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Composition of Four Selected Arkansas-Grown Non-GMO Soybean Cultivars and the Development of High Protein Beverage Using a Selected Soybean Protein Hydrolysate

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Composition of Four Selected Arkansas Grown Non- GMO Soybean Cultivars and the Development of High Protein Beverage Using a Selected Soybean Protein Hydrolysate

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By

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Abstract

Protein beverages made with non-genetically modified (non-GM) ingredients have been in demand due to the increasing consumers' interest in healthy eating habit. Beverages formulated with non-GM soy protein are rare in the United States market since 93% soybeans planted in U.S (2013) are labeled as GM. This study aimed to develop protein hydrolysate beverages using one cultivar among the four Arkansas grown non-GM soybean cultivars including Osage, R95-1705, R08-4004 and R05-4969. R08-4004 was selected since it had the highest amounts of the three branched amino acids including Phenylalanine (51.4 mg/g), Leucine (74.9 mg/g), and Isoleucine (49.7 mg/g) with lowest amount of anti-nutrients including stachyose (42.0 mg/g) and trypsin inhibitor (23.0 TUI/mg). Soy protein isolate (SPI) was prepared using the aqueous extraction (pH = 9), and hydrolyzed at optimal condition of Alcalase concentration 1.0 AU (3.2 µL/g), 50 ºC, and 120 min incubation. The result was protein hydrolysate with low S_0 value (35.4), low turbidity (0.9), high soluble protein content (46.7%), and highest Angiotensin-I Converting Enzyme (ACE-I) inhibition activity (66.6%).

Protein beverages containing 20 g SPH per 500 mL serving size were developed with Chai tea, tangerine, and mixed berries flavors. The sensory evaluation showed that the tangerine flavor had highest likeness followed by mixed berries, and Chai tea flavor. It also indicated that the usage of citric acid alone in tangerine flavor and the combination with bitter blocker and masking agent in mix berries flavor effectively minimized the bitter taste of SPH and increased the consumer acceptability. Shelf-life stability testing was at refrigerated storage (5 °C) over a 42 days period. Pasteurization (90-95 °C, 5 min) effectively prevented microbial growth. Turbidity increased significantly only in Chai tea drink, but not in tangerine and mixed berries flavored ones. Soluble protein content of three beverages decreased significantly caused by the precipitation of SPH over

the storage period. Total color difference values changed during 42 days of storage due to the degradation of natural colors in the three beverages. Overall, the tangerine and mix berries flavored beverages received high consumer acceptance and have potential for commercial application.

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Chapter 1: Introduction

1.1 Introduction

Soy foods have gained more attention among the U.S consumers for wellness, especially since the Food and Drug Administration (FDA) approved the claim about the association between soy protein and the reduced risk of coronary heart disease (CHD). According to Food and Agricultural Organization (FAO), soybean is the only plant food source that contains an amino acid nutritional profile similar to those derived from animal sources such as casein and egg white in regard to their protein digestibility corrected amino acid score (PDCAAS). In addition, health benefits of soy protein include decreasing blood cholesterol levels, body fat, bone loss, and the incidences of some cancers (Friedman & Brandon, 2001). According to the "Consumer attitudes about nutrition 2013" report, 75% of American consumers associate soy- based products with a healthy lifestyle and 40% are aware of FDA claim in which consuming 25 grams of soy protein per day reduces the risk of coronary heart disease (United Soybean Board, 2013). In "My Plate" consumer information, the USDA has stressed that choosing a variety of lean proteins such as plant proteins, particularly soy, rather than animal sources- can provide additional benefits such as high fiber and low saturated fats (Granato, 2011).

Crops labeled as genetically modified organisms (GMO) make up 93% of soybeans grown in the United States in 2013 (Cornejo 2013). Despite all the benefits of soy, this label is perceived negatively by consumers due to their concern about the safety of GMO foods and ingredients to their health (Organic & Non-GMO, 2007). Recently, a trend to grow non-GMO soybeans has been gaining in popularity amongst growers and a number of soybean companies have been joining the non-GMO Project (Organic & Non-GMO, 2009). The state of Arkansas has been recognized as a potential leader in the production of non-GMO soybean (Roseboro 2012); therefore, there is a need

to study soybean composition and develop new, innovative, science- based products using Arkansas grown non-GMO soybeans to ensure the availability of non-GMO food products. The outcome of this study may lead to commercial interest in utilizing the non-GMO soybeans and encourage breeders/growers to select nutrient-dense, high protein, and high yielding non-GMO soybean lines with potential end uses.

The protein nutritional market is on the rise and provides tremendous potential for new products (Levesque, 2014). The nutritional drink and performance beverage industry now caters to a growing number of health-conscious consumers who are trying to add more protein to their diets through beverages (Gerdes, 2012). The U.S sale for protein drink reached US$ 2.2 billion at the end of 2013, and is expected to reach US$ 4.1 billion in 2018, a 90% growth rate increase (Levesque, 2014). In addition, soy protein was placed as second top trend in functional product launches and proved that countless manufacturers are turning to plant-based nutrition products as well as more consumers are seeking healthier alternative choice (Sepessy, 2013).

Solubility, viscosity, and pH play major roles in ready to drink (RTD) beverages. Formulating high protein beverage can be challenging because isolated soy proteins, in their native state, are insoluble at high concentrations (Hazen, 2010). Many studies have shown that limited hydrolysis of isolated soy proteins using proteolytic enzymes can improve soy proteins' functional properties, including solubility, emulsification, gelation, and foaming (Damodaran & Paraf, 1997). In addition, pH is another important factor for developing a successful high protein beverage, so "control of pH is essential and must be tested in each beverage to maximize solubility" (Hazen, 2010). Taken all together, controlling the limited enzymatic hydrolysis condition, including optimal pH, and the concentration of soy protein hydrolysates are important to enhance a desirable solubility for the high protein drink. Even though the enzyme treatment provides targeted desirable

solubility characteristic to soy protein, it also introduces undesirable attributes to the final product (Cho et al., 2004). Bitterness is the major concern and obstacle when working with soy protein hydrolysates. Recent research has focused on the usage of bitter blocking agents and bitter masking agents to reduce or eliminate bitterness of soy protein hydrolysates (Sun, 2011). Additionally, adding flavor agents seems helpful to reduce the production cost as well as enhance the acceptability of high protein soy beverages.

1.2 Hypothesis

This study attempted to prepare a shelf stable protein drink (500mL) containing 20 gram of soy protein hydrolysate that is targeted for all consumers. The central hypothesis for this study includes:

1. Limited/controlled hydrolysis of soy protein in which hydrolyzed protein would be soluble and suitable for making clear protein drink.
2. The bitter taste would be masked by using the combination of bitter blocker, sweetener, and natural flavors to achieve the consumers' sensorial acceptance.

1.3 Objectives

This study aimed to develop a novel high-protein drink by utilizing an Arkansas grown non-GMO soybean cultivar. Specific objectives are as follows:

1. Conduct compositional analysis, including protein, amino acid composition, sucrose content, isoflavones and anti-nutritional factors such as phytic acid, and trypsin inhibitor, of the four Arkansas grown non-GMO soybean cultivars: Osage, R95-1705, R08-4004 and R05-4969.
2. Prepare protein isolates and optimize enzymatic hydrolysis conditions to obtain optimum solubility and acceptable bitterness of soy protein hydrolysates.

3. Develop a high protein drink formulation using the selected non-GMO soybean cultivar that possesses a premium amino acid profile among the above four cultivars.
4. Conduct sensory evaluation and consumer acceptability of the product as well as investigate the shelf life stability of the protein drink.

Chapter 2: Literature review

2.1 Soybean production

Soybean is the most predominant oilseed and the second major crop grown in the United States (U.S Environmental Protection Agency, 2013; Ash, 2012). In the year 2012, total value of the U.S soybean crop was more than $43 billion and total harvest areas was 77.2 million acres (American Soybean Association, 2013). Most of U.S soybeans are grown in the upper Midwest (Ash, 2012). The top ten soy-producing states are Iowa, Illinois, Minnesota, Indiana, Ohio, Nebraska, Missouri, South Dakota, North Dakota, and Arkansas (U.S Department of Agriculture, 2008). In Arkansas, soybean production has traditionally been one of the largest agricultural enterprises and was valued at 1,956,672 thousand U.S dollar in the year 2012 (University of Arkansas, 2013; Arkansas Soybean Promotion Board). Soybeans are grown in more than 50 of the 75 counties in the state, but most highly concentrated in eastern Arkansas (Arkansas Soybean Promotion Board). Some soybeans are also produced in the Arkansas River Valley in the west and the Red River Valley in the southwest (Arkansas Soybean Promotion Board).

After the soybeans are harvested, the beans are used for several purposes. About 85% of the world's soybeans are processed into soybean oil and meal (American Soybean Association, 2013). Soybean oil is the number one edible oil and represents about 65% of all the edible oil in the U.S (U.S Environmental Protection Agency, 2013). The soybean meal is a major ingredient in livestock feed with over 30 million tons of consumption per year (North Carolina Soybean Producers Association; U.S Environmental Protection Agency, 2013). Only a small percentage of whole soybean production is processed for human consumption or made into soy-based foods such as soymilk, soy flour, soy protein, and functional ingredients for many retail food products (North Carolina Soybean Producers Association). There is wide range of soybean seed varieties that are

grown around the world under many different climatic and conditions (SoyaTech). The first genetically modified (GM) soybeans were planted in the U.S in 1996. Genetically modified soybeans have become predominant in major soy producing countries (SoyaTech). According to 2013, the US Department of Agriculture reported that 93% of the US soybeans planted in the U.S were GM (Cornejo, 2013). Soybeans have been modified to become herbicide resistant or to carry the interested traits for oilseed processors (SoyaTech). However, with the increasing demand toward soy food as well as consumer's sensitive concern about the GM food, soybean industry has witnessed a trend of non-GMO soybean. The recent survey, titled "2010 Analysis of the U.S GMO Food Soybean Variety Pipeline" funded by U.S Soybean Export Council, found that public and private non-GMO food soybean breeding programs have been increasing in size and scope, and been developing a variety of food-grade soybean suitable for wide range of geographic regions (Roseboro, 2010). This showed that U.S soybean producers have a renewed interest in support non-GMO variety development to provide farmer more choice. Indeed, more soybean farmers have planted non-GMO since 2009, and the trend is expected to continue in the near future (Farm & Dairy, 2009). In addition, the traits targeted for development in new non-GMO seed varieties, include disease resistance, higher protein/oil, low phytate, high yield, and general seed improvement (Roseboro, 2010). In brief, significant demand growth for soy foods, especially soy protein, will ensure the new trend for food-grade non-GMO soybeans (SoyaTech).

2.2 Consumer attitude toward GMO and non-GMO food products

Consumer attitudes toward GMO and non-GMO food are dynamic and this issue is heavily debated. However, it is reasonable to emphasize that the number of consumers who are concerned about ingredients made from GMO is growing fast. In addition, food manufacturers have been providing products without GM ingredients in response to the increasing demand for GMO-free

products. For instance, General Mills dropped GMO ingredients in Cheerios cereal from the beginning of 2014 (Horovitz, 2014). In addition, Whole Foods Market publicized that all products in their stores must have clear label about GMOs content by 2018 (Cernivec, 2014b). Packaged Facts reported that the U.S retail sales of non-GMO food and beverages would grow at a compound annual growth rate of 12.9 percent over the next five year and by 2017, non GMO products could account for 30 percent of the total food and beverage market with a total value of $264 billion (Latif, 2013). This report supported that consumer attitudes, currently, are negative toward GM food. Consumers are afraid of the long-term effects of consuming GM foods; and they also believe that GM foods will disadvantage developing countries as well as disturb the ecological balance et cetera (Tenbült et. al, 2005).

2.3 Nutritional and performance beverages

2.3.1 Market trends (high protein, vegetarianism, lactose intolerance)

According to the "Nutritional and Performance Drinks-Us-January 2014" report conducted by Mintel Groups (Chicago, US), the nutritional and performance drink market has reached US$12.3 billion in the end of the year 2013, reflecting 48% growth from 2008 to 2013 (Levesque, 2014) . This strong growth has been driven by the success on the all three segments including sport drinks, nutritional drinks, and protein drinks (Levesque, 2014). During this five-year period, the protein drink segment has experienced an incredible growth (233%) compared to the sport drinks and nutritional drinks, 30% and 38% respectively, despite that protein drink is the smallest one among the three segments (Levesque, 2014). The protein drink segment is also expected to have a highest forecasted growth rate, 90%, through the year 2018 (Levesque, 2014). It is projected to reach US$ 4.1 billion in 2018 due to the continuing consumers' interest in healthy and nutritious eating habits (Levesque, 2014).

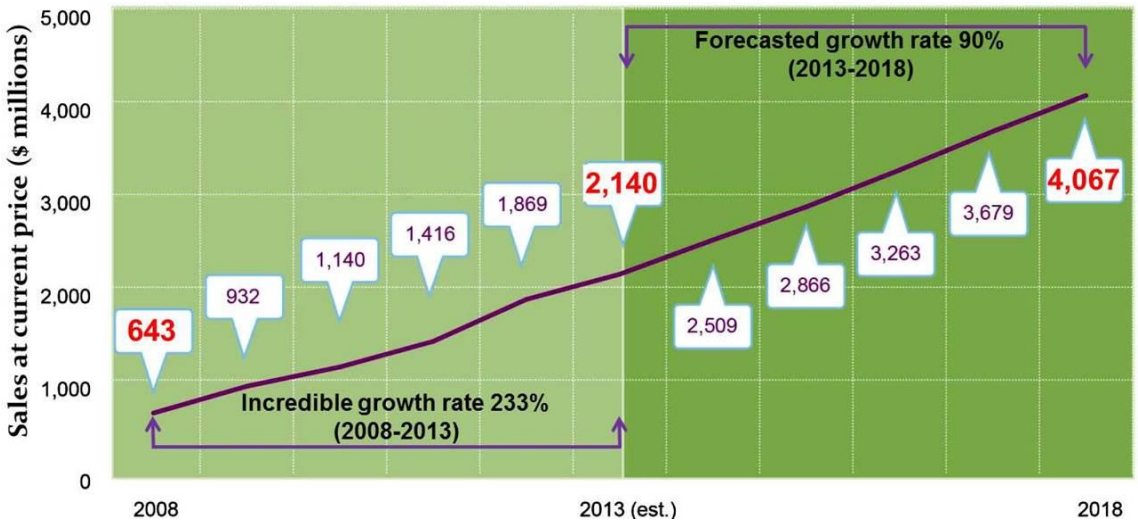


Figure 1: Total US retail sales and forecast of protein drinks from 2008 to 2018 (Adapted from Levesque, 2014)

Protein drinks are defined as a liquid drinks and drink mixes marketed for the health and other benefits of their protein content (Levesque, 2014). Protein was rated fourth of the top ten functional food trends of the year 2012 and have been maintaining its strong mega trend in 2014 (Sloan, 2012; Sloan, 2014). There has been an expansion of protein beverages towards general non-athlete consumers. In fact, moving away from its traditional target of bodybuilders, protein-rich drinks can find a niche with other consumer groups such as Baby Boomers, casual exerciser, or even health-conscious consumers (Lane, 2012; Zegler, 2013). The high demand of high protein beverages is the result of the increasing awareness about the valuable role of protein in building up lean bodies and maintaining ideal body weights (Scott, 2010). It has been proved by the double number of U.S nutritional and performance drink products claiming high protein content from 2009 to 2013 (Levesque, 2014). In addition, the quality of protein, the presence of branched amino acids (BCAAs) including leucine, isoleucine, and valine, and the overall amino acid absorption are the most important factors when the protein drinks are formulated (Berry, 2013). Furthermore, compared to the powder delivery form, the use of protein in ready-to-drink format offers

manufacturers a good way to bring protein-rich products to a mass market appeal due to its convenience, flexibility, and less preparation time benefits (Scott, 2010; Lane, 2012).

Another trend that is happening in food market currently is vegetarianism. Vegetarianism is the belief and practice of eating mainly or entirely of plant-sourced foods and abstaining from animal-sourced foods (American Cancer Society, 2010). In 2012, a nationwide poll conducted by the Vegetarian Resource Group (VRG) found that approximately 9 million (4% U.S adult population) were vegetarian (Stahler, 2012). They also reported that 47% of U.S adults follow a vegetarian-inclined diet (Stahler, 2012). Furthermore, according to the recent market study reported on August 2013 by Mintel, "No animal ingredients" claim climbed up very fast by 200% from 2008 to 2012 (Mintel, 2013). In summation, these researchers have illustrated that the demand for vegetarian products have been on the rise.

According to American Dietetic Association (ADA), consuming of soy proteins, nuts, and plant-sterol have a beneficial cholesterol-lowering effect (American Dietetic Association, 2009). In addition, consumers, these days, are making major shift from dairy products. According to the recent USDA research published in May 2013, Americans are consuming progressively less fluid milk (Stewart, Dong & Carlson, 2013). Decreasing U.S fluid milk consumption is influenced by: changes in the food environment, wider selection of non-dairy beverage choices, consumer's ethics and perspectives (Stewart, Dong & Carlson, 2013). As a result, dairy-free products have expanded and skyrocketed in the U.S food market as alternative for dairy beverages. Driving forces are not only consumers with dairy-related intolerances and allergies but also vegans, ethnic cultures, and shoppers with concerns about hormones and antibiotics (Sund, 2013). Among many non-dairy beverages, only soy beverage is nutritionally similar to dairy. Soybeans potassium content is comparable to the amount of potassium in cow's milk; this nutrient is essential to manage blood

pressure (Sund, 2013). Recently, there has been an increased interest for soy protein to replace milk protein in many food products because of dairy's elevated prices and supply issues in order to reduce the cost while still maintaining consumer acceptance of the end products (Cosgrove, 2005).

2.3.2 The power of protein

Proteins have been historically recognized as one of the most essential macronutrients for the human diet. They are the components of many structures, such as bones, hair, muscles, teeth, and organs and act as regulators of cell functions, transport substances throughout the body, and provide energy if needed (Fink, Burgoon & Mikesky, 2008). Among these functions, protein plays an important role in the skeletal muscle that comprises about 40% of the body mass. The cellular mechanism of muscle mass regulation evidently proves that the combination of exercise and specific nutrition intervention, particularly protein, provide an effective way to build up muscle mass (Wolfe, 2002; Koopman, 2007; Wackerhage & Rennie, 2006; Weinert, 2009).

Since some amino acids cannot be synthesized by the body itself, they must be provided through dieting order to manufacture proteins for the body. After ingestion, dietary proteins are digested firstly into random coils in the stomach by pepsin proteolytic enzyme (Berg et. al, 2007). The degradation continues in the lumen of the intestine by various proteolytic enzymes secreted by the pancreas (Berg et. al, 2007). The result is free amino acids, di-and tripeptides that are transported into the intestinal cell and then released into the blood (Berg et. al, 2007). The amino acids in the blood and extracellular fluids make up the free amino acid pool (Mauro, 2007). From this source, free amino acids are absorbed by other tissues for the continuous synthesis of new proteins replaced for the degraded ones (Mauro, 2007; Wilson, 2013). All twenty amino acids are found in the pool. However, lysine, threonine, and BCAAs (valine, leucine, and isoleucine) are

present in the highest concentrations among the essential amino acids while glutamine, glycine, and alanine make up the highest concentrations among the nonessential amino acid (Mauro, 2007). The largest amount of free amino acid (approximately 80%) is used by the protein synthesis in skeletal muscle (Rennie & Tipton, 2000; Wilson, 2013). Both protein synthesis and protein breakdown in muscle tissues are occurring constantly, so their relative rates, called protein turnover, determine whether the muscle is in anabolic or catabolic state (Lowery & Antonio, 2012; Weinert, 2009). Muscle hypertrophy, which is the increase mass of muscle, only happens when the rate of muscle protein synthesis is higher than the rate of muscle protein breakdown; as the result, the overall net protein balance is positive (Fink, Burgoon & Mikesky, 2008; Burd, et al, 2008; Mauro, 2007). On the other hand, due to the fact that the body does not store protein, the body, without daily intake of protein, will face with net negative protein balance. The result could be no growth has occurred and vital body function has shut down (Cabrone, McClung & Pasiakos, 2012). This scenario is even worse in athletes whose protein breakdown is higher caused by training. Athletes who have chronic low protein diets may face with imperfect sport performance as well as pose a high risk of injury (Fink, Burgoon & Mikesky, 2008; Tipton & Witard, 2007; Kreider, 2010).

