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Isoflavone Distribution in Soy Seed and Antioxidant Activity of Defatted Soy Flour Extract

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ISOFLAVONE DISTRIBUTION IN SOY SEED AND ANTIOXIDANT ACTIVITY OF DEFATTED SOY FLOUR EXTRACT

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ABSTRACT

Soy isoflavones distribution in soy cotelydon, coat and germ were determined. The β-Glucoside form of isoflavones was the major class of isoflavones in all three soy parts. Soy germ was the richest source of the isoflavones among the three soybean parts. Distribution of the isoflavones in soy cotyledon was different from that in soy coat or germ.

As defatted soy flour is a main byproduct in the soy oil refining process, utilizing the low value byproduct as a valuable food antioxidant was investigated in this study. The level of isoflavones in the defatted soy flour extract was 55 mg/g, which was over 100 times higher than in crude oil or gum. Defatted soy flour extract demonstrated the greatest activity in preventing menhaden fish oil oxidation. It could retain over 60% of DHA (docosahexaenoic acid) and 65% of EPA(eicosapentaenoic acid) in the fish oil after heated at 150^{o}C for 30 min, while only 30% of DHA and 37% of EPA were retained in the fish oil with no additive. The defatted soy flour extract also significantly inhibited the generation of rancid volatiles in the fish oil during storage. The antioxidant capability from highest to lowest was defatted flour extract > gum > degummed oil = crude oil.

The antioxidant capability of the defatted soy flour extract and the extract treated with heat and the enzyme were evaluated and compared with common synthetic food antioxidants. While EPA and DHA in the control menhaden oil were degraded to below 10 % after 4 days storage at room temperature, 64% EPA and 60% DHA in the menhaden oil mixed with 5% of the enzyme treated extract and 36% EPA and 28% DHA in the oil with 5% of the heated extract still remained. However, the capability of the heated or enzyme treated extract at 5% addition level was not greater compared to the synthetic antioxidants at 0.02% addition level,

The thermal stabilities of soy isoflavones were evaluated at temperatures of 100, 150, and 200°C. The degradation rate constants increased with increasing heating temperature. The order of thermal stability from low to high was glycitin < genistin < daidzin < glycitein < genistein < daidzein at temperature below 150 ℃. The energy of activation (Ea) of the six isoflavones was in a range from 15.9 to 37.6 KJ/mol. It indicated that the glucoside isoflavones were more liable than aglycone isoflavones at high temperature.

CHAPTER 1 INTRODUCTION

1.1 General Background

Soybean (*Glycine max L. Merrill*) is an important worldwide crop because it is a good source of vegetable oil and high-quality protein for both culinary and industrial usage (Lee et al., 2008). For example, in 2004, apporoximately 223 million tons of soybeans were produced globally and the United States alone produced 85.5 million tons (ASA, 2004). Soybean had been claimed to have medicinal function for some diseases in oriental countries hundreds years ago (Xu and Chang, 2008). The health function has been well studied and documented in the last several decades. Consumption of soybean has been linked to many health-promoting activities, especially in reducing the risk of various cancers (Adlercreutz, 1990; Lichtenstein, 1998; Messina et al., 1994) and coronary heart disease (Anthony et al., 1996). Many studies confirmed that unique components of soybean, isoflavones and phenolic compounds, such as anthocyanins and tocopherols play an important role in protecting against oxidative stress that causes the development of those chronic diseases (Djuric et al., 2001). Soy isoflavones, genistein, glycitin, and daidzein and their derivatives, have the potential to directly scavenge oxidants such as superoxide and nitric oxide, and other free radicals (Coward et al., 1993; Hogg et al., 1993; Stoll, 1997). Isoflavones are also able to decrease oxidative damage in cells via indirect mechanisms, such as induction of antioxidant-scavenging enzymes (Cai and Wei, 1996). In addition to isoflavones, soybean also contains various phenolic compounds and a significant amount of tocopherols, which have been reported to possess antioxidant function as well (Kim et al., 2006; Yoshida, 2003).

The physical structure of the soybean seed consists of germ, cotyledon, and coat. Proteins and carbohydrates are usually contained in the cotyledon of soybean. The major composition of the

germ is lipids (Liu, 1997). However, the information about the distribution of phenolic compounds, tocopherols, and isoflavones in the different structural parts of soybean seed is limited. In this study, the levels of total phenolic compounds, tocopherols, and isoflavones and antioxidant activities in soy germ, cotyledon, and coat were determined.

Soy oil occupies about 80 % of the food vegetable oil market (ASA, 2007). For producing refined soy oil, hexane is a common solvent used to extract about 20 % crude soy oil from the bean. Then the crude soy oil is refined through degumming, bleaching, deodorization, and other processing to obtain final refined soy oil (Erickson et al., 1980). During the oil processing, a large quantity of byproducts, such as defatted soy flour and gum, and intermediate oils, are generated. As chemical polarity of soy antioxidants, phenolic compounds, isoflavones, and tocopherols are different, they could have different distribution in the byproducts and intermediate oils after the hexane extraction and degumming process. Thus, an understanding of soy antioxidant distribution in those soy products, especially the defatted soy flour, could be helpful to utilize the lower value and abundant byproduct as a valuable source for producing effective and safe natural antioxidants. These could be used in either food preservation or nutritional supplements. In this study, the antioxidants capabilities of soybean crude oil, degummed oil, gum, and defatted soy flour produced during soy oil refining were investigated. The total phenolic compounds, isoflavones, and tocopherol contents in the oils and byproducts were determined. The antioxidant capabilities of those products were evaluated using the DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical quenching method and a menhaden fish oil oxidation model.

Fish oil is a rich source of polyunsaturated fatty acids, especially omega-3 fatty acids such as EPA and DHA (Wanasundara and Shahidi, 1998). Bang and Dyerberg (1972) found that low incidence of coronary artery disease among Eskimos is contributed by the nutritional properties

of omega-3 polyunsaturated fatty acids (PUFAs) in their diet. Numerous studies have reported that polyunsaturated fatty acids can reduce risk factors associated with degenerative diseases, including cancer, cardiovascular disorders, and other inflammatory conditions (Perez-Mateos et al., 2004). However, the omega-3 PUFAs in fish oil are readily oxidized to produce off- or rancid-flavor volatiles when exposed to light, oxygen, prooxidants, and high temperatures (McClements and Decker, 2000). Most lipid oxidation has a detrimental effect on the quality of lipid food, as it leads to the development of off-odors and off-flavors (Yinci et al., 1995; Brannan and Erickson, 1996). Thus, the quality of fish oil or foods fortified with fish oil usually deteriorates rapidly without any stabilizing treatments. The instability of omega-3 PUFA during processing and storage is becoming a serious hurdle for developing the fish oil functional foods. In order to prevent lipid oxidation, synthetic antioxidant such as butylated hydroxyanisole (BHA), butyl-4-methylphenol (BHT), tert-butylhydroquinone (TBHQ), and propylgallate (PG) were widely added to foods. However, use of synthetic antioxidants in food may cause potential health hazards. Several studies found that BHA caused conversion of ingested material into toxic substances or carcinogens due to increased secretion of microsomal enzymes of liver and extra-hepatic organs, such as the lungs and gastrointestinal tract mucosa (Ito et al., 1982; Wattenberg, 1986). Due to the safety concerns of synthetic antioxidants, discovery of natural antioxidants, especially of plant origin, which are generally recognized to be safe has notably increased in recent years (Wanasundara and Shahidi, 1998; Yanishlieva, and Marinova, 2001). As soy contains different types of antioxidants mentioned above, using soy antioxidants to serve as natural antioxidant ingredients to stabilize fish oil and other food systems containing omega-3 PUFA would be beneficial not only in prolonging the food storage stability but also supplementing the health promoting function from soy antioxidants as well. Therefore, in this

study, menhaden oil was used as an acatual food system to determine the antioxidant capabilities of soy products and synthetic anatioxidants.

Glucoside and aglycone isoflavones have different antioxidant activity and bioavailability. The aglycone isoflavones have more antioxidant activity and bioavailability compared to the glucoside isoflavones because they have more hydroxyl groups and lower molecular weight with higher absorption (Izumi et al., 2000). However, compared with glucoside isoflavones, only a small amount of the total isoflavones exists in aglycone forms in soy (Pham and Shah, 2009). To convert glucoside isoflavones to aglycone isoflavones in a soy product could increase its antioxidant activity and bioavailability. The glucoside form of isoflavone could be cleaved through heating or hydrolytic action of β-glucosidase to generate aglycone isoflavones (daidzein, glycitein, and genistein) (Chien et al., 2005). In this study, heating and enzymatic hydrolysis methods were used to convert the glucoside isoflavones to aglycone isoflavones. The changes of their antioxidant activity after the conversion were evaluated.

As thermal processing is the most common food preparation method, the changes of the content and distribution profile of soy isoflavones due to heat treatments have been confirmed by many researchers (Xu et al., 2002; Coward et al., 1998; Mahungu et al., 1999; Ungar et al., 2003; Pinto et al., 2005; Huang et al., 2006). These changes during heating could directly affect their overall antioxidant activity in the systems. Acetyl glucoside and malonyl glucoside are the aglycone derivatives, which could also convert to aglycone and other derivative types during heating. Most thermal lability studies of isoflavones were performed in soybean food systems. As the soy food system has four different types of isoflavones, the degradations of aglycone and their glycosolated derivatives and conversions of the glycosolated isoflavones to other forms of isoflavones could take place at the same time during heating. Those studies were not able to clearly reveal the true kinetics and thermal stability of each individual glucoside and aglycone in

such a dynamic and complicated actual system during heating. In this study, pure glucoside and aglycone forms of isoflavones were used to evaluate their thermal stabilities individually in order to eliminate any conversion from their precursor isoflavones during the heating process. The results of this study could clearly provide the information of the real kinetics and thermal stability of each glucoside or aglycone form of isoflavone at different heating temperatures.

1.2 Objectives

1. To determine total phenolic compounds, tocopherols, and isoflavones in soybean germ, cotyledon, and coat and evaluate the antioxidant activities of the extracts from the three soy parts.
2. To compare total phenolic compounds, tocopherols, and isoflavones in crude soy oil, degummed oil, gum, and defatted soy flour extract and their antioxidant activity in inhibiting menhaden oil oxidation and scavenging DPPH free radicals.
3. To determine the change of antioxidant activities of defatted soy flour extracts after heat treatment and enzymatic hydrolysis and compare their antioxidant activities with synthetic antioxidants in inhibiting menhaden oil oxidation and scavenging DPPH free radicals.
4. To evaluate the thermal dynamic properties of isoflavones using a kinetic model and pure standards.

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CHAPTER 2 LITERATURE REVIEW

2.1 Chemical Composition of Soybean

Soybean is a major commercial crop in over 35 countries with total production above 200 million metric tons annually. The major soybean production countries are the United States, Brazil, Argentina, China, India, Paraguay and Canada. Soybean and soy products are widely used in various food products and animal feeds. Soy oil represents one of the most important edible vegetable oils. Soy protein is broadly used for livestock feed in the United States. Although the quantity of direct soybean consumption as human diets is much lower than that for producing soy oil, it continues to increase due to improvements in taste of soy foods and recognition of health benefits of soybean. The world soybean production is expected to increase to 8-9 billion by 2050 (USDA, 2004).

Compared with other food legumes, soybean has a higher level of protein (about 40% on a dry weight basis) and lipid content (about 20%). It also contains other valuable components which include phospholipids, vitamins, and minerals (Liu, 1997). A whole soybean seed consists of three important parts, soy embryo, cotyledons, and coat. The proximate compositions of whole seed and the three parts are listed in table 2.1 (Liu, 1997).

2.2 Health Benefits of Soybean

Recently, many clinicians and researchers are interested in the potential role of soyfoods in preventing and treating chronic diseases (Liu, 1997). Dietary intake of soybean has been linked to prevention of osteoporosis, cardiovascular disease and cancer, including breast, colon, and prostate cancers (Adlercreutz, 1995; Stoll, 1997). Steele (1992) replaced milk with a soy beverage and found that soy beverage decreased blood cholesterol levels about 5 to 10 % and LDL cholesterol levels 10 to 20 % within 4 weeks. The proposed mechanism for the action of

Table 2.1 Proximate composition of soybeans and their structure parts

	Percentage in whole seed	Dry matter (%)			
		protein	lipid	carbohydrate	ash
Coat	8	9	1	86	4.3
Embryo	2	41	11	43	4.4
Cotyledons	90	43	23	29	5.0
Whole seed	100	40	20	35	5.0

Referred from (Liu, 1997)

soy is that isoflavones may be potent inhibitors of cholesterol synthesis and able to block cholesterol absorption from the diet or increase cholesterol excretion from the body (Potter, 1995). Soy isoflavones were also recognized to play the most important role in reducing the formation and progression of certain types of cancers and some chronic diseases such as Alzheimer's disease and osteoporosis (Messina, 1999).

Besides soy isoflavones, a higher level of soy phenolic compounds and tocopherols has been reported, which may perform as antioxidants to protect against oxidative stress from free radicals (Djuric et al., 2001; Fritz et al., 2003; Andlauer et al., 1999; Meng et al., 1999). Free radicals are oxidants and able to oxidize biomolecules and lead to mutagenic changes, tissue damage and cell death (Yang et al., 2000). They have a significant pathological role in the development of cancer, emphysema, cirrhosis, atherosclerosis, arthritis, and various other degenerative diseases. Soy isoflavones, phenolic compounds, and tocopherols are greater antioxidants and can reduce the access of oxidants and other deleterious molecules, due to their ability to quench oxygen-derived free radicals by donating a hydrogen atom or an electron, or to chelate redox-active metals and inhibit lipooxygenases (Tripathi and Misra, 2005).

2.3 Chemical Mechanism of Antioxidation and Natural Antioxidants

Autoxidation refers to the reaction with molecular oxygen via a self-catalytic mechanism (Fennema, 1996). Oxidation proceeds through a free-radical chain reaction mechanism involving three stages.

1. Initiation - formation of free radicals

$$RH \rightarrow R\cdot + H\cdot$$

2. Propagation - free-radical chain reaction, in which an unsaturated hydrocarbon loses a hydrogen to form a radical.

$$RH \rightarrow R\cdot + H\cdot$$

$$R\cdot + O2 \rightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

3. Termination - formation of nonradical products

$$R\cdot + R\cdot \rightarrow \text{Nonradical products}$$

$$R\cdot + ROO\cdot \rightarrow \text{Nonradical products}$$

Free radicals are produced during peroxide formation - hydroperoxides are the major initial reaction products of fatty acids with oxygen. Subsequent reactions control both the rate of reaction and the nature of products formed (Fennema, 1996).

Antioxidants are chemicals that can retard free radical oxidation reaction. Three mechanisms are considered for inhibition of oxidative reactions by antioxidants 1) interrupting the free-radical oxidation; 2) being preferentially oxidized; 3) can bind or deactivate trace metals that are free or salts of fatty acids by the formation of complex ions or coordination compounds. The first one is the main mechanism for most antioxidants.

An antioxidant (AH) reacts with free radicals to interrupt the oxidation as following (Fennema, 1996):

$R \cdot + AH \rightarrow RH + A \cdot$

$RO \cdot + AH \rightarrow ROH + A \cdot$

$ROO \cdot + AH \rightarrow ROOH + A \cdot$

$R \cdot + A \cdot \rightarrow RA$

$RO \cdot + A \cdot \rightarrow ROA$

The hydrogen donation to free radicals is the main mechanism of antioxidation action. The reaction starts in a lipid system with the formation of a complex between the lipid radical and the antioxidant (free radical acceptor) (Yanishlieva and Marinova, 2001). The antioxidant radicals $A \cdot$ formed in above reactions have a very low reactivity and do not propagate the chain reaction after forming a complex lipid antioxidant, which inhibits the formation of extremely reactive peroxy radicals and hydroperoxides. Thus, they terminate the free radical initiated reaction and protect the lipid from oxidation.

There are two major types of antioxidant based on their different sources: synthetic and natural antioxidants. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA**)**, propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are widely used in the past decades as food additives to increase food shelf life, especially in lipids and lipid-containing products by retarding the process of lipid oxidation. However, BHT and BHA are suspected to have not only carcinogenic effects on humans (Ito et al., 1986; Wichi, 1988), but also toxic effects on enzyme systems as well (Inatani et al., 1983). Therefore, there has been a considerable interest in the food industry to find natural or safe antioxidants to replace synthetic compounds in food applications.

Lately, the safety and health benefits of antioxidants from natural sources, such as fruits and cereals, have been reported by numerous studies and recognized by the FDA and many consumers. The antioxidants from natural sources are generally considered as safe food

ingredients. Two families of antioxidants, polar (hydrophilic) antioxidants, such as phenolic compounds, and non-polar (lipophilic) antioxidants, such as tocopherols, are found in the grains and cereal (Adom and Liu, 2005; Truswell, 2002). Those compounds have hydroxyl groups on a chromanol ring, which have the ability to scavenge free radicals and inhibit lipid oxidation (Steinmetz and Potter, 1996). Traditionally, antioxidants are extracted from grain and cereal using conventional organic solvent methods. However, phenolic compounds and tocopherols differ in polarity causing different recovery in the extraction. Because phenolics, like ferulic, vanillic, and caffeic acids, are hydrophilic and have small molecular weights, previous studies have shown that more polar solvents (e.g. methanol or acetone) extract greater amounts of phenolics from cereal bran (Kahkonen et al., 1999; Sun et al., 2006). Tocopherols and phytosterols are more lipophillic than phenolics and readily extracted with hexane or other non-polar solvents (Moreau et al., 2003; Nystrom et al., 2005; Xu and Godber., 1999).

Common grains, corn, oat, wheat, rice and their byproducts have been reported to have unique antioxidants (Kahkonen et al., 1999; Martinez-Tome et al., 2004; Oufnac et al., 2007; Sun et al., 2006; Xu and Godber, 2001; Xu et al., 2001). Cereal antioxidants, including phenolic acids, procyanidins, tocopherols, phytosterols, and other phytochemicals are capable of decreasing health problems like cardiovascular disease and certain cancers by hindering lipid oxidation, scavenging harmful free radicals, and slowing the arteriosclerosis process (Steinmetz and Potter, 1996). Not only do antioxidants in grains have potential health benefits, but they also are attributed to “maintaining the oxidative stability” of cereal products inhibiting rancidity (Peterson, 2001).

2.4 Antioxidants and Their Health Benefits in Soybean

Several important antioxidants have been identified in soybean, including isoflavones, tocopherols,and phenolic compounds, which contribute to the health benefit function of soybean.

2.4.1 Soy Isoflavones

Soy isoflavones are a group of flavonoids and have a particularly limited distribution in nature. They are mainly found in soybeans and its food products. The basic structures of soy isoflavones are C6-C3-C6 shown in the figure 2.1. Besides major isoflavones, genistin, daidzin, and glycitin, soy also contains different types of isoflavone derivatives which have an acetyl, malonyl, or glycoside group bonded on one C6 ring. Also more than one hydroxyl groups are connected to the two C6 rings. Ribeiro et al. (2007) reported **t**he total isoflavone content varied from 71.11 to 174.30 mg/100 g in early cultivars; 61.83–127.96 mg/100 g in semi-early cultivars, and 80.57–128.26 mg/100 g for the medium maturity group. The results showed that there were significant differences in the total isoflavone content among cultivars.

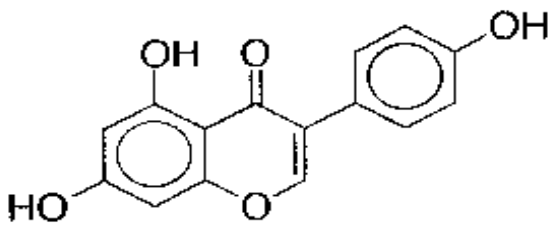


Figure 2.1 Basic structure of soy isoflavone

Uniquely, the higher level of isoflavones in soy is a key factor that contributes to soy health benefits. Many *in vitro* and *in vivo* studies demonstrated that soy isoflavone intake is consistent with those soy health benefits (Lee et al., 2005). Soy isoflavones are able to interact with cellular receptor of estrogens to reduce the risk of coronary heart breast cancer, due to their structural similarity (Tikkanen and Adlercreutz, 2000). They also are able to reduce the level of total cholesterol, low-density lipoprotein cholesterol, and LDL oxidation to prevent cardiovascular diseases (Meng et al., 1999). Their antioxidant capacities are associated with the number of hydroxyl groups in their structure (Naim et al., 1976).

