept in the dark at 4 °C for antioxidant analysis. After *in vitro* gastric or gastrointestinal digestions described below, soluble and insoluble (indigestible) fractions were collected and freeze dried.

Isoflavone Extraction. To determine the isoflavone composition, the milled soybean seed or cooked soybean were extracted by using a mixture of 0.1N hydrochloric acid (HCl), acetonitrile and distilled water (2/7/3, v/v/v) (*45*). After the extraction, the solution was centrifuged and the supernatant was filtered (0.45 μm). The filtrate was evaporated at 45°C using a Savant SPD 1010, Speed Vac Concentrator (Thermo Fishers Scientific, Ashville, NC). The residue was reconstituted in 1 ml methanol and filtered (0.2 μm, Fisher acrodisk) prior to HPLC analysis. For indigestible fractions after gastric or gastrointestinal digestion, fractions were freeze dried and extracted at describe above.

***In Vitro* Digestion.** *Gastric Digestion*. Figure 5.2 shows the procedure of obtaining extracts from an *in vitro* digestive system. In order to compare the effect of digestive enzymes on the release of antioxidants from cooked soybean after *in vitro* gastrointestinal digestion, denatured or active digestive enzymes were used for our study. Inactive digestive enzyme solutions were prepared by boiling the enzyme solutions for 15 min. One gram of freeze-dried, cooked soybean was treated with 20 mL of inactive or active pepsin solution (final concentration, 1mg/mL) in screw cap flasks. After mixing, the pH of the mixture of sample and pepsin was adjusted to 2.0 with 4N HCl to mimic gastric conditions. The mixture was then digested in a shaker water bath at 120 rpm at 37 °C for 2 hours. After gastric digestion, 5 mL of gastric digesta were centrifuged at 10,000 x g at 4 °C for 10 min. The supernatant (bioaccessible) was filtered using a 0.45μm disk filter. This is the bioaccessible fraction after gastric digestion but before intestinal digestion. After centrifugation,the remaining indigestible fraction was lyophilized and extracted with 50% aqueous acetone as described above. All filtrates or extracts were kept at -20 °C for antioxidant activity analysis. The digestion for isoflavone analysis was performed with the same procedure as describe above. After filtration of the bioaccessible fraction, the filtrate was evaporated by using a Savant SPD 1010, Speed Vac Concentrator (Thermo Fishers Scientific, Ashivile, NC). The residue was reconstituted with methanol and filtered with a 0.45μm disk filter prior to HPLC analysis. The gastric indigestible fraction was freeze dried and extracted for isoflavone analysis.

Intestinal Digestion. The remaining solution from gastric digestion, 15 mL, was adjusted to pH 8.0 to mimic intestinal pH. After addition of inactive (boiled) or active (not boiled) enzyme mixtures of pancreatin (0.27mg/mL, final concentration) and bile salts (2.94 mg/mL,

final concentration), the sample was digested in a shaking water bath at 100 rpm at 37 °C for 2 hours. After centrifugation, the supernatant (bioaccessible fraction) was collected and filtered as described above. The indigestible fraction was lyophilized and then extracted with 50% aqueous acetone as described above. All solutions were kept at -20 °C for further analysis. The digestion procedure for isoflavones was performed using the same procedure as describe above. After filtration of bioaccessible fraction, the filtrate was evaporated by using a Savant SPD 1010, Speed Vac Concentrator (Thermo Fishers Scientific, Ashville, NC). The residue was reconstituted with methanol and filtered with a 0.45μm disk filter prior to HPLC analysis. After freeze drying, the indigestible fractions of gastrointestinal digestion was extracted and prepared for isoflavone analysis.

Total Phenolic Content (TPC). The total phenolic content (TPC) of soybean extracts was determined using the Folin-Ciocalteu reagent with gallic acid as standard (*46*). In brief, the appropriate dilutions of extracts were mixed with Folin-Ciocalteu reagent and 20% sodium carbonate (Na_2CO_3) at ambient temperature. After incubation for 2 hours at room temperature, the absorbance was recorded at 760 nm (Thermo Electron Corporation, Genesys 10-UV, Madison, USA). The TPC value of the soybean extracts was expressed in milligrams of gallic acid equivalent (GAE) per gram of soybean (dry weight basis).

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) The $ORAC_{FL}$ assay was performed to measure the peroxyl radical scavenging activity of soybean samples with Trolox as an antioxidant standard according to a method reported previously (*47*). Briefly, a fluorescein stock solution (100 μM) in phosphate buffer (75mM, pH 7.4) was prepared and kept at 4° C, protected from light. A fresh working fluorescein solution (100 nM) was prepared daily by diluting the stock solution in phosphate buffer (75mM, pH 7.4). Two hundred microliters of the working fluorescein solution was added to 40μL of sample or Trolox standard prepared in the phosphate buffer (20, 40, 80,100, and 200 μM) in a black 96-well plate and incubated for 20 minutes at 37°C. The assay was initiated by adding the peroxyl radical generator prepared in the phosphate buffer. Specifically, 35μL of 0.36 M 2,2'-azobis-2-amidinopropane (AAPH) was added and the fluorescence was measured (λ_{ex} = 485 nm and λ_{em} = 535 nm) every minute using a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) maintained at 37° C until the reading had

declined to less than 5% of the initial reading. Standards and samples were run in triplicate. Results for ORAC were determined by using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve (AUC). The $ORAC_{FL}$ value of each soybean extract was expressed in micromoles of Trolox equivalents per gram sample (μM TE/g).

DPPH• Scavenging Activity. This high-throughput DPPH• assay, based on the reduction of the free radical DPPH•, was slightly modified from a previous report (*48*) and carried out using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Briefly, the reaction mixture contained 100 μL of soybean extracts and 100 μL of 0.208 mM DPPH• solution. The absorption at 515 nm was determined immediately when the reaction was initiated. Absorbance was measured every minute for 30 minutes. The relative DPPH• scavenging capacities were expressed as millimoles of Trolox equivalents (TE) per gram of sample (mM TE/g), calculated using the area under the kinetic curve (AUC).