2.4 Soybean

2.4.1 Soybean composition including the anti-nutritional factors

The soybean [Glycine max (L.) Merrill] is the broad bean classified under plant legume (Dwevedi & Kayastha, 2011). From its early Southeast Asian origins, soybean cultivation has been expanded and become one of the most important global crops in market place (Reinwald, Akabas & Weaver, 2010). Unlike other legume beans, soy has stood out to consumer's attention as a valuable source of nutrients, phytochemicals, and bioactive compounds that provide health

promoting benefits (Schryver, 2002; Reinwald, Akabas & Weaver, 2010). In fact, the mature soybean contains all three macronutrients (carbohydrate, lipid, and protein), vitamins, minerals required for normal growth and for the building of body tissues (American Soybean Association, 2000). The macronutrients, in average, are found in soybeans as following: 38% protein, 30% carbohydrate, 18% oil, and 14% moisture, ash, and hull (American Soybean Association, 2000). It is worth to mentioning that soybean composition may vary according to varieties, geographical locations, and growing conditions (Berk, 1992). Additionally, in order to improve soybean-desired characteristics' yield, soybean breeders have successfully developed many soybean cultivars whose protein content is about 40% to 45% and lipid content is about 18% to 21% (Berk, 1992; Anderson and Wolf, 1995).

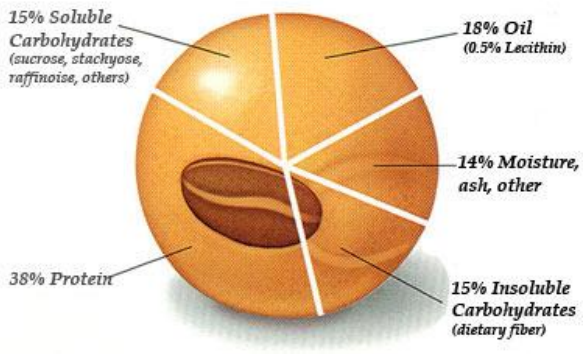


Figure 2: Proximate Composition of a mature soybean.
(Adapted from American Soybean Association)

Soybean oil is low in saturated lipids, rich in essential fatty acids, and a good source of vitamin E (Jooyandeh, 2011). Polyunsaturated fatty acids make up 61% to 63% of its total fat content (United Soybean Board; Reinwald, Akabas & Weaver, 2010). Soybean not only provides a good source of omega-6 fatty acid (50% of total fat content) but also is one of the few plant sources of omega 3 linolenic fatty acid (7-8% of total fat content) (United Soybean Board; Jooyandeh, 2011). With its natural high content of polyunsaturated fatty acid, soybean oil is classified as semi-drying oil and highly possible to undergo oxidative deterioration (Berk, 1992).

The problem is solved by using advance technologies during the oil refining process and soybean oil, presently, is considered as good, multi-purpose, comparative pricing, and versatile edible oil (Berk, 1992).

Thirty percentage carbohydrate found in whole soybean can be divided into two main groups: soluble sugars (10%) and insoluble non-starch polysaccharides (20%) (Berk, 1992; Choct et. al, 2010). Interestingly, less than1% of starch found in soybean makes it unique and different to other legumes (Berk, 1992; Choct et. al, 2010). Soluble sugars consist of 5 % of sucrose, 4% of stachyose, and 1% of raffinose (Berk, 1992, Choct et. al, 2010). Stachyose and raffinose both are α-galactosided oligosaccharides; but stachyose is a tetrasaccharide consisting of galactose-galactose-glucose-fructose while raffinose is a trisacccharide consisting of galactose-glucose-fructose (Berk, 1992). As humans lack the α-galactosidase enzyme to break down these oligosaccharides, they are not hydrolyzed in human gastrointestinal tract. Instead, they are fermented to short-chain fatty acids by microorganism in colon, form intestinal gas production, and lead to uncomfortable flatulence symptom (Berk, 1992; Adnerson, et al, 1999). The insoluble non-starch polysaccharides consist of cellulose (8%) and pectic polysaccharides (Choct, et al, 2010). All the indigestible carbohydrates are characterized as dietary fibers that are able to absorb water and swell considerably (Berk, 1992).

Soybean has higher protein content compared to other legumes, average 38% and 40% respectively (United Soybean Board). In addition, soybean is also considered as the only complete protein plant source that has similar biological value to milk, beef, and egg proteins (Fink, Burgoon & Mikesky, 2008; Bucci & Unlu, 1999). Soy protein is high in lysine and limited in methionine contents (Wolf, 1970). Interestingly, soy protein contains a higher percentage (35%) of the five amino acids, including glutamine, lysine, and the BCAAs (valine, leucine, isoleucine) than other

proteins, such as whey, casein, egg, and beef (Bucci & Unlu, 1999). Different forms of soy proteins, such as soy flour, soy protein concentrate, and soy protein isolate can have different content of amino acid (Berk, 1992). According to their sedimentation properties, four main protein fractions are 2S, 7S, 11S, 15S that cover 22%, 37%, 31%, and 11% of total protein content respectively in soybeans (Barac' et. al, 2004; Ohio State University). The 2S fraction comprises about 8% to 22% of the extractable soybean protein and number of enzymes. The most predominant protein of the 2S has been studied are the trypsin inhibitors, particularly Bowman-Birk inhibitor and Kunitz inhibitor (Ohio State University; Berk, 1992). Trypsin inhibitors are reported as principal antinutrional compounds due to their large quantities in the soybean (El-Shemy et al., 2000). Bowman-Birk inhibitor is composed of 71 amino acid residues and 7 disulfide bonds ("Soybean protein"). The high number of disulfide bonds in the small protein molecular weight (786 Dalton) makes Bowman-Birk inhibitor a very rigid structure as well as a very resistant to denaturation (Ohio State University). This protein can inhibit the activity of both trypsin and chymotrypsin (Ohio State University). The trypsin-inhibiting site is the bond between lysine-16 and serine-17 and the chymotrypsin-inhibiting site is the bond between leucine 43 and serine 44 (Ohio State University). Another main trypsin inhibitor is Kunitz inhibitor (KI) which consists of 181 amino acid and only 2 disulfide bridges (Ohio State University). The two disulfide bonds in a large protein molecular weight (21,5 kDa) make Kunitz inhibitor not as rigid as Bowman-Birk inhibitor (Ohio State University). The inhibiting site is found at the bond of arginine 63 and isoleucine 64 (Ohio State University). These trypsin inhibitors can be inactivated without adverse effect in nutrition using thermal treatment (Barać et. al, 2004). Exceptionally, dry heat treatment has showed no significant reducing effect on trypsin inhibitors even at 121°C (Barać et. al, 2004). Most effective treatments to deactivate trypsin inhibitors are steam jet cooking and autoclaving

methods (Barać et. al, 2004). The 7S fractions are divided into three major groups based on their different physicochemical properties: β-conglycinin, γ-conglycinin, and basic 7S globulin (Damodaran & Paraf, 1997). The principal component of 7S fraction is β-conglycinin that is a sugar containing globulin (glycoprotein), exhibits molecular heterogeneity, and accounts for 30-50% of the total seed proteins (Damodaran & Paraf, 1997). 7S fraction also contains some enzymes, such as β-amylase and lipoxygenase, and hemagglutinins (Berk, 1992). Lipoxygenase forms hydroperoxides in polyunsaturated fatty acids, particularly linoleic and linolenic acid, by the addition of oxygen to their double bonds (Ohio State University). Further lipid oxidation of breaking down of hydroperoxides result in the generation of the undesirable off-flavors such as "beany", "green", and "grassy" aroma of the soy protein during the processing and storage (Chedea & Misaka, 2011). In contrast, lipoxygenase is useful in baking industry in which it effectively bleaches the wheat carotenoids to produce the bread with a whiter crumb (Chedea & Misaka, 2011; Wolf, 1970). Similar to trypsin inhibitors, lipoxygenase can be inactivated by heat treatment, either by high pressure processing (800 MPa) or by direct milling and ultra-high temperature (135ºC in 2 minute) during soymilk production (Geronazzo et. al, 1998; Van der Ven et. al, 2005). Hemagglutinins, in theory, are able to cause the aggregation of red blood cells, but they seem to have no harm on the protein quality of the soybean proteins (Ohio State University). The 11S fraction is composed mainly of 11S globulin which is also called glycinin, main component of soybean protein (Ohio State University; Beck, 1992; Food and Agriculture Organization, 1992). Glycicin consists of six basic and six acidic subunits (Damodaran & Paraf, 1997; Ohio State University). Each subunit has an acid and basic polypeptide linked through disulfide bonds, except for the acid polypeptide -A_4- (Barać et. al, 2004). The final 5S fraction is poorly characterized and is considered as dimer of glycinin (Berk, 1992).

The main storage protein of soybean are 7S (β-conglycinin) and 11S (glycinin) account for about 50-90% of seed protein (Damodaran & Paraf, 1997; Barać et. al, 2004). Both proteins are able to form disulfide linked polymers which contribute to insolubility of soy protein isolates at their isoelectric points (Wolf, 1970). Nevertheless, globulins are soluble in water or dilute salt solutions at pH values above or below the isoelectric region which is about pH 4.2 to 4.6 (Ohio State University; Berk, 1992). Insolubility in the isoelectric pH zone can be solved by hydrolyzing the protein with pepsin to attain a much lower molecular weight than one of original (Wolf, 1970). Heating soy protein above 70ºC denatures its protein structure and assists the protein aggregates through electrostatic, hydrophobic, and disulfide interchange mechanisms (Barać et. al, 2004). Glycinin has higher thermal susceptibility than β-conglycinin when comparing their transition temperature, 92ºC and 72ºC respectively (Barać et. al, 2004). At 100ºC, soy protein approaches minimum solubility, after which solubility increases with continued heating because high molecular weight aggregates are formed during heating with gels appearing at protein concentrations near 8%. At 8-12% concentrations, the gels break down at 125ºC.

Phytic acid has been recognized as an antinutrient factor found in soybean. In the free acid from, phytic acid is unstable but in the dry form such as its salt, phytic acid is relatively stable (Reddy et. al, 1989) In general, phytic acid potentially binds to positively charged proteins and/or multivalent cations or minerals in foods because phytic acid is such a strongly negatively charged molecule over a wide range of pH values (Reddy et. al, 1989). In particularly, the interactions of phytic with soy protein can lead to the decreasing of soy protein solubility at pH values below its isoelectric point. It has been suggested that the solubility of low phytate acid soy protein below the isoelectric pH is improved because the unmasking of positive charged proteins provide additional sites for protein hydration process. 11S protein does not bind to phytic acid significantly

in comparison with 7S protein and whey protein (Reddy et. al, 1989; Barać et. al, 2004). In addition, phytic acid also shows strong ability to chelate metal ions, such as zinc, calcium, and iron (Zhou & Erdman, 1995). At high concentration, their insoluble complex salts cannot be absorbed in the gastrointestinal tract, so the result is the poor bioavailability of minerals in the humans (Barać et. al, 2004). Phytic acid is removable from soy protein isolate either by decreasing the pH of mother solution to 2.4 to 4.0 in the presence of Ca^{2+} or Mg^{2+} ions and ultrafiltration or by increasing the pH to 7.0 to 11.0 in the presence of EDTA (Barać et. al, 2004).

Soybean is also known as a rich source of isoflavones, a group of naturally occurring plant polyphenolic compounds that possess estrogen-like effects (96 paper, Munro et. al, 2003; Messina, 1999). In non-fermented soy products, isoflavones are presented predominantly as β-glycosides which are bounded to a sugar molecule; whereas, in fermented soy products, isoflavone aglycones dominate because the sugar molecule is released (Higdon, 2006; Messina & Messina, 2010). Isoflavones glycosides are also conversed to aglycone forms in the small intestine where they undergo the enzymatic hydrolysis process (Cesar, et al, 2008). A typical chemical structure of isoflavone compound has two benzene rings (A and B) bonded through a heterocyclic pyrane C ring (Messina, 1999). Among various isoflavone compositions found in soy-based, the three primary isoflavone aglycones are genistein (4', 5, 7-trihydroxyisoflavone), daidzein (4', 7-dihydroxyisoflavone), and glycitein (7, 4'-dihydroxy 6-methoxyisoflavone) (Munro et. al, 2003, Messina, 1999). Their glycosides are called genistein, daidzin, and glycitin respectively (Higdon, 2006). Wang and Murphy (1994) showed that the content of isoflavones found in commercial soybean foods are various depended on variety of soybeans, processing conditions as well as the dilution with nonsoy ingredients of typical product (Wang & Murphy, 1994). Particularly, second generation soy foods (soy hotdog, soy bacon, tofu yogurt, soy cheeses, and soy noodles) content

only 6-60% of the total isoflavones found in whole soybeans. Furthermore, traditional non-fermented soy foods (roasted soybeans) and instant soy beverage powder have 2-3 time the isoflavone contents as compared with fermented soy foods (tempeh, bean paste, and miso), 1625 µg/g and 1183 µg/g compared to 625 µg/g, 593 µg/g, and 294 µg/g respectively. In agreement, Mantovaoni et al. (2009) reported the average isoflavone contents extracted of textured soy protein was higher than the one extracted from defatted soy flour, 32.05mg/100g SPI and 13.32 mg/100g SPI respectively.

2.4.2 Health benefits of soy food consumption

According to the Consumer Attitude Survey conducted by United Soybean Board in 2013, 75% consumers rated soy as healthy food source. Health benefits of soy that consumers were aware of included weight management (31%), reducing the risk of heart disease (31%), and reducing the risk of some cancers (22%). However, in deed, more and more researches are continuing to prove that soybean provides more health benefits than previously presumed.

The cardiovascular protective effect of soybean has been confirmed since 1999 when FDA approved the heart health claim which states "25g of soy protein a day as a part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease" ("Code of Federal Regulation- Title 21"). To obtain this claim, the food product should contain at least 6.25g of soy protein and meet the nutrient content required for low fat and low cholesterol foods ("Code of Federal Regulation- Title 21"). The most consistent benefit of soybean on heart health is due to the cholesterol-lowering effects of soy protein that has been proved in several studies. In a meta-analysis of 38 controlled clinical trials conducted by Anderson et al. (1995), the result shown that the consumption of soy protein rather than animal protein significantly decreased serum concentrations of total cholesterol, low-density-lipoprotein (LDL) cholesterol, and triglycerides.

Furthermore, Venter (1999), in his review about the health benefits of soybean and soy products, stated that consume soy beverage instead of milk has been shown to decrease total serum cholesterol by 5% to 10% and (LDL) cholesterol by 10% to 20% within four weeks (Venter, 1999). The effects of soybean on atherosclerosis, the primary cause of cardiovascular disease, are possible related to both soy protein and isoflavones (Zhou, 2004; Cena & Steinberg, 2011). The interaction of soy protein and isoflavones seem to perform a strongest hypo-cholesterolemic effects compared to either soy isoflavones or soy protein isolate alone (Mortensen et. al, 2009; Zhou, 2004). Another proposal mechanism that explains the cholesterol-lowering effect of soy is that soybean is able to reducing cholesterol and bile acid absorption from the gastrointestinal tract and increasing bile acid excretion (Omoni & Aluko, 2005; Venter, 1999). Fukui et al. (2002) observed the plasma total cholesterol concentration of rats of fed isoflavone-rich and isoflavone-depleted isolated soy protein diets were comparable and both significantly lower than of rats fed casein. Interestingly, the fecal steroid excretions of soy protein diet groups were higher than of casein group, although the addition of isoflavone concentrate showed no effect. These results suggested the cholesterol-lowering effect of isolated soy protein could be credited to the protein content while insoflavones and other minor constituents may play as assistant roles (Fukui et. al, 2002). In summary, according the American Heart Association advisory, daily consumption of minimum 25g of soy protein, along with its associated phytochemicals such as isoflavones, can improve the serum lipid profiles by lowering the total and LDL cholesterol and increasing HDL cholesterol; thereby, soybean possibly reduces the risk of coronary heart disease (Erdman, 2000).

The role of soybean intake in weight management has been increased recently. Many studies in animals and humans favor the positive effect of soy protein on obesity and lipid metabolism. High protein intake has been considered to suppress appetite as it induced an increase

of plasma peptide YY (PYY), a satiety related protein (Astrup, 2005). Konig et al (2012) reported that a high soy protein meal replacement was associated with lower glycemia and insulinemia and higher fat oxidation compared with the high glycemic index and low protein content breakfast (Konig et. al, 2012). While diets rich in animal products are usually associated with cholesterol and high risk of cardiovascular disease, soy protein is a healthier alternative choice due to its association of polyunsaturated fatty acid and phytochemicals, namely as isoflavones. Compare to animal-derived protein sources, soy protein offers similarly influence on the magnitude of food intake suppression, energy expenditure, and insulin sensitivity (Velasquez & Bhathena, 2007). Lukaszul et al. (2007) compared the effect of soy milk and skim milk in promoting weight loss. The result exposed that 720 ml soy milk or an equivalent volume of skim milk promoted statistically equivalent losses in weight, body fat, and abdominal circumference while preserving fat-free mass (Lukaszul et. al, 2007). Anderson et al. observed that, during a 12 week weight loss trial, consumption of soy based meal replacements was linked to significant reductions in serum triglycerides at six and twelve weeks while milk based meal replacements was not (Anderson & Hoie, 2005). Furthermore, Deibert et al suggested that high soy protein along with low fat diet could improve the weight loss without losing muscle mass in overweight and obese people (Deibert et. al, 2004). The authors also concluded that soy protein enriched diet limited in carbohydrates and fat was easy to follow.

Several studies have proved that soy protein is able to enhance energy, enduring strength and sport performance. Consumption of 40g of soy protein per day for three to four weeks can enhance exercise recovery that results in speeding up the training progress as well as inhibiting muscle soreness incidences (DiSilvestro et. al, 2006). In a study on young male adults fed either soy protein or whey protein, Rossi et al. found that soy and whey protein performed equally;

however, soy fed subjects showed a rise in plasma total antioxidants status while the whey fed subjects did not have any increase (Rossi et at, 1998). As a result, beside its muscle building effect, soy protein can provide another advantage to reduce the oxidative stress and damage happening in muscle tissues (DiSilvestro et. al, 2006). Taken this benefit, athletes can reduce their recovery time and continue their training sooner. In a double blind study of young badminton player, Husaini et al. found that consumption of soy protein beverage powder over 4-month period could significantly improve the VO_{2max} and iron status (Husaini et al, 1998). VO_{2max} is defined as maximal oxygen uptake that can be used by subject during exercising and it is related to athlete's capacity to perform sustained aerobic endurance.