2.4.2 Phenolic Compounds

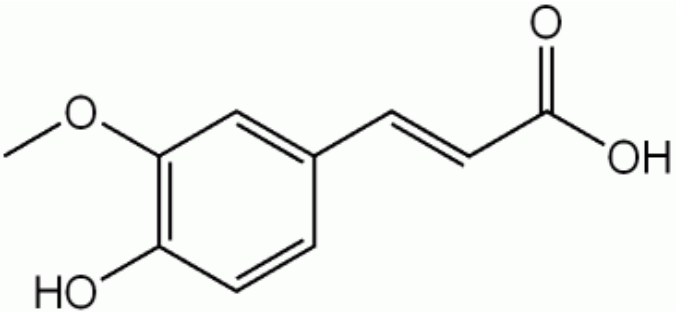


Figure 2.2 Basic structure of phenolic compound

Phenolic compounds belong to a class of compounds with a great variety of biological effects, including anti-inflammatory, antimicrobial and antioxidant activities (López-Vélez et al., 2003; Wen et al., 2003). Phenolic compounds have an aromatic ring bearing one or more hydroxyl groups (Figure 2.2). They are classified into three major groups based on their chemical structures: simple phenol and phenolic acid derivates, hydroxycinnamic acid derivates, and flavonoids. The simple phenol group includes vanillic acid, gallic acid with other derivative forms. The phenolic acid derivatives group contains *ρ*-coumaric acid, *trans*-cinnamic acid, caffeic acid, ferulic acid and their conjugated forms, ester or glucoside (Kim et al., 2006).

These phenolic compounds have many antioxidative effects and health promoting functions. They have been reported to exhibit medicinal properties such as antitumor, antiviral, antimicrobial, anti-inflammatory, hypotensive and antioxidant activities (Robbins, 1980; Matsubara et al., 1985; Robak and Gryglewski, 1988). They are gaining popularity because of their demonstrated effects to reduce the risk of certain cancers, stroke, and heart disease (Keli et al., 1995).

2.4.3 Tocopherols

Tocopherols, synthesized by photosynthetic organisms, are the antioxidants that occur naturally in vegetable oils to maintain oil quality by terminating free radicals (Yoshida et al., 2003). The

chemical structure of tocopherols is shown in figure 2.3. Tocopherols play important roles in human and animal nutrition. In soybean, γ-tocopherol, the biosynthetic precursor to α-tocopherol, is the predominant form found in the seed (Liu and Brown, 1996). On a dry basis, the amounts of α-, γ- and δ-tocopherols in the soybeans ranged from 10.9 to 28.4, 150 to 191, and 24.6 to 72.5 ug/g, respectively (Eskin et al., 1996; Speek et al.,, 1985; Jung et al., 1989).

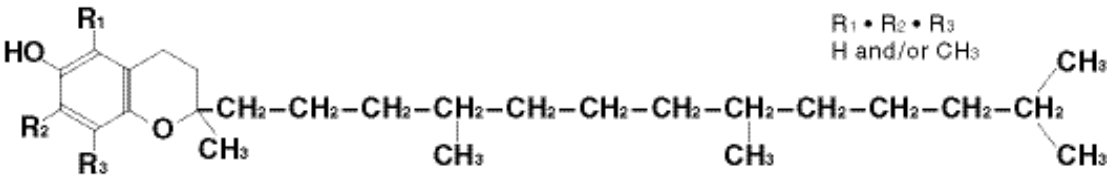


Figure 2.3 Chemical structures of tocopherols

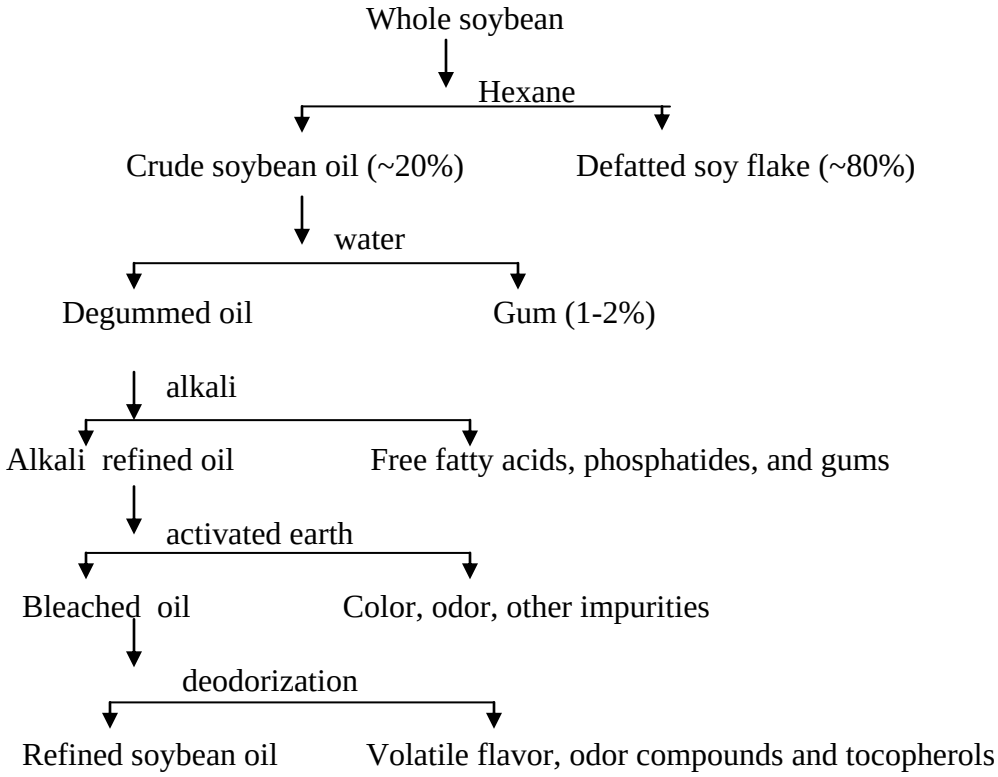
2.5 Soy Oil Refining Process and Byproducts

In the soybean oil industry, soybean oil (soy oil) is extracted by hexane from whole soybean. The flow chart of soybean oil refining process is listed in table 2.2. The oil content of soybeans is around 20% dry basis (Erickson et al., 1980). The composition of fatty acids of soy oil is listed in table 2.3.

Defatted soy flour is the residue produced after the nearly complete removal of the oil from soybean. Defatted soy flour occupied about 80% of dry soybean flour. Defatted soy flour contains most soy protein and carbohydrates and is usually used for animal feed. Only a small portion of it is further processed into different types of soy protein products for human consumption (Liu, 1997).

Degumming is a process that involves mixing crude soybean oil with 2-3% water and gently agitating for 30 - 60 minutes at a temperature of 70˚C (Erickson et al., 1980). Thus, soy phosphatides and other impurities can be settled, filtered, or centrifuged out from the degummed oil. Around 1~2% phospholipids can be removed by degumming from crude oil.

Table 2.2 Flow chart of soybean oil refining process



Tocopherols in crude and deodorized soybean oil were 1670 and 1138 ppm, respectively (Player et al., 2006). About 4.3% of α-tocopherol was lost during hexane extraction and loss of 15% in bleaching and 20 ~51% in deodorization (Kanematsu et al., 1983). This loss of tocopherols does not reduce the oxidative stability of the processed soybean oil because its final tocopherol content is between 700 and 1000 ug/g, which is still above the optimum range of 400 - 600 ug/g (Kanner et al., 1994).

Table 2.3 Fatty acid composition of soybean oils (Percent by weight of total fatty acids)

Fatty acid		% total fatty acids
Unsat./Sat. ratio		5.7
Saturated	Capric Acid (C10:0)	-
	Lauric Acid (C12:0)	-
	Myristic Acid (C14:0)	-
	Palmitic Acid (C16:0)	11
	Stearic Acid (C18:0)	4
Mono- unsaturated	Oleic Acid (C18:1)	24
Poly- unsaturated	Linoleic Acid (ω6) (C18:2)	54
	Alpha Linolenic Acid (ω3) (C18:3)	7

Referred from (Zamora, 2005).

*Percentages may not add to 100% due to rounding and other constituents not listed

2.6 Antioxidant Activity and Bioavailability of Isoflavones and Conversion of Glucoside to Aglycone Isoflavones

Aglycone isoflavones have a higher antioxidant capability and bioavailability than the glucoside isoflavones (Pyo et al., 2005; Kao and Chen, 2006). Xu et al. (1994) have reported that daidzein was found to have higher bioavailability than genistein in adult women. Izumi et al. (2000) found that aglycone soy isoflavones were absorbed faster and in higher amounts than their glucosides in a human study. Thus, conversion of glucoside isoflavones to aglycone isoflavones using a heating method could increase soy health benefits (Mathias et al., 2006). However, heating could also cause the degradation of aglycone and other forms of isoflavones. To optimize the heating condition for maximizing the conversion without isoflavones loss is difficult because it involves many factors such as temperature, time, soy variety, sample size, etc.

2.7 Enzymatic Interconversion of Glucoside Isoflavone to Aglycone Isoflavone

Chuamkhayan et al. (2007) reported that *D. cochinchinensis* β-glucosidases could hydrolyze both conjugated and non conjugated glycosides in crude soy extract and greatly increased the aglycone isoflavone. The β-glucoside form of isoflavone could be cleaved through the hydrolytic action of β-glucosidase to generate daidzein, glycitein, and genistein (Chien et al., 2005). Lee and Chou (2006) had noted that the content of the aglycone was significantly increased (daidzein, from 30 to 150 mg/g; genistein, from 30 to 120mg/g) in black soybean and β-glucoside isoflavone content was markedly reduced after fermentation at 30°C for 3 days. Kim et al. (2004) reported a significant increase in daidzein and genistein during 8 days germination. It was concluded that glucosidases existing in soybean also could hydrolyze glucoside isoflavones to aglycone isoflavones. Compared with using heating method, enzymatic conversion may be more easily optimized to yield aglycone isoflavones without isoflavone degradation.

2.8 Thermal Stability of Soy Isoflavones

Isoflavones are found to have four chemical forms: aglycone (daidzein, glycitein and genistein), glucoside (daidzin, glycitin and genistin), acetylglucoside (acetyldaidzin, acetylglycitin and acetylgenistin), malonylglucoside (malonyldaidzin, malonylglycitin and malonylgenistin) (Huang et al., 2006). In general, isoflavones were either degraded under normal thermal processing conditions or subject to conversions between the different forms (Huang et al., 2006). Chien et al. (2005) reported that the pathway of aglycone isoflavone interconversion could be from malonyl β -glucosides → acetyl β–glucosides → β-glucosides → aglycone structures of soy isoflavones during heating without oxygen in a model system. β-glucoside form isoflavones are the intermediate products in the degradation chain. They could be decomposed by losing the β-glucoside groups or produced by de-esterification from malonyl and acetyl glucoside soy

isoflavones at the same time (Xu et al., 2001). Murphy et al. (2002) also reported that malonylglycosides and acetylglycosides are thermally unstable and can readily be converted to their respective more heat-stable non-conjugated β-glycosides. Park et al. (2002) measured the isoflavone profiles in soy flour and showed a decrease in total isoflavones (20.6% ± 7.9%) for all cultivars after heating at 121^{o}C for 40 min. Daidzein and glycitein were not stable and sensitive to UV – Vis light and malonyl isoflavone degraded with prolonged storage (Rostagno et al., 2005). Heating could also cause the total degradation of isoflavones without forming aglycone. Chiarello et al. (2006) observed that 20% of total isoflavone content decreased and the isoflavone distribution profile remained nearly unchanged in soy milk after heat treatment. They also found a decrease in aglycone content and low levels of acetyl forms after 15 min autoclaving. Grun et al. (2001) also reported that total isoflavone content decreased during aqueous heating of tofu because of a great decrease in daidzein.

Also, thermal stabilities of isoflavones under acid or alkali condition during heating were studied. Wang et al. (1990) reported that heating daidzin and genistin conjugated forms under acidic conditions released free isoflavones. However, Mathias et al. (2006) found that no conjugated isoflavones could be converted to their corresponding aglycones form under alkaline or acidic condition with heating treatment.

2.9 Application of Natural Antioxidants in Inhibiting Fish Oil Oxidation

2.9.1 Omega-3 Long Chain Fatty Acids and Their Oxidation in Menhaden Oil

Marine fish oils from menhaden, mackerel, tuna, salmon, cod and others have been recommended in the daily diet for preventing heart diseases because they contain polyunsaturated omega- 3 fatty acids, such as EPA and DHA. Polyunsatureated omega-3 (or n-3) fatty acids (PUFA) are all cis polyenoic fatty acids with at least three double bonds and have the first double bond located between carbons 3 and 4 counting from the methyl (omega) end of the

chain. The other double bonds are each separated by a methylene (-CH_2) group. Table 2.4 gives the composition of fatty acids in Menhaden fish oil. Menhaden oil is one of the fish oils containing higher level of omega-3 PUFAs. DHA and EPA in the oil are 11.5 and 13.5 %, respectively.

Table 2.4 The composition of fatty acid in Menhaden fish oil

Fatty acid		Fish oil (%)
saturated	C14:0	5.6
	C16:0	21.6
	C18:0	9.0
	C20:0	—
	C22:0	5.3
mono-unsaturated	C16:1	7.9
	C18:1 *n*-9	15.5
	C20:1	—
poly-unsaturated	C18:2 n-6 (linoleic acid)	1.5
	C18:3 n-3 (α-linolenic acid)	1.4
	C20:5 *n*-3 (eicosapentaenoic acid, EPA)	13.5
	C22:6 *n*-3 (docosahexaenoic acid, DHA)	11.5
	Other	5.8

Referred from (Chen et al., 2006)

*Values are expressed as percentages of the total amount of fatty acids.

2.9.2 Oxidation of Omega-3 Polyunsaturated Fatty Acids

Fish oil is readily spoiled in two major ways: oxidative and hydrolytic degradation, due in part to the high unsaturated levels of EPA and DHA (Cmolik and Pokorny, 2000; Huss, 1988). The

lipid oxidation of fish oils is the most important cause of deterioration in fish oil quality (Lundberg, 1967). Undesirable flavors and odors develop at an early stage of oxidation, even during the induction period (Stansby, 1967). The oxidation of PUFA results in generation of a mixture of aldehydes, peroxides, and other oxidation products, which contribute to off-flavors and substantial reduction in the shelf life of products.

The oxidation of fish oil have been evaluated by a number of research groups. Huss (1988) reported the acceptability limit for PV (peroxide value) of crude fish oil was 7~8 meq O_2/kg oil. Boran (2006) reported the fish oil samples of horse mackerel, shad, garfish and golden mullet stored at 4 ℃ reached this limit in 90 days. The samples stored at −18 ℃ did not exceed the acceptability limit within 150 days. Verma et al. (1995) reported that peroxide value (PV) of sardine oil stored at −20 ℃ increased from 4.12 to 18.6 during frozen storage for 150 days. In another study, PV of whole fish of whiting and horse mackerel stored at −18 ℃ for 360 days showed an increase from 3.46 and 8.46 to 28.3 and 24.8, respectively (Simeonidou et al., 1997). Frankel et al. (2002) investigated the oxidation stability of fish oil and algae oil and found that temperature had a significantly greater effect on them. Between 40 and 50˚C, the peroxide values increased 2.1-fold in the fish oil and 1.2-fold in the algal oil after oxidation for 48 h. Between 50 and 60 ˚C, the peroxide value increased 20.8-fold in the fish oil and 2.2-fold in the algal oil.

TBA value of fish oil samples is an important index of the PUFA oxidation and increases during storage, like AV (acid value) and PV. Karacam and Boran (1996) reported that TBA values of whole or gutted anchovy increased from 0.3 to 3.1 ppm during storage for 180 days at −18 ℃. In another study, TBA of whole fish of *M. mediterraneus* and *T. trachurus* increased from 0.026 to 0.192 and from 0.129 to 0.264 mg/kg, respectively, after storage for 360 days at −18 ℃ (Boran, 2006).

However, these studies are mainly based on the level of lipid oxidation products, such as peroxide value (PV) and thiobarbituric acid reactive substances (TBA). The direct observations on the changes or oxidation of omega-3 PUFA during the fish oil or meat storage are less often reported. Only Borquez et al. (1997) reported that the total level of omega-3 fatty acids dropped from 120 mg to 40 mg/g oil after 7-day storage at 25˚C.

2.9.3 Using Natural Antioxidants in Preventing PUFA Oxidation

Adding antioxidants from plant extracts to retard or prevent lipid oxidation in a variety of food products has been studied (Boyd et al., 1993; Frankel et al., 1998; Birch et al., 2001; Rababah et al., 2004). Plant phenolics and flavanoids effectively minimized lipid oxidation in lipid-based food products (Rababah et al., 2004). Pe ŕez-Mateos (2006) investigated the quality of two different sources of omega-3 fatty acids (fish oil concentrate and menhaden oil) with or without the addition of natural antioxidants (rosemary and green tea). They found that all fish oil samples containing antioxidants were fairly stable during frozen storage. Pazos et al. (2005) reported grape extract could effectively inhibit the formation of hydroperoxydes in fish oils at 40 ℃. Medina (1999) found the extract from virgin olive oil containing 400 ppm polyphenols was an effective antioxidant as compared with 100 ppm of a 1:1 mixture of the synthetic antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) when tuna was oxidized at 40 and 100 ℃. It indicated that natural antioxidants could be more effective than synthetic antioxidants in preventing PUFA oxidation.

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CHAPTER 3 DISTRIBUTION OF ISOFLAVONES AND ANTIOXIDANT ACTIVITIES OF SOYBEAN COTYLEDON, COAT AND GERM

3.1 Introduction

Soybean is an important commodity in the world and also one of the two leading crops in the United States. It is a rich source of a group of flavonoids, isoflavones. The health functions of soy isoflavones in preventing development of heart disease and cancers have been confirmed by many studies (Anderson and Garner, 1997; Messina et al., 1994; Booth et al., 1999; Coward et al., 1993; Caragay, 1992). Daidzein, glycitein, and genistein are the three basic aglycones isoflavones without sugar moiety. Daidzin, glycitin, and genistin are β-glucoside isoflavones which are derivative forms from their corresponding aglycones. The β-glucoside isoflavones are the major form of isoflavone found in unprocessed soy flour (Xu et al., 2002). They could be decomposed by losing the β-glucoside group to form aglycone isoflavones during heating and fermentation (Xu et al., 2002).

The chemical structural differences of soy isoflavones may result in variable bioavailabilities in biological systems. Isoflavones with daidzein aglycone was found to have higher bioavailability than the isoflavones with genistein aglycone in adult women (Xu et al., 1994). Aglycone soy isoflavones were absorbed faster and in higher amounts than their glucoside isoflavones in a human study as well (Izumi et al., 2000). The content of each isoflavone was reported to be fairly different between soybean variety and cultivation condition, such as area, year and temperature (Lee et al., 2003; Zhu et al., 2005; Riedl et al., 2007; Kim et al., 1997; Kim et al., 2006). Also, chemical composition, such as lipids, proteins and carbohydrates, in the three soy parts, cotyledon, coat and germ are very different. Soy cotyledon mainly contains protein, while soy germ is rich in carbohydrates (Liu, 1997). In the same variety of soybean, the amount

of each isoflavone in the three soy parts could be significantly different as well. In this study, the levels of different isoflavones in soy cotyledon, coat and germ from four soy varieties were determined and compared.

Similar to other seeds, soybean contains a number of different types of phenolic antioxidant compounds, such as ferulic, vanillic, caffeic, coumaric, and syringic acids (Cheigh and Lee, 1993). Those phenolics in soybean are generally believed to be responsible for the positive effects on cardiovascular disease and cancers. The phenolics and soy isoflavones are also able to prevent undesirable lipid oxidation reactions in the body that lead to the risk of these diseases (Cheigh and Lee, 1993). In this study, total phenolic contents in the soy cotyledon, coat and germ of the four soy varieties were also determined and compared. The DPPH (2,2'-diphenyl-1-picrylhydrazyl) method, as a measurement of the capability of scavenging free radicals, was used to measure the antioxidant activity of the extracts of different soy parts. The results of this study would be helpful in the development and utilization of different soy parts as food antioxidants or nutritional supplements. They also provided useful information for effectively processing all parts of soybean based on their unique isoflavone composition and antioxidant activity.

3.2 Materials and Methods

3.2.1 Materials

Hexane and methanol were HPLC grade and purchased from Fisher Scientific Inc. (Fair Lawn, NJ). DPPH (2,2'—diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, catechin, and 2-thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, Mo). Soy isoflavones standard daidzin, glycitin, genistin, daidzein, glycitein, and genistein were purchased from Sigma-Aldrich (St. Louis, Mo). Four different varieties (we used simple letters A, B, C, D to represent the four varieties) of soybean were obtained because they were harvested and available from local farmer markets.

3.2.2 Soy Parts Separation and Extraction

Whole soybean seed was manually separated into three parts: cotyledon, coat and germ. Then each part was ground into powder using a coffee bean blender. The particles of the ground soy part were able to pass through a 1 mm sieve. The ground soy part (3.00 g) was weighed into a 25-mL test tube and extracted using 10 mL methanol. The mixture was incubated at 60 ℃ in a water bath and stirred gently for 20 min. After the incubation, the solvent layer was separated from the solid residue by centrifuging at 2000 ×g for 10 minutes using a Hermel Z383 K table top centrifuge (National Labnet Company, Woodbridge, NJ). The clear supernatant was transferred to a clean test tube. Then the solid residue was extracted with another 10 mL of methanol. The separated methanol layers were combined and dried using a vacuum evaporator at 50 ℃. The dried soy part extract was weighed and stored at -20 ℃.