HPLC Analysis of Isoflavone Composition. Isoflavone analysis in the soybean extracts was performed using an Agilent 1200 quaternary HPLC system (Agilent Technologies, CA) equipped with a photodiode array detector. The isoflavone standards were separated by using a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size 5μm) using a linear gradient program with a mobile phase containing solvent A (0.1% glacial acetic acid in H_2O) and solvent B (0.1% glacial acetic acid in acetonitrile) (*49*). The solvent gradient was linear, programmed from 15 to 35% solvent B in 50 minutes with a flow rate of 1.0 mL/min. Identification of isoflavones in each soybean sample was accomplished by comparing the retention time and absorption spectra of peaks in the extracts to that of the standard compounds. Quantification of individual isoflavones was conducted using external standard procedures.

Statistical Analysis. Data were reported as mean ± SD for duplicate determinations. The results were analyzed by one way ANOVA and means separated using Tukey's multiple comparison using SPSS 13.0. Differences were considered statistically significant when the P value was < 0.05.

RESULTS AND DISCUSSION

Total Phenolic Contents. The total phenolic contents (TPC) of the soybean extracts from uncooked soybean (US), cooked soybean (CS), bioaccessible (soluble) and indigestible (insoluble) fractions after gastric or gastrointestinal digestion with inactive (boiled) or active (not boiled) enzymes are presented in Figure 5.3. Compared to uncooked soybean, TPC (dry weight basis) of cooked soybean was significantly decreased by about 29.3 % ($p < 0.05$). This is in agreement with Xu and Chang (*26*), who observed that thermal processing, including boiling, caused a decrease in TPC value in soybeans. This loss of TPC after cooking is either due to degradation of polyphenols or leaching of the more soluble phenolic compounds from soybean into the water during the soaking and boiling steps. In the bioaccessible fraction, TPC values were significantly higher than those of uncooked or cooked soybean extracts ($p < 0.05$). Phenolic compounds are located and accumulated in the cell walls. Although gastric digestion causes some release of phenolic compounds from the soybean matrix, significantly higher TPC were observed in soluble fractions during gastrointestinal digestion, since hydrolysis of carbohydrate and protein by pancreatin likely caused the release of bound phenolic compounds. This is in agreement with previous studies using commercial soy protein isolate and whole soy powder (*50*). The effect of digestive enzymes on release of phenolic compounds from soybean matrix was evaluated using inactive and active digestive enzymes. As shown in Figure 5.3., both gastric and intestinal digestive enzymes were associated with significantly increased TPC ($p < 0.05$) compared to treatments with denatured enzymes.

TPC values of bioaccessible fractions were considerably higher than in cooked soybeans. This suggests that high amount of phenolic compounds can be extracted and are stable in the small intestine. Moreover, these bioaccessible phenolic compounds may reach the colon and be further metabolized by microflora. TPC within indigestible fractions during digestion were also observed with similar patterns to those for the soluble fractions. This indicates that the some phenolic components still remain in the soybean matrix. Overall, most phenolic components can be released and posses antioxidant activities during gastrointestinal digestion. Grain phytochemicals are present in the free soluble conjugate and insoluble bound forms. For example, most phenolic acids in cereals such as wheat are in the insoluble bound forms and there

is a release of bound phenolic compound during digestion (*51, 52*). It appears that significant amounts of bound phenolic compounds of soybean may be extracted during digestion.

Oxygen Radical Absorbance Capacity. Oxygen radical absorbance capacity (ORAC) of soybean extracts is exhibited in Figure 5.4. The patterns of ORAC data of soybean extracts were similar to those of TPC. Compared to uncooked soybean, cooking soybean caused a significant decrease by about 28.6 % in ORAC value ($p < 0.05$). This pattern was similar to a previous study (*26*). However, compared to bioaccessible fractions after digestion, the ORAC values of the bioaccessible fraction after gastrointestinal digestion was significantly higher than cooked soybean ($p < 0.05$), but not significantly different from the uncooked soybean. This is in agreement with previous work (*50*). Similar to TPC results, the intestinal digestive enzymes (pancreatin – bile salt) significantly increased ORAC values within the bioaccessible fractions compared to pepsin used in the gastric digestion ($p < 0.05$). Since gastric digestion may cause protein coagulation, bound phenolic compounds may not be liberated from the soybean matrix, which would result in the observed low antioxidant activities. When comparing bioaccessible and indigested fractions, ORAC values were significantly higher in bioaccessible fractions after gastrointestinal digestion ($p < 0.05$), although antioxidant extracts from indigestible fractions still exhibited some ORAC. Similar to observations for TPC, digestive enzymes promoted the release of antioxidants during digestion.

DPPH$^{\bullet}$ radical scavenging activity. The DPPH$^{\bullet}$ radical scavenging activity of soybean extracts are shown in Figure 5.5. Although cooking soybeans significantly increased the TPC and ORAC values, the DPPH$^{\bullet}$ scavenging activity of cooked soybean was not significantly different from uncooked soybean. Xu and Change reported that thermal processing slightly increased the DPPH$^{\bullet}$ scavenging activity of soybeans (*26*). Antioxidants do not always degrade during thermal processing. Some work has suggested that thermal processing can induce either loss or formation of antioxidant compounds (*53*). In previous studies, cooking promoted an increase in phenolics in green beans, pepper, and broccoli (*54*). The total flavonoid and condensed tannin contents of boiled soybeans were higher than in uncooked soybean (*26*). Therefore, increased DPPH$^{\bullet}$ scavenging properties of cooked soybean may be understandable. The DPPH$^{\bullet}$ of the bioaccessible fraction after gastric digestion was significantly higher than