2.4.3 Soy protein isolate preparation

Soy protein isolate (SPI) is considered as a highly purified form of soy protein products due to its high content of protein on dry basis, approximately 90-95% of protein (Wolf & Cowan, 1971). The high protein concentration of SPI provides maximum formulation flexibility when it is used in food products (Berk, 1992). Beside the advantage of being almost pure protein, isolation of soybean is practically free of objectionable odor, color, flavor, and anti-nutritional factors (Berk, 1992). Before the protein extraction, whole soybeans are milling and removed the naturally occurring soybean oils to get defatted soy flour (Scheide & Brand, 1987). Soy protein extraction falls into two general categories: the aqueous extraction method and the non-aqueous methods (Scheide & Brand, 1987). The non-aqueous methods separate the protein component from the nonprotein component using organic solvents (Scheide & Brand, 1987). However, organic solvents usually cause undesirable effects to protein; for example, they can cause serious denaturation of the protein resulting in denatured protein whose is less palatable and has poor functionalities such as poor heat gel ability, water binding, and heat coagulation compared to

undenatured one (Scheide & Brand, 1987). In contrast, aqueous extraction techniques generally result in palatable undenatured protein with good heat gel ability, water binding, and heat coagulation properties (Scheide & Brand, 1987). The principle of aqueous extraction is taking advantage of the insolubility in water at iso-electric point (pI) of the glycinin proteins found in soy (Scheide & Brand, 1987). At its pI (pH about 4.0 to 4.8), the protein is insoluble and precipitated while a large portion of soy flour remains in aqueous solution; and the protein-rich precipitate can be separated from the supernatant, yielding a high quality protein concentration (Scheide & Brand, 1987). Wolf (1983) described SPI process using defatted soy flour, which had oil extracted using hexane, as starting material. Protein was extracted by solubilizing the defatted meal in water at 60ºC with the meal solvent ratio of 1:10 and pH 8-11. The insoluble fiber was removed from the solution by centrifuging. The solution was, then, adjusted pH to 4.2- 4.5 so that the protein would be precipitated. The protein curd was separated from soluble sugars by centrifuging. Water-wash and centrifugation had been applied several times before the washed-protein was neutralized to pH 6.8 and spray-dried. According to Russin et al. (2007), the particle size of soy flour could affect the yield of SPI. Particularly, the smaller the particle size was, the higher the recovery of protein could be achieved. The pH range between 7.5 and 9.0 is most commonly preferred to extremely high pH during the protein extraction because the excessively high pH can stimulate the protein-carbohydrate interaction that results in loss of protein as well as in formation of dark pigments in solution (Berk, 1992). The temperature used for protein extraction was said not to cause any affect to protein yields (Nazareth, 2009).

2.4.4 Enzymatic soy protein hyrolysates

2.4.4.1 Definition

Hydrolysis is the reaction by which a molecule of water breaks the peptide bond (Kumagai, 2012). Protein hydrolysates are produced from purified protein sources by heating with acid or, preferably, addition of proteolytic enzymes, followed by purification procedures (Bucci & Unlu, 1999). Because the hydrolysis increases the ionizable groups in the protein, the produced peptide has smaller size and becomes more soluble and more amenable to chemical modification (Kumagai, 2012). Enzymatic hydrolysis is greatly preferred because acid hydrolysis oxidizes cysteine and methionine, destroys some serine and threonine, and converts glutamine and asparagine to glutamate and aspartate respectively, lowering protein quality and biological value (Bucci & Unlu, 1999). In addition, proteolytic enzymes hydrolyze proteins more gently than acid, do not require high temperature, and usually target specific peptide bonds (Pasupuleti & Braun, 2010). Each protein hydrolysate is a complex mixture of peptides of different chain length together with free amino acid, which can be determined by a global value known as degree of hydrolysis (DH) (Manninen, 2009). DH is defined as the percentage of peptide bonds cleaved per gram of protein compared with the total number of peptide bonds per gram of protein (Chen et. al, 2011). Generally, protein hydrolysates are digested faster and more even uptake of amino acids when compared with isonitrogenous amounts of whole proteins or free form amino acid mixtures (Bucci & Unlu, 1999). Protein hydrolysates are considered as safe when they are derived from proteins with a history of safe use in human nutrition, such as casein, soy, or whey proteins, and when they are made with food-grade proteolytic enzymes and commonly used food- processing methods ("The benefits of ", 2004).

2.4.4.2 Enzymes used in protein hydrolysis

In food protein hydrolysis, endopeptidases are mostly used; however, sometimes the endopeptidases and exopeptidases have been used together to achieve a higher desired degradation

(Adler-Nissen, 1986). Based on the nature of their catalytic side, proteolytic enzymes are divided into four major classes: serine proteases, cysteine proteases, metalloproteases, and aspartic proteases. A wide variety of proteolytic enzymes are commercially available from animal, plant, and microbial sources (Pasupuleti & Braun, 2010). Enzymes' abilities to release peptide varies and depends on many factors as listed: the choice of enzyme such as the origin of enzymes, enzymes activity, and their typical pH range; protein source such as casein, whey, or soy protein; and the hydrolysis conditions such as temperature, pH, and time. Particularly, the most common choices of enzymes for the production of soy protein hydrolysate are trypsin, alcalase, chymotrypsin, pepsin, substilisin, papain, bromelain, ficin, and neutrase (Barać et. al, 2004; Barrett et al., 2004). Alcalase is a commercial food-grade enzyme derived from select strain of *Bacillus licheniformis* (Nielsen & Olsen, 2002). This enzyme is preferred to other enzymes due to its broad specificity to peptide bond cleavage, especially hydrophobic –COOH, to improve the solubility of soy protein in beverage application (Adler-Nissen, 1986). Alcalase has been reported as one of the highly efficient bacterial protease to prepare vegetable protein hydrolysates (Gao & Zhao, 2012; Yang et al, 2011, Zmudziński & Surówka, 2003). Recently, alcalase is used preferably **(**U.S. Pat. No. 7,332,192 B2 filed by Solae (St. Louis, MO) to produce soy protein isolate hydrolysate that can be used in acidic beverage formulations (Cho, Shen, & Mooshegian, 2008).

Table 1: List of protease common used for soy protein hydrolysates.
(Adapted and modified from Adler-Nissen, 1986)

Source	Type of protease	Common names	pH range	Preferential specificity
Ox, pig	Aspartic	Pepsin	1-4	Aromatic-COOH and -NH_2, Leu-, Asp-, Glu-COOH
	Serine	Trypsin	7-9	Lys-, Arg-COOH
	Serine	Chymotrypsin	8-9	Phe-, Tyr-, Trp-COOH
Papaya fruit	Cysteine	Papain (pure)	5-7	Lys-, Arg-, Phe-X-COOH
Figure latex	Cysteine	Ficin	5-8	Phe-, Tyr-COOH
Pineapple stem	Cysteine	Bromelain	5-8	Lys-, Arg-, Phe-, Tyr-COOH
Bacillus licheniformis	Serine	Alcalase	6-10	Broad specificity, mainly hydrophobic –COOH
Bacillus amyloliquefaciens	Serine	Substilisin	6-10	Broad specificity, mainly hydrophobic –COOH
(*B. subtilis*)	Metalloprotease	Neutrase	6-8	Leu-, Phe-NH_2, and other

2.4.4.3 Food application of soy protein hydrolysates

Food industry classifies protein hydrolysates and their application based on the DH: protein hydrolysates with DH lower than 10% are used to improve the functional properties of foods; those with DH between 20% and 40% are utilized in the manufacturing of sauces, meats, and soups due to their ability to enhance flavor profile; and those with DH higher than 40% are manufactured into supplements and medical diets to treat certain diseases such as phenylketonuria (Darmawan, 2010).

Enzymatic soy protein hydrolysates can enhance soy protein functional properties such as an increase in protein solubility, a decrease in molecular size, or an exposure of hydrophobic groups, factors which influence emulsifying capability and emulsion-stabilizing ability of protein hydrolysates (Chen et. al, 2011). It is worthy to note that DH is the key parameter to determine the tastes and functional properties of protein hydrolysates (Adler-Nissen & Olsen, 1979). Soy protein hydrolysates prepared with alcalase (pH = 8) and with neutrase (pH = 7) increase their iso-electric

solubility, emulsifying capacity, and foaming capacity at low DH values (Adler-Nissen, 1986). They also concluded that the pH solubility curve of soy isolate changed from the usual U shape to a flat curve which was relatively independent of pH (Adler-Nissen, 1986). Such solubility properties seem to be advantageous in slightly acid food systems because the complete solubility retain at pH values at least down to pH, 2.5. This complete solubility is still achieved until above pH, 8 from which some precipitation has been observed to occur. Therefore, it is possible to use isolated soy protein hydrolysates in soluble high nutritional products, particularly high protein sport drink. Even though the enzymatic modification offers several improvements to the functional properties of soybeans, it also delivers undesirable bitter notes to the products. Bitterness has been the major limitation in utilizing protein hydrolysates in various applications, particularly in beverages (Cho et. al, 2004). The intensity of bitterness and DH are positively correlated at low DH values, and the relationship is generally expected to show a maximum at medium DH value (Adler-Nissen & Olsen, 1979). Furthermore, Cho et al., also concluded that the bitterness of fractionated soy protein hydrolysates depended on the DH of their parent proteins, and was found predominately associated with the medium molecular mass range peptides at 1000-4000 Da (Cho et. al, 2004). Pedersen reported that the bitter peptides could be removed using many methods such as adsorption, extraction, masking with other flavors, formation of plastein, or release of hydrophobic amino acid from bitter peptides by exopeptidases (Pedersen, 1994). Emulsifying property of protein is also improved using the proteolytic modification. Hettiarachy & Kalapathy (1997) found that the product of soybean protein isolate hydrolyzed with papain demonstrated foaming property similar to hen egg protein.

Vaughn et al. (2008) suggested that increased consumption of soy protein hydrolystaes might cause body weight loss as it can decrease the rate of body weight gain independently of food

consumption; therefore, soy peptides could play a role on body weight regulation, possibly by increasing energy utilization. Protein hydrolysates are commonly used as the major protein in clinical dietary management of phenylketonuria, food allergy, and chronic liver disease (Clemente, 2000). Protein hydrolysates with low or free of phenyalanine amino acid have been used for treatment of phenylketonuric infants ensuring their physical growth and mental development (Clemente, 2000). For sport nutrition, protein hydrolysates are interesting compounds because they are rapidly digested and absorbed; in this manner, the amino acid are transported to plasma and muscle more rapidly compared to intact proteins ("The benefit of", 2004). For active people, it is important to have protein synthesis started quickly in order to compensate for the muscle tissues damaged during physical activity period; hence, the faster the protein is refilled into the body, the sooner the muscle protein synthesis can be trigger in the body. Furthermore, protein hydrolysates also promote a strong insulinotropic effect, which reduces protein breakdown and enhances muscle and tissue uptake of branched-chain amino acids (Thomson & Buckley, 2011). These effects provide protein hydrolysates a role in enhancing repair of tissue damage caused by surgery, ulcers, burns and muscle-damaging exercise (Thomson & Buckley, 2011). Taken all together, it is reasonable to say that enzymatic hydrolysis can be a choice to enhance the functional and nutritional properties of the soy proteins.

2.4.4.4 Antihypertensive activity of soybean hydrolysate

Hypertension happens when systolic blood pressure is higher than 140 mm Hg and/or diastolic blood pressure is higher than 90 mm Hg (Izzo & Black, 2003) According to Centers for Disease Control and Prevention (CDC), there are about 70 million people have high blood pressure, but only about 52% of these people have it under control (Nwankwo et al., 2013).

Hypertension could increase the risk of heart failure and stroke, the two of leading causes of death in American (Farley et al., 2010; Nwankwo et al., 2013).

Inhibiting angiotensin-I-converting enzyme (ACE) activity, which plays physiologically important role in the blood pressure regulation, is considered as key target of combating hypertension (Hong et al., 2008). The angiotensinogen from the liver is converted by renin-angiotensin system (RAS) into angiotensin-I which in turn is transported to the lungs where ACE changes the inactive form of angiotensin-I into the potent vasoconstrictor octapeptide angiotensin-II and inactivates the vasodilator bradykinin (Hong et al., 2008; Mallikarjun Gouda et al., 2006). Potent synthetic ACE inhibitors such as Captopril, Enalapril, and Lisinopril are widely used for hypertension treatment (Hong et al., 2008; Hernández-Ledesma et al., 2011). As these synthetic compounds cause various side effects such as coughing, taste disturbances, and skin rushes, food derived ACE inhibitor peptides may be an alternative choice for both treatment and prevention of hypertension due to their advantages such as no harmful side effects and lower cost (García et al., 2013; Hong et al., 2008). Enzymatic hydrolysis of food protein is the efficient method to produce potent bioactivity peptides (Nalinanon et al., 2011). Alcalase and pepsin are the most popular enzymes among different enzymes that have been employed to isolate antihypertensive peptides (García et al., 2013). Since the first ACE inhibitor peptide was extracted from snake venom, many other peptides from different food sources including casein, eggs, fish, garlic, buckwheat, mushroom have been isolated though enzymatic hydrolysis (Hong et al., 2008; García et al., 2013). Among these plant sources, soybean protein is the novel and cheap source to produce ACE inhibitory peptides (Campos and other, 2013). Chiang et al. (2006) concluded that hydrolysis of soy protein isolate for 0.5-6 hours with Alcalase produced the highest ACE inhibitory activity in comparison to ones with Flavourzyme, trypsin, chymotrypsin, and pepsin. The pentapeptide (Val-

Leu-Ile-Val-Pro) derived from soybean 11S globulin, or glycinin, treated with Protease P was reported to be a potent competitive inhibitor of ACE and to be resistant to digestion by proteases of the gastrointestinal tract (Mallikarjun Gouda et al., 2006). Some authors explored the antihypertensivity of 7S globulin, or β-conglycinin, and observed that the papain-hydrolyzed β-conglycinin-rich fraction had more than double the ACE inhibitory activity of one of pepsin-hydrolyzed glycinin-rich fraction (Margatan et al., 2013).

Various methods to measure the in vitro ACE inhibition that have been employed include spectrophotometric, fluorometric, radiochemical, high-performance liquid chromatography (HPLC), and capillary electrophoresis methods (Li et al., 2004). Among these methods, the spectrophotometric method described by Cushman and Cheung (1971) is utilized very often for determination ACE activity and inhibition in vitro (García et al., 2013; Li et al., 2004). The principle of this method is the hydrolysis of hippuryl-L-histidyl-L-leucine (HHL) by ACE to produce hippuric acid (HA) and histidyl-leucine (HL). The HA, which is related directly to the ACE activity, is extracted with ethyl acetate, evaporated, re-dissoluted in water, and measured its absorbance at 228nm (García et al., 2013; Li et al., 2004). Beside these steps are tiresome, overestimating ACE activity could happen because the ethyl acetate used to extract HA is able to extract unhydrolyzed HHL which also absorbs at 228nm (García et al., 2013). Li et al. (2004) developed a method without separation of HA from ACE reaction mixture based on the specific colorimetric reaction of HA with benzene sulfonyl chloride (BSC) in the presence of quinolone. This method provided the reliable results in comparison to Cushman and Cheung method with R= 0.9891 and $P<0.01$ when authors determined the ACE inhibitory activities of mung bean and rice protein hydrolysates. The extraction-free spectrophotometric method proposed by Li et al. (2004)

is preferred to screen for ACE inhibitory peptides derived from Arkansas growth non-GMO soybeans due to its direct, sensitive, reproducible, and less expensive.

2.4.5 The use of soy protein isolate and hydrolysate in beverages

Soy protein has been used in many beverages. Soy protein has been included in 9 to 53% of the beverages depending on the region around the world between January 2001 and October 2007 (Lu, 2007). Soy protein contented beverages can be categorized in sport performance, weight management, milk alternative, infant nutrition, medical nutrition, and senior adult nutrition (Paulsen, 2009). Some of these beverages contain multiple protein sources and isolated soy proteins are primarily economical alternative to other high-quality proteins (Paulsen, 2009). In addition, beverages containing soy are positioned to appeal to all health-conscious consumers rather than just to those trying to avoid dairy products (Paulsen, 2009; Post, 2005). However, native soy protein cannot be used effectively as a supplement in beverages, especially acidic beverages, because the unmodified soy proteins are substantially insoluble (Cho et al, 2008). As a result, unmodified soy proteins are precipitated after being stationary for a period of time in the grocery's shelf or in consumer's refrigerator (Cho et al, 2008). Additionally, consumers are asked to shake the product prior to consumption in order to re-suspend the soy protein contained in the beverages (Cho et al, 2008). Despite of well shaken, not all the soy protein in the beverage would be dissolved in the product and still stuck in the bottom or side of the container; therefore, consumers may not feel satisfied receiving the full benefits of the soy protein presented in the beverage (Cho et al, 2008). Soy protein researchers have modified soy protein isolate to overcome the problem above. It is generally known in the art to modify soy protein by hydrolyzing since soy protein hydrolysates are relatively more soluble than unmodified soy protein (Cho et al, 2008). Soy protein hydrolysates have been used in beverages since 1970s (Adler-Nissen, 1986). Soy protein can be delivered

simply by liquid ready-to-drink or by dry blended powder that must be mixed into designed fluid for consumption (Paulsen, 2009). Ready-to-drink beverage application requires soy protein with high solubility, low dusting, and controlled viscosity (Paulsen, 2009). In order to be considered as "soluble", the hydrolysate has to remain in the solution/ suspension under practical application (Adler-Nissen, 1986). Williams (1974) made the milk-type beverages/ bouillon in which contained the soy protein hydrolysates derived from applying *Aspergillus oryzae* protease at pH 2.8 and then neutralization (Adler-Nissen, 1986). In 1971, Sugimoto et al developed a lemon-flavored beverage made with 2% concentration of souble soya isolate hydrolysated by acid protease from the fungus *Trametes sanguinea* (Adler-Nissen, 1986). This drink was said to be quite acceptable at that time. In 2008, Solae patented its soy protein hydrolysate which possessed excellent suspension stability and flavor and could be used in acidic beverage formulations (Cho et al, 2008). In this invention, soy protein isolates were treated with alkaline proteases, preferred Alcalase, from about 30 minutes to about 60 minutes at temperature ranged from 48°C to 53ºC and pH ranged from 9.5 to 10.5 (Cho et al, 2008). This protein hydrolysate had DH ranged from 55 to 62 that were determined by using the Simplified Trinitrobenzene Sulfonic Acid (STNBS) method (Cho et al, 2008). This inventive soy protein hydrolysate was suggested to use from 0.5% to about 10% concentration in ready-to-drink acidic beverages (Cho et al, 2008). Recently, Lee (2011) developed a tea beverage with 5% of soy protein hydrolysate. In this study, the alcalse-hydrolyzed SPI was preferred to papain, bromelain, and trypsin- hydrolyzed ones because it had the highest solubility, thermal stability, and antihypertensive activity. The sensory test also shown that the lemon-flavored iced tea beverages fortified with alcalase-hydrolyzed SPI at 1-7% concentrations were acceptable.