3.2.3 Determination of Isoflavone Content Using HPLC

Soy part extract solution (5 mg/mL methanol) was transferred to HPLC vials. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm x 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium32 chromatography manager. The mobile phase was a mixture of water and ethanol, with percentage of water in ethanol ramped from 90% to 50% in 40 min with a constant flow rate of 0.3 mL/min (Xu et al., 2002). The chromatograms obtained at a wavelength of 254 nm were used to quantify the isoflavones. The concentrations of the three major isoflavones, daidzin, genistin, and glycitin and daidzein, glycitein, and genistein were calculated based on their standard curves. The total isoflavone content was calculated by summing the six isoflavone concentrations.

3.2.4 Determination of Total Phenolic Content

The total phenolic content of the soy extracts was determined using the Folin-Ciocalteu reagent (Velioglu et al., 1998). Folin-Ciocalteu reagent was diluted 10 times with deionized water. The

cotyledon, coat, or germ extract (20 mg) was dissolved in 10 mL methanol. The extract solution (0.10 mL) was mixed with 0.75 mL diluted Folin-Ciocalteu reagent. The reaction solution was left at 25°C for 5 min. Then 0.75 mL of sodium bicarbonate solution (60 g/L) was added. The mixture was incubated at 25°C for 90 min and filtered through a 0.45 μm syringe filter (Pall Corporation, Ann Arbor, MI). The absorbance of the solution was determined at 750 nm using a UV-Visible SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Catechin was used to prepare a standard curve. The total phenolic content was calculated and expressed as μg catechin equivalent / gram of soy part extract.

3.2.5 Determination of Antioxidant Activity Using DPPH Method

The concentration of DPPH solution was 0.025 g in 1000 mL of methanol. The cotyledon, coat, or germ extract (20 mg) was dissolved in 10 mL methanol. One mL of the DPPH solution was mixed with 200 μL of each extract methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm for 30 min at room temperature (about 25°C) using a UV-Visible SpectraMax Plus^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA). The inhibition percentage was calculated using the following equation (Moure et al., 2000):

$$\text{Inhibition\%} = (\text{Abs}_{t=0\ \text{min}} - \text{Abs}_{t=30\ \text{min}}) / \text{Abs}_{t=0\ \text{min}} \times 100$$

Where $\text{Abs}_{t=0}$ min was the absorbance of DPPH at zero time and $\text{Abs}_{t=30}$ min was the absorbance of DPPH after 30 min of incubation.

3.2.6 Statistical Analysis

The extraction of each soy part from different soybean varieties was performed in triplicate. The determination of total isoflavone and total phenolic content and DPPH free radical scavenging activity for each extract were performed in duplicate. The means and standard deviations were calculated and the data were analyzed by one-way ANOVA with duncan's multiple range test comparisons at $\alpha = 0.05$ by using SAS Statistical Analysis System (SAS Inst., Cary, NC).

3.3 Results and Discussions

3.3.1 Isoflavones in Soy Cotyledon, Coat, and Germ

Table 3.1 is the extraction yields of soy cotyledon, coat, and germ from the four varieties. The highest yield among the three extracts was soy germ, which was in a range of 20.37 to 27.59%. It was followed by cotyledon extract, with a yield between 7.77 and 11.30%. The extraction yield of soy coat was from 2.05 to 4.26 %. The average extract yield of germ was about 2.3 and 7 folds higher than cotyledon and coat, respectively. The weight percentages of soy cotyledon, coat, and germ were widely different and about 90, 8, and 2% of total soy seed weight, respectively (Liu, 1997). Thus, based on the weight percentages, the highest total extract weight among the three extracts was soy cotyledon extract.

Table 3.1 Extract yields (%) of different soy parts from four varieties

Variety	cotyledon	coat	germ
A	7.77 ± 0.97	2.05 ± 0.45	22.66 ± 1.02
B	9.93 ± 1.05	2.42 ± 0.51	21.18 ± 1.38
C	11.30 ± 1.25	4.26 ±0.36	27.59 ± 1.58
D	9.32 ± 1.26	3.31 ± 0.76	20.37 ± 1.49
Average	9.58 ± 1.13	3.01 ± 0.52	22.70 ± 1.37

Concentration and percentage of each isoflavone in the soy cotyledon, coat, and germ extracts are listed in Table 3.2 and 3.3, respectively. Daidzin and glycitin were the major isoflavones in the germ and coat extracts. The levels of daidzin and glycitin in the germ extract were 9.39 – 37.13 and 11.20 – 39.26 mg/g, respectively (Table 3.2). The levels of daidzin and glycitin in the coat extract were 2.48 – 5.82 and 1.33 – 8.21 mg/g, respectively (Table 3.2). Although the levels

of daidzin and glycitin in germ extracts were higher than that in coat extracts, the percentage of the two isoflavones occupied total isoflavones in the germ or coat extract was similar and approximately 80% (Table 3.3). The soy cotyledon had the lowest level of glycitin among the three soy part extracts. It was lower than the detectable level in two of the four varieties in this study. Genistin and daidzin were the leading isoflavones in the soy cotyledon extract and in a range of 1.43 – 5.90 and 0.8 – 3.51 mg/g, respectively (Table 3.2). Genistin in the cotyledon extract was over 60% of total isoflavone while daidzin was up to 30% (Table 3.3).

In the study of Liu et al. (2005), the three glucoside isoflavones were the major soy isoflavones and up to 70% of the total soy isoflavones. Kim et al. (2007) also reported that the distribution and concentration of isoflavones in different parts of soy seed produced in Korea were widely variable. He reported that isoflavone content was highest in Geomjeongkong 2 embryo (5701 ug/ g), Heugcheongkong cotyledon (951 ug/ g), and Keunolkong seed coat (56 ug/g). Isoflavone was least present in Keunolkong embryo (341 ug/ g), Hwaeomputkong whole seed (175 ug/ g), and Seonheukkong cotyledon (81 ug/ g). These results were similar to this study in that the highest level of daidzin and glycitin were found in soy germs and the lowest level of glycitin were found in cotyledon.

Compared with the glucoside isoflavones, aglycone isoflavones daidzein, glycitein, and genistein in cotyledon, coat, and germ extracts were very low (Table 3.2). They usually occupied less than 5% of total isoflavones in the three soy part extracts. The differences of the aglycone isoflavone levels in the three extracts were not as significant as the differences of the glucoside isoflavones. It was in agreement with the results of other studies that aglycone isoflavones were the lowest level in raw whole soybean and soybean parts (Kim et al., 2007; Zhu et al., 2005).

Table 3.2 Concentrations(mg/g) of isoflavones in different soy part extract from four varieties

		Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein	Total
Germ	A	37.13 ±0.32	21.08 ±0.19	5.86 ±0.12	0.62 ±0.03	0.44 ±0.05	0.20 ±0.02	65.32 ±0.73
	B	28.50 ±1.29	39.26 ±1.80	9.15 ±1.22	1.77 ±0.07	1.89 ±0.11	0.85 ±0.04	81.43 ±4.53
	C	11.16 ±0.22	11.20 ±0.29	3.77 ±0.02	1.20 ±0.11	0.35 ±0.09	0.09 ±0.01	27.76 ±0.74
	D	9.39 ±0.11	22.18 ±1.15	4.67 ±0.09	0.13 ±0.02	0.35 ±0.05	1.77 ±0.23	38.49 ±1.44
Coat	A	5.82 ±0.39	2.76 ±0.13	1.77 ±0.04	0.29 ±0.01	0.27 ±0.03	0.12 ±0.01	11.02 ±0.61
	B	5.64 ±0.23	8.21 ±0.05	2.01 ±0.21	0.49 ±0.01	0.32 ±0.01	0.28 ±0.02	16.94 ±0.52
	C	2.48 ±0.04	1.33 ±0.05	1.40 ±0.08	0.20 ±0.05	0.08 ±0.01	0.07 ±0.01	5.56 ±0.24
	D	2.75 ±0.54	4.95 ±0.70	2.19 ±0.26	0.05 ±0.01	0.09 ±0.01	0.09 ±0.01	10.13 ±1.54
Cotyledon	A	3.51 ±0.01	n.d.	5.90 ±0.21	0.14 ±0.04	n.d.	0.16 ±0.02	9.71 ±0.27
	B	0.80 ±0.05	0.35 ±0.04	1.43 ±0.05	0.10 ±0.01	n.d.	0.05 ±0.02	2.73 ±0.17
	C	2.77 ±0.02	n.d.	3.59 ±0.31	1.67 ±0.04	n.d.	0.24 ±0.10	6.67 ±0.48
	D	2.96 ±0.54	0.84 ±0.06	4.62 ±0.17	0.10 ±0.01	0.30 ±0.01	0.15 ±0.01	8.97 ±0.82

n.d. = not detected.

Table 3.3 Percentage (%) of each isoflavone occupied the total isoflavone content in three soy part extracts from four varieties

		Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
Germ	A	56.84 ± 0.49	32.28 ± 0.29	8.97 ± 0.19	0.95 ± 0.05	0.67 ± 0.07	0.30 ± 0.03
	B	35.00 ± 1.58	48.21 ± 2.21	11.24 ± 1.50	2.18 ± 0.09	2.32 ± 0.14	1.05 ± 0.05
	C	40.19 ± 0.78	40.33 ± 1.03	13.58 ± 0.08	4.32 ± 0.41	1.26 ± 0.31	0.32 ± 0.05
	D	24.39 ± 0.29	57.63 ± 2.98	12.14 ± 0.22	0.33 ± 0.04	0.91 ± 0.14	4.59 ± 0.06
Coat	A	52.77 ± 3.51	25.05 ± 1.14	16.03 ± 0.39	2.64 ± 0.11	2.43 ± 0.28	1.08 ± 0.10
	B	33.28 ± 1.34	48.46 ± 0.31	11.89 ± 1.23	2.89 ± 0.04	1.86 ± 0.04	1.63 ± 0.09
	C	44.60 ± 0.72	23.92 ± 0.94	25.18 ± 1.36	3.60 ± 0.98	4.40 ± 0.16	1.26 ± 0.17
	D	27.17 ± 5.36	48.90 ± 6.90	21.60 ± 2.53	0.47 ± 0.06	0.93 ± 0.13	0.92 ± 0.13
Cotyledon	A	36.20 ± 0.09	0	60.77 ± 2.21	1.43 ± 0.37	0	1.60 ± 0.16
	B	29.32 ± 1.89	12.89 ± 1.36	52.39 ± 1.91	3.48 ± 0.27	0	1.83 ± 0.81
	C	30.88 ± 0.37	0	40.02 ± 4.59	0.78 ± 0.67	0	2.68 ± 1.53
	D	33.02 ± 6.00	9.37 ± 0.93	51.49 ± 1.97	1.15 ± 0.14	3.33 ± 0.16	1.65 ± 0.14

The total isoflavone content in the soy germ extract was from 27.76 to 65.32 mg/g. It was 5.56 – 11.02 and 2.73 – 9.71 mg/g in the soy coat and cotyledon extracts, respectively. Soy germ had the most concentrated isoflavone among the parts in the four soybean varieties. Although the soy cotyledon extract had slightly lower total isoflavone content than the coat extract, the total amount of isoflavones in soy cotyledon was actually much higher than soy coat due to the higher amount of soy cotyledon than soy coat. Kim et al. (2007) indicated that the total isoflavone content in soy cotyledon was 10 times higher than in soy coat.

3.3.2 Total Phenolic Content in Soy Cotyledon, Coat and Germ Extract

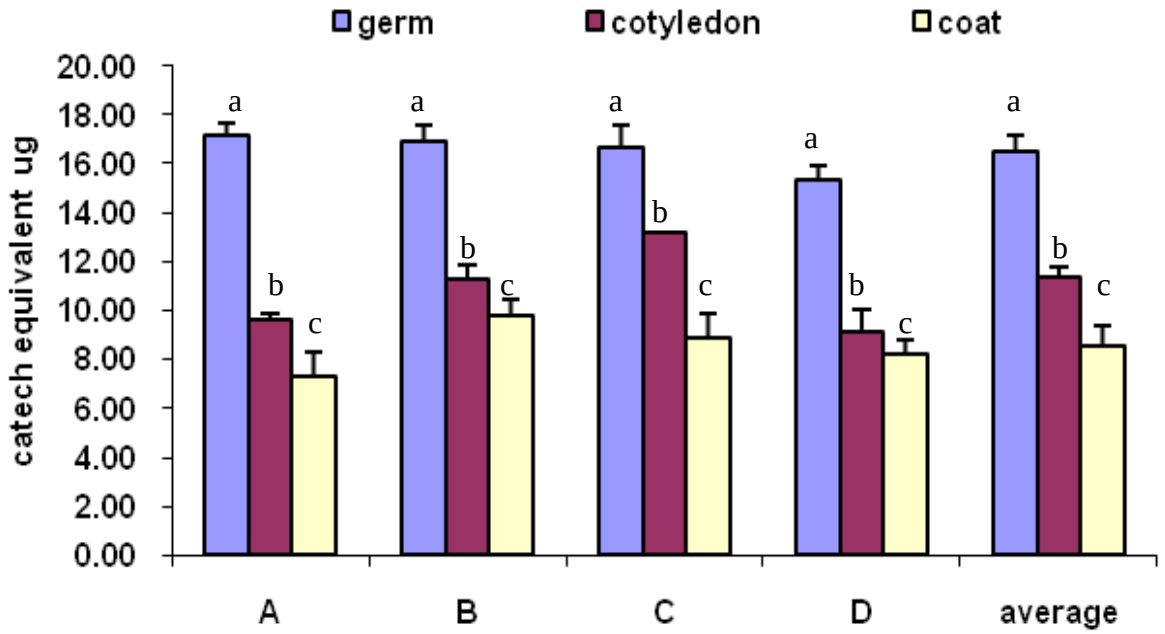

Figure 3.1 Total phenolic contents of different soy part extracts from four varieties
*Significant difference ($P<0.05$) in one group is expressed by different letters.

The total phenolic contents of soy cotyledon, coat and germ extracts are shown in Figure 3.1.The phenolic content in the soy germ extracts was from 15.34 to 17.17 μg/g of catechin equivalent and significantly higher than the cotyledon or coat extracts. The ranges of total phenolic contents in the cotyledon and coat extracts were 9.15 – 13.24 and 7.30 – 9.78 μg/g of catechin

equivalent, respectively. The total phenolic content in the cotyledon extract was significantly higher than the coat extract within the same variety. The difference of total phenolic contents in 11 cultivars of soybean harvested from India and Bulgaria were up to 5 folds (Sakthivelu et al., 2008). It was also observed that the lowest and highest level of phenolic compounds varied between 13 and 101 μg/g in the cotyledon tissues of nine soybean varieties in Korea (Kim et al., 2006). It is similar to this study that the total phenolic content in either soy cotyledon or coat was much lower than that in soy germ (Kim et al., 2006). Besides the isoflavones, other phenolic compounds, such as chlorogenic, caffeic, ferulic, and p-coumaric acid, were found in soybean (Cheigh and Lee, 1993). They all could significantly contribute the total phenolic content in soy cotyledon, coat, and germ part.

3.3.3 Antioxidant Activities of Soy Cotyledon, Coat, and Germ Extract

Several studies suggested that the total phenolic content may have positive correlation with antioxidant activity (Emmons and Perterson, 1999; Velioglu et al., 1998). Higher antioxidant activity of soy extract was reported by using a Rancimat and DPPH scavenging methods (Liu et al., 2005; Lee et al., 2002). In this study, antioxidant activities of the three soy part extracts measured using the DPPH free radical scavenging method are shown in Figure 3.2. The ranges of free radical inhibition percentage of the soy cotyledon, coat, and germ extracts in this study were 59.00 – 68.75%, 53.00 - 66.44%, and 43.48 – 51.61%, respectively. The soy germ extracts that had the highest phenolic content among the three part extracts exhibited significantly higher antioxidant activity than cotyledon or coat extracts (Figure 3.2). The higher antioxidant activity of the soy germ than coat was found as well by Kim et al. (2006). Although the cotyledon extract had much lower total phenolic content than the germ extract, its free radical scavenging activity was only slightly lower than the germ extract. Chen et al. (1998) found that soy protein

had the capability to scavenge free radicals. It could be the reason why the cotyledon extract had relatively higher free scavenging activity, although it had lower total phenolic content. Also, Sakthivelu et al. (2008) found that the scavenging DPPH activity did not correlate with total isoflavone content and very weakly with total phenolic content in eleven soy varieties. Thus, it is possible that other antioxidants besides of isoflavones and phenolic may contribute the antioxidant activity of soy cotyledon extract.

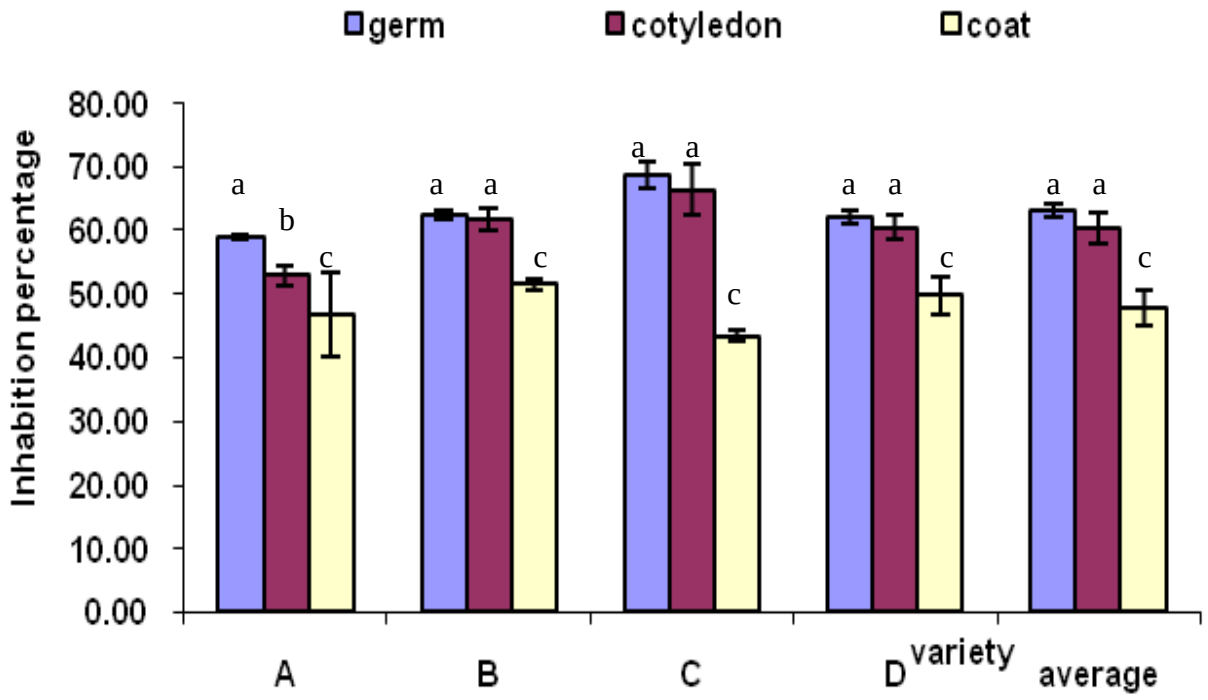

Figure 3.2 Antioxidant activity of three soy part extracts from four varieties
* Significant difference ($P<0.05$) in one group is expressed by different letters.

3.4 Conclusion

The distribution and content of isoflavones varied greatly in different soy parts. Soy germ extract had several fold higher level of total isoflavones than soy coat or cotyledon extract. The distribution profile of soy isoflavones in soy cotyledon was different from soy coat or germ. In soy germ and coat, daidzin and glycitin were the dominating isoflavones while genistin and

daidzin were the major isoflavones in soy cotyledon. Aglycone isoflavones were much lower than glucoside isoflavones in each of soy parts. The total phenolic content from high to low was soy germ, cotyledon, and coat extract. Although the cotyledon extract had lower total phenolic content than the germ extract, its antioxidant or scavenging free radical activity was equivalent to the germ extract and higher than the coat extract, which had the lowest antioxidant activity among the three soy part extrac ts.

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CHAPTER 4 COMPARISON OF SOYBEAN OILS, GUM, AND DEFATTED SOY FLOUR EXTRACT IN STABILIZING MENHADEN OIL DURING HEATING*

4.1 Introduction

Many epidemiological and clinical studies confirmed that daily intake of omega-3 long chain polyunsaturated fatty acid (PUFA) from fish oil is beneficial for preventing various cardiovascular diseases (Crombie et al., 1987; Dyerberg et al., 1978; Wang et al., 2006). Food products enriched with fish oil or omega-3 PUFA as a health promoting component offer potential in the burgeoning area of functional food product development. However, the omega-3 PUFAs in fish oil are readily oxidized to produce off- or rancid- flavor volatiles when exposed to light, oxygen, prooxidants and high temperatures (McClements and Decker 2000). Thus, the quality of fish oil or foods fortified with fish oil usually deteriorates rapidly, if not stabilized. While the instability of omega-3 PUFA has been solved for some food applications, there are serious hurdles for using fish oil in other foods, such as many functional foods, that still need to be overcome. Synthetic antioxidants, such as TBHQ (tertiary butyl hydroquinone), BHA (butylated hydroanisole) and BHT (butylated hydroxytoluene), propyl gallate (PG), alpha-tocopherol acetate, and EDTA (ethylenediaminetetraacetic acid) are used for retarding the fish oil oxidation. However, potential toxicity and mutagenicity of these artificial chemicals have been concerns for many years (Wanasundara and Shahidi, 1998). BHA could convert ingested material into toxic substances or carcinogens due to increased secretion of microsomal enzymes of liver and extra-hepatic organs, such as the lungs and gastrointestinal tract mucosa (Wattenberg, 1986). Nakagawa et al. (1995) found that PG was cytotoxic to isolated rat hepatocytes because it impaired mitochondria and led to ATP depletion. Kotsonis et al. (2001)

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have suggested that even small amounts of artificial antioxidants could have potentially harmful health effects from long-term consumption.