cooked soybean ($p < 0.05$), while the bioaccessible fraction after gastrointestinal digestion was significantly decreased compared to the one after gastric digestion. But the release of antioxidants was affected by digestive enzymes, with significant increases after both gastric and intestinal enzymes ($p < 0.05$). Comparing indigestible fractions during digestion, the DPPH$^{\bullet}$ values of all indigestible fractions were similar in pattern to the bioaccessible fractions. The digestive enzymes did not affect the release of antioxidants from soybean matrix within indigestible fractions. Interestingly, the bioaccessible fraction after gastric digestion with inactive enzyme showed negative values for DPPH$^{\bullet}$. Characteristics of the bioaccessible fraction, such as protein coagulation, may cause turbidity in the DPPH$^{\bullet}$ solution and may cause interference and negative measurements. In DPPH$^{\bullet}$ assays performed spectrophotometrically, turbidity of the sample solution or the mixture of sample and DPPH$^{\bullet}$ solution will affect the results, which is one of the drawback of using this assay (*55*). Moreover, DPPH$^{\bullet}$ radical scavenging capacity can be affected by the sample solution in acid or basic conditions. This may be one of the reasons that we observed a significantly higher scavenging activity in the gastric bioaccessible fraction than in the intestinal bioaccessible fraction (*56*). Another reason may be that protein precipitation during intestinal digestion may affect the results of DPPH$^{\bullet}$ scavenging property of the bioaccessible fraction. Although Bolling et al. used low concentrations of various soybean extracts from *in vitro* digestion, the antioxidant activity of digestion extracts were not evaluated by using the DPPH$^{\bullet}$ assay because the values were too low (*50*). DPPH$^{\bullet}$ radical is a non-biological radical which means it is not generated in body. However, the DPPH$^{\bullet}$ radical scavenging assay has been widely used for assessing antioxidant activity of various food samples such as grains, fruits, and food products since it is relatively simple and easy to perform. Overall, in the DPPH$^{\bullet}$ radical scavenging capacity assay, bioaccessible antioxidants were released by active gastric digestive enzymes, but the antioxidant activity of bioaccessible fraction after gastrointestinal digestion was not stable.

HPLC Analysis of Isoflavone Contents. The bioaccessibility of isoflavones from soy based bread and custard have been investigated using *in vitro* digestion (*37, 57*). Soy isoflavones have been also investigated for their bioavailability, and their aglycone forms, including genistein and daidzein, have been found to be more bioavailable than their glucoside forms. Table 5.1 shows the isoflavone contents of uncooked, cooked soybean, bioaccessible fractions,

and indigestible fractions with inactive (boiled) or active (not boiled) digestive enzymes during *in vitro* digestion. The amounts of daidzein, genistein, and their corresponding glycosides, and malonylgenistin were estimated from soybean extracts. The total isoflavone contents were calculated as the sum of all isoflavones measured in this study. Since daidzein and genistein have been reported as the most bioavailable aglycones (*58, 59*), the sum of daidzein and genistein as a percentage of the total isoflavones was also estimated. Previous studies have reported that the profiles of isoflavones were affected by processing methods, including production of soymilk, tofu, and tempeh (*26, 28*). In this study, in agreement with a previous study by Xu and Chang (*26*), cooked soybean showed a 19 % loss of total isoflavones compared to the original soybean seed. The cooking process considerably increased daidzein, genistein, and their glycosides and caused a decrease of malonylgenistin by about 65%. These results can be explained as the conversion of malonylgenistin to genistin, and the hydrolysis of glycosides to aglycones, in agreement with previous work (*27*).

Figure 5.6 presents the chromatogram of bioaccessible isoflavones during *in vitro* digestion. After *in vitro* gastrointestinal digestion, the bioaccessible fractions showed significant decreases in contents of all isoflavones measured. The total isoflavone contents after gastrointestinal digestion were decreased in 46.6 % from cooked soybean. Hydrolysis of malonylgenistin, daidzin, and genistin by pancreatin-bile salt in aqueous solutions might result in a decrease in daidzein and genistein in the bioaccessible fraction after digestion. Daidzin and genistin were only 15% and 10% stable in the bioaccessible fraction after gastric digestion, respectively, and these values were slightly lower than glycosides digested with inactive pepsin. During gastric digestion, the low pH may cause protein coagulation and lower the release of isoflavones, because isoflavones are tightly associated with protein (*60*). After gastrointestinal digestion with active enzymes (pancreatin – bile salt), recoveries of daidzin, genistin, and malonylgenistin were estimated to be 83.3 %, 59.4 %, and 10.7 %, respectively. Daidzein and genistein were found to have 37 % and 73.7 % recovery after digestion, respectively. The results are somewhat different from previous work (Walsh et al) who studied bioaccessibility of isoflavones from soy based bread (*37*). They reported that daidzin, genistin, and malonylgenistin were primarily contained (more than 80 %) in the aqueous fraction of digesta. Moreover, aglycones including daidzein and genistin, were found to be recovered at about 59 % and 33 %, respectively. However, in our study except for daidzin, both genistin and malonylgenistin were

present at relatively lower levels than reported in the previous study. Daidzein was estimated to be lower, but genistein was relatively higher than those from the earlier work. When compared to only the stability of genistein, our results are in agreement with the previous study, which reported 92% stability of genistein after digestion (*57*), but they did not compare the bioaccessibility of the other isoflavones to genistein, so it is hard to compare our results to this study. When comparing solubility between aglycones, genistein is more lipophilic than daidzein. A previous study has reported that the presence of bile salts promoted more release of aglycones by producing micelles during digestion (*37, 61*). In our study, genistein was not only more diffused into the soluble fractions than daidzein, but also was more hydrolyzed from genistin. Intestinal digestive enzymes caused the release of isoflavones into the bioaccessible fraction when compared to bioaccessible fraction with inactive digestive enzymes. The bioaccessible fraction with inactive enzyme after gastrointestinal digestion also showed significantly higher glucosides than the one from gastric digestion. This may be the reason that longer aqueous extraction caused more water soluble daidzein and genistein release from the soybean matrix.