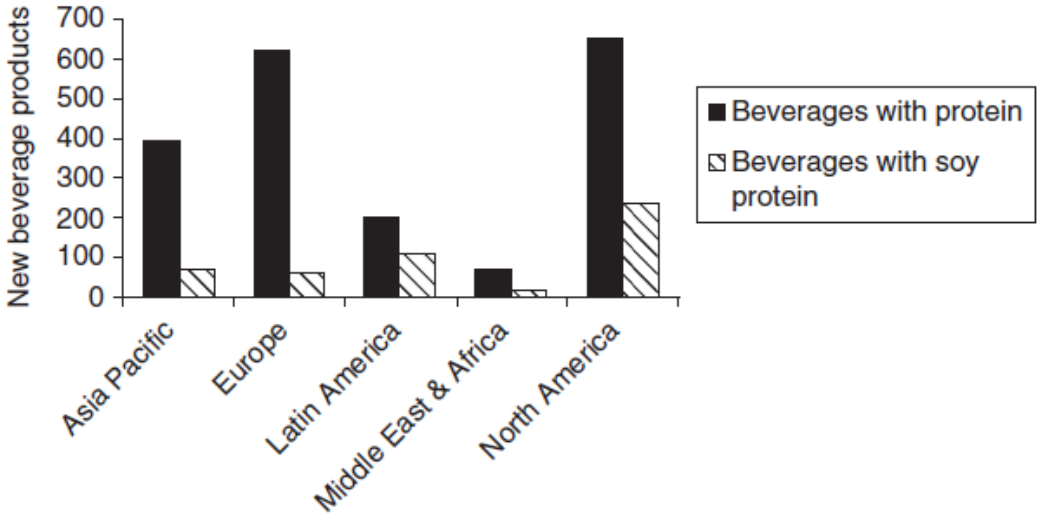


Figure 3: New beverage launches with any "protein" or "soy protein" on the ingredient label for different world regions between January 2001 and October 2007.
(Adapted from Paulsen, 2009)

Table 2: Health and nutrition benefit of soy protein versus whey protein in beverage.
(Adapted from Paulsen, 2009)

Health and nutrition benefits	Soy protein	Whey protein
High quality protein (PDCAAS = 1.0)	✓	✓
Benefits in weigh management	✓	✓
Contain isoflavones, other botanical compounds associated with health benefits	✓	
Reduces cholesterol, can maintain healthy arteries	✓	✓
May reduce high blood pressure	✓	✓
Increase glutathione concentrations-may reduce the risk of canner	✓	
Can increase muscle mass, when combined with exercise	✓	✓
Reduces inflammation in muscle following exercise	✓	

2.5 Sensory evaluation

2.5.1 Sensory aspects of soy protein hydrolysates and their products

Despite that soy foods have drawn more attention for their health benefit, the undesirable beany flavor notes, especially in soy beverage, is the greatest obstacle. The enzymatic extraction hydrolyzes the soy protein to expose some bitter peptides that contribute to unpleasant bitter taste

(Riaz, 2005). Bitterness has been the major limitation in utilizing protein hydrolysates in various applications, especially in beverages (Cho et al, 2004). The flavor of hydrolysed vegetable proteins is generally associated with the presence of free amino acids, small peptides, salt, and other various volatile compounds (Aaslyng et al, 1998; Sun, 2011). The bitterness seems a problem when peptides with low molecular weight whose terminal amino acids are hydrophobic, such as isoleucine, tyrosine, phenylalamine, and tryptophan, are produced (Sun, 2011). Therefore, the bitterness of enzyme hydrolysed soy protein is caused by its hydrophobicity (Belitz et at, 1979; Cho et al, 2004). In native proteins, hydrophobic amino acids do not display bitterness because they are usually buried inside the molecule thus have no chance to contact with taste receptors when ingested (Sun, 2011). In contrast, hydrolysis disrupts protein structure, so it enables hydrophobic amino acid residues to contact with taste receptors (Sun, 2011). Methods for debittering of protein hydrolysates include: selective separation of bitter peptides from hydrolysates; masking bitter taste by addition of various component such as polyphosphates, specific amino acids such as Asp and Glu, α-cyclodextrins; treatment of hydrolysates with exo- and endo-peptidases; and modification of taste signaling to block bitter taste perception (Sun, 2011). Selective separation of bitter peptides causes the loss of some amino acid residues from hydrolysates resulting in the decrease of the nutritious value of the hydrolysates (Sun, 2011). Furthermore, selective separation methods using activated carbon, extraction with alcohol, or hydrophobic interaction chromatography are not applicable for producing food products (Sun, 2011). Enzymatic debittering is another method to reduce bitterness; for example, Nishiwaki et al, 2002, treated bitter peptide solution of soy protein hydrolysates with amino-peptidase derived from *Grifola frondosa* and concluded that hydrophobic amino acids such as valine, leucine, phenylalanine, tyrosine, and isoleucine were released from the bitter solutions by the action of the

amino-peptidase (Sun, 2011). However, the cost for enzymatic debittering in conventional, batch-type hydrolytic systems is high and only can be used once; and the heat treatment for enzymatic deactivation also increases the cost of production (Sun, 2011). Recent research has focused on the usage of bitter blocking agents and bitter masking agents to reduce or eliminate bitterness of soy protein hydrolysates (Sun, 2011). It may be possible to predict the efficiency of these blockers with advance in understanding the molecular mechanism underlying bitter taste perception and how bitter blockers function to suppress bitterness (Sun, 2011). Additionally, adding flavor agents seems helpful to reduce the production cost as well as enhance the acceptability of high protein soy beverages. Potter et al. (2007) reported that the flavor of blueberries could mask the beany flavor in soy beverage. The result showed that only the isolate-juice blend received mean hedonic score "above slightly"; furthermore, there was a strong correlation between the overall acceptability of the beverages with flavor because the consumer acceptability decreased as the soy's sensory attributes, such as green grassy aroma, painty aroma, bitterness, and chalkiness, increased (Potter et. al, 2007). Only a few studies have been reported about the sensory acceptability of soy protein beverages. Therefore, more research needs to be conducted to improve the sensory characteristics of soy protein beverages.

2.5.2 Consumer sensory acceptance tests

Sensory evaluation is defined as a scientific method used to evoke, measure, analyze, and interpret those responses to products as perceived through the senses of sight, smell, touch, taste, and hearing (Lawless & Heymann, 2010). Sensory evaluation has essential role in food industry for product development, recipe modification, quality control, and evaluation of products. The three main categories of sensory tests are difference tests, descriptive analyses, and affective testing (Lawless & Heymann, 2010). The sensory affective tests are used to assess consumer

response to products (Walker, 2004). Affective tests can be qualitative or quantitative, depending on purpose (Walker, 2004). Quantitative methods, including preference and acceptance tests, are utilized to identify the overall preference or liking for a product or sensory attributes of a product (Walker, 2004). Typical question of preference testing is "Which product do you prefer?" whereas those of acceptance testing are "Do you like this product?", "How much do you like it?", or "What do you like about this product?" (Walker, 2004). Hedonic scale, along with paired preference, is the most common used to evaluate consumer preference and acceptance (Lyon et al. 1992). Hedonic scale is the scale with ruler-like properties whose equal intervals indicate the extent of liking from extreme dislike to extreme like (Lawless & Heymann, 2010). The 9-point hedonic scale is the popular scale (Peryam and Pilgrim 1957). Typically, a hedonic test should have a sample of 50-150 untrained panelists who are randomly selected and represent the target consumer population (Lawless & Heymann, 2010). Because of the high variability of individual preferences, the large panel size is required to insure statistical power and test sensitivity; furthermore, it also provides a chance to investigate the reason for liking or disliking a product (Lawless & Heymann, 2010).

2.6 Beverages' shelf-life stability

The shelf life of food product is defined as the time during which the product remains safe, retains certain desired sensory, chemical, physical, and biological characteristics, as well as complies with any label declaration of nutritional data (Yong & O'Sullivan, 2011).There are many factors that influence the shelf life of product such as temperature changes, exposure to light, mechanical stress transmission of gases, humidity changes, and contamination with microorganisms and spores (Yong & O'Sullivan, 2011). These factors should be controlled to minimize their effects, so the shelf life of product will be increased (Brown, 2011).

In order to prevent/minimize the microbial growth, beverages usually undergo microbiological tests and heat treatment process. In order to determine the efficacy of thermal processes total plate count, yeast count, and mold count are performed (Krebbers et al., 2003). Thermal process provides food a sufficient heat treatment to destroy pathogenic and/or spoilage causing microorganisms, anti-nutrients such as antitrypsin and lectins, and enzyme systems that cause degradation in the food (David & Graves, 1996). Desired temperature is held for a specific length of time in order to either pasteurize or aseptically process (sterilize) the product (Micro Thermics, n.d). FDA set the minimum log reduction of bacteria for beverage/ liquid food processing; however, manufacturers usually exceeded the "5 log reduction microorganism" requirements to attain lower failure rates, longer shelf life, and product quality and image (Micro Thermics, n.d). Preservative-free beverages require harsher thermal processing than chemically preserved products to guarantee microbiological stability (Juvonen et. al, 2011). Protein drinks are preferred to be shelf-stable; therefore, they should be sterilized and packaged in sterile container. Aseptic products fall into 2 categories: high acid products (pH < 4.6) that are typically processed between 102-113°C and low acid products (pH >4.6) that are typically processed above 138ºC (Micro Thermics, n.d). High temperature/short time processing or ultra-high-temperature processing is suggested for ready-to-drink, high pH (pH>4.5) protein beverages (Eckert & Riker, 2007).

Table 3: Examples of typical thermal processes in the beverage industry.
(Adopted from Juvonen et al. (2011))

Thermal processing	Temp.	Time	Inactivation of microbes
Flash pasteurization (pH < 4.6)	75-85°C 90-96°C	1-4 minute 30-90 second	Heat-resistant moulds, alicyclobacilli may survive.
Hot filling, pH < 4.6	83-88°C 92-95°C	0.5-1.5 minute 10-15 second	Heat-resistant moulds, alicyclobacilli may survive.
Tunnel pasteurization (pH < 4.6)	72-80°C	5-20 minute	Heat-resistant moulds, alicyclobacilli may survive.
High temperature short time (HTST) treatment (pH < 4.6)	105-115°C	0.5-4.2 minute	Sterile
Ultra high temperature (UHT) treatment (pH > 4.6)	130-150°C	1-9 second	Sterile, ambient storage in hermetic package possible

The role of packaging in extending the shelf-life of beverages are to ensure delivery of food to consumers in the best condition as well as to protect the products from outside environmental effects from the moment they are processed through storage and retailing to ultimate consumers (Robertson, 2011). In addition, a product, especially a beverage, will not sell, no matter how innovative and on-trend it is, if its packaging is not well-designed or have noticeable shelf presence (Canning, 2009). According to Steven Fay, executive vice president at Roscoe Berner Food & Beverage, beverage packaging has represented a volatile issue with sustainability atop the mind of modern consumers (Canning, 2009). Despite that plastic packaging is attractive due to its form and shape versatilities, consumers have been concerned about the safety of plastic because of possibility of undesirable chemicals leaching into beverage itself (Canning, 2009). Therefore, glass and aluminum are better choices for beverages because they are more recyclable than plastic containers (Canning, 2009). Glass containers with an appropriate closure provide almost perfect protection to beverages without the agreed shelf life (Ashurst, 2011). In addition, glass containers are safely suitable for in-bottle pasteurization and hot-filled thermal processes which are widely applied to beverage products (Girling, 1999). The advantages of glass containers

are its quality image, brand image differentiation, pamper evidence, recyclability and re-use possibilities (Ashurst, 2011). The effects on shelf-life of beverages packed in glass are effectively confined to the physical-chemical changes that occur as a result of accelerated storage time, the transmission of light, storage temperature, and even the presence of dissolved oxygen (Ashurst, 2011).

After the heat treatment and packaging have been done, conducting sensory and storage period is required for a new product development in order to validate it market life (Brown, 2011). It can be assess in many ways including chemical, physical, and sensory analysis after processing and during realistic storage by challenge testing, and consumer feedback on product quality (Brown, 2011). Instrumental techniques can be used to analyze any changes or appearance of trace amount of chemical components (Brown, 2011). Storage condition for the shelf-life test should be the condition in which the product is experienced the most in the market and under which shelf life date will be applied to the bulk of future production (Man, 2011). For cost reasons, beverages' shelf life test trial tend to employ fixed conditions in the absence of universal standards such as chilled storage (0-5ºC, high relative humidity 90%+) or temperate storage (25°C, 75% relative humidity) (Man, 2011). During its storage period test, a product is expected to have minimum or no deteriorating effect on its quality parameters such as color, pH, and cloudiness (turbidity. Turbidity is used to describe the presence of cloudiness or haze in the solution caused by the suspended solids that are generally invisible to naked eyes (Sharma & Kalonia, 2010; Goodner, 2009). Relative increases in the turbidity during the storage period causes by the formation of protein aggregates and results in the undesirable turbidity and sedimentation (LaClair & Etzel, 2009). Turbidity of protein solutions can be measured using UV-Visible spectrophotometer (Sharma & Kalonia, 2010). Wavelengths ranged from 360-800nm are used because the protein

does not absorb at these wavelengths (Sharma & Kalonia, 2010). According to Lee (2011), turbidity of SPI and hydrolysates significantly increased with heat treatment (95ºC; 60 minutes) versus without heat treatment ones. Additionally, this author also concluded that alcalase-hydrolysated SPI, compared to bromelain, trypsin, papain-hydrolyzed SPIs, has the lowest turbidity and would be a great ingredient to incorporate into protein fortified beverages in order to make relatively translucent beverages (Lee, 2011). Furthermore, the color of product may have a huge influence on the decision of consumer purchase because color is used as indicator for flavor and freshness of beverages. According to Francis (1995), a product's color affects other sensory characteristics that result in consumers' acceptability, choice, and preference toward that product. Product discoloration is one of the most commonly observed defects in beverages (Ashurst, 2011). The pH is a common parameter used during the shelf life determination. Just like any other beverages, the protein-rich drink can be unstable during storages; therefore, shelf life determination is required.

References

Aaslyng, M.D., Elmore, J.S. & Mottram, D.S. (1998). Comparison of the aroma characteristics of acid-hydrolyzed and enzyme-hydrolyzed vegetable proteins produced from soy. *Journal of Agricultural and Food Chemistry*, 46: 5225–5231.

Adler-Nissen, J. (1986). *Enzymatic hydrolysis of food proteins*. Elsevier Applied Science Publisher, New York.

Adler-Nissen, J. & Olsen, H.S. (1979). Chapter 7: *The influence of peptide chain length on taste and functional properties of enzymatically modified soy protein*. Book: Functionality and protein structure. ACS Symposium Series. 92:125-146.

American Cancer Society. (2010). *Vegetarianism*. Retrieved from http://www.cancer.org/treatment/treatmentsandsideeffects/complementaryandalternative medicine/dietandnutrition/vegetarianism.

American Dietetic Association. (2009). Position of the American Dietetic Association: Vegetarian diets. *Journal of American Dietetic Association*, *109*(7): 1266-1282.

American Soybean Association. (2013). 2012 highlights. Retrieved from http://soystats.com/2012-soybean-highlights/.

American Soybean Association. (2000). Composition of Soy. Retrieved from http://www.wishh.org/aboutsoy/composition.html.

Anderson, J.W., & Hoie, L.H. (2005). *Weight Loss and Lipid Changes with Low-Energy Diets: Comparator Study of Milk-Based versus Soy-Based Liquid Meal Replacement Interventions*. 24. Taylor & Francis Group.

Anderson, R.L. & Wolf, W.J. (1995). Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *The Journal of Nutrition*, 125(35): 581S-588S.

Anderson, J.W., Johnstone, B.M., & Cook-Newell, M.E. (1995). Meta-analysis of the effects of the soy protein intake on serum lipids. *New England Journal of Medicine*, 333 (5): 276-282.

Anderson, J.W., Smith, B.M., & Washnock, C.S. (1999). Cardiovascular and renal benefits of dry bean and soybean intake. *American Journal of Clinical Nutrition*, 70 (3): 464s- 474s.

Arai, S., Noguchi, M., Kurosawa, S., Kato, H., & Fujimaki, M. (1970). Applying proteolytic enzymes on soybean. 6. Deoderization effect of aspergillopeptidase A and debittering effect of aspergillus acid carboxypeptidase. *Journal of Food Science*, 35(4): 392-395.

Arkansas Soybean Promotion Board. (n.d.). Soybean statistics. Retrieved from website: http://www.themiraclebean.com/soybean-statistics.

Ash, M. (2012). Soybeans & oil crops. U.S Department of Agriculture, Economic Research Service. Retrieved from website: http://www.ers.usda.gov/topics/crops/soybeans-oil-crops.aspx.

Ashurst, P. (2011). The stability and shelf life of fruit juices and soft drinks. In Kilcast, D., & Subramaniam, P. (2nd Ed). *Food and Beverage Stability and Shelf Life*. Cambridge: Woodhead Publishing.

Astrup, A. (2005). The satiating power of protein- a key to obesity prevention? American Journal of Clinical Nutrition, 82: 1-2.

Barać, M.B, Stanojević, S.P., Jovanović, S.T. & Pešić, M.B. (2004). Soy protein modification- A review. *Acta Periodica Technologica (APTEFF)*, 35:3-16. doi:10.2298/APT0435003B.

Berk, Z. (1992). Technology of production of edible flours and protein products from soybeans. Vol. 97. FAO, Viale delle Terme di Caracalla. Rome, Italy.

Barrett, A.J, Rawlings, N.D, & Woessner, J.F. (2004). Handbook of proteolytic enzymes. 2nd ed. London: Elsevier.

Belitz, H. D., Chen, W., Jugel, H., Treleano, R., Wieser, H., Gasteiger, J., & Marsili, M. (1979). Sweet and bitter compounds: Structure and taste relationship [Food flavor]. In *ACS Symposium series American Chemical Society, 115*: 93-131. doi:10.1021/bk-1979-0115.ch004.

Bernstien, A., Treyzon, L., & Li, Z. (2007). Are high protein, vegetable based diets safe for kidney function? *Journal of American Dietetic Association*, 107: 644-650.

Berry, D. (2011). A sweet dilemma: Sweeteners for healthful foods and beverages. *Food Product Design*, 25-33.

Berry, D. (2013). Sports drink evolution. Retrieved from http://www.foodbusinessnews.net/articles/news_home/Consumer_Trends/2013/08/Sports_drink_evolution.aspx?ID={6AA703FC-F917-444F-9581-C48A48C2E3DD}&cck=1.

Brown, M. (2011). Processing and food and beverage shelf life. In Kilcast, D., & Subramaniam, P. (2nd Ed). *Food and Beverage Stability and Shelf Life*. Cambridge: Woodhead Publishing.

Bucci, L. R., & Unlu, L. (1999). *Energy-yielding macronutrients and energy metabolism in sport nutrition*. *Chapter 10: Protein and amino acid supplements in exercise and sport* (1st ed). CRC Press, Boca Raton, FL, 191-210.

Burd, N. A., Tang, J. E., Moore, D. R., & Phillips, S. M. (2008). Exercise training and protein metabolism:Influences of contraction, protein intake, and sex-based differences. *Journal of Applied Physiology, 106(5)*: 1692-1701.

Cabrone, J., McClung, J., & Pasiakos, S. (2012). Skeletal Muscle Responses to Negative Energy Balance: Effects of Dietary Protein. *Advances Nutrition*, *3*.

Campbell, B., Kreider, R.B., Ziegenfuss, T., La Bounty, P., Roberts, M., Burke, D., Landis, J., Lopez, H., & Antonio, J. (2007). International Society of Sports Nutrition Position Stand: Protein and Exercise. *Journal of the International Society of Sports Nutrition*, 4(1):4-8.

Campos, M.R.S., González, F.P., Guerrero, L.C., & Ancona, D.B. (2013). Angiotensin I-converting enzyme inhibitory peptides of chia (Salvia hispanica) prodiced by enzymatic hydrolysis. *International Journal of Food Science*, article ID 158482, 8 pages.

Canning, K. (2009). Alternative universe. *Private Label Buyer*, *23*(11), 42-46.

Cena, E.R. & Steinberg, F.M. (2011). Soy may help protect against cardiovascular disease. *California Agriculture*, 65(3): 118-132.

Cernivec, S. (2013). Stevia sweetens the deal. *Beverage Industry*, 104(3): 48-53.

Cernivec, S. (2014). Taking beverages back to basics. *Beverage Industry*, 105(2): 20-24.

César, I.C., Braga, F.C., Vianne-Soares, C.D., Nunan, E.A., Pianetti, A., & Moreira-Campos, L.M. (2008). Quantitation of genistein and genistin in soy dry extracts by UV-Visible spectrophotometric method. *Química Nova*, *31*(8):1933-1936.