Lately, the safety and health benefits of antioxidants from natural sources, such as grains and cereals, have been reported in numerous studies and recognized by the FDA and many consumers (Martinez-Tome et al., 2004; Nystrom et al., 2005; Truswell, 2002). The antioxidants from natural sources are generally considered as safe food ingredients. However, information on using the natural antioxidants to replace synthetic antioxidants in preventing fish oil oxidation is limited. Several antioxidants, including isoflavones, have been identified in soybeans (Meng et al., 1999). Soy isoflavones were well recognized to play an important role in reducing the formation and progression of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer's disease, and osteoporosis (Messina, 1999). They are effective antioxidants because of their phenol structure and redox potential (Meng et al., 1999). Thus, the antioxidants in soybean could be used as a natural "green" antioxidant ingredient to effectively stabilize fish oil and other food systems containing omega-3 PUFA.

In this study, the capabilities of intermediate products and byproducts of soybean oil refining, including crude soy oil, degummed oil, gum and defatted soy flour, for preventing long chain fatty acid (DHA and EPA) oxidation and stabilizing menhaden oil during heating were investigated. The DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical quenching activity, total phenolic, isoflavone, and tocopherol contents in the oils, gum, and defatted soy flour extract were determined. Also, the levels of rancid volatiles, hexanal and octen-3-ol were determined during storage at room temperature. This information would be helpful in the development and utilization of soy products as a food antioxidant or nutritional supplements.

4.2 Materials and Methods

4.2.1 Materials

Hexane, methanol, and butanol were HPLC grade and purchased from Fisher Scientific Inc. (Fair Lawn, NJ). BCl_3 - methanol and 2,2 - dimethoxypropane were purchased from Supelco (Bellefonte, PA). Menhaden fish oil, DPPH (2,2'—diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, catechin, and 2-thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, Mo). Heptadecanoic acid (C17:0), docosahexaenoic acid C22:6 (DHA), eicosapentaenoic acid C20:5 (EPA), soy isoflavones (daidzin, glycitin, and genistin), and alpha and gamma tocopherol standards were from Sigma-Aldrich (St. Louis, Mo). Soybeans were purchased from a local farmers market.

4.2.2 Crude Soy Oil, Degummed Oil, Gum, and Defatted Soy Flour Extract Preparation

4.2.2.1 Crude Soy Oil: Dry soybeans(D) were ground for 3 minutes at a medium speed in a kitchen blender. The particles of the ground whole soy flour were able to pass through a 1 mm sieve. The soy flour (200 g) was weighed into a 1000-mL glass beaker and extracted using 400 mL hexane. The mixture was incubated at 60^{o}C in a water bath and stirred gently for 20 min. After the incubation, the solvent layer was separated from solid residue by centrifuging at 2000 $\times g$ for 10 minute using a Hermel Z383 K table top centrifuge (National Labnet Company, Woodbridge, NJ). The clear supernatant was transferred to a clean round bottom flask. Then the solid residue was extracted with another 400 mL of hexane. The separated hexane extracts were combined and dried using a vacuum evaporator at 50^{o}C. The dried extract was crude soy oil, which was weighed to obtain the crude oil yield. The solid residue was dried under a ventilation hood overnight to obtain defatted soy flour.

4.2.2.2 Defatted Soy Flour Extract: Methanol (400 mL) was mixed with the defatted soy flour(150g) and incubated at 60^{o}C in a water bath and stirred gently for 20 minutes. After the incubation, the supernatant was separated from the residue by centrifuging at 2000 × g for 10 min. The defatted soy flour extract was obtained after the supernatant was dried using the vacuum evaporator at 50^{o}C. The extract was weighed to obtain the defatted soy flour extract yield.

4.2.2.3 Degummed Oil and Gum: Twenty grams of crude oil was weighed and transferred into a clean centrifuge test tube and degummed using 3% (w/w) distilled water in the oil. The mixture of oil and water was vortexed for 5 min and incubated at 60 ℃ in a water bath for 30 min with shaking. The incubated crude oil and water mixture was centrifuged at 3000 × g for 10 min. The separated upper oil phase was degummed oil, which was transferred to a clean test tube and weighed. The lower phase was a mixture of white gum and water, and subsequently was dried using the vacuum evaporator at 80^{o}C to remove the moisture and was then weighed.

4.2.3 Determination of Total Phenolic Content

The total phenolic contents of the oils, gum, and defatted soy flour extract were determined using the Folin-Ciocalteu reagent (Velioglu et al., 1998). The Folin-Ciocalteu reagent was diluted 10 times with deionized water. The oils, gum, or defatted soy flour extract (50 mg each) were re-dissolved in 10 mL methanol, and 0.1 mL of this solution was mixed with 0.75 ml diluted Folin-Ciocalteu reagent. The reaction solution was left at 25^{o}C for 5 min. Then 0.75 mL of sodium bicarbonate solution (60 g/L) was added. The mixture was incubated at 25^{o}C for 90 min and filtered through a 0.45 μm syringe filter (Pall Corporation, Ann Arbor, MI). The absorbance of the solution was determined at 750 nm using a UV-Visible SpectraMax Plus^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA). Catechin was used to prepare a

standard curve. The total phenolic content was calculated and expressed as μg catechin equivalent / gram of the oil, gum, or defatted soy flour extract.

4.2.4 Determination of Tocopherol Content Using HPLC

The total tocopherol concentration was determined using the method of Xu (2002). The HPLC system consisted of Waters (Milford, MA) 510 pumps, a 715 Ultra WISP injector, and 470 fluorescence detector. Chromatograms were recorded and processed using Waters Millennium chromatography software. Samples were injected into a 25 cm x 4.6 mm i.d. 5-μm Supelcosil LC-Si (Supelco, Bellefonte, PA) column. The column was preceded by a 5 cm x 4.6 mm I.D. guard column packed with 40-μm pellicular silica. The mobile phase consisted of 0.5% ethyl acetate and 0.5% acetic acid in hexane at a flow rate of 1.5 mL/min. The fluorescence detector was set at 290 nm excitation and 330 nm emission. The oils, gum, and defatted soy flour extract (50 mg each) were dissolved in 10 mL of hexane and vortexed. One hundred μL of the solution was injected into the HPLC system. Each tocopherol concentration was calculated based on their standard curves. The total tocopherol content was calculated by summing each tocopherol concentration.

4.2.5 Determination of Isoflavone Content Using HPLC

The oils, gum, or defatted soy extract solution (50 mg in 10 mL methanol) was transferred to HPLC vials. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (id 3 mm x 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium32 chromatography manager. The mobile phase was a mixture of water and ethanol, with percentage of water in ethanol ramped from 90% to 50% in 40 min with a constant flow rate of 0.3 mL/min (Xu et al., 2002). The chromatograms obtained at a wavelength of 254 nm were used to quantify the isoflavones. The concentration of the three major isoflavones, daidzin,

glycitin, and genistin, was calculated based on their standard curves. The total isoflavone content was calculated by summing the three isoflavone concentrations.

4.2.6 Determination of Free Radical Scavenging Capability Using DPPH Method

The solution for the DPPH test was prepared by re-dissolving 50 mg of each crude oil, degummed oil, gum, and defatted soy flour extract in 20.0, 10.0, 5.0, and 2.5 mL methanol. The concentration of DPPH solution was 0.025 g in 1000 mL of methanol. Two mL of the DPPH solution was mixed with 100 μL of each methanol solution and transferred to a cuvette. The reaction solution was monitored at 515 nm for 30 min at 25^{o}C using a UV-Visible SpectraMax Plus^{384} spectrophotometer (Molecular Devices, Sunnyvale, CA). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation (Moure et al., 2000):

$$\text{Inhibition\%} = (Abs_{t=0\ min} - Abs_{t=30\ min}) / Abs_{t=0\ min} \times 100$$

Where $Abs_{t=0\ min}$ was the absorbance of DPPH at zero time and $Abs_{t=30\ min}$ was the absorbance of DPPH after 30 min of incubation.

4.2.7 Preparation of Fish Oil Samples and Accelerated Oxidation by Heating

Menhaden fish oil solution was prepared by dissolving 1000 mg oil in 100 mL of hexane. Each 250 mg of crude oil, degummed oil, gum, and defatted soy flour extract was dissolved in 50 mL of methanol, respectively. Two milliliter of the menhaden fish oil solution (10.0 mg /mL of hexane) was added to each test tube (13 ×100 mm). Two tenths (for 5 % w/w addition) or 1.0 mL (for 25 % w/w addition) of the crude oil, degummed oil, gum, or defatted soy flour extract solution (5.0 mg/ mL of methanol) was mixed with the fish oil solution by vortexing for 1 min. Then, all solutions in the test tubes were evaporated at 30^{o}C using a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, MO). Thus, the dried samples

are the fish oil mixed with 5 or 25 % addition of the soy oils, gum, and defatted soy flour extract. The dried samples from only 2.0 mL of menhaden oil without any additive served as the control.

Twelve fish oil samples from each level of addition of the crude and degummed oils, gum, and defatted soy flour extract and control were prepared. Three of them were used for obtaining an average initial TBA value and another three for measuring average initial DHA and EPA concentrations before heating. The remaining six samples were heated at 150^{o}C in a sand bath for 30 min. Then, three of them were used for determining their final TBA value and another three for measuring their final DHA and EPA concentrations after heating.

4.2.8 Determination of Fish Oil Oxidation Using TBA Method

The TBA values were determined using the method of Pegg (2002). Fish oil sample in the test tube was dissolved in 4 mL butanol and mixed thoroughly. Four milliliter of TBA (0.2% in butanol) solution was added to the tubes. The tubes were capped and vortexed for 1 min. The TBA reaction was carried out at 95^{o}C in a water bath for 1 hr. After the reaction, the tubes were cooled down in ice water. The absorbance of each solution was measured at 523 nm using the UV-Visible SpectraMax Plus^{384} spectrophotometer. Each fish oil oxidation after heating was expressed by the increase of TBA absorbance value, which was the difference of the final TBA absorbance value from each heated sample and its corresponding average initial TBA absorbance value before heating.

4.2.9 Determination of DHA and EPA Changes Using GC Method

The DHA and EPA were determined using the method of Li and Watkins (2002). Fish oil sample was mixed with heptadecanoic acid (C17:0) (0.1 mg/mL in hexane), as an internal standard, for the DHA and EPA analysis. After adding 2 mL BCl_3-methanol and 1 mL 2, 2'-dimethoxypropane, all test tubes were capped and incubated at 60^{o}C in a water bath for 10 min to

perform the derivatization of fatty acid methyl esters. Then, 1 mL hexane and 1 mL water were added to the tubes and vortexed for 30 seconds. The upper hexane layer was transferred to another tube, dried with anhydrous sodium sulfate and transferred to a GC vial.

A gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA) with a FID detector was used to determine the DHA and EPA concentration. Helium was used as a carrier gas with a column flow rate of 1.2 mL/min. The injection volume was 5 μl and the split ratio was 1:100. The injector and detector temperature was 250 and 270°C, respectively. The oven temperature program was set to hold at 50°C for 3 min and then increased at 4.0°C/min to 250°C. The column was a Supelco SP2380 (30m × 0.25mm) (Bellefonte, PA). The concentrations of DHA and EPA were calculated using the C17:0 internal standard as a reference. The percentage of retained DHA or EPA in the fish oil after heating was obtained by comparing its final concentration in each heated sample to its corresponding average initial concentration before heating.

4.2.10 Determination of Headspace Rancid Volatiles in Fish Oil

Fish oil sample (200 mg) mixed with 10 mg defatted soy flour extract was placed into 2-ml vials as treatment group. Vials with only 200 mg fish oil was the control group. The vials were capped and stored at room temperature. Headspace volatile sample was taken using a syringe at day 0, 2, 4, 6, 8 and 10.

A Varian CP-3800 gas chromatography was used for the headspace volatiles analysis. The GC column was a high polarity supelcowax 10 fused silica capillary column (30m x 0.32 internal diameter, 0.1 um film) and helium was the carrier gas. Initial oven temperature was set at 60°C for 5min, raised to 110°C at 5 °C/min and maintained for 5 min. The mass detector temperature was set at 200 °C. Hexanal and octen-3-ol were identified by comparing their mass spectra and

retention time with their standards and quantified using total ion peak area. The inhibition rate =100 x (1- peak area of treatment group sample/peak area of the control group sample).

4.2.11 Statistical Analysis

The determination of the total phenolic, tocopherol, and isoflavone contents and the DPPH free radical scavenging test were performed in triplicate. The TBA, DHA and EPA analysis were performed three times as well. The means and standard deviations were calculated and the data were analyzed by one-way ANOVA with duncan's multiple comparisons at $\alpha = 0.05$ by using SAS Statistical Analysis System (SAS Inst., Cary, NC).

4.3 Results and Discussions

4.3.1 Total Phenolic, Isoflavone, and Tocopherol Contents of the Crude and Degummed Oils, Gum, and Defatted Soy Flour Extracts

Table 4.1 Total phenolic, tocopherol, and isoflavone contents in crude and degummed oils, gum, and defatted soy flour extract

Samples	Crude oil	Degummed oil	Gum	Defatted soy flour extract
Phenolics (μg/g)	7.1 ± 0.4 a	6.1 ± 0.6 a	6.0 ± 0.6 a	11.3 ± 1.5 b
Tocopherols (μg/g)	387 ± 16 a	414 ± 23 a	238 ±15 b	215 ± 10 b
Isoflavones (mg/g)	0.2 ± 0.1 a	N.D.	0.3 ± 0.1 a	55.0 ± 4.0 b

*N.D.: not detected; Data with different letters in a row are significantly different ($P<0.05$).

The yield of soybean crude oil, degummed oil, and gum was 16.2 ± 0.7, 12.5 ± 1.8, and 1.9 ± 0.4 % (based on whole dry soy flour weight), respectively. These values are similar to the yields produced by industrial scale soybean oil extraction and refining (Erickson et al, 1980). Soybean usually contains 17 - 22 % lipid and 1.5 – 2.5 % of gum (Liu, 1997). Soybean also consists of about 35% protein and 30% carbohydrate, which are hexane insoluble components and the major

composition of the defatted soy flour (Liu, 1997). In this study, the yield of defatted soy flour extract using methanol was 7.5 ± 0.8 % (based on whole soy flour weight).

The defatted soy flour extract had the highest concentration of phenolic compounds and isoflavones among the soy products (Table 4.1). The total phenolic content in the defatted soy flour extract was 11.3 μg catechin equivalent /g and approximately 1.5 times higher than that of the crude oil and twice as high as degummed oil or gum (Table 4.1). The concentration of isoflavones in defatted soy flour extract was 55 mg/g, with 0.3 and 0.2 mg/g in the gum and crude oil, respectively, but was under the detection limit in degummed oil (Table 4.1). The polarities of the phenolics and isoflavones are higher than triglyceride and other lipids. They are readily extracted by more polar solvents, such as methanol rather than hexane. A similar observation was reported in the study by Sun et al. (2006), in which the total phenolic content in oat extract obtained by methanol was over three times higher than the extract produced by hexane, although the yield of hexane extract was higher than the methanol extract. Opposite to the total phenolic and isoflavone contents, the total tocopherol content (215 μg/g) in the defatted flour extract was significantly lower than the oils or gum (Table 4.1). The degummed oil had the highest tocopherol concentration (414 μg/g), followed by crude oil (388 μg/g) and gum (238 μg/g). The tocopherol content in soybeans varies significantly by different varieties and usually ranges from 190 to 300 μg/g (dry basis) (Liu, 1997). Compared to phenolics and isoflavones, tocopherols are less polar and largely extracted by non-polar solvent, such as hexane. Higher tocopherol content in the extract by using hexane than acetone or methanol was reported by several studies (Moreau et al., 2003; Sun et al., 2006). Thus, in this study, more hydrophilic antioxidants may be highly concentrated in defatted soy flour extract while more lipophilic antioxidants may be concentrated in the oils and gum.

4.3.2 Free Radical Scavenging Capability of the Crude and Degummed Soy Oils, Gum, and Defatted Soy Flour Extract

The results of free radical quenching capabilities (TEAC) of the oils, gum, and defatted soy flour extract measured by the DPPH method are shown in Figure 4.1. The free radical scavenging capability of defatted soy flour extract was significantly higher than the oils or gum, and increased to 71.5 % at 20 mg/mL of concentration. At that concentration, the scavenging capabilities were 50.7, 42.3, and 34.2 % in the crude oil, degummed oil, and gum, respectively. The order of the free radical scavenging capability from high to low was defatted soy flour extract, crude oil, degummed oil, and gum. The DPPH free radical scavenging test has been widely used for evaluating antioxidant activity of grain and cereal extracts (Bryngelsson et al., 2002; Sun et al., 2006). These studies also indicated that antioxidant activities of methanol extracts from grains and cereals determined using the DPPH test was positively correlated with the concentration of phenolic acids in the extracts. In this study, the order of the DPPH radical quenching capabilities is in agreement with their concentration of phenolic compounds (Table 4.1). The higher DPPH radical scavenging capability of defatted soy flour extract suggests that most antioxidants in soybean are hydrophilic compounds and not likely extracted by hexane. Even though the hexane extract (the crude oil) would have contained a higher level of the lipophilic tocopherols, their antioxidant activity was not as important as the hydrophilic antioxidants, which remained in the defatted soy flour. Another possible reason could be a dilution effect brought about by greater extraction of neutral lipid material, such as triglyceride and fatty acids. Adom and Liu (2005) found that the hydrophilic antioxidant activity in grains and cereals contributed over 98% of the total antioxidant activity.

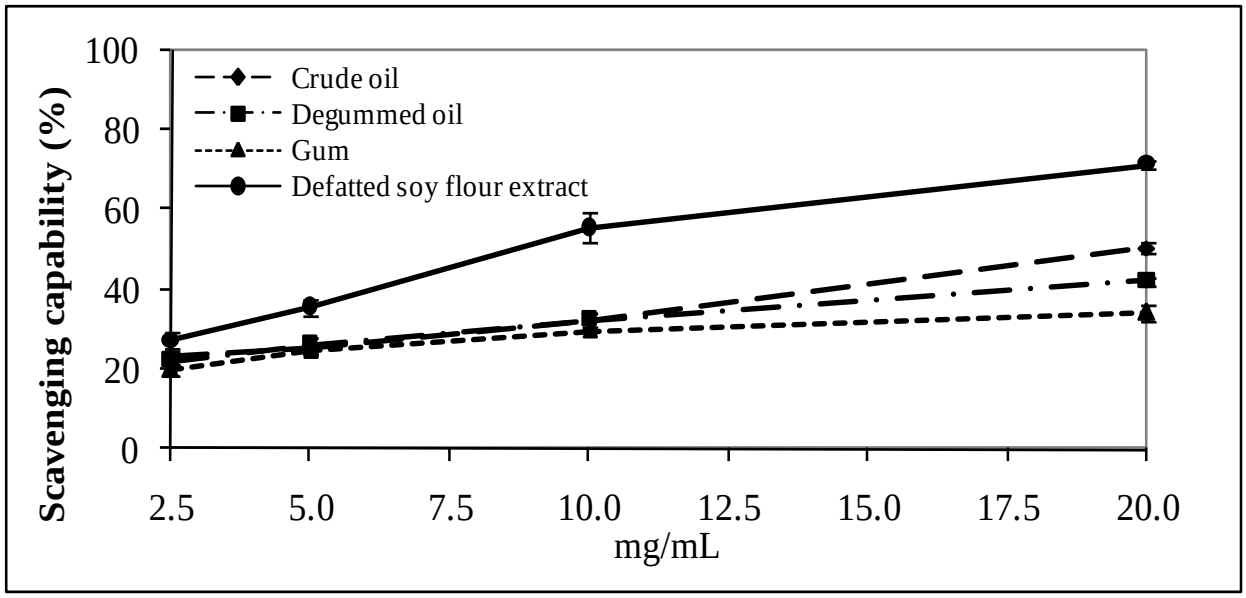

Figure 4.1 Free radical quenching capabilities (TEAC) of crude oil, degummed oil, gum, and defatted soy flour extract at different concentrations measured by DPPH method.

4.3.3 Capabilities of the Crude and Degummed Oils, Gum, and Defatted Soy Flour Extract in Preventing DHA and EPA Oxidation and Stabilizing Menhaden Oil during Heating

The capabilities of the oils, gum, and defatted soy flour extract in preventing DHA and EPA oxidation during heating are shown in Table 4.2. The initial content of EPA and DHA in menhaden oil were 13.5 % and 11.5 %, respectively. Significantly higher retained DHA and EPA were found in the fish oils mixed with the gum and defatted soy flour extract. In the control group, only 29.9 % of DHA and 37.2 % of EPA in the fish oil were retained after being heated at 150^{o}C for 30 min, while 49.6 – 71.5 % DHA and 55.6 – 75.9 % EPA were retained in the fish oils mixed with 25 % gum or 25 % defatted soy flour extract. For the crude and degummed oils, the fish oil stabilizing capabilities were achieved only when they were added to the fish oil at a level above 25 %. It has been reported that crude soybean oil showed a stronger capacity to inhibit lipid oxidation than refined oil because it contains higher phenolic pigments, which are removed during oil refining (Xu et al., 2005).