The isoflavone contents of indigestible fractions were also evaluated during digestion. Isoflavones contained in indigestible fractions were compared to bioaccessible fractions in order to estimate how much isoflavones still remained in the indigestible fractions after gastric and gastrointestinal digestions. The total isoflavone contents of indigestible fraction after digestion were 36.8 % in cooked soybean. Malonylgenistin was still retained in indigestible fraction at around 31 %. This may indicate that malonylgenistin might be degraded and converted to genistin during digestion, and so would not be detected as much as in bioaccessible fractions. This may be caused by more extractable malonylgenistin in indigestible fractions. Indigestible fractions contained significantly lower daidzin and genistin than bioaccessible fractions ($p < 0.05$). This indicated that more glycosides were transferred into the aqueous phase (bioaccessible) during digestion. Aglycones in the indigestible fractions showed no significant differences compared to those in bioaccessible fractions ($p < 0.05$), and yet were still extracted in relatively high amounts from the indigestible fraction. This can be explained by more water soluble glycosides being extracted from the soy matrix. This might cause a relatively high stability of aglycones extracted in bioaccessible fractions, even though higher amounts were extracted in indigestible fractions during digestion. The effect of digestive enzymes on release of isoflavones from indigestible fractions was not significant. These indigestible fractions can

reach the colon and then further liberate and convert aglycones from glucosieds by β-glycosidase from microflora in the intestine.

A previous study reported that daidzein was more water soluble, and thus more bioaccessible, than genistein in a soy based bread (*37*). However in our study, despite being less water soluble, genistein was more bioaccessible during *in vitro* digestion. Some reports have suggested that genistein is more bioavailabile than daidzein (*58, 59*), whereas others have reported the opposite (*33*). Both bioaccessibility and bioavailability of aglycones may depend on the types of soy-based food matrix. For example, aglycones, including daidzein and genistein, from soy milk were absorbed faster than those from tempeh and textured vegetable protein (*62*).

In conclusion, relatively high levels of phenolic compounds in soybeans were extracted and recovered during a model *in vitro* digestion process. The gastrointestinal bioaccessible fraction exhibited significantly higher ORAC values than the others. Digestive enzymes, especially small intestinal enzymes such as pancreatin – bile salt, promoted the bioaccessibility of cooked soybean after gastrointestinal digestion. For isoflavones in soybean extracts, malonylgenistin, glycosides and aglycones were recovered after the digestion process. Levels of both daidzein and genistein were significantly different from those in cooked soybean, and genistein was found to be the most extractable, stable, and bioaccessible isoflavone during *in vitro* digestive study. Although an *in vitro* digestive system can not exactly mimic *in vivo* digestive processes, bioaccessibility of soybean extracts during *in vitro* digestion were estimated to be high in TPC, ORAC, and isoflavones, and this result may help understand relative bioavailabilities of antioxidant compounds in soybean products.

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Table 5.1. Isoflavone profiles of soybean extracts[a]

	Daidzin (μg/g)	Genistin (μg/g)	Malonylgenistin (μg/g)	Daidzein (μg/g)	Genistein (μg/g)	Total (μg/g)	(Den+Gen)/total (%)
US	126.7b,c ± 17.2	129.9a,b,c ± 7.5	2313.3c ± 279.5	11.9a,b ± 0.7	9.1a ± 2.3	2590.9	0.8
CS	531.6g ± 27.4	696.1f ± 15.1	818.3b ± 111.8	20.8b ± 3.9	32.7b ± 92	2099.6	2.5
Soluble fraction (digestible and bioaccessible)							
G-I	121.5b,c ± 9.5	118.4a,b ± 8.9	4.9a ± 0	n.d.[b]	n.d.	244.8	n.d.
G-A	79.7a,b ± 2.6	69.6a ± 1.4	9.2a ± 0.2	n.d.	n.d.	158.5	n.d.
GI-I	256.7e ± 25.8	216.4b,c ± 21.8	73.5a ± 5.0	n.d.	n.d.	546.6	n.d.
GI-A	443.0f ± 12.0	416.1e ± 7.6	88.3a ± 3.5	7.7a ± 0.1	24.1a ± 1.3	979.2	3.2
Insoluble fraction (indigestible)							
G-I	75.6a,b ± 1.3	112.8a,b ± 0.1	153.3a ± 18.4	8.1a ± 0.5	11.9a ± 0.5	387.4	5.2
G-A	64.5a ± 0.2	113.2a,b ± 4.6	152.3a ± 8.7	7.1a ± 0.2	13.6a ± 0.3	373.9	5.3
GI-I	195.5d ± 5.2	332.9d,e ± 38.8	386.3a ± 55.2	14.9a,b ± 5.6	25.0a ± 12.7	1030.5	3.9
GI-A	147.4c,d ± 7.5	268.5c,d ± 4.4	254.5a ± 6.2	14.0a,b ± 0.3	27.2a ± 0.9	777.3	5.3

[a] Soybean extracts were prepared from US, uncooked soybean; CS, cooked soybean; G-I, gastric digestion - inactive enzyme; G-A, gastric digestion -active enzyme; GI-I, gastrointestinal digestion - inactive enzyme; GI-A, gastrointestinal digestion - active enzyme.

Den, daidzein; Gen, genistein.

[b] n.d., not detected.

Data marked by the same letter in each column are not significantly different ($P < 0.05$).