CFR - Code of Federal Regulations Title 21. (2013). Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.82.

Chedea, V.S., & Jisaka, M. (2011). Inhibition of soybean lipoxygenases – Structural and activity models for the lipoxygenase isoenzymes family. In *Recent Trends for Enhancing the Diversity and Quality of Soybean Products*, Prof. Dora Krezhova (Ed.), ISBN: 978-953-307-533-4, InTech, doi: 10.5772/17976.

Chen, L, J. Chen, J. Ren & M. Zhao. (2011). Effects of ultrasound pretreatment on the enzymatic hydrolysis of soy protein isolates and on the emulsifying properties of hydrolysates. *Journal of Agriculture and Food Chemistry*, 59(6): 2600-2609.

Chiang, W.D., Tsou, M.J., Tsai, Z.Y., & Tsai, T.C. (2006). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry*, 98(4): 725-732.

Cho, M.J., Shen, R., & Mooshegian, R. (2008). Soy protein isolate. *U.S. Patent No. 7,332,192 B2*. Washington, DC: U.S. Patent and Trademark Office.

Cho, M.J., Unklesbay, N., Hsieh, F.H., & Clarke, A.D. (2004). Hydrophobicity of bitter peptides from soy protein hydrolysates. *Journal of Agriculture and Food Chemistry*, *52(19)*: 5895-5901.

Choct, M., Dersjant-Li, Y., McLeish, J., & Peisker, M. (2010). Soy Oligosaccharides and Soluble Non-starch Polysaccharides: A Review of Digestion, Nutritive and Anti-nutritive Effects in Pigs and Poultry. *Asian-Australasian Journal of Animal Sciences, 23*(10): 1386-1398.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Journal of Food Science & Technology,* 11: 254-262.

Cornejo, J.F. (2013). *Adoption of genetically engineered crops in the U.S.* USDA, Economic Research Service. Retrieved from website: http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx.

Cosgrove, J. (2005). Protein-fortified beverages. *Beverage Industry*, 96(7): 55-57.

Cushman, D.W., & Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Journal of Food Science*, 46: 1035-1042.

Damodaran, S., & Paraf, A. (1997). *Food proteins and their applications*. New York: Marcel Dekker.

Darmawan, R. (2010). *Antioxidant and antihyperglycemin potential of soybean hydrolysates*. Master's thesis, University of Illinois at Urbana-Champaign.

David, J.R.D., & Graves, R.H. (1996). Aseptic Processing and Packaging of Food and Beverages (1st ed). Boca Raton, FL: CRC Press.

Deiber, P., König, D., Schmidt-Trucksaess, A., Zaenker, K.S., Frey, I., Landmann, U. & Berg, A. (2004). Weight loss without losing muscle mass in pre-obese and obese subjects induced by a high- soy- protein diet. *International Journal of Obesity*, 28: 1349-1352.

DiSilvestro, R.A., Mattern, C., Wood, N., & Devor, S.T. (2006). Soy protein intake by active young adult men raises plasma antioxidant capacity without altering plasma testosterone. *Nutritrion Research*, 26(2): 92-95.

Dwevedi, A., & Kayastha, A. (2011). Soybean: A Multifaceted Legume with Enormous Economic Capabilities (Chapter 11). *Soybean - Biochemistry, Chemistry and Physiology*. ISBN 978-953-307-219-7.

Eckert, M., & Riker, P. (2007). Overcoming challenges in functional beverages. *Food Technology*. 61(3). Retrieved from http://www.ift.org/food-technology/past-issues/2007/march/features/overcoming-challenges-in-functional-beverages.aspx?page=viewall.

El-Shemy, H., Abdel-Rahim, E., Shaban, O., Ragab, A., Carnovale, E., & Fujita, K. (2000). Comparision of nutritional and antinutritional factors in soybean and faba bean seeds with and without cortez. *Soil Science and Plant Nutrition*. 46(2), 515-524.

Erdman, J.W. (2000). Soy protein and cardiovascular disease: A statement for healthcare professionals form the nutrition committee of the AHA. *Circulation*, 102(20): 2555-2559.

Farley, T.A., Dalal, M.A., Mostashari, F., & Frieden, T.R. (2010). Deaths preventable in the US by improvements in the use of clinical preventive services. *American Journal Preventive Medicine*, 38(6): 600-609.

Farm & Dairy. (2009). More crop producers to plant non-genetically modified soybeans. Retrieved from https://www.farmanddairy.com/news/more-crop-producers-to-plant-non-genetically-modified-soybeans/11642.html.

Feder, D. (2012). How Sweet It Is. *Prepared Foods*, 181(8), 61-73.

Fink, H.H., Burgoon, L. & Mikesky, A.E. (2008). *Practical applications in sports nutrition*. Sudbury, MA, USA. Jones & Bartlett Learning, print.

Food and Agricultural Organization-World Health Organization. (1991). Protein quality evaluation:Report of the joint FAO/WHO expert consultation. Rome, Italy. FAO Food and Nutrition (51).

Friedman, M., & Brandon, D.L. (2001). Nutritional and health benefits of soy proteins. *Journal of Agricultural and Food Chemistry*, *43*(3): 1069-1086. Retrieved from http://pubs.acs.org/doi/abs/10.1021/jf0009246.

Fukui, K., Tachibana, N., Wanezaki, S., Tsuzaki, S., Takamatsu, K., Yamamoto, T., Hashimoto, Y. & Shimoda, T. (2002). Isoflavone-free soy protein prepared by column chromatography reduces plasma cholesterol in rats. *Journal of Agricultural and Food Chemistry*, 50(20): 5717-5721.

Jooyandeh, H. (2011). Soy Products as Healthy and Functional Foods. *Middle-East Journal of Scientific Research, 7*(1): 71-80.

Francis, F. (1995). Quality as influenced by color. *Food Quality & Preference*. 6(3); 149-155.

García, M.C., Puchalska, P., Esteve, C., & Marina, M.L. (2013). Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrences bioactivities. *Talanta*, 106: 328-349.

Gerdes, S. (2012). Consumers have a thirst for protein beverages. *Dairy Foods*, 113(8), 22.

Geronazzo, H., Macoritto, A., Mercado, A., Toro, M.A., & Cuevas, C.M. (1998). Lipoxygenase and trypsin inhibitor inactivation in soymilk processing by direct milling and ultra high temperature (DM-UHT). *Archivos latinoamericanos de nutricion*, 48(1):52-57.

Goel-lal, G. (2012). Attitudes toward beverage sweeteners- US- March 2012. *Mintel*. Retrieved from http://0-academic.mintel.com.library.uark.edu/display/590264.

Goodner, K.L (2009). Estimating turbidity (NTU) from absorption data. *Sensus technical note*: SEN-TN-0010. Retrieved from http://www.sensusflavors.com/t-r-pdf/SEN-TN-0010-Turbidity,_Tea,_And_UV-VIS.pdf.

Girling, P.J. (1999). Packaging of beverages in glass bottles. In Giles, G. A. (1999). Handbook of Beverage Packaging. Sheffield, England: Blackwell Publishing.

Granato, H. (2011). Exploring opportunities in proteins. Retrieved from http://www.naturalproductsinsider.com/articles/2011/07/exploring-opportunities-in-proteins.aspx.

König, D., Muser, K., Berg, A., & Deibert, P. (2012). Fuel selection andappetite-regulating hormones after intake of a soy protein-based meal replacement. *Nutrition*. 28(1): 35-39.

Krebbers, B., Matser, A.M., Hoogerwerf, S.W., Moezelaar, R., Tomassen, M.M., & Van den Berg, R.W. (2003). Combined high-pressure and thermal treatments for processing of tomato puree: evaluation of microbial inactivation and quality parameters. *Innovative Food Science & Emerging Technologies*, *4*(4): 377-385.

Hazen, C. (2010). *Mixing up high protein beverages*. *Food product design*. Retrieved from http://www.foodproductdesign.com/articles/2010/04/mixing-up-high-protein-beverages.aspx?pg=7#content.

Hernández-Ledesma, B., Del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science*, 165(1), 23-35.

Higdon, J. (2006). Soy Isoflavones. *Linus Pauling Institute*. Retrieved from http://lpi.oregonstate.edu/infocenter/phytochemicals/soyiso/.

Hong, F., Ming, L., Yi, S., Zhanxia, L., Yongquan, W., & Chi, L. (2008). The antihypertensive effect of peptides: A novel alternative to drugs? *Peptides*, 29: 1062-1071.

Horovitz, B. (2014). Cheerios drops genetically modified ingredients. USA Today. Retrieved from http://www.usatoday.com/story/money/business/2014/01/02/cheerios-gmos-cereals/4295739/.

Hughes, G. J., Ryan, D. J., Mukherjea, R., & Schasteen, C. S. (2011). Protein digestability-corrected amino acid scores (PDCAAS) for soy protein isolates and concentrate: criteria for evaluation. *Journal of Agricultural and Food Chemistry*. 59(23): 12707-12712.

Husaini, M.A, Moeloek, D. Utamin, S.S, & et al. (1998). Supplementation of "Supro" beverage powder on body size, iron status, and physical performance of growing badminton athletes. Final report. *Nutrition Research and Development Centre*, Bogor, Indonesia.

Juvonen, R., Virkajärvi, V., Priha, O. & Laitila, A. (2011). *Microbiological spoilage and safety risks in non-beer beverages*. VTT Technical Research Centre of Finland. Retrieved from http://www.vtt.fi/publications/index.jsp.

Koopman, R., Saris, W.H., Wagenmakers, A.J., & van Loon, L.J. (2007). Nutritional interventions to promote post-exercise muscle protein synthesis. *Sports Medicine*, 37(10): 895-906.

Kreider, R.B., Wilborn, C.D., Taylor, L., Campbell, B., Almada, A.L., Collins, R., ... & Antonio, J. (2010). ISSN exercise & sport nutrition review: Research & recommendations. *Journal of the International Society of Sports Nutrition*, *7*(7): 1-43.

Kumagai, H. (2012). Chemical and enzymatic protein modifications and functionality enhancement. In book: Food proteins and peptides: chemistry, functionality, interactions, and commercialization (1st ed). CRC Press, Boca Raton, FL, 165-198.

LaClair, C.E. & Etzel, M.R (2009). Turbidity and protein aggregation in whey protein beverages. *Journal of Food Science*, 74(7): C526-C535.

Lane, J. (2012). *The Next Chapter in Sports Nutrition*. Retrieved from http://www.nutraceuticalsworld.com/issues/2012-05/view_features/the-next-chapter-in-sports-nutrition/.

Latif, R. (2013). Packaged facts: Non- GMO products could reach $264 billion in U.S. sales by 2017. *Bevnet-The Beverage Industry's Source*. Retrieved from http://www.bevnet.com/news/2013/packaged-facts-non-gmo-products-could-reach-264-billion-in-u-s-sales-by-2017.

Lawless, H.T, & Heymann, H. (2010). Sensory evaluation of food: principles and practices. 2nd ed. New York: Springer.

Lee, J. (2011). *Soy protein hydrolysate: solubility, thermal stability, bioactivity, and sensory acceptability in a tea beverage*. University of Minnesota. Retrieved from http://purl.umn.edu/117047.

Lemus-Mondaca, R., Vega-Gálvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, 132(3): 1121-1132.

Levesque, S.D. (2014). *Performance food and drink – U.S-January 2014*. Retrieved from http://academic.mintel.com/display/679959/.

Li, G.H., Liu, H., Shi, Y.H., & Le, G.W. (2005). Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. *Journal of Pharmaceutical and Biomedical Analysis*, 37(2): 219-224.

Lowery, L.M., & Antonio, J. (2012). *Dietary Protein and Resistance Exercise*. (1 ed.). Boca Raton, Florida: Taylor & Francis Group.

Lukaszuk, J.M., Luebbers, P., & Gordon, B.A. (2007). Preliminary study: soy milk as effective as skim milk in promoting weight loss. *Journal of American Dietetic Association*, 107(10): 1811-1814.

Lyon, D.H, Francombe, M.A, Hasdell, T.A, & Lawson, K. (1992). Guidelines for sensory analysis in food product development and quality control. New York: Chapman & Hall, Van Nostrand Reinhold, Inc.

Lu, G. (2007). Internal memo, Solae LLC, St. Louis, MO

Maheshwari, P., Ooi, E, & Nokolov, Z. (1995). Off-flavor removal from soy-protein isolate by using liquid and supercritical carbon dioxide. *Journal of the American Oil Chemists' Society*, 72(10):1107-1115.

Mallikarjun Gouda, K.G., Gowda, L.R., Rao, A.G., Rao, A.G., & Prakash, V. (2006). Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max). *Journal of Agriculture and Food Chemistry*, 54(13): 4568–4573.

Man, C.M.D (2011). Food storage trials: an introduction. In Kilcast, D., & Subramaniam, P. (2nd Ed). *Food and Beverage Stability and Shelf Life*. Cambridge: Woodhead Publishing.

Manninen, A.H. (2009). Protein hydrolysates in sport nutrition-Review. *Nutrition & Metabolism*, 6: 38-42.doi: 10.1186/1743-7075-6-38.

Mantovani, D., Filho, L.C., Santos, L.C., de-Souza, V.L.F., & Watanabe, C.S. (2009). Chromatographic Quantification of Isoflavone Content from Soy Derivates Using HPLC Technique. *Journal of Chromatographic Science*, 47(9): 766-769.

Margatan, W., Ruud, K., Wang, Q., Markowski, T., & Ismail, B. (2013). Angiotensin converting enzyme inhibitory activity of soy protein subjected to selective hydrolysis and thermal processing. *Journal of Agricultural and Food Chemistry*, 61(14): 3460-3467.

Matoba, T., Hidaka, H., Narita, H., Kitamura, K., Kaizuma, N., & Kito, M. (1985). Lipoxygenase-2 isozyme is responsible for generation of n-hexanal in soybean homogenate. *Journal of Agricultural and Food Chemistry*, 33(5):852- 855.

Mauro, G.D.P. (2007). *Nutrition in Exercise and Sport*. (2nd ed.). Boca Raton, Florida: Taylor & Francis Group.

Messina, M.J. (1999). Legumes and soybeans: overview of their nutritional profiles and health effects. *The American Journal of Clinical Nutrition*, 70(3): 439s-500s.

Messina, M., & Messina, V. (2010). The role of soy in vegetarian diets. *Nutrients*, 2(8): 855-888.

Micro Thermics. (n.d). Development basics for pasteurized (HTST), aseptic and similar products: Integrating commercial processes into product development. Retrieved from http://storage.globalcitizen.net/data/topic/knowledge/uploads/20120220142732533.pdf.

Mintel. (2013). More than one-third of Americans consume meat alternatives, but only a fraction are actually vegetarians. Retrieved from http://www.mintel.com/press-centre/food-and-drink/meat-alternatives-market-trend/.

Mortensen, A., Kulling, S. E., Schwartz, H., Rowland, I., Ruefer, C. E., Rimbach, G., ... & Sontag, G. (2009). Analytical and compositional aspects of isoflavones in food and their biological effects. *Molecular Nutrition & Food Research*, 53(S2): S266-S309.

Munro, I.C., Harwood, M., Hlywka, J.J., Stephen, A.M., Doull, J., Flamm, W.G. & Adlercreutz, H. (2003). Soy Isoflavones: A Safety Review. *Nutrition Review*, 61(1): 1-33.

Nakamura, M., Nishitani, T., Mihashi, T., & Tomizawa, A., inventors. (1994). Method for deodorizing soybean proteins with ion exchangers. Japan patent 94276955.

Nalinanon, S., Benjakul, S., Kishimura, H., & Shahidi, F. (2011). Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. *Food Chemistry*, 124(4): 1354–1362.

Nazareth, Z.M (2009). *Compositional, functional and sensory properties of protein ingredients*. Master's thesis, University of Iowa State Univesity. Retrieved from http://lib.dr.iastate.edu/cgi/viewcontent.cgi?article=1485&context=etd.

Nielsen, P.M., & Olsen, H.S. (2002). Chapter 7: *Enzymatic modification of food protein*. Book: Enzymes in food technology (1st ed). CRC Press, Boca Raton, FL, 109-140.

Nishiwaki, T., Yoshimizu, S., Furuta, M. & Hayashi, K. (2002). Debittering of enzymatic hydrolysates using an aminopeptidase from the edible basidiomycete Grifola frondosa. *Journal of Bioscience and Bioengineering*, 93(1), 60–63.

North Carolina Soybean Producers Association. (n.d.).*How soybeans are used*. Retrieved from http://www.ncsoy.org/ABOUT-SOYBEANS/Uses-of-Soybeans.aspx.

Nwankwo, T., Yoon S.S., Burt V., & Gu Q. (2013). Hypertension among adults in the US: National health and nutrition examination survey, 2012-2012. NCHS Data Brief, No. 133.

Hyattsville, MD: National Center for Health Statistics, Centers for Disease Control and Prevention, US Department of Health and Human Services.

Ohio State University. (n.d). Soybean proteins. Retrieved from http://class.fst.ohio-state.edu/FST822/lecturesab/Soy.htm.

Omoni, A.O., & Aluko, R.E. (2005). Soybean foods and their benefits: potential mechanisms of action. *Nutrition Review*, 63(8): 272-283.

Organic & Non-GMO. (2009).*More U.S farmers planting non-gmo soybeans*. Retrieved from http://www.non-gmoreport.com/articles/mar09/farmers_planting_non-gmo_soybeans.php.

Organic & Non-GMO. (2007). *US consumer concerned about safety of GM foods*. Retrieved from http://www.non-gmoreport.com/articles/jan07/GM_food_safety.php.

Paulsen, P.V. (2009). Isolated soy protein usage in beverages. In Paquin, Paul (2009). Functional and Speciality Beverage Technology. Cambridge: Woodhead Publishing.

Pasupuleti, V. K., & Braun, S. (2010). *Protein hydrolysates in biotechnology*. *Chapter 2: State of the art manufacturing of protein hydrolysates* (1st ed). New York: Springer, 11-32.

Pedersen, B. (1994). Removing Bitterness from Protein Hydrolysates. *Food Technology*, 48: 96-98.

Peryam, D.R., & Pilgrim, F.J., (1957). Hedonic scale method for measuring food preferences. *Food Technology*, 11(9): 9-14.

Post, G. (2005). Soy beverages steadily gaining popularity. *Fruit processing: Journal for the Fruit Processing and Juice Producing European and Overseas Industry*, 15(4): 236-237.

Potter, R.M, Dougherty, M.P., Halteman, W.A., & Camire, M.E. (2007). Characteristics of wild blueberry-soy beverages. *LWT - Food Science and Technology*, 40(5):807-814.

Qi, M., Hettiarachchy, N. S., & Kalapathy, U. (1997). Solubility and emulsifying properties of soy protein isolates modified by pancreatin. *Journal of Food Science*, 62(6): 1110-1115.

Reinwald, S., Akabas, S., & Weaver, C. (2010). Whole versus the piecemeal approach to evaluating soy. *Journal of Nutrition*, 140(12): 2335S-2343S.

Rennie, M. J., & Tipton, K. D. (2000). Protein and amino acid metabolism during and after exercise and the effects of nutrition. *Annual Review of Nutrition*, 20(1): 457-483.

Riaz, M.N. (2005). *Soy applications in food* (1st ed). Boca Raton, FL: CRC Press.

Roberston, G.L. (2011). Packaging and food and beverage shelf life. In Kilcast, D., & Subramaniam, P. (2nd Ed). *Food and Beverage Stability and Shelf Life*. Cambridge: Woodhead Publishing.