Table 4.2 Retained DHA and EPA(%) in menhaden oils mixed with crude oil, degummed oil, gum, and defatted soy flour extract after heating at 150°C for 30 min

Sample	Control	Fish oil mixed with 5 % (w/w)			
		Crude oil	Degummed oil	Gum	Defatted soy flour extract
DHA (%)	29.9 ± 4.1 a	31.5 ±1.6 a	32.8 ± 2.4 a	46.7 ± 1.0 b	62.8 ± 9.2 c
EPA (%)	37.2 ± 2.1 a	38.0 ± 0.6 a	38.9 ± 1.3 a	52.9 ± 0.7 b	67.7 ± 3.7 c
Sample	Control	Fish oil mixed with 25 % (w/w)			
		Crude oil	Degummed oil	Gum	Defatted soy flour extract
DHA (%)	29.9 ± 4.1 a	36.4 ± 2.2 b	39.0 ± 2.1 b	49.6 ± 0.3 c	71.5 ± 2.3 d
EPA (%)	37.2 ± 2.1 a	45.6 ± 0.2 b	46.0 ± 3.5 b	55.6 ± 1.3 c	75.9 ± 4.1 d

* Initial levels of DHA and EPA are 2.3 and 2.7 mg before heating, respectively.
*Data with different letters in each row are significantly different (P<0.05).

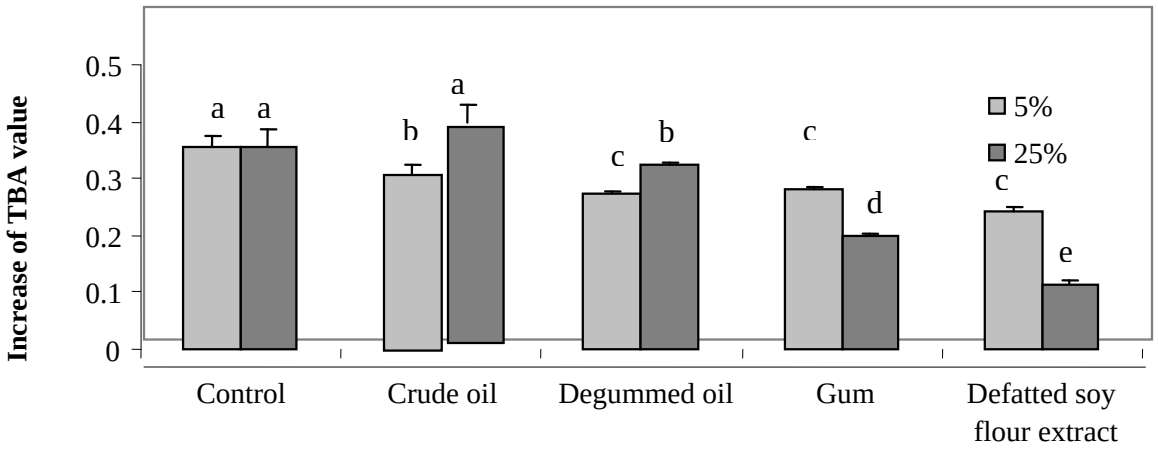

Figure 4.2 Changes of TBA values in the menhaden oils mixed with crude oil, degummed oil, gum, and defatted soy flour extract (5 and 25%, w/w) after heating at 150°C for 30 min.
(*Bars with different letters on top are significantly different (P<0.05))

However, because of a high concentration of susceptible unsaturated fatty acids (C18:1 and C18:2) in the soy oils, the increase of TBA reactive oxidation products in the fish oil mixed with 25 % crude or degummed oil was significantly higher than the fish oil with the 5 % addition level after heating (Figure 4.2). The significantly higher antioxidant capability of defatted soy flour extract in stabilizing fish oil was also reflected by the lower increase of TBA reactive oxidation products than the oils or gum (Figure 4.2). Some studies indicated that the TBA reactive substances in fish oil were increased 3 to 4 times at 4^{o}C and 2 to 3 times at -18^{o}C during 30 days of storage (Boran et al., 2006). The level of n-3 fatty acids dropped from 120 mg to 40 mg/g oil after 7-day storage at 25˚C (Borquez et al., 1997). In this study, the change of TBA value in the fish oil mixed with 25% of defatted soy flour extract was three times lower than the control group, although the fish oil oxidation was accelerated at 150^{o}C. The higher level of phenolics in defatted soy flour extract may have contributed to its higher antioxidant capability in stabilizing the menhaden oil during heating. It was reported that the phenolic antioxidants are most likely responsible for preventing bulk oil oxidation (Frankel, 1996). The significantly higher soy isoflavones in defatted soy flour extract are the most important antioxidants because of their phenol structure and redox potential that provides excellent antioxidant activity (Meng et al., 1999). The capabilities of Chardonnay grape and black raspberry seed flour extracts rich in phenolic antioxidants in preventing n-3 fatty acids in menhaden oil oxidation were reported by Luther et al. (2007). In their study, the oxidation of EPA and total n-3 fatty acids in menhaden oil mixed with 1.5% of Chardonnay grape and black raspberry seed flour was retarded after 80^{o}C incubation for 4.5 hour. They also found that the DHA and EPA in fish oil with tocopherols at 130 ppm were oxidized as fast as the control group without any stabilization. This result is in agreement with our study in that a lower capability of the crude and degummed oil in stabilizing

the fish oil was observed, although the oils had higher level of tocopherols. Sun et al. (2006) also found that a methanol extract of oat demonstrated greater capability in inhibiting the DHA oxidation during heating than the hexane extract with higher tocopherol content. Some studies found that an oat extract with higher level of phenolics significantly improved the stability of vegetable oils at a frying temperature of 180 ℃ and reduced peroxide values during 26 days of storage at 60 ℃ (Duve and White, 1991; Tian and White, 1994).

4.3.4 Production of Rancid Volatiles in Fish Oil Mixed With and Without the Defatted Soy Flour Extract during Room Temperature Storage

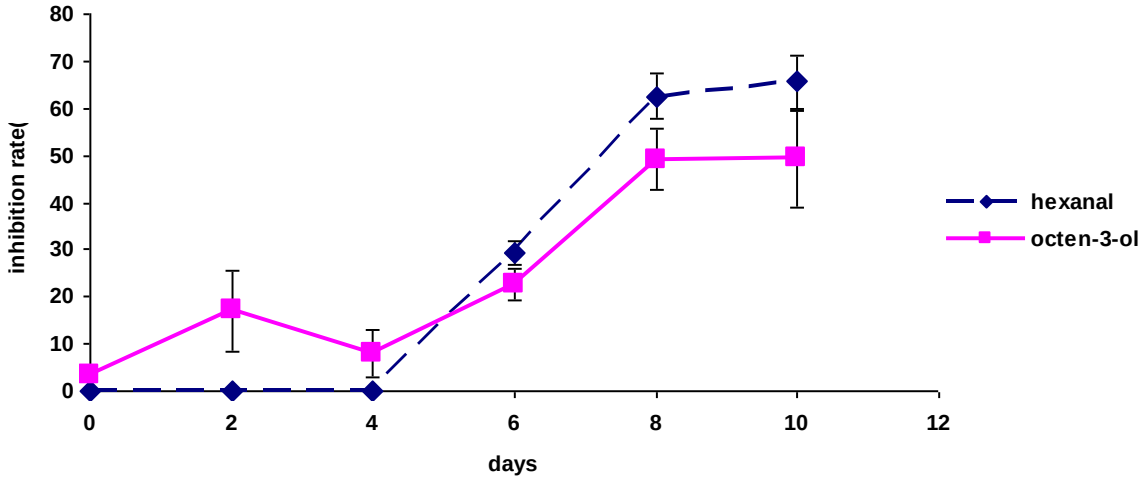

Figure 4.3 The inhibition rate of hexanal and octen-3-ol in fish oil with soy extract during storage

Undesirable flavor and odor, such as aldehydes, ketones and alcohols were produced due to degradation of unsaturated fatty acids during oxidation (Drumm and Spanier, 1991). Hexanal and octen-3-ol are major rancid volatiles related to the lipid oxidation of the fish oil. The level of the two rancid volatiles reflects the development of the oxidation degree during lipids oxidation (Jayathilakan et al., 2007). Compared with control group, the production of hexanal and octen-3-

ol in fish oil during storage at room temperature was significantly reduced by adding 5% defatted soy extract after 4 days (Figure 4.3). The inhibition rates of hexanal and octen-3-ol formation were about 65.6% and 49.3% respectively after 10 days. Wettasinghe and Shahidi (1999) reported that defatted borage seeds extract (200 ppm) could reduce the formation of hexanal in bulk corn oil after 7 days when stored at 4°C. Jayathilakan et al. (2007) reported that cinnamon (250mg/100g) could inhibit about 40% hexanal formation when added in beef sample during storage at 5°C. Our results indicated that defatted soy flour extract could reduce the production of rancid volatiles in fish oil during storage at room temperature as well.

4.4 Conclusion

The defatted soy flour extract showed the greatest capability for stabilizing menhaden oil and preventing DHA and EPA oxidation during heating. The extract could significantly reduce the degradation of omega-3 long chain polyunsaturated fatty acids in foods during cooking. It also prevent the production of undesirable and toxic lipid oxidation products. This would maintain the health function and quality of foods fortified with fish oil. Therefore, defatted soy flour could be utilized to extract soy antioxidants, in order to stabilize fish oil and also enhance health promoting potential with the inclusion of soy isoflavones.

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CHAPTER 5 COMPARISON OF CAPABILITIES OF DEFATTED SOY EXTRACT WITH HEAT AND ENZYMATIC TREATMENT AND SYNTHETIC FOOD ANTIOXIDANTS IN INHIBITING MENHADEN OIL OXIDATION

5.1 Introduction

Isoflavones are a group of diphenolic secondary metabolites produced in a very limited distribution of plants, most frequently in the Leguminosae. Among the common dietary legumes, soybean contains the highest level of isoflavones (Chuankhayan, 2007). Many studies reported that soybean isoflavones may have many health benefits, such as anticarcinogenic, antiatherosclerotic, antibacterial and antioxidant activities (Chuankhayan, 2007). Their capability in preventing lipid oxidation was demonstrated in the Chapter 4. As the antioxidant activity of a compound is correlated to the number of hydroxyl group on benzene ring, the more hydroxyl groups, the higher the antioxidant activity would be. Thus, aglycone isoflavone may have higher antioxidant capability than its glucoside isoflavone because it has one more hydroxyl group. Heat treatment and enzyme hydrolysis could break down isoflavone glucosides and convert them into aglycones (Ishihara, 2007). The glucosidases (β-D-glucoside-o-glucohydrolase, EC. 3.2.1.21) is a heterogeneous group of enzymes, specific to glucosides. They catalyze the hydrolysis of the β–glucosidic linkage of oligosaccharides and other glucoside conjugates to release glucose. Thus, the enzyme was used to hydrolyze glucoside isoflavones in the defatted soy flour extract to increase its antioxidant activity in this study.

Lipid oxidation is one of the major causes of quality deterioration in lipid-containing foods. It affects the color, flavor, texture and nutritive value of food (Hettiarachychy et al, 1996). In order to prevent lipid oxidation, synthetic antioxidant such as butylated hydroxyanisole (BHA), butyl-4-methylphenol (BHT), tert-butylhydroquinone (TBHQ), and propylgallate (PG) were widely

added into foods. However, use of synthetic antioxidants in food may cause potential health hazards. There are several recent reports on the effect of BHA on conversion of ingested material into toxic substances or carcinogens due to increased secretion of microsomal enzymes of liver and extra-hepatic organs, such as the lungs and gastrointestinal tract mucosa (Ito et al., 1982; Wattenberg, 1986). Furthermore, Ito et al. (1985) reported that BHT is carcinogenic in rats and is also in the process of being carefully scrutinized. Nakagawa et al (1995) found that PG was cytotoxic to isolated rat hepatocytes because it impairs mitochondria and led to ATP depletion. PG also could induce sisterchromatid exchanges and chromosomal aberrations in CHO-K1 cells (Tayama and Nakafawa 2001). In addition, TBHQ has not been approved for food use in Europe, Japan and Canada because it might induce forestomach tumour (Van Esch, 1986). BHA and BHT are also banned from use in food products in Europe. The Food and Drug Administration (FDA) in the United States is examining possible removal of BHA from the GRAS (generally recognized as safe) list (Wanasundara and Shahidi, 1998). Due to the safety concerns of synthetic antioxidants, interest in natural antioxidants, especially of plant origin, has notably increased in recent years (Yasoubi, 2007). Natural antioxidants are generally viewed as being safe (Yanishlieva and Marinova, 2001).

Therefore, the objective of this study was to evaluate the potential of using defatted soy flour extract treated with heat or enzyme to increase of the aglycone isoflavones as a food antioxidant to replace synthetic antioxidants in various food application. Menhaden oil with high content of susceptible long chain unsaturated fatty acids was used as a lipid model to measure and compare the capability of treated extracts with synthetic antioxidants. The information could be useful to expand the utilization of defatted soy flour as a value-added material.

5.2 Materials and Methods

5.2.1. Chemicals

Hexane and methanol were HPLC grade and purchased from Fisher Scientific Inc. (Fair Lawn, NJ). BCl_3 - methanol, 2, 2 - dimethoxypropane were purchased from Supelco (Bellefonte, PA). Menhaden fish oil and DPPH (2, 2'—diphenyl-1-picrylhydrazyl) were purchased from Sigma-Aldrich (St, Louis, Mo). Heptadecanoic acid (C17:0), docosahexaenoic acid C22:6 (DHA), eicosapentaenoic acid C20:5 (EPA), soy isoflavone (Genistein, Daidzin, Genistin, Daidzein, and Glycitin), and alpha tocopherol standards were from Sigma-Aldrich (St, Louis, Mo). Dried soybean was purchased from a local farmers market. Synthetic antioxidants (BHA, BHT, TBHQ, PG and α-T) were purchased from sigma-Aldrich (USA). Glucosidase from *Aspergillus niger* was purchased from Sigma-Aldrich (USA).

5.2.2. Preparation of Defatted Soy Flour Extract

Soybean seeds was ground in a home style coffee grinder. The ground material was then passed through a standard 20-mesh sieve (particle size <0.825 mm) to obtain soy flour. The soy flour was mixed thoroughly and subdivided into multiple glass bottles and stored in a freezer (-20 ℃) until analyzed.

Soxhlet extraction was carried out to remove fat from soy flour. Soy flour (150g ± 1 g) was placed in a Whatman cellulose extraction thimble (60 mm internal diameter and 180 mm external length; Whatman International Ltd, Maidston England) with a filter paper on top of the flour. The thimble was put in a Soxhlet extraction apparatus. Approximately 500 ml of hexane was added into the Soxhlet apparatus. The soy flour was extracted for 8 h to remove fat. The defatted soy flour was taken out and spread on an aluminum foil to dry under a hood. After it was dried, the defatted soy flour was put back into the extraction thimbles and placed back into

the extraction apparatus. Then, methanol was used as extraction solvent to extract the defatted flour for 8 h. Then, the extract solvent was evaporated under a vacuum rotoevaporator. The defatted soy flour extract was obtained after solvent was completely evaporated. The extract was stored at -20 ℃.

5.2.3 Preparation of Heat and Enzyme Treated Defatted Soy Flour Extracts

One gram of defatted soy flour extract was transferred into a clean test tube. The test tube was then put into a 150^{o}C sand bath for 30 min. Heated defatted soy flour extract was obtained after the test tube was cooled down at room temperature. The heat treated extract was prepared twice and mixed together and stored at at -20 ℃.

Twenty mg of glucosidase was dissolved in 50 ml buffer solution (pH=4.0, 69 ml 0.1M sodium citrate and 131 ml 0.1M citric acid) to obtain enzyme solution. One gram of soy extract was weighed into a clean round flask and mixed with 10 ml the enzyme solution. The reaction mixture was incubated at 37°C for 24 hours and then extracted using hexane. The hexane layer was dried by a vacuum evaporator to obtained the enzyme treated defatted extract. The enzyme treated extract was prepared twice and mixed together and stored at at -20 ℃ .

5.2.4 Preparation of Fish Oil Samples with Defatted Soy Extracts and Synthetic Antioxidants

Menhaden fish oil solution was prepared by dissolving 1000 mg oil in 100 mL of hexane. Each 250 mg of soy extract, heated soy extract, enzyme treated extract and synthetic antioxidants were dissolved in 50 mL of methanol to make a 5 mg/ml stock solution of each. One milliliter of the menhaden fish oil solution (10.0 mg/mL of hexane) was added to each test tube (13 × 100 mm). One-tenth mL (for 5 % addition level) of the soy extracts or one-fifth mL (for 1 % addition level) synthetic antioxidant solution (5.0 mg/mL of methanol) was mixed with the fish oil solution by vortexing for 1 min. One-fifth mL of synthetic antioxidant solution (0.1 mg/mL of methanol)

was mixed with the fish oil to obtain a 0.02 % addition level. A 1.0 mL aliquote of menhaden oil solution without any additive served as the control. Then, all solutions in the test tubes were evaporated at 30^{o}C by a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, Mo., U.S.A.). The dried samples were stored at room temperature. Two of each group were taken to determine the change of DHA and EPA concentrations at diffenent storage times.

5.2.5 Determination of Isoflavones in Defatted Soy Flour Extract and Heat and Enzyme Treated Extracts

Isoflavones in the soy extract and enzyme treated defatted soy flour extract were carried out using an Agilent 1100 HPLC system consisting of a binary pump with a vacuum degasser, an auto-sampler, and a diode array detector (DAD, Agilent Technologies, Palo Alto, CA). Separation of isoflavones was achieved using a reversed phase C_{18} column (Phenomenex, Lorance, CA, USA, 150 × 4.6 mm; particle size 5 μm), preceded by a guard column (Phenomenex, 4 × 3.0 mm) of the same stationary phase. The HPLC conditions were solvent A 100% acetonitrile and solvent B 10% acetonitrile and 90% water (v/v); the gradient was 0 – 5 min B at 15% and 5 - 44 min B from 15% to 45%. Flow rate was set to 1.0 ml / min. HPLC chromatograms were detected using a photo diode array UV detector at 254 nm. The concentration of each isoflavone was calculated based on its standard curve structures.

5.2.6 Determination of EPA and DHA Contents in Fish Oil during Storage

Ten mg of fish oil sample was added to each test tube (13x100mm). Heptadecanoic acid (C17:0) (5mg/mL, add 0.2 ml to each sample) as an internal standard for the fatty acid analysis was added to each tube, then evaporated to dryness using a vacuum centrifuge at 50^{o}C. After adding 2 mL BCl_3-methanol and 1 mL 2, 2-dimethoxypropane and tubes were votexed to perform the derivatization of fatty acid esters. The test tube was capped and incubated in a 60^{o}C water bath

for 30 mins then transferred to an ice water bath for 10 mins to terminate the reaction. Then, 2 mL hexane and 1mL water were added to the tube and vortexed for 30 seconds. The upper hexane layer was transferred to another tube, dried with anhydrous sodium sulfate and transferred to a GC vial.

A gas chromatograph (Hewlett Packard 5890, Agilent Technologies, Palo Alto, CA**)** with a FID detector was used to determine the DHA and EPA concentration. Helium was used as a carrier gas with a column flow rate of 1.2 mL/min. The injection volume was 5 μl and the split ratio was 1:100. The injector and detector temperature was 250 and 270°C, respectively. The oven temperature program was set to hold at 50°C for 3 min and then increased at 4.0°C/min to 250°C. The column was a Supelco SP2380 (30m × 0.25mm) (Bellefonte, PA). The concentrations of DHA and EPA were calculated using the C17:0 internal standard as a reference. The percentage of retained DHA or EPA in the fish oil during storage was obtained by comparing their concentration in each sample with the corresponding initial concentration.

5.2.7 Determination of Free Radical Scavenging Capability Using DPPH Method

The free radical scavenging activity was determined using the DPPH method. The concentration of DPPH solution was 0.025 g in 1000 mL of methanol. One milliliters of the DPPH solution was mixed with 200 μL of sample (5 mg/ml soy extract and 0.2mg/ml synthetic antioxidant solution respectively) in methanol solution and transferred to a cuvette. The reaction solution was incubated for 30 min at 25 °C in the dark and then monitored at 515 nm using a UV-Visible SpectraMax Plus384 spectrophotometer (MolecularDevices, Sunnyvale). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation (Moure et al, 2000):

$$\text{Inhibition\%} = (Abs_{t=0min} - Abs_{t=30min})/Abs_{t=0min} \times 100$$

Where $Abs_{t=0min}$ was the absorbance of DPPH at zero min and $Abs_{t=30min}$ was the absorbance of DPPH after 30 min of incubation.