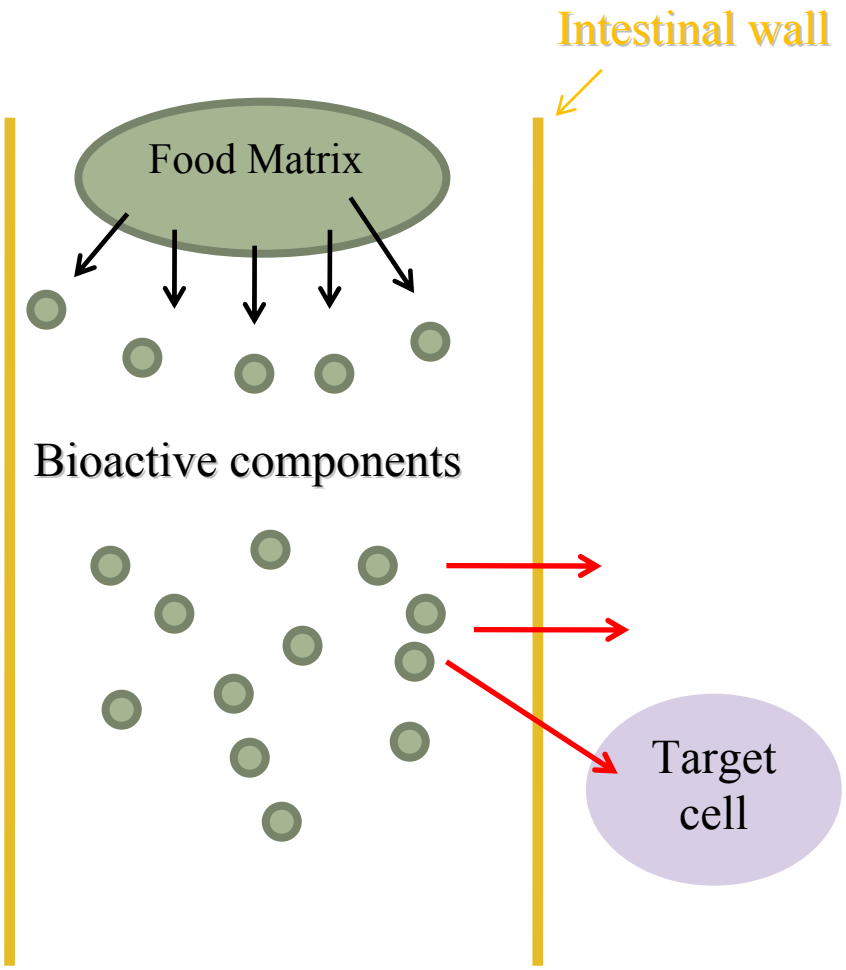

Figure 5.1. Process of bioaccessibility, absorption, and bioavailability from a food matrix during digestion

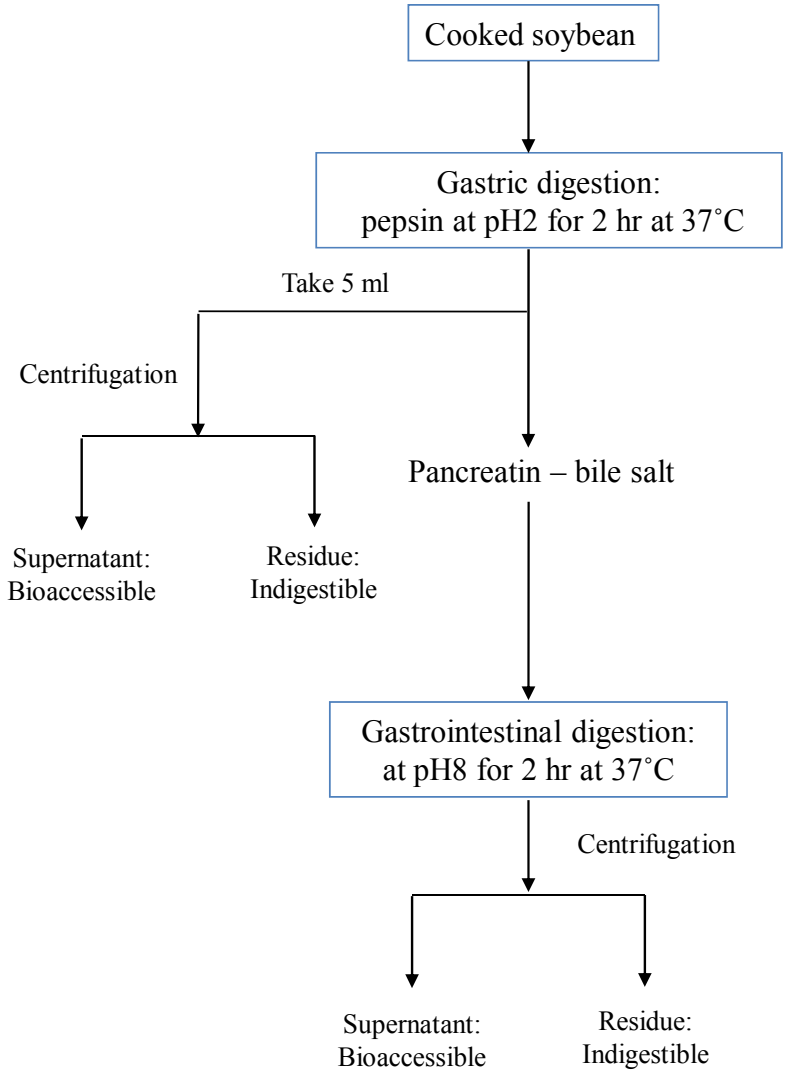

Figure 5.2. Schematic diagram of an *in vitro* digestion of cooked soybean.