Roseboro, K. (2010). *Survey finds many non-gmo food soybean seed breeding programs.* Retrieved from http://www.non-gmoreport.com/articles/sept10/non-gmo_food_soybean_seed_breeding_programs.php.

Roseboro, K. (2012). *Arkansas could become leader in non-GMO soybeans*. Retrieved from http://www.non-gmoreport.com/articles/july2012/arkansasleaderinnongmosoybeans.php.

Rossi, A., DiSilvestro, R.A., & Blostein-Fujii, A. (1998). Effects of soy consumption on exercise induced acute muscle damage and oxidative stress in young adult males. In FASEB Journal, (Vol 12, No.5, pp. A653-A653). 9650 Rockville Pike, Bethesda, MD 20814-3998 USA: FEDERATION AMER SOC EXP BIOL.

Russin, T.A., Arcand, Y., & Boye, J.I. (2007). Particle size effect on soy protein isolate extraction. *Journal of Food Processing and Preservation*, 31(3): 308-319.

Scheide, J.D., & Brand, K.E. (1987). Process for the extraction of protein from soy flour. *U.S. Patent No. 4,704,289*. Washington, DC: U.S. Patent and Trademark Office.

Schryver, T. (2002). Increasing health benefits using soy germ. *Cereal Foods World*, 47(5): 185.

Scott, S. (2010). Protein appeals to the masses. *Beverage Industry*, 101(2), 14-20.

Sepessy, C. (2013). *State of the game: special report about the sports nutrition market*. Retrieved from http://www.naturalproductsinsider.com/reports/2013/08/State-of-the-Game.aspx.

Sharma, V.K., & Kalonia, D.S. (2010). Experimental detection and characterization of protein aggregates. In Wang, W. & Roberts, C.J. (2010). Aggregation of therapeutic proteins. Hoboken, New Jersey: John Wiley & Sons.

Sloan, E.A. (2014). The top ten functional food trends. *Food Technology*. 68 (4) Retrieved from http://www.ift.org/~/media/food%20technology/pdf/2014/04/0414_feat_1_toptentrends.pdf.

Sloan, E.A. (2012). Top 10 Functional Food Trends. *Food Technology*. 66 (4) Retrieved from http://www.sloantrend.com/Articles/IFT/2012-04Top10FoodTrends.pdf.

SoyaTech. (n.d.). *Soy facts*. Retrieved from http://www.soyatech.com/soy_facts.htm.

Stahler, C. (2012). *How often do Americans eat vegetarian meals? And how many adults in the U.S. are vegetarian?* Retrieved from http://www.vrg.org/blog/2012/05/18/how-often-do-americans-eat-vegetarian-meals-and-how-many-adults-in-the-u-s-are-vegetarian/.

Stewart, H., Dong, D., & Carlson, A., (2013). *Why Are Americans Consuming Less Fluid Milk? A Look at Generational Differences in Intake Frequency.* USDA -Economic Research Report No. 149. Retrieved from: http://www.ers.usda.gov/media/1118789/err149.pdf.

Sun, X.D. (2011). Enzymatic hydrolysis of soy proteins and the hydrolysates utilisation. *International Journal of Food Science and Technology*, 46(12): 2447-2459.

Sund, E. (2013). *Got Non-dairy Beverage?* Retrieved from http://www.foodandnutrition.org/July-August-2013/Got-Non-dairy-Beverage/.

The benefits of protein hydrolysates in sportmen. (2004). Retrieved from http://www.vital-news.com/phocadownloadpap/vital24.pdf.

Thomson, R.L., & Buckley, J.D. (2011). Protein hydrolysates and tissue repair. *Nutrition Research Reviews*, 24(02): 191-197.

Tenbült, P., de Vries, N. K., Dreezens, E., & Martijn, C. (2005). Perceived naturalness and acceptance of genetically modified food. *Appetite*, 45(1): 47-50.

Tipton, K.D., & Wolfe, R.R. (2004). Protein and amino acids for athletes. *Journal of Sports Sciences*, 22(1), 65-79.

Tipton, K., & Witard, O. (2007). Protein requirements and recommendations for athletes: relevance of ivory tower arguments for practical recommendations. *Clinics in Sports Medicine*, 26(1): 17-36.

U.S Department of Agriculture. (2008). *Soybean statistics*. National Agricultural Statistics Service. Retrieved from website: http://www.nass.usda.gov/Statistics_by_State/Oklahoma/Publications/Media_Resources/Soybean_Factsheet.pdf.

U.S Environmental Protection Agency. (2013). *Major crops grown in the united states*, Agriculture. Ag101. Retrieved from website: http://www.epa.gov/agriculture/ag101/cropmajor.html.

United Soybean Board. (2013). Consumer Attitudes about Nutrition 2013. Retrieved from http://www.soyconnection.com/sites/default/files/Soy_Connection_Consumer_Attitudes.pdf.

United Soybean Board. (n.d).Soy nutritional content. Retrieved from website http://www.soyconnection.com/soy_foods/nutritional_composition.php.

University of Arkansas. (2013). *Economic contribution of arkansas agriculture*. (2013). Division of Agriculture Research and Extension. Retrieved from website: http://division.uaex.edu/news_publications/Economic_Contribution_2013.pdf.

Van der Ven, C., Ariette M.M, & van den Berg, R.W. (2005). Inactivation of soybean trypsin inhibitors and lipoxygenase by high-pressure processing. *Journal of Agriculture and Food Chemistry, 53*(4): 1087-1092.

Vaughn, N., Rizzo, A., Doane, D., Beverly, J.L., & de Mejia, E. (2008). Intracerebroventricular administration of soy hydrolysates reduced body weight without affecting food intake. *The FASEB Journal*, 21(5): A372.

Velasquez, M.T., & Bathena, S. J. (2007). Role of dietary soy protein in obesity. *International Journal of Medical Sciences*, 4(2): 72-82.

Venter, C.S. (1999). Health benefits of soybean and soy products: a review. *Journal of Family Ecology and Consumer Sciences/Tydskrif vir Gesinsekologie en Verbruikerswetenskappe*. 27 (2): 24-33.

Wackerhage, H. & Rennie, M. (2006). How nutrition and exercise maintain the human musculoskeletal mass. *Journal of Anatomy*, 208(4): 451-458.

Walker, G. (2004). Affective test, also known as hedonic or consumer tests. Retrieved from http://sst-web.tees.ac.uk/external/U0000504/Notes/Sensory/AffectiveTests.html.

Wang, H., & Murphy, P.A. (1994). Isoflavone content in commercial soybean foods. *Journal of Agriculture and Food Chemistry*, 42(8): 1666-1673.

Weinert, D. (2009). Nutrition and muscle protein synthesis: a descriptive review. *Journal of the Canadian Chiropractic Association*, *53*(3): 186-193.

Wilson, M. (2013). Molecular signals and skeletal muscle adaptation to exercise. *International Journal of Applied Exercise Physiology*, *2*(1):1-10.

Wolf, W.J. (1970). Soybean Proteins: Their Functional, Chemical, and Physical Properties. *Journal of Agriculture and Food Chemistry, 18*(6): 969-976.

Wolf, W.J., & Cowan, J.C. (1971). Soybean as a food source. *Critical Review in Food Science & Nutrition*, 2(1): 81-158.

Wolfe, R. (2002). Regulation of muscle by amino acids. *Journal of Nutrition*. 132(10): 3219S-3224S.

Wright, R. (2011). The Nutraceutical Beverage Market: Thirsting for New Ideas. Retrieved from http://www.nutraceuticalsworld.com/issues/2011-07/view_features/the-nutraceutical-beverage-market-thirsting-for-ne/#.dpuf.

Yong, N.W.G., & O'Sullivan, G.R. (2011). The influence of ingredients on product stability and shelf life. In Kilcast, D., & Subramaniam, P. (2nd Ed). *Food and Beverage Stability and Shelf Life*. Cambridge: Woodhead Publishing.

Zhou, J.R. (2004). Soy and the prevention of lifestyle-related diseases. *Clinical and Experimental Pharmacology and Physiology*, 31(s2): S14-S19.

Zhou, J.R., & Erdman, J.W. Jr. (1995). Phytic acid in health and disease. *Critical Reviews in Food Science & Nutrition*, 35(6): 495-508.

Chapter 3: Compositional analyses of the Four Selected Arkansas Grown Non-Genetically Modified Soybean Cultivars

Abstract

Soybean is considered as one of the important crops economically for the United States because of its tremendous versatility. However, most of soybean grown in the United States labeled as genetically modified organism (GMO) has raised consumers concern about the safety of GMO foods and impact to their health.

Maximizing the nutrient content while minimizing the anti-nutrient factors is important in selecting a cultivar of non-GMO soy beans; therefore, four Arkansas grown non-GMO cultivars, including Osage, R95-1705, R08-4004, and R05-4969, were evaluated for their nutrient and anti-nutrient profiles. The components considered in this study included moisture, lipid, carbohydrate, total mineral, protein with an amino acid profile, and isoflavone, as well as anti-nutrient components such as phytic acid content and trypsin inhibitor contents.

The results showed that R08-4004 cultivar would provide the best nutrient to anti-nutrient ratio. R08-4004 had comparable high protein content of 51.1% of dry basis with no significant difference with the other three cultivars (Osage 52.4%, R95-1705 53.3%, R05-4969 51.6%), lowest content of stachyose (42.0 mg/g), and lower trypsin inhibitor content (23.0 TUI/mg). Among the four cultivars evaluated, R08-4004 had the highest amounts of favorable essential amino acid composition, especially the three branched-chain amino acid (Isoleucine 49.7 mg/g, Leucine 74.9 mg/g, and Phenylalanine 51.4 mg/g). Therefore, R08-4004 cultivar was selected for further study to develop a high protein beverage.

3.1 Introduction

Soybean has been considered as an important economic oilseed crop in the United States (Ash, 2012). From the nutritional point of view, soybean protein is an alternative choice for people with allergenic to animal milk proteins such as casein and whey proteins. Since 1995, the Food and Drug Administration (FDA) approved the claim about the association between soy protein and reduced risk of coronary heart disease (CHD); therefore, soybean has been increasingly recognized and consumed due to its high nutritional values such as proteins, isoflavones, and dietary fiber (Garcia et al., 1997; Nutraceutical World, 2010; United Soybean Board, 2013). Soybean is also known as the richest source of isoflavones. Isoflavones belong to the families of phytoestrogens that are considered to be responsible for many potential health benefits of soy foods (Prabhakaran et al., 2006; Gardner et al., 2009; Shi et al., 2010). Despite that the protein content and amino acid profile of soybean, it is also comparable to those derived from animal sources such as egg, milk, and meat. The presence of anti-nutritional factors, such as trypsin inhibitor, phytic acid, and oligosaccharides, may adversely affect protein digestibility and amino acid availability (Gilani, Cockell, & Sepehr, 2005). Particularly, high level content of trypsin inhibitor can affect the nitrogen balance in the intestine through the loss of amino acids from endogenous secretions (El-Shemy et al., 2000). Phytic acid, with its metal ion chelating property, can interact with protein resulting in phytate protein complex formation and interfere with the absorption of minerals, especially zinc (Anderson & Wolf, 1995). Oligosaccharides, such as stachyose and raffinose, may interfere the digestion of nutrients or cause flatulence and abdominal discomfort for monogastric animals (Baker 2007; Chen et al., 2013).

In addition, most of soybean grown in the United States in 2013 was labeled as genetically modified organisms (Cornejo, 2013); hence, it can have a negative impact in consumers due to

their concern about the safety of GMO foods and ingredients to their health (Organic & Non-GMO, 2007). Recently, a trend to grow non-GMO soybeans is seen among growers and a number of soybean companies have been joining the non-GMO Project (Organic & Non-GMO, 2009). Arkansas has been recognized as a potential leading state in the production of non-GMO soybean (Roseboro, 2012); therefore, there is the need to study the composition of Arkansas grown non-GMO soybeans, including the anti-nutritional factors such as trypsin inhibitor, phytic acid, and oligosaccharides.

The objective of this study was to determine the composition of the four selected Arkansas grown non- GMO soybean cultivars (Osage, R95-1705, R08-4004, and R05-4969) including moisture, lipid, protein, ash contents, as well as sucrose, oligosaccharides, isoflavones, phytic acid, trypsin inhibitors and amino acid content. The result will be used to select one out of the four Arkansas grown non- GMO soybean cultivars that has high protein content, and higher amounts of branched amino acid for high protein beverage preparation.

3.2 Materials and methods

3.2.1 Materials

Four selected Arkansas grown non-GMO cultivars: Osage, R95-1705, R08-4004, and R05-4969 were provided by the Department of Crop, Soil, and Environmental Sciences at the University of Arkansas, Fayetteville.

Petroleum ether, sulfuric acid, and chemicals for Kjeldahl digestion were purchased from VWR (Radnor, PA). Standard glucose, sucrose, raffinose, and stachyose were purchased from Sigma Chemical Co. (St. Louis, MO). All the other reagents and chemicals were purchased from VWR (Radnor, PA) and Sigma Chemical Co. (St. Louis, MO).

3.2.2 Proximate Analyses

All of the four cultivars of soybean were ground, passed through 60 mesh (250 μm) standard testing sieve (VWR International, USA), and stored under refrigeration condition (5ºC).

Moisture content was determined using AACC Official Method [44-19] by measuring the mass of 2.0g of sample before ($M_{Initial}$) and after (M_{Dried}) 2h of drying in a hot air oven at 135°C (AACC 2000). The percentage of moisture content was calculated using the formula:

$$\% \, Moisture = \frac{M_{initial} - M_{dried}}{M_{initial}} \times 100 \quad (1)$$

Lipid content was determined using AACC Official Method [30-25] as follows: Ground dried sample (M_{Sample}) was treated with petroleum ether solvent (1:5 ratio) in Soxhlet extraction apparatus to extract the lipid. After 8h of extraction, the solvent was evaporated and crude fat (M_{Lipid}) was dried to constant weight at 100ºC (AACC 2000). The percentage of lipid content (by weight) in each sample was calculated using the formula:

$$\% \, Lipid = \frac{M_{lipid}}{M_{sample}} \times 100 \quad (2)$$

Ash content (Total mineral) was determined using AACC Official Method [08-03] by measuring the mass of exactly 2g of ground sample before (M_{Sample}) and after (M_{Ash}) 2h of placing in a muffle furnace, which was preheated to 600°C (AACC 2000). The percentage of total mineral content (by weight) in each sample was calculated using the formula:

$$\%Ash \; (wet \; basis) = \frac{M_{ash}}{M_{sample}} \times 100 \quad (3)$$

Protein content was determined using AACC Method [46-11A] "Improved Kjeldahl method, copper catalyst modification". Approximately 0.2g of defatted sample was digested at 410º C in the Digestor (Foss Tecator, Hillerod, Denmark) with the addition of the copper catalyst tablet and 10 mL of 10N sulfuric acid for 60 min. Sample in Kjeldahl digestion flask, then, was

cooled to ambient temperature in a fume hood. The protein content of each sample was determined in an automatic Kjeltec^{TM} 2300 Distillation Unit (Foss Tecator, Hillerod, Denmark).

Total carbohydrate content was calculated by the difference (BeMiller, 2010) using percentage of moisture, total fat, crude protein, and ash based on following formula:

100% - (% moisture + % fat + % protein + % ash) (4)

3.2.3 Oligosaccharide and sucrose content determination

Oligosaccharide and sucrose contents were determined using high performance liquid chromatography (HPLC). Ground sample was screened through a 100 mesh (100 μm) standard testing sieve (VWR International, PA, USA). Sugar extraction was conducted using the procedure described by Giannoccaro et al. (2006). Samples were weighed (approximately 0.15g) and transferred into 2mL Eppendorf microcentrifuge tube, which contained 1.5 mL DI water. The content was vortexed and incubated at ambient temperature in a horizontal shaker for 20 min before centrifugation at 16,000g for 10 min. Five hundred μL aliquot from the supernatant was transferred into a new 2mL Eppendorf microcentrifuge tube, mixed with 700 μL 100% acetonitrile at ambient temperature for 30 min, and then centrifuged at 16,000g for 10 min. Approximately 70 μL was filtered through a 0.2 μm membrane disc filter (Pall Co., Mich, USA) using a 1mL syringe. The filtered sample was transferred into a new 2.0 mL centrifuge tube and 24 μL of filtered sample was diluted with 576 μL DI water before the HPLC injection. Blank and validated standard samples were prepared at concentrations of 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 μg/600 μL for calibration curves of the four major sugars: glucose, fructose, sucrose, and stachyose. A Dionex DX500 HPAEC-PAD system including GS50 pump, an ED40 pulsed amperometric detector, an AS40 automated sampler with a 25 μL injection loop, and a chromeleon chromatography management system (Dionex, CA, USA) was used to identify and quantity sugar content of

sample. Sugar was separated by a system in which had an analytical CarboPac PA10 pellicular anion-exchange resin column (250 × 4 mm), a CarboPac PA10 guard column (50 × 4mm), and an AminoTrap column (30 × 3 mm). Sugars were eluted under isocratic condition with 90 mM NaOH at a flow rate of 1.0 mL/min. Each sample needed about 30 min to be analyzed. The sugars in filtered sample**s** were identified by comparing their retention times to those of the standard sugars, including glucose, fructose, sucrose, and stachyose. Sugar contents were expressed as mg/g of dried samples.

3.2.4 Phytic acid content

Phytic acid contents were determined by the measurement of inorganic phosphorous (P_i) concentration using colorimetric assay method based on the inverse relationship between phytate concentration and P_i concentration in soybean (Chen et al., 1956; Wilcox et al., 2000; Scaboo et al., 2009). For phytate extraction, 1.0 mL of P_i extraction buffer was added to 2mL Eppendorf microcentrifuge containing approximately 0.1 g of ground sample. The solution was shaken before incubated for 6h at 4°C. During the incubation period, a vortex one or two time was needed in order to mix extraction buffer and ground seed tissue. At the end of incubation period, sample was mixed again, stood for 30 min for tissue to settle, and centrifuged at 4000g for 4 min. Chen's reagent was prepared as following ratio: 2mL of DI water: 1mL of 10% ascorbic acid: 1mL of 2.5% ammonium molybdate: 1mL of 6N H_2SO_4. In addition, known samples included a high and low phytate soybean cultivars were prepared in a similar manner as samples. Samples and known samples were replicated three times using flat-bottomed Corning Costar microliter plate (VWR International, USA) along with 8 standards that will be prepared to have concentrations of 155, 465, 930, 1395, 1860, 2325, and 2635 ngP respectively. Each well of samples and checks contained 90 μL of DI water, 10 μL prepared sample/or check, and 100 μL of Chen's reagent while each

standard well contained 100 μL of prepared standard and 100 μL of Chen's reagent. The plate had been incubated at ambient temperature for exactly one hour before it was read. A Biotek Sinergy HT plate reader (BioTek Instruments, Winooski, VT) set at a wavelength of 510 nm was used to determine the concentration of P_i. The calibration curve was established based on the absorbance of 8 standards against the P_i. The inorganic phosphorous content of each sample was determined based on the calibration curve. Sample with high content of P_i would have low content of phytic acid while sample with low content of P_i would have high content of phytic acid.