5.2.8 Statistical Analysis

The determination of the isoflavones contents and the DPPH free radical scavenging test were performed in triplicate. The DHA and EPA analysis were performed three times as well. The means and standard deviations were calculated and the data were analyzed by one-way ANOVA with duncan's multiple comparisons at $\alpha = 0.05$ by using SAS Statistical Analysis System (SAS Inst., Cary, NC).

5.3 Results and Discussions

5.3.1 Capabilities of Defatted Soy Flour Extract and Heat and Enzyme Treated Extracts in Inhibiting Menhaden Fish Oil Oxidation during Storage at Room Temperature

Table 5.1 Retained DHA and EPA in fish oil with or without different soy extract(5%) stored at room temperature

days		0	2	4
control	EPA (%)	97.07± 1.52	49.28 ± 0.80	9.64 ± 0.53
	DHA (%)	97.26 ± 1.66	43.52 ± 1.07	6.27 ± 0.43
Original soy extract	EPA (%)	100.03± 2.52	59.55± 0.22	16.46± 0.25
	DHA (%)	100.00± 6.13	54.29± 0.53	12.13± 0.60
Heat treated extract	EPA (%)	99.85± 3.78	69.30± 3.52	35.53± 2.91
	DHA (%)	95.63± 3.76	63.61± 2.98	28.11± 2.67
Enzyme treated extract	EPA (%)	98.24±4.11	85.51±3.17	64.33±1.68
	DHA (%)	97.92±4.56	83.73±2.94	60.89±2.02

Table 5.1 showed that EPA and DHA in menhaden oil were not stable and both decreased below 50% in 2 days and 10% in 4 days storage at room temperature. Retained EPA and DHA in

heated control were below 18% after heat treatment (data not shown). The EPA and DHA oxidation were inhibited by adding 5% of defatted soy extract. Both EPA and DHA in the menhaden oil mixed with 5% defatted soy extract were still over 50% and 10% after 2 and 4 days storage (Table 5.1). This is in agreement with the findings in Chapter 4, which reported higher antioxidant capability of defatted soy extract in preventing the EPA and DHA oxidation. In addition to soy isoflavones as a major contributor to the antioxidant capability of soybean (Kim and Lee, 2004; Lin and Lai, 2006; Ishihara, 2007), other antioxidant constituents such as saponins, phytic acid, and lecithin could also have contributed to the antioxidant capacity (Ishihara 2007).

It was found that the EPA and DHA in the menhaden oil mixed with 5% heated defatted soy extract had higher retained levels during storage than that in the menhaden oil with 5% untreated (original) defatted soy extract (Table 5.1). After 4 days storage, the retained EPA and DHA in the menhaden oil with heated defatted soy extract were 35.5 and 28.1% respectively and two times higher than that in the menhaden oil mixed with untreated defatted soy extract. These findings suggest that thermal treatment could effectively improve the antioxidant activity of defatted soy flour extract. The reasons might be that 1) the level of aglycone isoflavones in the heated defatted soy extract was increased because the sugar moiety of glucoside isoflavones was released during heating; 2) the aglycone isoflavones could have higher antioxidant activity than glucoside isoflavones as they have an additional –OH group on the benzene ring. Kwak (2007) reported that heating or fermentation changed total isoflavones content and its composition in soy products. Mathias et al. (2006) and Barnes et al. (1998) indicated in general, isoflavones were not destroyed under normal thermal processing conditions but rather were subjected to interconversions between the different forms. However, some aglycones (especially daidzein)

were thermally degradable (Huang et al. 2006; Murphy et al. 2002). To avoid the thermal liability of aglycone isoflavones during heating treatment we used enzyme to hydrolyze the glucoside group and convert glucoside isoflavones to to its aglycone form.

β-Glucosidase extracted from Aspergillus niger (β -glucoside glucohydrolase, EC 3.2.1.21) is an enzyme that is able to cleave the β-glucosidic linkages of di- and/or oligo- saccharides or other glucose conjugates. β –Glucosidases play pivotal roles in many biological processes, such as the degradation of cellulosic biomass, cyanogenesis and the cleavage of glucosylated flavonoids (Kuo and Lee, 2008). It was also reported to be able to break down glucoside groups from glucoside isoflavones to form aglycones isoflavones (Tsangalis et al, 2002). Since aglycone isoflavones are vulnerable under thermal treatment, using the enzyme might be a gentle way to break down the glucoside group on glucoside isoflavones and convert them to be aglycone isoflavones.

From Table 5.1, it was found that the capability of inhibiting EPA and DHA oxidation of defatted soy flour extract was significantly enhanced after enzyme treatement. Both EPA and DHA in the menhaden oil mixed with the enzyme treated defatted soy extract remained over 80% in 2 days and 60% in 4 days during room temperature storage. The retained levels were five times and twice higher than those in the control and menhaden oil mixed with heated soy extract, respectively. The results supported the study of Naim et al. (1976) who reported that aglycone isoflavones had greater antioxidant activities than their glucoside isoflavones. Also, Rimbach et al. (2004) used enzymes to catalyze genistein to monoglucuronides and monosulfates, diglucuronides and disulfates, or mixed conjugates with one site glucuronidated and one site sulfated, and demonstrated that aglycone isoflavones had higher antioxidant ability than their conjugated forms. Ishihara (2007) found that the genistein had the highest antioxidant activity

followed by total isoflavone content, daidzein series, aglycone conjugates and glucosidic conjugates using the ABTS (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) radical cation decolorization assay.

5.3.2 Conversion of Glucoside Isoflavones to Aglycone Isoflavones after the Enzyme Treatment

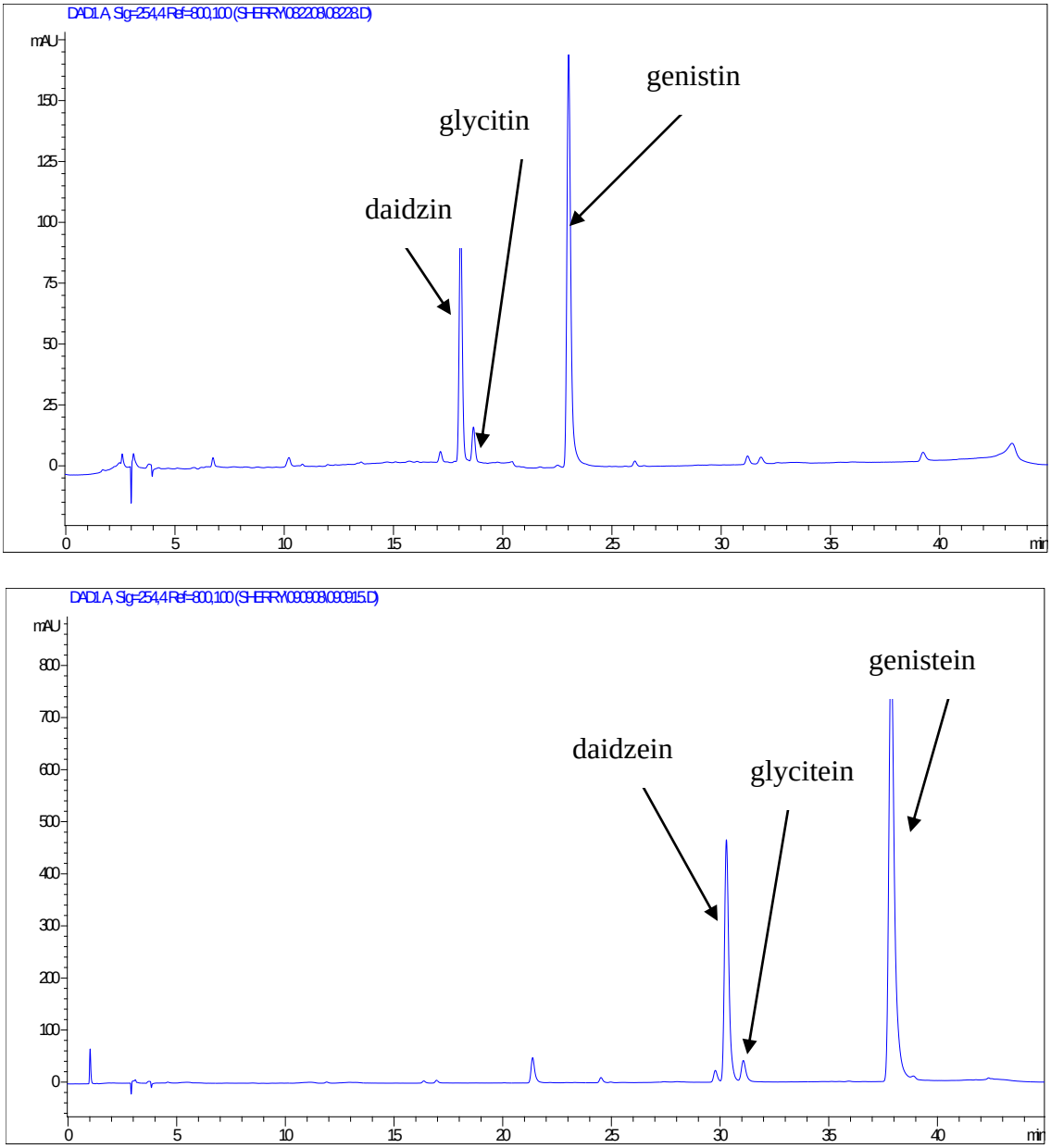

Figure 5.2 Isoflavones profile of defatted soy flour extract before (above) and after (below) enzyme treatment

The major isoflavones in defatted soy flour extract were daidzin and genistin. However, after the enzyme treatment, the glucoside isoflavones in the extract were converted to aglycone isoflavones. Figure 5.2 shows that isoflavones profile in defatted soy flour extract before and after the enzyme treatment. In the enzyme treated defatted soy flour extract, daidzein and genistein are the major isoflavones.

Before the enzyme treatment, the percentages of glucoside and aglycone isoflavones in the extract were 94.5 % and 5.47 %. After the treatment, the percentage of glucoside isoflavones decreased to 5.54 %, while the percentage of aglycone isoflavones increased to 94.46 %. The ratio between aglycone and glucoside content changed from 0.06 to 17.05.

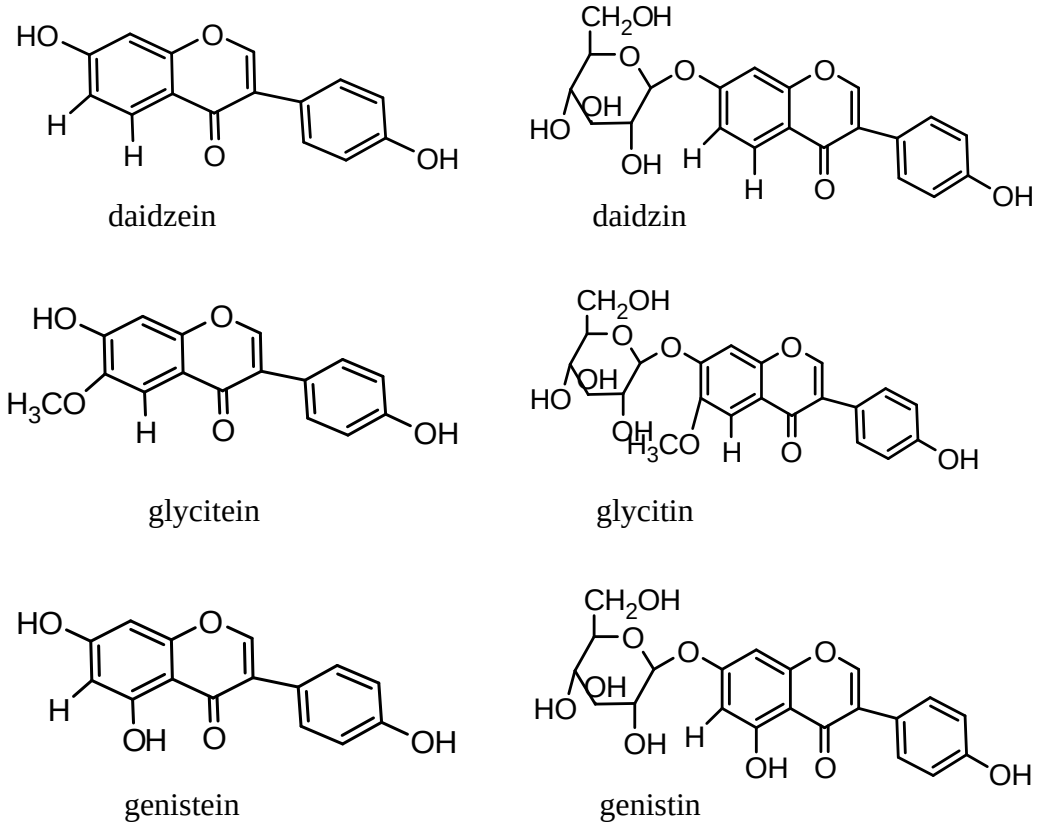

Figure 5.1 Chemical structures of isoflavones (From Nielsen and Williamson, 2007)

The diagram of the conversion mechanism is shown in Figure 5.1. Zhang et al. (2007), Izumi et al. (2000) and Setchell et al. (2002) reported that glucoside isoflavones in soybean have less biological activities and are more difficult to be absorbed by the body than the corresponding aglycones. The aglycone isoflavones are more bioactive than the glucoside isoflavones since they are readily absorbed across the enterocyte of the intestine (Ishihara et al., 2007). Kim and Lee (2004) reported the antioxidant capacity of isoflavones decreased as follows: genistein > daidzein > genistin > daidzin by using vitamin C equivalent antioxidant capacity.

5.3.3 Comparison of Free Rradical Scavenging Capabilities of the Defatted Soy flour Extract with the Enzyme Treated Extract and Synthetic Antioxidants

The free radical scavenging capabilities of the defatted soy flour extract, the enzyme treated defatted soy flour extract and synthetic antioxidants commonly used in food products using the DPPH method are shown in Figure 5.3. The order of the capability from high to low was 0.02 % PG ≈ 0.02 % TBHQ > 0.02 % BHA ≈ 0.02 % α-T > 0.02 % BHT ≈ 5 % enzyme treated defatted soy flour extract > 5 % defatted soy flour extract. The capabilities of PG and TBHQ were over 90 %, while the defatted soy flour extract with and without enzyme treatment were approximately 20 % and 10 %, respectively. The difference of their capabilities may be related to the number of hydroxyl groups on the benzene ring. Based on their chemical structures, both TBHQ and PG have more than two hydroxyl groups and BHA, BHT and α-T have one hydroxyl group. Also, the hydroxyl group of BHT is between two propyls, which may weaken the effectiveness of antioxidation. Loo (2007) found a similar order of free radical scavenging activity as BHA> BHT. Although most of the isoflavones in the enzyme treated extract were aglycone isoflavones that have two or more hydroxyl groups, because commercial synthetic antioxidants had 95% purity, the concentration of isoflavones in the enzyme treated defatted soy flour extract would be much lower.

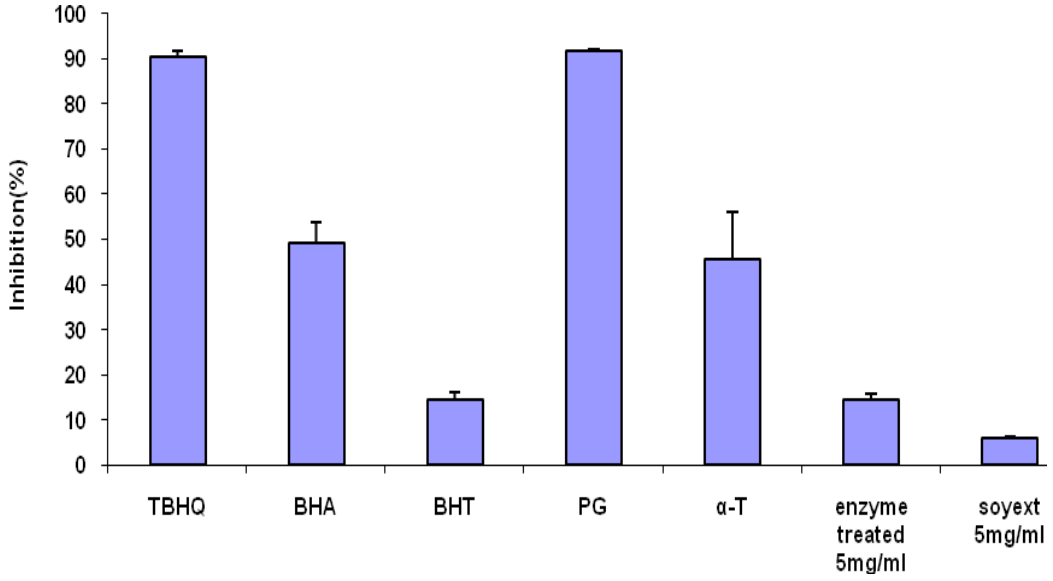

Figure 5.3 Antioxidant activities of natural and synthetic antioxidants (all antioxidants unmarked concentration was 0.2mg/ml)

5.3.4 Comparison of the Capabilities of the Enzyme Treated Defatted Soy Flour Extract and Synthetic Antioxidants in Inhibiting Menhaden Fish Oil Oxidation during Storage at Room Temperature

Figure 5.4 shows that the capabilities of the enzyme treated defatted soy flour extract and synthetic antioxidants in preventing the EPA and DHA oxidation in menhaden oil during storage at room temperature. The menhaden oils were mixed with the enzyme treated defatted soy extract and each synthetic antioxidant at 1% level. The EPA and DHA in the control group decreased to below 5% of their original level, whereas the enzyme treated extract group still retained more than 10% after 5 days storage.

The synthetic antioxidants had significantly higher antioxidant capability in inhibiting menhaden fish oil oxidation. From Figure 5.4, at 1% level, the order of the activity of the

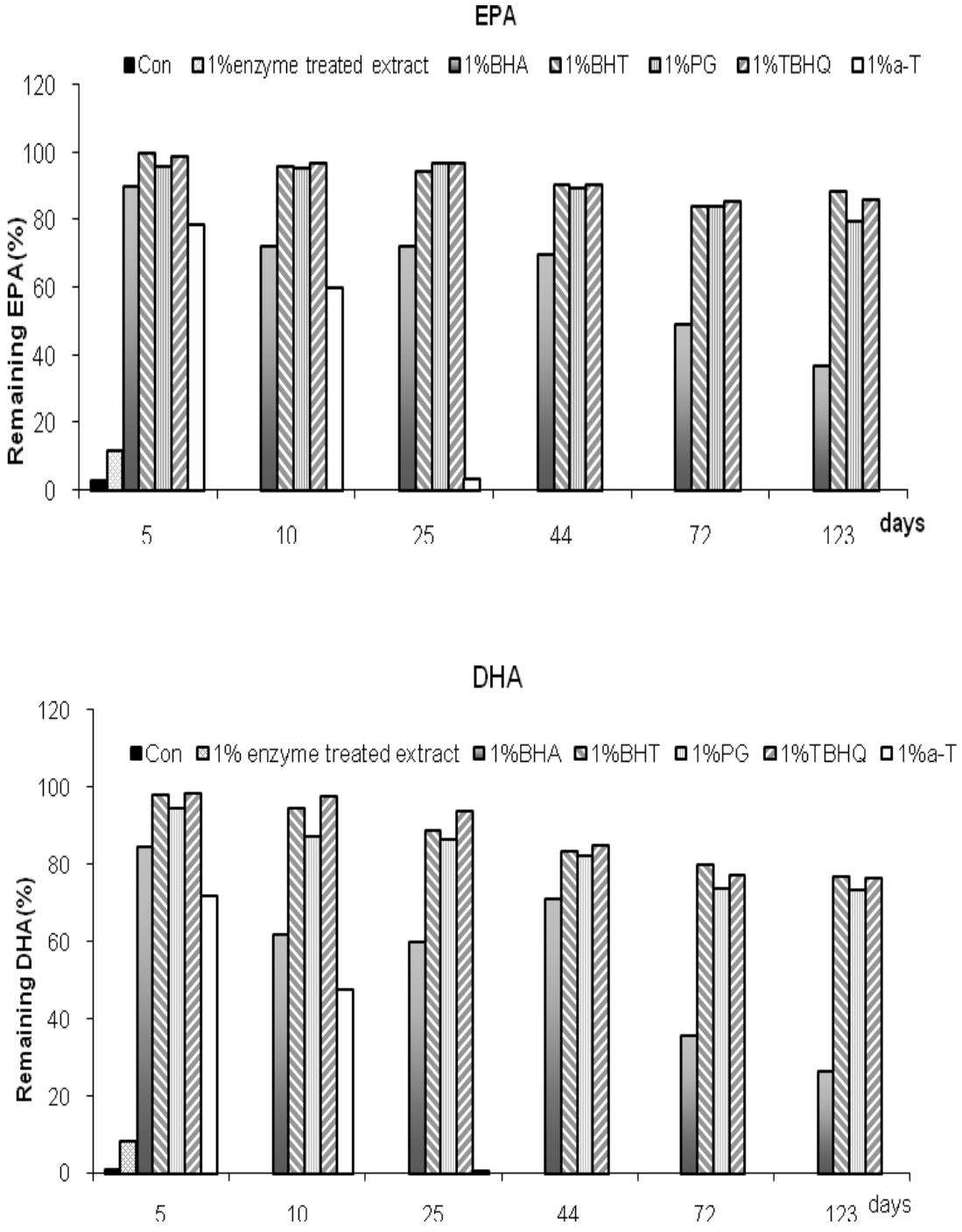

Figure 5.4 Retained EPA (above) and DHA (below) in menhaden fish oil mixed with 1% synthetic antioxidant or enzyme treated extract at room temperature storage

antioxidants in stabilizing menhaden fish oil omega-3 fatty acids from high to low was: TBHQ ≈ BHT > PG > BHA > α-T > the enzyme treated extract. The inhibiting capability of α-T was the lowest among these synthetic antioxidants. The EPA and DHA in the fish oil mixed with α-T decreased to undetectable level after 15 days storage at room temperature. However, after 123 days storage, the fish oil mixed with TBHQ or BHT still retained over 85% of EPA and 75% DHA respectively. For the fish oil with PG, 80% EPA and 73% DHA remained after 123 days storage.