	Bioaccessible fraction		Indigestible fraction	
	Gastric digestion	Gastro-intestinal digestion	Gastric digestion	Gastro-intestinal digestion

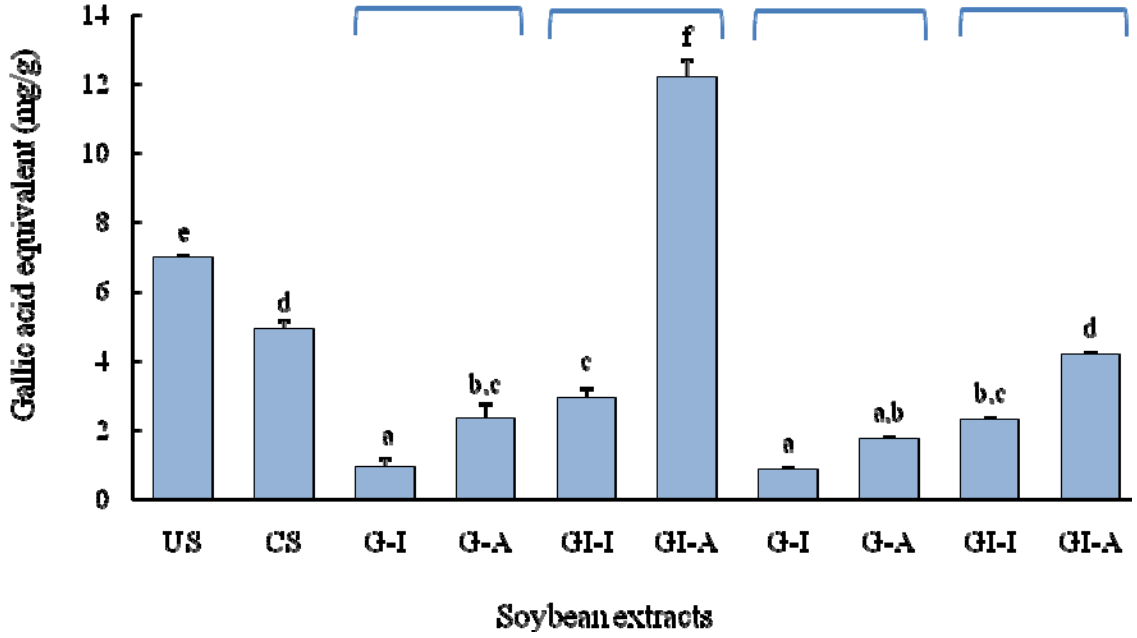

Figure 5.3. Total phenolic content (TPC) of soybean extracts. US, uncooked soybean; CS, cooked soybean; G-I, gastric digestion - inactive enzyme; G-A, gastric digestion -active enzyme; GI-I, gastrointestinal digestion - inactive enzyme; GI-A, gastrointestinal digestion - active enzyme. Results are expressed as milligrams of Trolox equivalents (TE) per gram of soybean seed (mean ± SD, $n = 4$). Bars marked by the same letter are not significantly different ($P < 0.05$).

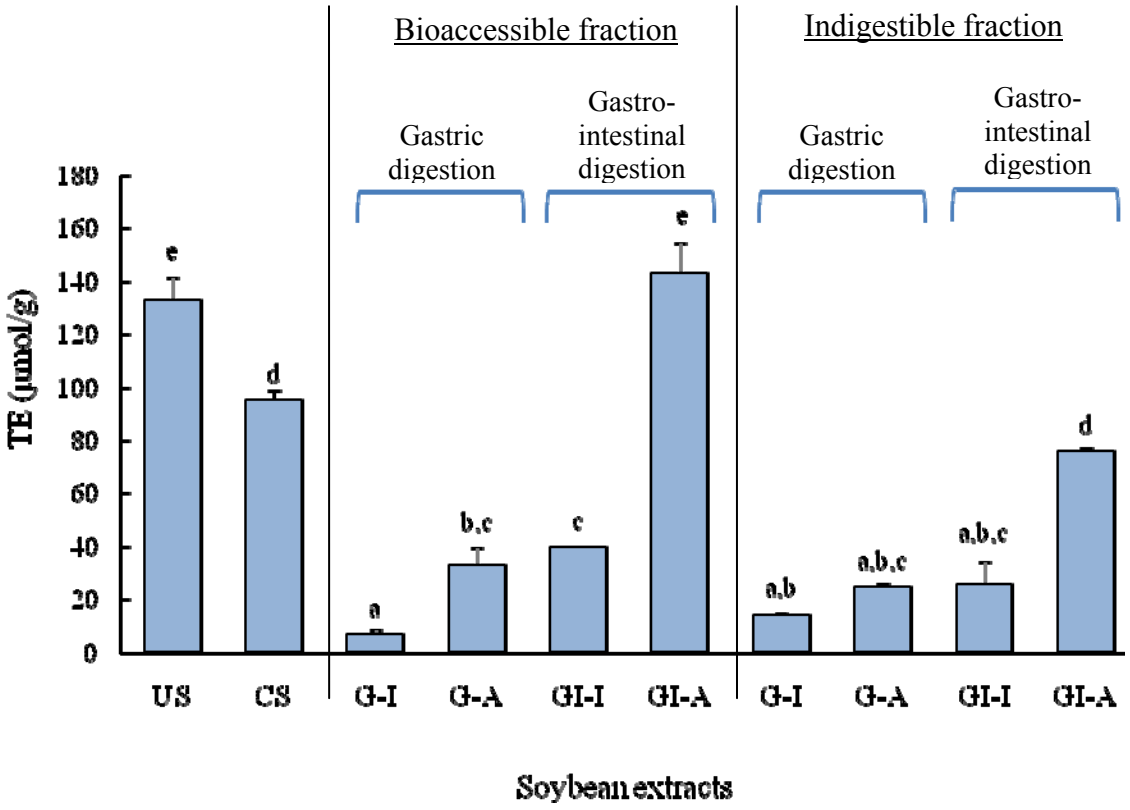

Figure 5.4. Oxygen radical absorbance capacity (ORAC) of soybean extracts. US, uncooked soybean; CS, cooked soybean; G-I, gastric digestion - inactive enzyme; G-A, gastric digestion -active enzyme; GI-I, gastrointestinal digestion - inactive enzyme; GI-A, gastrointestinal digestion - active enzyme. Results are expressed as micromoles of Trolox equivalents (TE) per gram of soybean seed (mean ± SD, $n = 4$). Bars marked by the same letter are not significantly different ($P < 0.05$).