3.2.5 Isoflavones Contents

The twelve isoflavones (daidzin, malonyl daidzin, acetyl daidzin, daidzein, glycitin, malonyl glycitin, acetyl glycitin, glycitein, genistin, malonyl genistin, acetyl genistin, genistein) contents were determined using the reversed-phase HPLC (Griffith and Collison 2001). Samples, in triplicate, were extracted as follows. Approximately 0.2g of sample was mixed with 2mL solvent which contained 53% acetonitrile. Sample tubes were shaken for 2h at ambient temperature. After 2h, samples were centrifuged at 7000 g for 5 min. Supernatants were collected with syringe, filtered through a 0.45 μm polyvinylidene diflouride (PVDF), and filtered (obtained from Scientific Resources ,Eatontown, NJ, USA) before analyzed using HPLC. Injection volume was 5.0 μL/sample. Each sample was extracted twice separately and evaluated independently for all 12 isoflavones. A YMC ODS-AM column, 250 x 3 mm with 5.0 μm packing (Waters, Milford, MA, USA), was used. Mobile phase contained solvent A, which was 0.1% (v/v) trifluoro acetic acid in acetonitrile, and solvent B, which was 0.1 % (v/v) triflouro acetic acid in Millipore water. Mobile phase was set up at 100% at initial, changed to 50% during the first 30 min, and finally returned to 100% within the last 5 min. The flow rate was 1mL/min at 37ºC. Effluent detection was

measured by UV absorbance at 260 nm. The isoflavones standards, including daidzin, genistin, daidzein, and genistein, were obtained from Sigma Chemical Co. (St. Louis, USA).

3.2.6 Trypsin Inhibitor Content

Trypsin contents were determined using the improved colorimetric method described by Liu & Markakis, 1989. Reagents were prepared as follows. Assay buffer was 50 mM Tris buffer (pH = 8.2) containing 10 mM $CaCl_2$ and was kept at 5°C. Stock trypsin was the 50mL solution of 1 mM HCl (pH = 2.5) containing 2.5 mM $CaCl_4$ and 10mg of crystalline porcine trypsin (Sigma Chemical Co., St. Louis, MO, USA) and was kept at 5ºC. Stock BAPA (N-benzoyl-DL-arginine p-nitroanilide hydrochloride) solution consisted of 400mg of BAPA in 10 mL of dimethyl sulfoxide. Fresh working trypsin solution was prepared by diluting 2 mL of stock trypsin to a total volume of 25 mL using the above HCl solution. A working BAPA solution was prepared by diluting 0.25 mL of stock BAPA to a total of 25 mL using the 37°C preheated assay buffer. Approximately 500mg of sample was extracted with 50 mL DI water for 30 min using shaker. 10 mL suspension was mixed with 10 mL of assay buffer, shaken for 3 min, and filtered through a Whatman N°. 2 paper. The reaction was conducted at 37ºC. Testing solution was mixed with 2.0 mL of BAPA, 1.0 mL of sample, and 0.5 mL of trypsin enzyme (10000 unit/mg), incubated exactly for 10 min, and stopped by adding 0.5 mL of 30 % acetic acid. The absorbance of the trypsin activity in sample (A^S) and the absorbance of blank sample of 1 mL of water (A^R) were recorded at the wavelength 410 nm. Trypsin inhibitor was calculated and expressed in terms of either trypsin unit inhibited (TUI) or international units inhibited (IUI) as follows:

$$\text{TUI/mg sample} = \frac{(A^S - A^R) \times 100}{M_{sample}} \quad (5a)$$

$$\text{IUI/g} = \text{TUI/mg sample} \times 1000 \times 0.000516 \quad (5b)$$

3.2.7 Preparation of soy protein isolate

Ground soybean samples passed through 60 mesh (250 μm) standard testing sieve (VWR International, USA) were defatted with n-hexane (1:4, w/v, soy flour to solvent ratio) by constant mixing for 6h at ambient temperature. The solvent with the lipid was removed by filtration using a porcelain funnel with Whatman No. 4 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK) under the hood. The defatting process was repeated twice to remove traces of soybean oil and the defatted flour was dried overnight under the hood to remove residual hexane.

Soy protein isolate (SPI) was prepared using the aqueous extraction method. Defatted soybean flour was mixed with deionized (DI) water (1:10 w/w) and stirred until uniform slurry was obtained. The pH was adjusted to 9.5±0.05 with 6N or 1N sodium hydroxide (NaOH) to solubilize the protein in the flour while stirring for three hours. The solubilized protein was separated from the suspension by centrifugation at 3,000g for 20 min. After centrifugation, the soluble protein in the supernatant was subjected to isoelectric precipitation at pH 4.5±0.05 with 6N or 1N hydrochloric acid (HCl) and kept overnight in a cool room (5 ± 0.5ºC) for protein precipitation. The precipitated protein was separated from solution by centrifugation at 3,000 × g for 20 min, washed 3 to 4 times with DI water, adjusted to pH 7.0 with 1N NaOH, freeze-dried and stored at 5ºC.

Four cultivars' SPI samples were sent to Proteomics & Mass Spectrometry Facility of Donald Danforth Plant Science Center (St. Louis, MO, USA) for amino acid analysis using UPLC (Ultra Performance Liquid Chromatography) method. In this method, the SPI samples were oxidized to convert Cys and Met into cysteic acid (CyA) and methionine sulfone (MetS) prior the HPLC procedure in order to prevent their degradation during hydrolysis. In addition, hydrolysis converted Asn and Gln into Asp and Glu, respectively.

3.2.8 Statistical analysis

The reported data are expressed as mean of triplicate observations ± standard derivation. Data were subjected to one way analysis of variance (ANOVA) using JMP Pro 12.0 software (SAS Institute, Cary, NC, USA). Student's t test was also use to separate the means of four cultivars at $P \leq 0.05$.

3.3 Results and discussion

3.3.1 Proximate Analysis

The results of the approximate compositions of the four selected Arkansas grown non-GMO soybeans are shown in Table 4. The moisture contents of the four soybean cultivars ranged from 5.5 to 7.0%. Although there were statistically significant differences among these cultivars ($P < 0.0001$), their moisture contents were all lower than both the recommended moisture content for short term as well as long term storages which are 12-13% and 10-11% respectively (Herbek & Bitzer, 1997; Hurburgh, 2008). Because the storage moisture contents of these cultivars were low, chemical and microbial spoilage reactions can be minimized with longer shelf life (Boge, Boylston, and Wilson 2009).

On a dry weight basis, the protein content of the four cultivars ranged from 51.1 to 53.3 % with no significant differences ($P = 0.4 > 0.05$). Compared to the common range of 29-51 % protein content of other soybean cultivars reported by Hafez (1983), Giami (2002), and Balisteiro, Rombaldi & Genovese (2013), all of these four cultivars are considered as high protein soy bean cultivars. These high protein Arkansas grown non-GMO soybean cultivars can be used as good protein sources for food, especially for non-GMO high protein food products. The total lipid content of the four cultivars ranged from 19.2 to 24.4 % and was statistically significant differences ($P < 0.0001$) among the four cultivars. The total lipid content of cultivar R05-4969 was highest

(24.4 %) while cultivar R95-1705 was the lowest (19.2 %). Total mineral content determined by the ash method were 5.8 to 5.9 % with no significant differences among the four cultivars (P = 0.25 > 0.05). Generally, the values of protein, lipid, and ash contents of the four Arkansas growth non GMO soybean cultivars in this study were higher than those of the Roundup Ready GM soybean (34.6 % protein content, 19.0 % lipid content, and 4.6 % ash content respectively), which has been contributing 93-94 % of the USA soybean production (Bøhn et al., 2014). The difference in composition could be due to the different genetic backgrounds, different environmental conditions, and possibly different agricultural practices (Wolf et al., 1982; Bøhn et al., 2014).

3.3.2 Oligosaccharide and sugar contents

The oligosaccharide and sugar contents of the 4 cultivars are shown in Table 5 and are categorized in terms of glucose, fructose, sucrose, and stachyose. Glucose content of the 4 cultivars ranged from 2.2 to 3.0 mg/g. R05-4969 had significantly higher glucose content (3.0 mg/g) (P = 0.02 < 0.05) in comparison with the other three cultivars' values (Osage 2.2 mg/g; R08-4004 2.4 mg/g; and R95-1705 2.5 mg/g). Fructose content of the 4 cultivars ranged from 2.3 to 2.4 mg/g. Osage had significantly lowest fructose content (2.3 mg/g) (P = 0.0002 < 0.05) in comparison with the other three cultivars' values (2.4 mg/g). Sucrose, a disaccharide, was found in a higher concentration in comparison to the two monosaccharides (glucose and fructose) that **is** in agreement with Saldivar et al. (2011). Sucrose content also had the greatest variances and significantly differed among the four cultivars ranging from 30.5 to 44.8 mg/g (P < 0.0001). R08-4004 had highest sucrose content (44.8 mg/g) while Osage has lowest one (30.5 mg/g). Stachyose content of the four cultivars ranged from 42.0 to 45.8 mg/g. R08-4004 had significantly lowest stachyose content (42.0 mg/g) (P = 0.0072 < 0.05) in comparison to other cultivars' (45.8, 44.1, and 43.2 mg/g respectively for R05-4969, R95-1705, and Osage). The different proportion of

oligosaccharide and sugar content of the four cultivar could be explained by their genetic variation (Kennedy, Mwandemele, & McWhirter, 1985). Since oligosaccharide, particularly stachyose, is non-digestible and results in abdominal discomfort when soy food products are consumed, cultivar R08-4004 has an advantage to be used as food or feed soybean due to its lowest stachyose and highest sucrose (Callyway, Colasito, & Mathews, 1966; Saldivar et al., 2011).

3.3.3 Phytic acid content

In this study, inorganic phosphrous content, (Table 6), was used to estimate the phytic acid content in the four soybean cultivars. This method was used because measuring P_i is easier, quicker, and less expensive than measuring phytate (Scaboo et al, 2009). The amount of phytic acid content is inversely proportionate to the amount of P_i (Wilcox et al., 2000) so higher P_i reflects lower the phytic acid content. Osage and R08-4004 had low content of P_i (156.2 µg/g and 168.8 µg/g, respectively) ($P < 0.0001$) which indicated their high estimated phytic acid contents in contrast to R05-4969 and R95-1705 cultivars which had higher P_i values (184.6 µg/g and 234.5 µg/g respectively) and lower estimated phytic acid contents.

3.3.4 Trypsin inhibitor

The trypsin inhibitor activity for each of the 4 cultivars determined is showed in Table 7. Trypsin inhibitor content of the 4 cultivars ranged from 22.6 to 25.5 TUI/mg which is lower than the data reported by Balisteriro et al. (2013), but is in agreement with the other 19 cultivars conducted by Anderson & Wolf (1995). The differences could be explained by the genetic variation of soybean cultivars. Among the 4 cultivars, Osage had significantly higher trypsin inhibitor content (25.5 TUI/mg) while the other three cultivars had similar amounts of trypsin inhibitors, 22.6 TUI/g, 22.9 TUI/g, and 23.0 TUI/g respectively for R95-1705, R05-4969 and R08-4004) ($P = 0.02 < 0.05$). Since higher levels of trypsin inhibitor can negatively affect the nitrogen

balance in the intestine through the loss of amino acids from endogenous secretions, it is important to select cultivars with the lowest possible trypsin inhibition (El-Shemy et al., 2000).

3.3.5 Isoflavones

Soybean is the richest source of isoflavones that are thought to be responsible for many potential health benefits of soy foods (Prabhakaran et al., 2006; Gardner et al., 2009; Shi et al., 2010). Isoflavones contents of the four Arkansas-grown non-GMO cultivars analyzed by HPLC are showed in Table 8. There were significant differences in the total isoflavone content of the four cultivars ranging from 4437.0 to 6860.6 µg/g ($P < 0.0001$). The highest and lowest total isoflavones contents were found in R08-4004 (6860.6 µg/g) and R95-1705 (4437.0 µg/g). All four cultivars had higher isoflavone contents higher than reported by Balisteriro et al. (2013), Paucar-Menacho et al. (2010) and Sakthivelu et al. (2008).

The group of malonyl glucosides including malonyl genistin, malonyl daidzin, and malonyl glycitin were found as major components of the 12 isoflavones of the 4 cultivars. Malonyl genistin was found in highest amounts ranging from 3658.8 to 5496.8 µg/g and significant differences were observed among the four cultivars ($P < 0.0001$). Malonyl genistin was found in largest amounts of the total genistein which is the most common isoflavone. The result, once again, was similar to other studies of Balisteriro et al. (2013), Paucar-Menacho et al. (2010), Sakthivelu et al. (2008), and Zhang et al. (2006). Glycitein was not found in R05-4969 cultivar, while both glycitein and malonyl glycitin were not detected in R08-4004 cultivars. The influences of planting location, growning year, and genotype of cultivars could have contributed to the variations of the 12 isoflavones contents observed in the four cultivars (Sakthivelu et al., 2008).

3.3.6 Amino Acid Profile

Amino acid profiles of the 4 cultivars of soybean are showed in Table 9. The amino acid profile was used as a parameter to select the cultivar that had a desirable amounts of essential amino acid (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) for the protein drink. Tryptophan content of the 4 cultivars ranged from 11.3 to 11.7 mg/g with no significant difference among the 4 cultivars ($P = 0.49 > 0.05$). Histidine content of the 4 cultivars ranged from 24.4 to 26.0 mg/g. The cultivar R95-1705 had significantly highest histidine content (26.0 mg/g) than the other three cultivars (24.6 mg/g, 24.5 mg/g, and 24.4 mg/g respectively for R08-4004, R05-4969, and Osage) ($P = 0.05$). Methionine content of the 4 cultivars ranged from 19.2 mg/g to 20.3 mg/g with no significant differences among the 4 cultivars ($P = 0.24 > 0.05$). Threonine content of the 4 cultivars ranged from 32.8 to 35.3 mg/g. The cultivar R08-4004 had significantly highest threonine content (35.3 mg/g) than the other three cultivars (33.9, 33.2, and 32.8 mg/g respectively for R05-4969, Osage, and R95-1705) ($P = 0.02 < 0.05$). Lysine content of the 4 cultivars ranged from 60.5 to 71.1 mg/g. The cultivar R95-1705 had significantly lowest lysine content (60.5 mg/g) than the other three cultivars (66.4 mg/g, 68.0 mg/g, and 71.1 mg/g respectively for Osage, R08-4004, and R05-4969) ($P = 0.01 < 0.05$). Valine content of the 4 cultivars ranged from 48.1 to 50.6 mg/g. The cultivar R05-4969 and R08-4004 had significantly higher valine contents (50.6 mg/g and 50.2 mg/g respectively) than the cultivar R95-1705 and Osage (48.1 mg/g and 49.0 mg/g respectively) ($P = 0.003 < 0.05$). Isoleucine content of the 4 cultivars ranged from 46.1 to 49.7 mg/g. The cultivar R08-4004 had significantly higher isoleucine content (49.7 mg/g) than the Osage and R05-4969 (47.3 mg/g and 46.1 mg/g respectively) ($P = 0.01 < 0.05$). Leucine content of the 4 cultivars ranged from 74.1 to 75.7 mg/g. The cultivar R95-1705 had significantly lowest leucine content (74.1 mg/g) than the other two cultivars Osage and R05-4969 (75.6 mg/g, and 75.7 mg/g respectively) ($P = 0.02 < 0.05$).

Phenylalanine content of the 4 cultivars ranged from 51.4 to 52.8 mg/g with no significant differences among them ($p = 0.85 > 0.05$). In general, the 4 cultivars had comparable amino acid profiles with minor variances. This result was similar to other studies conducted on soybean cultivars by (Bayford, 2002, Rayaprolu *et al.*, 2015). However, among the four cultivars, the R08-4004 cultivar had the highest content of essential amino acids, especially branched amino acid such as Valine, Isoleucine, and Leucine that are preferable amino acids in protein beverages. Therefore, R08-044 was the selected cultivars for further development of high protein drink.

3.4 Conclusion

The compositions of four selected Arkansas grown non-GMO soybean cultivars (Osage, R09-1705, R08-4004, and R05-4969) were determined in order to select the cultivar which has high protein content and essential amino acid, and low content of anti-nutrient factors including stachyose and trypsin inhibitor. R08-4004 was selected for the developing of high protein drink due to its comparable high protein content (51.1% of dry basis), essential amino acids (tryptophan 11.6 mg/g, histidine 16.8 mg/g, methionine 20.3 mg/g, threonine 35.3 mg/g, lysine 68.0 mg/g, valine 50.2 mg/g, isoleucine 49.7 mg/g, leucine 74.9 mg/g, phenylalanine 51.4 mg/g), lowest stachyose (42.0 mg/g) in comparison to the other three cultivars.

References

AACC. 2000. Approved Methods of the American Assiciation of Cereal Chemists 10th edition. Methods: 08-03, 30-25, 44-19, and 46-11A. AACC International. St. Paul, Minn.

Anderson, R. L., & Wolf, W. (1995). Compositional changes in trypsin inhibitors, phytic acid, saponins, and isoflavones related to soybean processing. *Journal of Nutrition*, *125*(3 suppl), 581-588. Retrieved from http://jn.nutrition.org/content/125/3_Suppl/581S.full.pdf.

Ash, M. (2012). *Soybeans & oil crops*. U.S Department of Agriculture, Economic Research Service. Retrieved from website: http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/background.aspx.

Baker, K. M. (2007). *Nutritional value of high protein and low oligosaccharide varieties of soybean fed to pigs and poultry*. Master's thesis, University of Illinois at Urbana-Champaign. Retrieved from http://nutrition.ansci.illinois.edu/sites/default/files/ThesisBakerK.pdf.

Balisteiro, D.M.,Rombaldi, C.V., & Genovese, M.I. (2013). Protein, isoflavones, trypsin inhibitory, and in vitro antioxidant capacities: comparison among conventionally and organically grown soybeans. *Food Research International*, 51(1), 8-14.

Calloway, D.H., Colasito, D.J., & Mathews, R.D. (1966). Gases produced by human intestinal flora. *Nature*, 212, 1238–1239.

Chen, P.S, Toribara, T.Y., & Warner, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry*, 28(11): 1756-1758

Chen, L., Li, L., Vadlani, P.V., Madl, R.L., & Wang, W. (2013). Value-added products from soybean: removal of anti-nutritional factors via bioprocessing. In H. El-Shemy (Ed.), *Soybean- Bioactive compounds.* Retrieved from http://www.intechopen.com/download/get/type/pdfs/id/42947

Cornejo, J. F (2013). *Adoption of genetically engineered crops in the U.S.* USDA, Economic Research Service. Retrieved from website: http://www.ers.usda.gov/data-products/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-ge-adoption.aspx.

El-Shemy, H., Abdel-Rahim, E., Shaban, O., Ragab, A., Carnovale, E., & Fujita, K. (2000). Comparision of nutritional and antinutritional factors in soybean and faba bean seeds with and without cortez. *Soil Science and Plant Nutrition*, 46(2), 515-524. Retrieved from http://www.tandfonline.com/doi/pdf/10.1080/00380768.2000.10408804.

Garcia, M.C, Torre, M., Marina, M.L. & Laborda, F. (1997). Characterization of soyabean and related products. *Critical Revviews in Food Science and Nutrition*, 37(4): 361-391.

Gardner, C., Chatterjee, L. M., & Franke, A.A. (2009). Effects of isoflavone supplements vs. soy foods on blood concentrations of genistein and daidzein in adults. *The Journal of Nutritional Biochemistry,* 20(3): 227-234.

Gilani, G.S., Cockell, K.A., & Sepehr, E. (2005). Effects of antinutritional factors on protein digestibility and amino acid availability in foods. *Journal of AOAC International*, 88(3): 967-987.

Giannoccaro E., Wang Y. J., & Chen P. (2006). Effects of solvent, temperature, time, solvent-to-sample ratio, sample size, and defatting on the extraction of soluble sugars in soybean. Journal of Food Science, 71(1): C59–C64.

Kennedy, I.R., Mwandemele, O.D., & McWhirter, K.S. (1985). Estimation of sucrose, raffinose, and stachyose in soybean seeds. *Food Chemistry,* 17(2): 85-93.

Liu, K. & Markakis, P. (1989). An improved colorimetric method for determining antitryptic activity in soybean products. *Cereal Chemistry*. 66(5): 415-422.