Since most food applications of synthetic antioxidants employ an addition level of 0.02 %, we evaluated their capabilities to prevent menhaden oil oxidation at 0.02 % addition level compared with 5 % of additional level for the enzyme treated defatted soy extract (Figure 5.5).

Among the synthetic antioxidants, TBHQ had the highest antioxidant activity in preventing the long chain fatty acids oxidation, which is the same as when an addition level at 1% was used. Gordon and Kourimska (1995) reported that TBHQ showed higher antioxidant activity than other synthetic antioxidants when added to rapeseed oil and heated at 80℃. Khan and Shahidi (2001) also reported that TBHQ was the most effective antioxidant among the all synthetic antioxidants in protecting borage and evening primrose oil triacylglycerols when heated at 60℃. The chemical structure of TBHQ contains two ortho hydroxyl and two para hydroxyl groups, which might be responsible for its superior antioxidant activity in preventing oxidation in various edible oils (Khan and Shahidi, 2001).

BHA and BHT are the most widely used synthetic antioxidants. In Figure 5.5, the EPA and DHA in the fish oil mixed with 0.02 % BHA decreased to undetectable level after 10 days storage, which was two days longer than that of the fish oil with 0.02 % BHT. Moure et al (2000) reported that BHA had a higher antioxidant activity than BHT in soybean oil when evaluated by

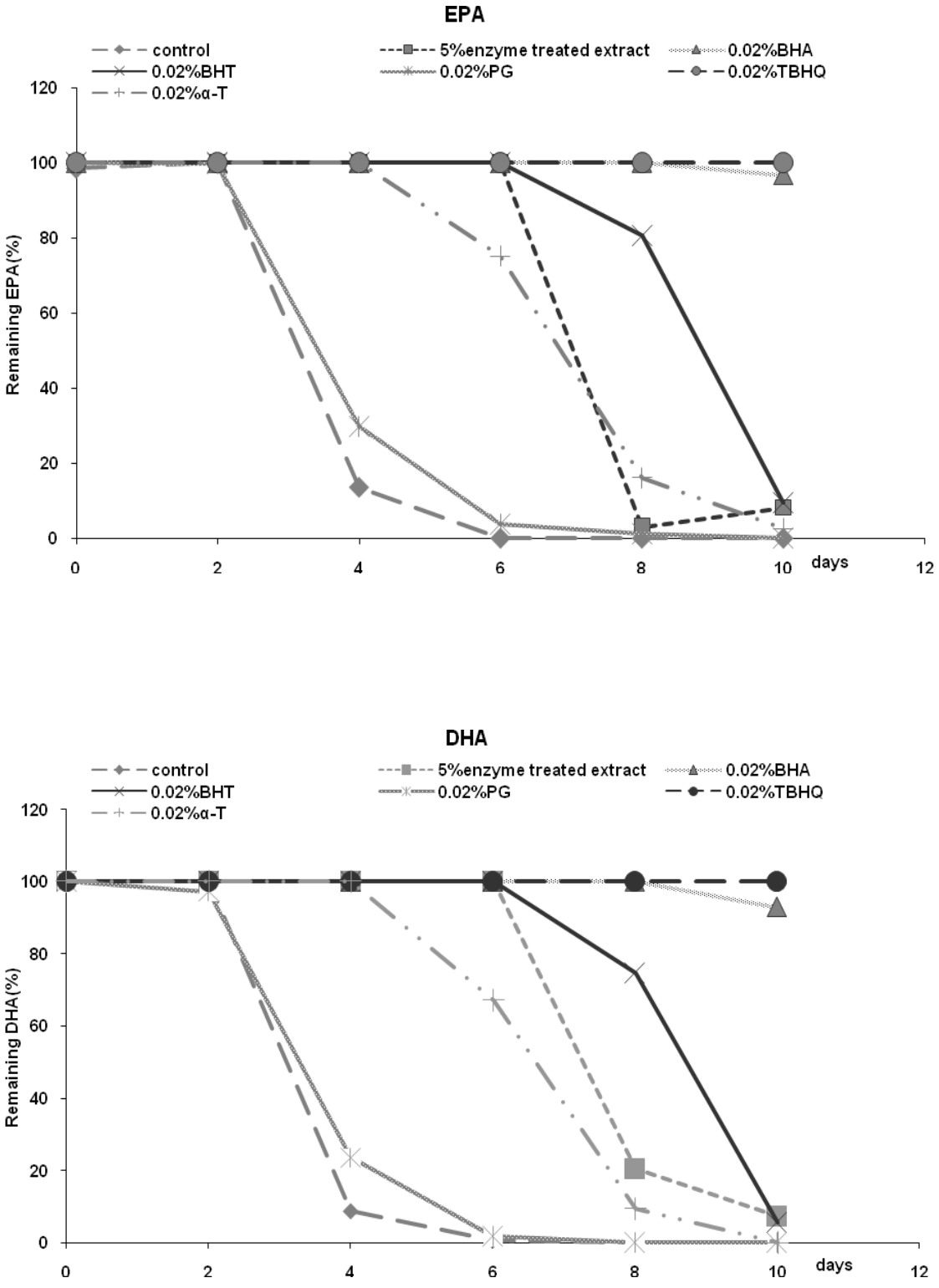

Figure 5.5 Retained EPA and DHA in menhaden fish oil mixed with synthetic antioxidants (0.02 %) and the enzyme treated extract (5 %) at room temperature storage

using antioxidant activity coefficient and inhibition of oil oxidation. Stuckey (1972) and Chipault (1962) reported that BHA was particularly effective in protecting the short chain fatty acids and flavor and color of essential oils.

The fish oil mixed with 5% of the enzyme treated soy extract also significantly prevented the EPA and DHA oxidation, compared with the control group. The retained EPA and DHA was still at 100% in the sixth day storage. Compared with most synthetic antioxidants, the capability of 5 % enzyme treated extract was lower. However, it was very close to 0.02 % BHT during 10 days storage and greater than that of 0.02 % α-T or PG. PG had the lowest antioxidant activity among the synthetic antioxidants at 0.02% addition level. This was consistent with the results of Jayathilakan et al (2007) who reported that PG showed the lowest antioxidant potential among synthetic antioxidants added in meat, beef and pork after storage at 4℃ for 6 days. Huang and Frankel (1997) reported that the activity of antioxidants depended on the lipid system, the presence of metal catalysts, the temperature of oxidation, the antioxidant concentration, the oxidation stage, and the method used to evaluate lipid oxidation. Furthermore, the effectiveness of an antioxidant in a food system is dependent on a range of factors such as concentration, the physical state of the substrate, solubility, phase partitioning, processing and storage (McBride, 2007). In general, 5 % enzyme treated soy extract could extend the EPA and DHA life 3 times longer than the control group.

5.4. Conclusion

The enzyme treated defatted soy flour extract had a higher antioxidant activity in inhibiting the EPA and DHA oxidation in menhaden oil than the extract with or without heat treatment. At 1% addition level, the order of the activity of synthetic antioxidants and the extract in stabilizing menhaden fish oil omega-3 fatty acids from high to low was: TBHQ ≈ BHT > PG > BHA > α-

T > the enzyme treated defatted soy extract. However, the antioxidant activity at 0.02 % addition level for synthetic antioxidants from high to low was TBHQ > BHA > BHT > 5% the enzyme treated extract > α-T > PG. Although the enzyme treated soy extract are still less effective compared to those synthetic antioxidants at the same addition level, it could be used at relatively high concentration in food products since it is safe and from natural origin.

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CHAPTER 6 THERMAL DYNAMIC PROPERTIES OF ISOFLAVONES EXTRACT DURING DRY HEATING

6.1 Introduction

The primary chemical structure of isoflavones, aglycone, has three different types named daidzein, glycitein and genistein. The three aglycone forms of isoflavones are differentiated by the number and position of hydroxyl on A, B rings and side group on their A-ring (Figure 6.1). Also, the aglycone can link with different sugar moieties to form other types of isoflavones. In addition to the aglycone form, three other types of isoflavones in soybean are the glucoside form, daidzin, glycitin and genistin; the acetyl glucoside form, acetyl daidzin, acetyl glycitin, acetyl genistin; and the malonyl glucoside form malonyl daidzin, malonyl glycitin and malonyl genistin (Huang et al, 2006).

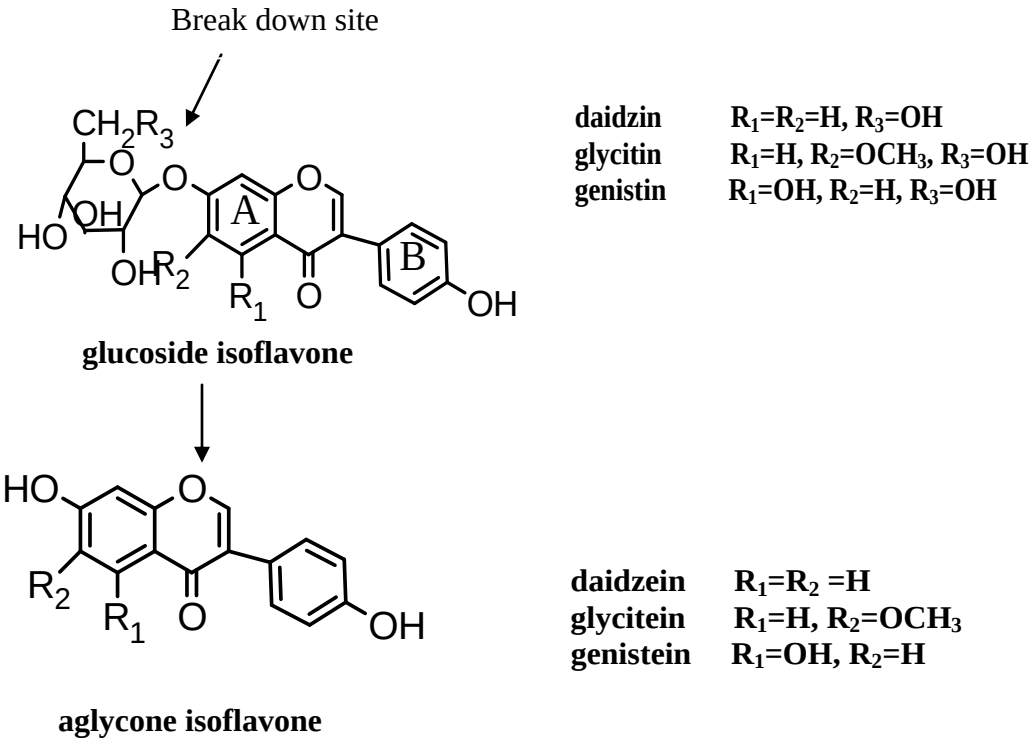

Figure 6.1 Chemical structures of glucoside and aglycone isoflavones

As thermal processing is the most common food preparation method, changes in the content and distribution profile of soy isoflavones in foods due to heat treatments have been studied by many researchers (Xu et al., 2002; Coward et al., 1998; Mahungu et al., 1999; Ungar et al., 2003; Pinto et al., 2005 Huang et al., 2006). Acetyl glucoside and malonyl glucoside are aglycone derivatives, so they could also convert to aglycone and other derivative types during heating. Wang et al. (1990) reported that heating the glucoside forms daidzin and genistin under acidic conditions released free aglycone isoflavones. Chien et al. (2005) studied the conversion and degradation of malonyl genistin and acetyl genistin during dry heating. Their results concluded that malonyl genistin was most susceptible to degradation and could convert to acetyl genistin or genistin, but did not report on the possible changes of glucoside and aglycone forms. Huang et al. (2006) found that daidzein and glycitein in soymilk decreased rapidly during the first 15 seconds of heating at 95°C or 121°C; however, after heating 45 min at 95°C, the content of genistein showed little increase. Huang et al. (2008) found that, generally, the content of individual aglycones (daidzein, glycitein, and genistein) decreased in steamed soybeans as the heating temperature was raised to 60 ℃ or higher, whereas the content of daidzin, glycitin, and genistin, the β -glucoside isoflavones, increased in steamed black soybeans after heating at 60-80 ℃ or higher for 30 min. However, Mathias et al. (2006) reported that no conjugated isoflavones could convert to the corresponding aglycone form under alkaline or acidic condition with heating treatment.

Most of those thermal stability studies were carried out in the whole soybean system, where there are four different types of isoflavones. The degradation of aglycone and their sugar moiety isoflavones and the conversion of the sugar moiety isoflavones to other types of isoflavones could take place at the same time during heating. Thus, those studies were not able to clearly

reveal the kinetics and thermal stability of glucoside and aglycone forms of isoflavones during heating. In this study, pure glucoside and aglycone forms of isoflavones were used to evaluate their thermal stabilities individually. This approach eliminated any conversion from precursor isoflavone during heating process. The results of this study could provide information related to the actual kinetics and thermal stability of glucoside and aglycone forms of isoflavones at different heating temperatures.

6.2 Materials and Methods

6.2.1 Chemicals

HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (Fair Lawn, NJ). Isoflavones daidzin, glycitin, genistin, daidzein, glycitein and genistein were purchased from Sigma-Aldrich (St. Louis, MO).

6.2.2 Standard Isoflavones Solution Preparation

The standard isoflavone (1.0 mg) was dispersed in 10 mL methanol and stirred for 10 min. The mixed solution was transferred to a 500 mL volumetric flask after it was passed through a filter paper (0.45μm x diameter 15 mm, Whatman, Maidstone, England). This solution was stored at -20 ^{o}C before use.

6.2.3 Sample Preparation and Dry Heat Processing

The standard solution (0.5 ml) was added to test tubes (13 ×100 mm) and the methanol solvent in the test tubes was evaporated at 50^{o}C by a centrifuge vacuum evaporator (CentriVap Mobile System; Labconco, Kansas City, MO). The evaporation resulted in a residue of isoflavone standard that thinly coated the bottom of each test tube. Then, test tubes were heated in a sand bath with a temperature controller. The temperatures were set at 100, 150 and 200^{o}C for each set of experiments. Two test tubes of each standard were randomly taken from the sand bath at 10,

20, and 30 min. Two test tubes that were not heated were considered 0 min samples. The experiment was replicated twice at each temperature.

6.2.4 Determination of Isoflavone Content Using HPLC Method

Analysis of isoflavones was carried out using an Agilent 1100 HPLC system consisting of a binary pump with a vacuum degasser, an auto-sampler, and a diode array detector (DAD, Agilent Technologies, Palo Alto, CA). Separation of isoflavones was achieved using a reversed phase C_{18} column (Phenomenex, Lorance, CA, USA, 150 × 4.6 mm; particle size 5 μm), preceded by a guard column (Phenomenex, 4 × 3.0 mm) of the same stationary phase. The mobile phase consisted of solvent A 100% acetonitrile and solvent B 10% acetonitrile and 90% water (v/v) at a Flow rate 0.8 ml/min. The gradient program was at 0- 5 min 15% B; 5-44min 15% to 45%B. HPLC chromatograms were monitored at wavelength 254 nm. The concentration of isoflavone was calculated based on its standard curve.

6.2.5 Thermal Dynamic Properties Calculation and Statistical Analysis

The degradation rate constant (k), half-life (t½), and energy of activation (Ea) of each isoflavone were determined using the method of Liu and Murphy (2007). The first-order degradation rate constant (k) was calculated by a linear regression plot of ln(concentration) vs. time (min). The half-life (t1/2) was predicted by –ln(2) / k. The energy of activation (Ea) was obtained using the Arrhenius equation through a linear regression plot of ln(k) vs. reciprocal of absolute temperature (K). The linear regression process and comparisons of the thermodynamic properties and production of isoflavone were performed using SAS PROC REG and ANOVA, respectively (SAS 9.1.3, Cary, NC). The duncan's multiple range test was performed for post-hoc multiple comparison.The statistical level of significant difference at two different experiment conditions was at $P<0.05$.

6.3 Results and Discussion

6.3.1 Thermal Stabilities of Isoflavones at Different Dry Heating Temperatures

The degradation rate constants of isoflavones heated at different heating temperatures are shown in Figure 6.2. The degradation rate constants of the isoflavones at temperature 100^{o}C were not significantly different. The degradation rate constants of all isoflavones increased with increasing heating temperature. The degradation rate constant (K) of daidzin and genistin at 150 ℃ were approximately 3 times higher than those at 100 ℃. The rate constant of glycitin at 150°C was almost 11 times higher than at 100°C. Glycitin is the most vulnerable among the three glucoside isoflavones at temperature 150^{o}C. At 200°C, the degradation rate constants of the three glucoside isoflavones increased greatly to the same level. It was about 8 times higher than that at 150°C for daidzin and genistin, while the constant value of glycitin at 200°C was 2.5 times higher than at 150 ℃. Likewise, the degradation rate constants of aglycone isoflavones increased with increasing heating temperature. At 150^{o}C, the degradation rate constant of glycitein was the highest among the three aglycone isoflavones. The degradation rate constant of daidzein was the lowest at the heating temperature at 200^{o}C. Compared with the three glucoside isoflavones, aglycone isoflavones were more stable at heating temperatures 150 and 200 ℃.

The half-lives of the six isoflavones at different temperatures are showed in Figure 6.3. The range of half-life of glucoside and aglycone isoflavones was from 144 to 169 min and 139 to 176 min at 100 ℃, respectively. When heating temperature increased to 150 ℃, the range of their half- lives was reduced to from 15.7 to 54.7 min for glucoside isoflavones and to from 40.0 to 90.6 min for aglycone isoflavones. At 200 ℃, their half-lives were reduced to from 5.8 to 6.0 min for glucoside isoflavones and to from15.7 to 21.2 min for aglycone isoflavones. Based on the shortest half-life, glycitin among the three glucoside isoflavones, and glycitein among the

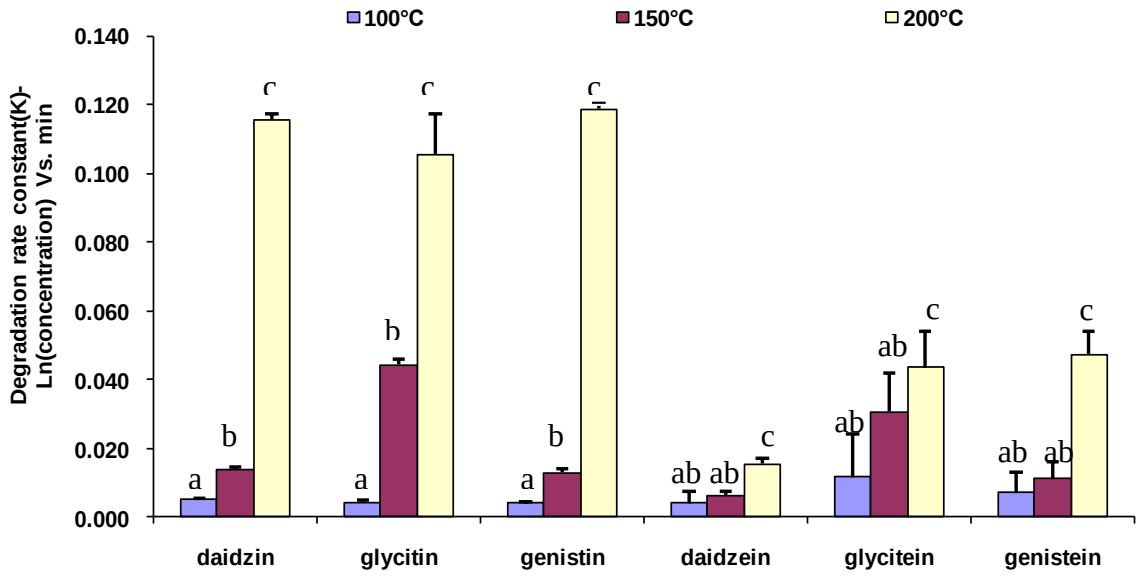

Figure 6.2 Degradation rate constants of each isoflavones heated at different temperatures
*Data with different letters in each row are significantly different (P<0.05)

aglycone isoflavones, had the lowest thermal stabilities. The half-lives of daidzin, glycitin and genistin at 100 ℃ was about 3.0, 9.2, and 3.1 times that of those at 150 ℃ and 25.2, 24.1, and 30.0 times that of those at 200 ℃, respectively. The half-lives of daidzein, glycitein, and genistein at 100 ℃ were about 1.9, 3.87, and 2.5 times that of those at 150 ℃ and 8.2, 7.2, and 11.3 times that of those at 200 ℃, respectively.