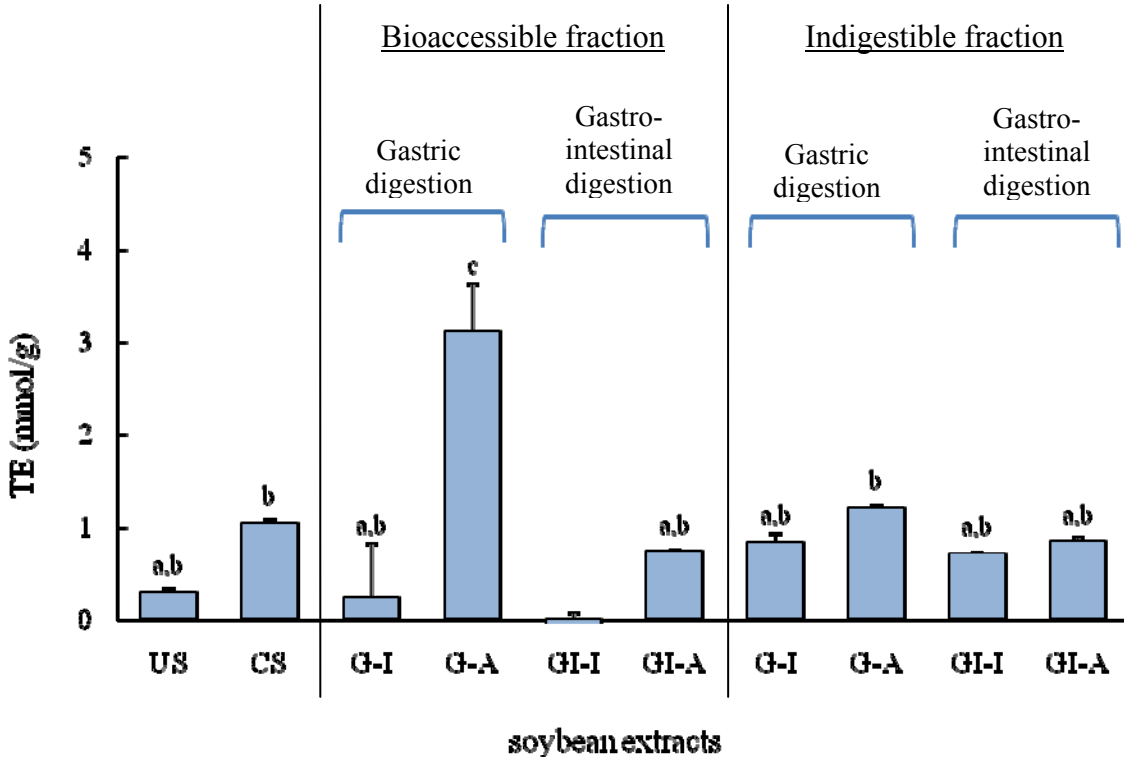

Figure 5.5. DPPH$^{\bullet}$ radical scavenging activity of soybean extracts. US, uncooked soybean; CS, cooked soybean; G-I, gastric digestion - inactive enzyme; G-A, gastric digestion -active enzyme; GI-I, gastrointestinal digestion - inactive enzyme; GI-A, gastrointestinal digestion - active enzyme. Results are expressed as millimoles of Trolox equivalents (TE) per gram of soybean seed (mean ± SD, $n = 4$). Bars marked by the same letter are not significantly different ($P < 0.05$).

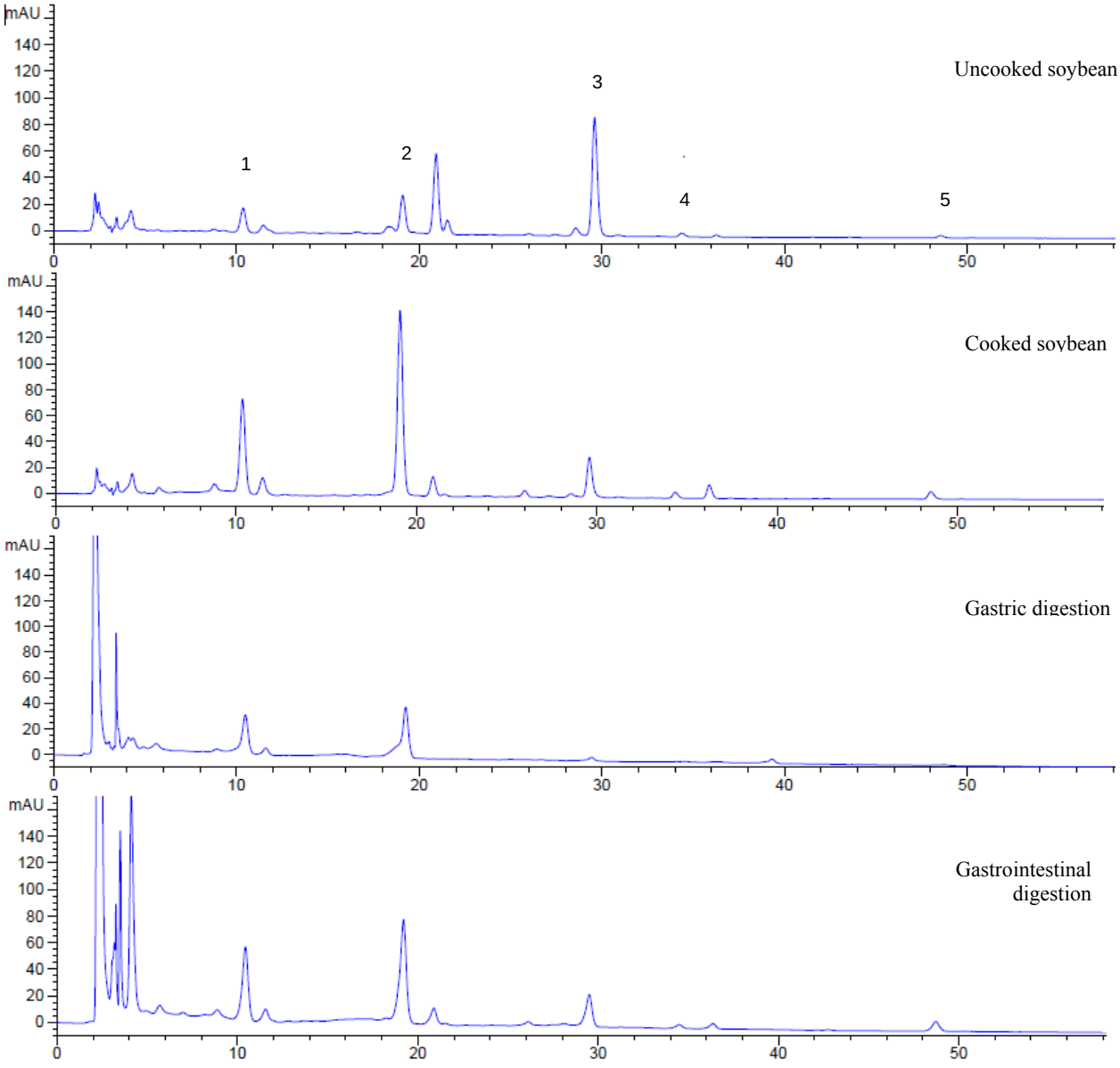

Figure 5.6. HPLC analysis of bioaccessible isoflavone with active enzymes after *in vitro* digestion. Chromatograms are the analysis of isoflavones of bioaccessible fractions from uncooked soybean, cooked soybean, gastric digested, and gastrointestinal digested soybean after *in vitro* digestion.

1. Daidzin; 2. Genistin; 3. Malonylgenistin; 4. Daidzein; 5. Genistein

CHAPTER 6

CONCLUSIONS AND FURTHER STUDY

The consumption of soy products rich in antioxidants, especially in isoflavones, has been shown to be associated with beneficial effects such as the prevention of cancers including breast and prostate cancers, cardiovascular diseases, diabetes and improvements in bone health (*1-5*). Soybeans and soy products are known as the best sources of isoflavones in foods. Besides isoflavones, soybean also contains micronutrients including phytic acid, saponins, phytosterol, vitamins and minerals. Moreover, soy antioxidant properties including isoflavones have been receiving an increasing body of interest since it has been recognized that soybeans have potential therapeutic and health promoting properties. Several studies have shown that contents of phenolic compounds including isoflavones in soybeans and soy products differ among cultivars, and under different conditions of growth and environment, as well as food processing methods (*6-15*).

In this study, isoflavone composition and antioxidant activities of soybean extracts were significantly different among nine Virginia-grown soybean cultivars. Overall, the V01-4937 cultivar could be recommended as it has the highest TPC, ORAC values, and isoflavone contents as well as the second highest DPPH· scavenging activity. The development of Virginia soybean cultivars with increased levels of isoflavones and/or natural antioxidants may potentially benefit Virginia soybean growers and local agricultural economies.

Our results also suggest that different extraction procedures have a remarkable effect on soybean antioxidant assessment, and UAE was more appropriate for soybean phenolic extraction because it is less time and solvent consuming than conventional solvent and soxhlet extractions. However, a conventional 70% aqueous ethanol extract had the highest ORAC values, while the soxhlet methanol extracts had the highest DPPH$^{\bullet}$ scavenging activities. This study may lead to the development of efficient extraction and preparation methods for soybean antioxidants that may lay groundwork for further nutraceutical development.

Our results also provide information that soybean bioaccessible extracts are stable enough to exert antioxidant activities. Isoflavones were stable during model digestion. It is

important to understand whether antioxidants are stable, bioaccessible, and then bioavailable in the body in order to exert their potential bioactivities in human body.

FURTHER STUDY

The soybean variety with the highest TPC, ORAC, and DPPH values was investigated and the stability and accessibility of antioxidant extracts during *in vitro* digestion was evaluated. This study needs to be further developed by investigating phenolic compound profiles including flavonoids and tannin contents in soybean antioxidant extracts. Since we were interested in soybean antioxidants besides isoflavones, these approaches can be valuable. In a previous study, Xu and Chang have evaluated phenolic acid compositions, total flavonoid content, and tannin contents among soybean cultivars and after thermal processing (*14, 16, 17*). There is a lack of information of soybean phenolic compound profiles during digestion. Therefore, this study can be further extended to evaluate phenolic acid compositions of bioaccessible antioxidants during *in vitro* digestion.

In the current study, we conducted the evaluation of bioaccessibility of soybean extracts during *in vitro* digestion. In order to enhance this study, the selected soybean with the highest TPC and isoflavone contents should be evaluated for bioaccessibility using *in vitro* digestion to mimic large intestinal digestion since phenolic compounds may be metabolized by microflora in the intestine. Moreover, the bioavailability of soy antioxidants can be further investigated using *in vitro* studies combining animal or human cell line such as Caco2, which is a human intestinal epithelial cell. These cell culture studies have been investigated for the bioavailability of carotenoids and isoflavones using *in vitro* studies (*18-20*). Even though most polyphenols can be absorbed in the intestine, and isoflavones are known as the most absorbable polyphenol class (*21*), there is limited information on the bioavailability of soybean antioxidants from soybean or soy-based products since most studies have been conducted with pure molecules.

As noted earlier, bioaccessibility and bioavailability are dependent on types of food and food processing. Previously, soy bread, custard, cookies, fruit juice, and chocolate bars have been investigated for assessing the bioaccessibility of isoflavones (*22-24*). Sugar, other carbohydrate, protein and fat contents in the food matrix are crucial factors affecting the bioaccessibility of soybean isoflavones or antioxidants since polyphenols are complex structures

associated with protein and starch, and include both hydrophilic and lipophilic components. With this information, food or nutraceutical products can be developed to increase TPC and isoflavones by using more efficient processing techniques to improve bioaccessibility of soybean antioxidants in the gut.

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