Park et al. (2002) measured the isoflavone profile in soy flour and showed a decrease in total isoflavones for all cultivars after heating at 121 ℃ for 40 min. Ungar et al. (2003) reported that daidzein was more labile to degradation than genistein from 70 to 120 ℃ for 20 min at pH 7 or 9. In our study, the thermal liabilities of daidzein and genistein were not significantly different during heating. The difference in results might be due to the lower heating temperature, higher water content, and possible interferences from the aglycone isoflavone precursors in those studies. Our result was consistent with Xu et al. (2002) who used mixed isoflavones purified

from soy flour during dry heating condition. They found that the order of stabilities of glucoside isoflavones from lowest to highest was glycitin, genistin, and daidzin and the stability of daidzein was higher than that of glycitein or genistein. The difference in the thermal stabilities of glucoside and aglycone isoflavones at high temperature might due to their different chemical structures. For some glucoside isoflavones, the linkage of glucoside group and aglycone could be broken at high temperature to convert relatively stable aglycone isoflavone before being totally degraded (Figure 6.1).

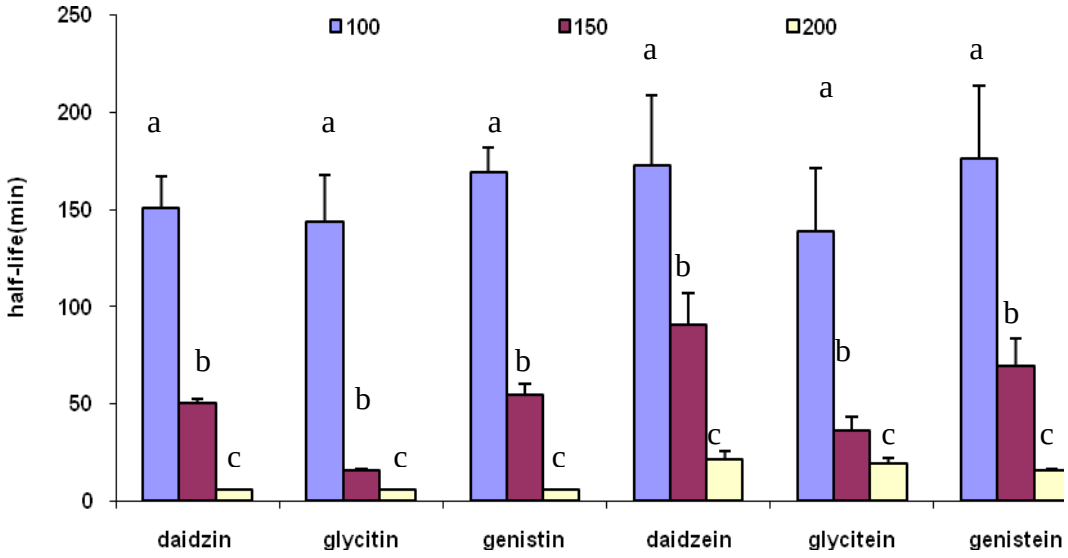

Figure 6.3 Half-life (t 1/2) of each isoflavone heated at different temperatures
*Data with different letters in each row are significantly different (P<0.05)

The energy of activation (Ea) of the six isoflavones are shown in Figure 6.4. The energy of activation (Ea) for the three glucoside isoflavones was from 34.0 to 37.6 kJ/mol. The energy of activation for aglycone isoflavone was from 15.9 to 33.4 kJ/mol. Glucoside isoflavone had a higher Ea than that of the aglycone form. The high Ea means that the rate of degradation was strongly temperature dependence (Fortea et al., 2009). In our study, the degradation of glucoside

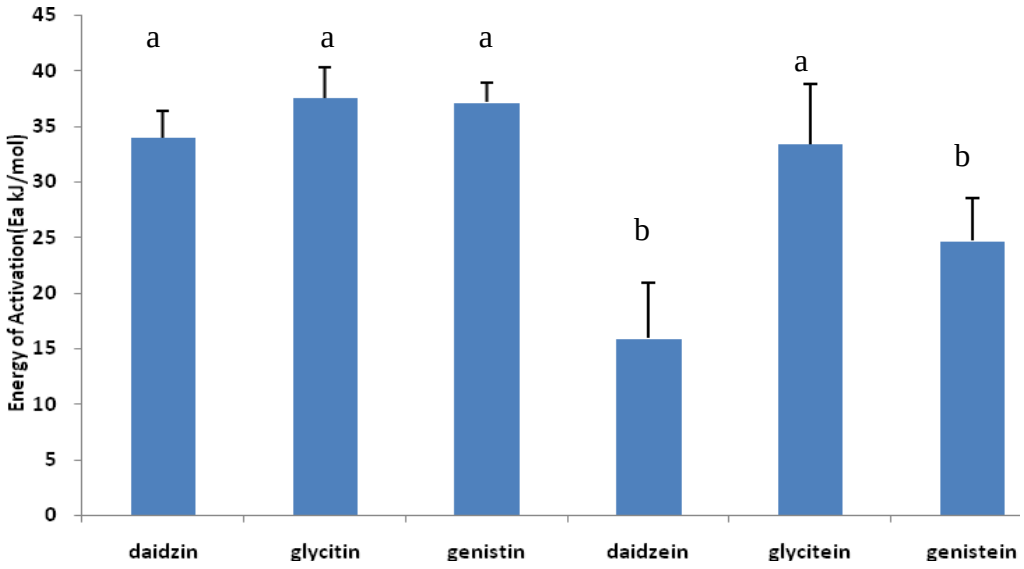

Figure 6.4 Energy of activation (E_a) of each isoflavone heated at different temperature for 30mins

*Data with different letters in each row are significantly different ($P<0.05$)

isoflavone was affected to a greater extent than aglycone isoflavone by the same higher temperature, which corresponded to higer activation energies that predict the effect of temperature in the reaction rate (Liu and Murphy, 2007). The lowest energy of activation of 15.9 kJ/mol was observed for daidzein (Figure 6.4). Ungar et al. (2003) reported that Ea for daidzein was 8.38 and 21.69 kJ/mol for pH 9 and 7; Ea for genistein was 11.57 and 3.88 kJ/mol at pH 9 and 7. In the study of Eisen et al. (2003), the Ea for genistin in tofu were 7.2 to 17.6 kJ/mol. The slightly greater Ea value of isoflavones in our study than those studies might result from the different study temperature and sample condition. Morrica et al. (2004) found a marked effect of temperature on the Ea value of the same compound at different environment factors, such as pH, temperature, humidity, initial concentration, pressure, media etc.

6.3.2 Conversion of Glucoside Isoflavones to Aglycone Isoflavones during Dry Heating

In this study, it was found that the conversion of the three glucoside isoflavones to their

corresponding aglycone isoflavones occurred at a heating temperature above 150^{o}C. Daidzin, glycitin and genistin decreased at a rate of 3.3 30.0 and 12.5 μg in 10 minutes, respectively (Figure 6.6). At the same time, daidzein, glycitein and genistein were formed to levels of 3.0, 25.0, and 4.9 μg in 10 min at 150 ℃ (Figure 6.5). After 30 min, about 24.5, 70.4, 30.3 μg of daidzin, glycitin and genistin were degraded, respectively, while daidzein, glycitein and genistein were generated to levels of 5.3, 67.1, and 6.7 μg, respectively. There was 95.3% conversion of glycitin to glycitein, while only 21.8% daidzin and 22.2% genistin were converted to their corresponding aglycone isoflavones at 150 ℃ after 30 min. This result indicated that degradation rate of glycitin was much faster than that of daidzin and genistin, and the glucoside group of glycitin was hydrolyzed much easier than that of daidzin and genistin. The reason might due to their chemical structure. From the chemical structure of these three glucoside compounds, on the 6 position of A ring, daidzin and genistin have a -H group, while glycitin has a –OCH_3 group. Methoxy substitutent on the A ring of glycitin might de-stabilize the glycosidic linkage, making hydrolysis of the glucoside group easier than that of daidzin and genistin during heating.

Generally, antioxidant activity depends on the number and positions of hydroxyl groups and other substituents, and glycosylation of flavonoid molecules (Cai et al, 2006). The presence of hydroxyl groups on the flavonoid structure enhances antioxidant activity (Cai et al, 2006). Comparing genistin with daidzin, they both have 4'-OH and a 7-glucoside group. Genistin has an extra –OH at 5 site of A ring. In turn, genistin showed a slightly higher degradation rate than daidzin (Figure 6.6) . The reason might due to their different molecular structure. Hamama and Nawar (1991) studied the thermal decomposition of some phenolic antioxidants using TLC and gas chromatograph mass spectrometry and reported that the order of stability of the four phenolic antioxidant at 185 °C from high to low was BHT>PG >BHA>TBHQ. The chemical structures

are the main factors lead to the stability extent. For example, BHT has one more $C(CH_3)_3$ group on 5 site; TBHQ had one –OH group while BHA had a OCH_3 at the same site, others was same for these two molecular structure. These situationa were similar to the results obtained for genistin and daidzin in this research. Nawar (1991) indicated that phenolic antioxidants exhibited significant decomposition at elevated temperature and gave rise to a number of breakdown products which in turn attacked another molecular or other intermediates.

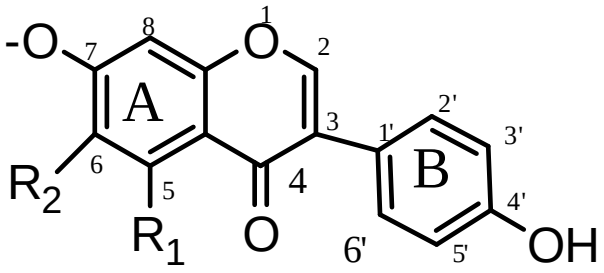

Figure 6.5 Chemical structure of isoflavones(referred from Heim et al, 2002)

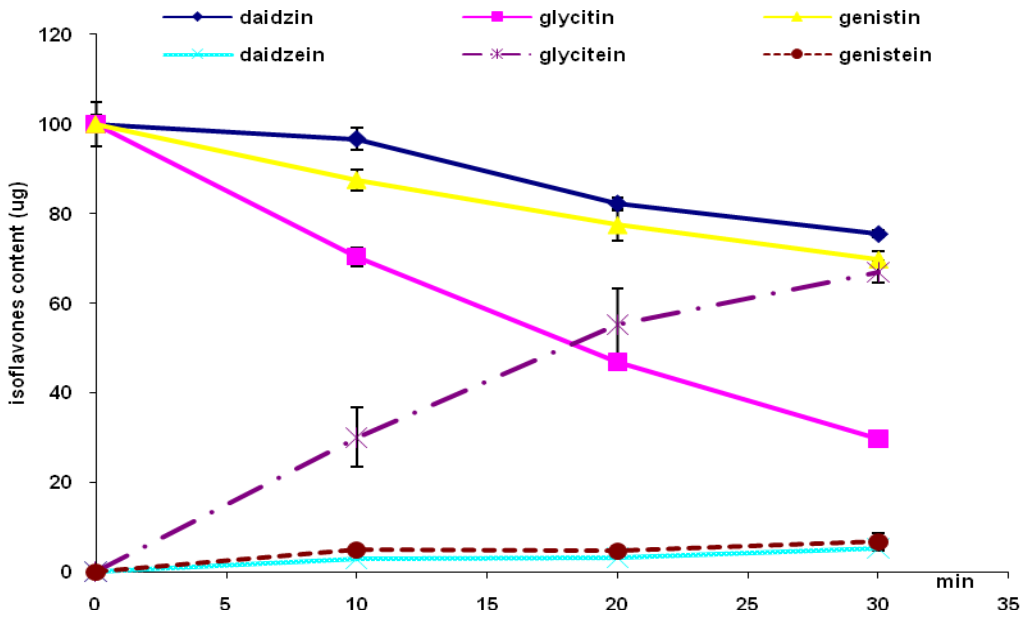

Figure 6.6 The content changes of glucoside and aglycone isoflavones at 150 °C

When heating at temperature 200 ℃, glycoside isoflavones decreased rapidly. There was only 2.4, 3.6, 2.2 μg daidzin, glycitin and genistin that remained from 100 μg of the original level after 10 minutes, respectively. Daidzein, glycitein and genistein were produced to levels of 19.1, 48.8, 9.3 μg after 10 min (Figure 6.7). Eventually, the levels of daidzein, glycitein and genistein were 18.3, 42.8 and 7.0 μg respectively after 30 min at 200 ℃. Similar to at 150 ℃, the conversion yield of glycitin to glycitein at 200 ℃ was higher than either of daidzin or genistin.

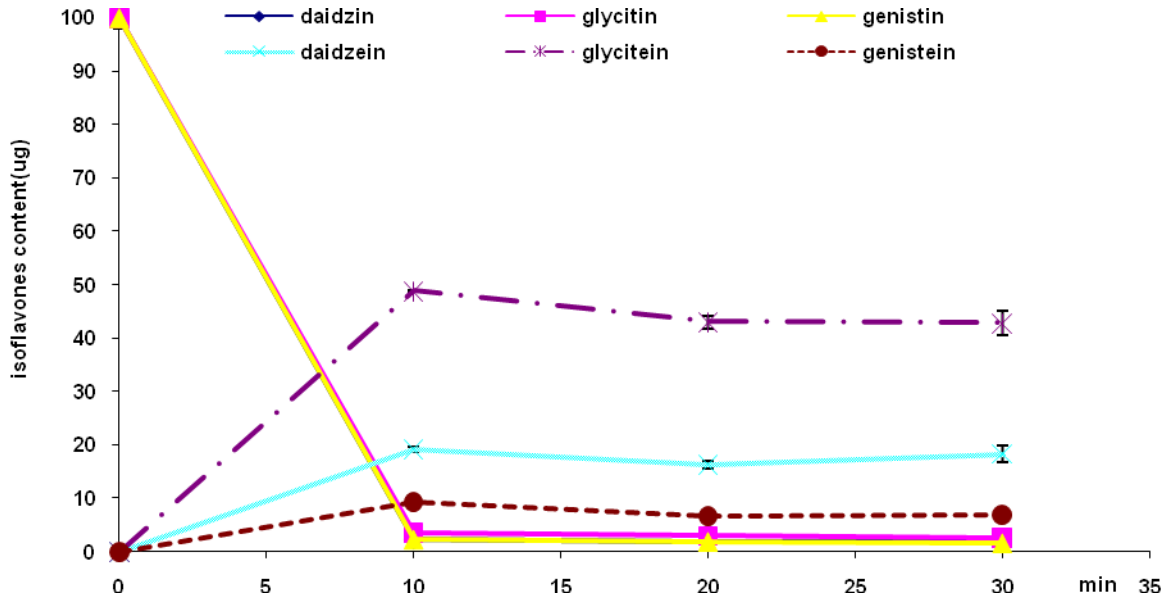

Figure 6.7 The content changes of glucoside aglycone isoflavones at 200 °C

The generation of aglycone from the acetyl- and malonly- glucoside form during heating was reported in several previous studies (Xu et al., 2002; Chien et al., 2005; Rostagno et al., 2005). Some studies also found that glucoside isoflavones could be produced from the degradation of malonyl forms of isoflavones (Coward et al., 1998; Mahungu et al., 1999). Chien et al. (2005) reported that genistein was generated at a slow rate after genistin was heated at 200 ℃. This was

different from our results in which the rate was much higher than in their study. The reason might be that Chien et al. (2005) used a methanol solution in a 2 mL ampoule which was flushed with nitrogen and sealed then heated at 200 ℃. Thus, degradation contributed from oxygen oxidation was not taking place in their study. However, the oxidation is very important reaction for most food processing. Our degradation study was preformed in an open air environment to include the oxidation degradation during heating.

6.4 Conclusion

From this study, thermal kinetics and stabilities of six major isoflavones during dry heating were revealed while eliminating possible interference from their precursors. Overall, aglycone isoflavones had higher thermal stability than glucoside isoflavones at dry heating temperature below 200^{o}C. The order of stabilities of aglycone and glucoside isoflavones from low to high was glycitin < genistin < daidzin < glycitein < genistein < daidzein at temperature below 150^{o}C. However, their thermal stabilities were not different at 200^{o}C. Also, conversion of glucoside isoflavone to aglycone isoflavone was found at heating temperature above 150 ℃. The conversion production of glycitein was higher than that of daidzein or genistein at 150 and 200^{o}C. The information obtained from this study could be helpful in maximally maintaining target isoflavone during high temperature food processing.

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CHAPTER 7 SUMMARY AND CONCLUSIONS

This research was designed to determine the total phenolic, isoflavones, tocopherols content and antioxidant activities in byproducts of soybean oil refining. We found defatted soy flour extract had high antioxidant activities. What's more, heated defatted flour extract showed a higher antioxidant activity than unheated extract. This compelled us to look for the reason and try to find a more effective method to enhance the antioxidant activity of defatted flour extract and to study the thermal properties of isoflavons during heating.

In chapter 3, total phenolic contents and isoflavone distribution in soy cotelydon, coat and germ and the antioxidant activity of their extracts were determined. The results showed that the β-Glucoside forms of isoflavones were the major class of isoflavones in all three soy parts. Soy germ was the richest source of the isoflavones among the three soybean parts. Daidzin and glycitin were the major isoflavones in the germ and coat, while daidzin and genistin predominated in the cotyledon. Therefore, distribution profiles of isoflavones in soy coat and germ are similar, but different from that in cotyledon. Compared with the β-glucoside form of isoflavones, aglycone isoflavones were at much lower levels in all three soy parts.

In chapter 4, total phenolic contents, tocopherols, and isoflavones in different byproducts were determined by HPLC. Their antioxidant activity in inhibiting menhaden oil oxidation were determined using the DPPH free radicals scavenging method and the headspace method. The results showed the level of isoflavones and phenolic contents in the defatted soy flour extract were much higher than that in other byproducts. The isoflavone concenteration was 55 mg/g in defatted flour extract, while it was only 0.2 or 0.3 mg/g in crude oil or gum and was undetected in degummed oil. Defatted flour extract demonstrated the greatest activity in preventing

menhaden fish oil oxidation. It was able to retain over 60% of DHA and 65% of EPA in the fish oil after it was heated at 150°C for 30 min, while only 30% of DHA and 37% of EPA were retained in the fish oil with no additive. The defatted flour extract also significantly inhibited the generation of rancid volatiles in the fish oil during storage. The antioxidant capability from highest to lowest was defatted flour extract > gum > degummed oil = crude oil.

In chapter 5, the changes of antioxidant activities of defatted soy extracts after heat treatment and enzyme treatment were determined. Their antioxidant activities were tested and compared with synthetic antioxidants at different addition levels. The results showed the antioxidant capability of the defatted soy flour extract was enhanced after it was heated or treated with glycosidase enzyme. The retained EPA and DHA in the menhaden oil mixed with 5% of the enzyme treated extract were about two times that of heated extract and 7 times that of control group after storage at room temperature for 4 days. However, the capability of the heated or enzyme treated extract at 5% addition level was not greater than any of the synthetic antioxidants at 0.02% addition level; only α-tocopherol (0.02%) and propyl gallate (0.02%) were lower than the enzyme treated extract (5%). The reason might be that the purity of the aglycone form of isoflavone in defatted soy extract was very low. Thus, in order to enhance the antioxidant activity of enzyme treated defatted flour extract, the purity of aglycone isoflavones should be improved.

In chapter 6, the thermal dynamic properties of isoflavones were evaluated at 100, 150, and 200°C using a kinetic model and pure isoflavone standards. The results showed that the degradation rate constants of soy isoflavones were not significantly different at 100°C. The degradation rate constants of each isoflavone increased as the temperature increased. The half-life decreased with increasing heating temperature. The energy of activation (Ea) of the β-

glucoside isoflavones was a little higher than those of aglycone isoflavones. Glucoside isoflavones were degraded and converted to their corresponding aglycone isoflavones at different rates. The order of thermal stability from low to high was glycitin < genistin < daidzin < glycitein < genistein < daidzein at a temperature equal or below 150 ℃.

The results of this research provided information relative to the distribution of soy antioxidants, isoflavones and phenolics and overall antioxidant activity in different soy parts and in the byproducts of soy oil refining. It also provided effective methods to ehhance antioxidant activity of defatted soy flour extract and the thermal properties of each isoflavone, as well as theirconversion from glucoside to aglycone isoflavones during heating. This information could be useful as a means to utilize low value byproducts that contain high level of soy antioxidants as a valuable antioxidant source.

APPENDIX
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This is Xiaohua Yue from Louisiana State University. In completion of my dissertation, I want to use the whole paper "Comparison of soybean oils, gum, and defatted soy flour extract in stabilizing menhaden oil during heating" as one chapter in my dissertation. So I am writing this letter to ask you for help. Could you give me a copyright permission so I can use the material described above in my dissertation?

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Thank you for your help.

Sincerely

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VITA

Ms. Yue was born and brought up in a small village of Wuxi, Jiangsu Province, China, which abounds with fish, rice and fruits. She completed her bachelor and master degrees in food science at Shanghai Fisheries University, China. In 2005, she began to persue her doctoral program in the Department of Food Science, Louisiana State University.

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