Nutraceuticals World. (2010). Soy. http://www.nutraceuticalsworld.com/issues/2010-07/view_monograph-center/soy-2010-07-01-00-00-00/.

Organic & Non-GMO. (2009).*More U.S farmers planting non-gmo soybeans*. Retrieved from http://www.non-gmoreport.com/articles/mar09/farmers_planting_non-gmo_soybeans.php.

Organic & Non-GMO (2007). *US consumer concerned about safety of GM foods*. Retrieved from http://www.non-gmoreport.com/articles/jan07/GM_food_safety.php.

Paicar-Menacho, L.M, Amaya-Farfán, J., Berhow, M.A., Mandarino, J.M.G., Mejia, E.G. & Chang, Y.K. (2010). A high-protein soybean cultivar contains lower isoflavones and saponins but higher minerals and bioactive peptides than a low-protein cultivar. *Food Chemistry*, 120(1): 15-21.

Prabhakaran, M., Hui, L.S., & Perera, C.O. (2006). Evaluation of the composition and concentration of isoflavones in soy based supplements, health products, and infant formulas. *Food Research International, 39*, 730-738.

Rayaprolu, S., Hettiarachchy, N., Horax, R., Satchithanandam, E., Chen, P., & Mauromoustakos, A. (2015). Amino acid profiles of 44 soybean lines and ACE-I Inhibitory activities of peptide fractions from selected lines. *Journal of the American Oil Chemists' Society*, 1-11.

Roseboro, K. (2012). *Arkansas could become leader in non-GMO soybeans*. Retrieved from http://www.non-gmoreport.com/articles/july2012/arkansasleaderinnongmosoybeans.php.

Sakthivelu, G., Akitha Devi, M.K, Giridhar, P., Rajasekaran, T., Ravishankar, G.A., Nikolova, M.T., Angelov, G.B., Todorova, R.M., & Bulgaria, G.P. (2008). Isoflavone composition,

phenol content, and antioxidanr activity of soybean seeds from India and Bulgaria. *Journal of Agricultural and Food Chemistry,* 56(6): 2090-2095.

Saldivar, X, Wang, Y.J., Chen, P & Hou, A. (2011). Changes in chemical composition during soybean seed development. *Food Chemistry*, 124(4): 1369- 1375.

Scaboo, A.M, Pantalone, V.R., Walker, D.R., Boerma, H.R., West, D.R., Wakekr, F.R., & Sams, C.E. (2009). Confirmation of molecular markers and agronomic traits associated with seed phytate content in two soybean RIL populations. *Crop Science*, 49(2):426-432.

Shi, H., Nam, P. K., & Ma, Y. (2010). Comprehensive profiling of isoflavones, phytosterols, tocopherols, mineals, crude protein, lipid, and sugar during soybean (glycine max) germination. *Journal of Agriculture and. Food Chemistry*, 58(8), 4970-4976.

United Soybean Board. (2013). Consumer Attitudes about Nutrition 2013. Retried from http://www.soyconnection.com/sites/default/files/Soy_Connection_Consumer_Attitudes.pdf.

Wilcox, J.R., Premachandra, G.S, Young, K.A., & Raboy V. (2000). Isolation of high seed inorganic P, low-phytate soybean mutants. *Crop Science.* 40(6):1601–1605.

Zhang, B., Hettiaracchy, N., Chen, P., Horax, R., Cornelious, B., & Zhu, D. (2006). Influence of the application of three different elicitors on soybean plants on the concentrations of several isoflavones in soybean seeds. *Journal of agricultural and food chemistry*, 54(15), 5548-5554.

Table 4: Proximate composition of the 4 Arkansas Grown Non-GMO Soybean Cultivars

Components (g/100g)	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
Moisture[b]	5.8 ± 0.1[b]	5.5 ± 0.1[c]	7.0 ± 0.0[a]	5.8 ± 0.1[b]
Protein[c]	52.4 ± 0.9[a]	53.3 ± 0.5[a]	51.6 ± 2.2[a]	51.1 ± 1.1[a]
Lipid[c]	23.7 ± 0.3[b]	19.2 ± 0.3[d]	24.4 ± 0.1[a]	20.6 ± 0.3[c]
Ash[c]	5.9 ± 0.0[a]	5.8 ± 0.0[a]	5.8 ± 0.0[a]	5.8 ± 0.0[a]
Total starch[b, d]	16.6 ± 1.0[b]	20.3 ± 0.4[a]	16.3 ± 1.0[b]	20.9 ± 1.1[a]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on wet-weight basis
[c] Values are reported on dry-weight basis
[d] Values are calculated by difference

Table 5: Oligosaccharide and Sugar Contents in the 4 Arkansas Grown Non-GMO Soybean Cultivars

Sugar Type[b] (mg/g)	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
Glucose	2.2 ± 0.1[b]	2.5 ± 0.2[b]	3.0 ± 0.3[a]	2.4 ± 0.2[b]
Fructose	2.3 ± 0.0[b]	2.4 ± 0.0[a]	2.4 ± 0.0[a]	2.4 ± 0.0[a]
Sucrose	30.5 ± 0.9[c]	35.5 ± 0.4[b]	44.6 ± 0.4[a]	44.8 ± 0.4[a]
Stachyose	43.2 ± 1.2[bc]	44.1 ± 0.5[b]	45.8 ± 0.2[a]	42.0 ± 0.5[c]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on dry-weight basis

Table 6: Inorganic Phosphorous Content in the 4 Arkansas Grown Non-GMO Soybean Cultivars

Content[b] (µg/g)	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
Inorganic P	156.2 ± 5.1[c]	234.5 ± 9.4[a]	184.6 ± 7.4[b]	168.8 ± 2.1[c]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on dry-weight basis

Table 7: Trypsin Inhibitor Content in the 4 Arkansas Grown Non-GMO Soybean Cultivars

Trypsin Inhibitor Content[bc]	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
TUI/mg	25.5 ± 0.7[a]	22.6 ± 0.7[b]	22.9 ± 0.9[b]	23.0 ± 0.7[b]
IUI/g	13.1 ± 0.4[a]	11.6 ± 0.3[b]	11.8 ± 0.5[b]	11.9 ± 0.4[b]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on dry-weight basis
[c] 1 TU = 0.000516 IU

Table 8: Isoflavone Contents in the 4 Arkansas Grown Non-GMO Soybean Cultivars

Isoflavones[b] (µg/g)	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
Daidzin	83.7 ± 2.9[c]	79.9 ± 0.5[c]	105.4 ± 1.5[b]	127.3 ± 1.1[a]
Malonyl Daidzin	255.5 ± 5.2[d]	299.6 ± 5.0[c]	366.1 ± 8.1[b]	982.1 ± 8.5[a]
Acetyl Daidzin	14.7 ± 5.4[c]	35.6 ± 4.4[ab]	53.2 ± 13.9[a]	18.8 ± 5.5[bc]
Daidzein	11.8 ± 0.7[c]	9.5 ± 0.6[c]	18.5 ± 0.5[a]	15.2 ± 1.7[b]
Glycitin	7.1 ± 0.7[b]	25.4 ± 0.3[a]	24.2 ± 0.5[a]	24.3 ± 0.8[a]
Malonyl Glycitin	97.5 ± 4.7[b]	149.5 ± 4.2[a]	158.7 ± 4.4[a]	0.0 ± 0.0[c]
Acetyl Glycitin	47.7 ± 0.6[b]	40.5 ± 0.4[d]	45.1 ± 0.4[c]	55.7 ± 0.6[a]
Glycitein	19.1 ± 0.6[b]	25.9 ± 1.8[a]	0.0 ± 0.0[c]	0.0 ± 0.0[c]
Genistin	120.2 ± 3.4[b]	85.3 ± 3.6[c]	119.4 ± 3.9[b]	130.0 ± 3.9[a]
Malonyl Genistin	4723.4 ± 188.2[b]	3658.8 ± 77.6[c]	4551.3 ± 71.8[b]	5496.8 ± 50.4[a]
Acetyl Genistin	8.2 ± 0.2[b]	19.0 ± 0.2[a]	5.1 ± 0.1[d]	6.3 ± 0.1[c]
Genistein	6.6 ± 0.1[b]	5.2 ± 0.4[c]	8.0 ± 0.3[a]	4.1 ± 0.1[d]
Total Daidzein[c]	365.6 ± 14.6[d]	427.5 ± 3.0[c]	543.2 ± 11.2[b]	1143.4 ± 5.1[a]
Total Glycitein[d]	171.3 ± 6.2[c]	241.3 ± 5.2[a]	227.9 ± 4.2[b]	80.0 ± 4.2[d]
Total Genistein[e]	4858.3 ± 186.1[b]	3768.3 ± 77.0[c]	4683.9 ± 74.8[b]	5637.2 ± 48.6[a]
Total Isoflavones[f]	5395.3 ± 168.7[b]	4437.0 ± 75.0[c]	5455.0 ± 66.2[b]	6860.6 ± 41.4[a]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on dry-weight basis.
[c] Total Daidzein = Daidzin + Malonyl Daidzin + Acetyl Daidzin + Daidzein
[d] Total Glycitein = Glycitin + Malonyl Glycitin + Acetyl Glycitin + Glycitein
[e] Total Genistein = Genistin + Malonyl Genistin + Acetyl Genistin + Genistein
[f] Total Isoflavones = Total Daidzein + Total Glycitein + Total Genistein

Table 9: Amino acid content (mg/g) in the 4 Arkansas Grown Non-GMO Soybean Cultivars

Amino acid[b] (mg/g)	Soybean Cultivars Type[a]			
	Osage	R95-1705	R05-4969	R08-4004
Trp[c]	11.6 ± 0.3[a]	11.7 ± 0.2[a]	11.3 ± 0.1[a]	11.6 ± 0.4[a]
CyA[e]	17.0 ± 0.4[a]	17.2 ± 0.7[a]	17.0 ± 0.4[a]	16.8 ± 0.9[a]
His[c]	24.4 ± 0.8[b]	26.0 ± 0.3[a]	24.5 ± 0.3[b]	24.6 ± 0.5[b]
Ser	46.2 ± 1.0[a]	47.8 ± 1.8[a]	45.1 ± 0.7[a]	45.3 ± 3.1[a]
Arg	73.5 ± 0.7[a]	73.9 ± 1.6[a]	72.1 ± 0.1[a]	72.1 ± 2.4[a]
Gly	38.6 ± 0.4[a]	39.0 ± 0.7[a]	39.0 ± 0.4[a]	39.1 ± 1.1[a]
Asp + Asn[f]	114.4 ± 0.7[a]	110.4 ± 2.0[c]	111.5 ± 0.7[bc]	114.2 ± 0.9[ab]
MetS[c,e]	19.8 ± 0.8[a]	19.2 ± 0.4[a]	20.3 ± 0.1[a]	20.3 ± 0.7[a]
Glu + Gln[f]	185.2 ± 0.6[a]	179.5 ± 2.2[b]	179.3 ± 0.5[b]	183.4 ± 1.6[a]
Thr[c]	33.2 ± 0.5[b]	32.8 ± 0.1[b]	33.9 ± 0.5[b]	35.3 ± 0.9[a]
Pro	49.1 ± 0.4[a]	49.3 ± 1.3[a]	49.5 ± 0.6[a]	48.5 ± 1.0[a]
Ala	35.6 ± 0.8[b]	37.7 ± 0.1[a]	37.4 ± 0.2[a]	36.9 ± 1.0[ab]
Lys[c]	66.4 ± 3.6[a]	60.5 ± 0.2[b]	71.1 ± 1.2[a]	68.0 ± 2.4[a]
Tyr	19.2 ± 0.2[b]	23.4 ± 1.3[a]	22.9 ± 1.2[a]	19.5 ± 0.5[b]
Val[c,d]	49.0 ± 0.2[b]	48.1 ± 0.3[b]	50.6 ± 0.4[a]	50.2 ± 0.6[a]
Ile[c,d]	47.3 ± 0.4[bc]	48.2 ± 0.8[ab]	46.1 ± 0.5[c]	49.7 ± 1.0[a]
Leu[c,d]	75.6 ± 0.1[a]	74.1 ± 0.0[b]	75.7 ± 0.7[a]	74.9 ± 0.8[ab]
Phe[c]	51.5 ± 2.4[a]	51.9± 2.6[a]	52.8 ± 0.6[a]	51.4 ± 0.7[a]

[a] Values are means ± SD of triplicates. SD = Standard Deviation. Values followed by the same letter in the same row are not significantly different ($P < 0.05$)
[b] Values are reported on dry-weight basis
[c] Essential amino acid
[d] Branched-chain amino acid
[e] Before hydrolysis, oxidation converts Cys and Met into CyA and MetS, respectively.
[f] Hydrolysis converts Asn and Gln into Asp and Glu, respectively

Chapter 4: Physicochemical Properties and Angiotensin-I converting enzyme inhibitory activity of Soy Protein Hydrolysates from a Non-genetically modified cultivar (R08-4004)

Abstract

Arkansas-grown non-genetically modified soybean cultivar, R08-4004, was selected to prepare protein isolate which was treated with Alcalase for limited enzymatic hydrolysis. The objective was to optimize Alcalase hydrolysis conditions to produce soy protein hydrolysate (SPH) with high yield, low bitterness and clarity for beverage applications. The degree of hydrolysis ranged between 14 and 52% during this study at varying incubation times using two different concentrations of Alcalase enzyme. Recovery of soluble protein, between 21% and 53%, was achieved with a decrease in turbidity. There was an increase in surface hydrophobicity (S_0) which is correlated to bitterness of SPH treated with 1.0 AU (3.2µL/g) of Alcalase 2.4 L. The Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis analysis showed a distinct hydrolysis pattern in which 7S globulin and the two acidic sub-units of 11S globulin were hydrolyzed extensively in comparison to the two basic sub-units of 11S globulin. Limited enzymatic hydrolysis produced low molecular weight peptides - <17000 Da. Among these SPHs, the one derived after 120 min incubation had DH of 25.5, production yield of 44.4%, high soluble protein content 46.7%, low S_0 value (35.4), low turbidity (0.9), and highest Angiotensin-I Converting Enzyme (ACE-I) inhibition activity (66.6%). This hydrolysate has potential as protein rich nutraceutical for developing many non-genetically modified food product applications.

4.1 Introduction

Soybean is widely used in the food industry for its quality protein profile (soy protein digestibility corrected amino acid score - PDCAAS = 1) and diverse functional properties (Michelfelder, 2009). Soy protein isolate (SPI) is preferred over soy flour or soy protein concentrate in the food industry due to its highest protein content which is normally over 90% on dry basis (Riaz, 2005). In addition, SPI also has a low flavor profile because it does not contain lipids and carbohydrates. Soy protein can be prepared either by aqueous or non-aqueous methods (Scheide & Brand, 1987). However, the aqueous method is preferred because the organic solvents used in the non-aqueous method can cause undesirable protein denaturation leading to non-palatability and poor functional properties (Scheide & Brand, 1987).

Proteins must be soluble in the food matrix for use in liquid foods or clear beverages (Adler-Nissen, 1976). However, the solubility of SPI varies depending on the pH, where the solubility is poor between 3.0 to 6.0 pH and least at its isoelectric point (pH = 4.5) (Utsumi & Kinsella, 1985). Quality of SPI needs to be improved for use in the development of high protein products, especially in beverages, in which solubility plays an important role. Modifying SPI using chemical or enzyme treatments have been tested previously (Bucci & Unlu 1999; Jung, Murphy & Johnson, 2005). Among the physical, chemical, and enzymatic modification methods, the enzymatic hydrolysis of the soy protein has several advantages (Deeslie & Cheryan, 1988; Qi, Hettiarachchy & Kalapathy, 1997). Additionally, enzymatic hydrolysis is preferred to acid hydrolysis since it produces few or no undesirable reaction products. It is a nutritionally safe method when the food grade enzymes are used, and also provides a uniform product (Campbell et al., 1996; Sun, 2011). The common enzyme used for enzymatic hydrolysis is Alcalase, which is an alkaline endopeptidase produced from *Bacillus licheniformis* (Vioque et al., 2000). Alcalase

contains serine in its active center that cleaves most peptide bonds, with a preference on the carboxyl side of hydrophobic amino acids (Walsh et al., 2003; Lorenzo, 2008). Alcalase has been used extensively to prepare a wide array of protein hydrolysates from soybean protein, chickpea protein, lima bean protein, and fish protein (Zmudziński & Surówka, 2003; Yang et al., 2011; Gao & Zhao, 2012; Chel-Guerrero et al., 2012; Ravichandran & Hettiarachchy, 2013; and Segura-Campos et al., 2013;). Furthermore, the functionality of the hydrolyzed product can be controlled by selection of specific reaction conditions (Sun, 2011). Various combinations of enzyme/substrate ratio, time, pH, and temperature were used in the production of protein hydrolysate to obtain various functional property enhancements. Optimizing enzymatic conditions is important to obtain the desired protein fragments with high solubility. The degree of hydrolysis (DH) has been used to determine the proportion of cleaved peptide bonds in a protein hydrolysate (Rutherfurd, 2010).

Another advantage of the enzymatic hydrolysis modification is the production of a peptide that can inhibit Angiotensin-I Converting Enzyme (ACE-I) which plays an important role, through the renin-angiotensin system, in controlling peripheral blood pressure (Mullally et al., 1996). Among many proteolytic enzymes, Alcalase has been reported to produce the highest ACE-I inhibiting peptides (Chiang et al., 2005; He et al., 2012). Researchers have reported several ACE-I inhibiting peptides from soybean sources (Chiang et al., 2005; Yang et al., 2011; and Lassissi et al., 2014), while there are no studies using non-genetically modified (non-GM) soybean cultivar. Hence, the objective of this study was to prepare non-GM soy protein isolate, which would be subjected to optimal hydrolysis using food grade Alcalase enzyme for producing large quantities of soluble peptides with acceptable bitterness and least opacity in solution, and to evaluate its potential ACE-I inhibitory activity.

4.2 Materials and methods

4.2.1 Materials

The Arkansas grown non-GM and high protein cultivar, R08-4004, was provided by Dr. Pengyin Chen, Professor, Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville. The R08-4004 soybean cultivar was selected due to the presence of high protein content and comparatively (with other non-GM cultivars) higher amounts of branched amino acids (data not shown) that were preferred for protein drink application.

Food grade enzyme Alcalase 2.4L obtained from Novozyme Inc. (Bagsvaerd, Denmark) was used to hydrolyze soy protein. ACE (from rabbit lung), hippuryl-histidyl-leucine (HHL) substrate, and Captopril were purchased from Sigma (St. Louis, MO). All laboratory chemicals were analytical grade and were purchased from Sigma (St. Louis, MO), VWR (Radnor, PA), and Fisher Scientific (Pittsburg, PA).

4.2.2 Preparation of soy protein isolate

Soybean was ground, passed through a 60 mesh (250 μm) standard testing sieve (VWR International, USA) before being defatted with n-hexane (1:4, w/v, soy flour to solvent ratio) with constant stirring for 6h at ambient temperature. The solvent with the lipid was removed by filtration using a porcelain funnel with Whatman No. 4 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK) under the hood. The defatting process was repeated twice to remove traces of soybean oil and the defatted flour was dried overnight under the hood to remove residual hexane.

Soy protein isolate was prepared using the aqueous extraction method (Rayaprolu et al., 2013). Defatted soybean flour was mixed with DI water (1:10 w/w) and stirred until uniform slurry was obtained. The pH was adjusted to 9.5 ± 0.05 with 6M or 1M sodium hydroxide (NaOH) to solubilize the protein in the flour while stirring for three hours. The solubilized protein was

separated from the suspension by centrifugation at 3,000g for 20 min. After centrifugation, the soluble protein in the supernatant was subjected to isoelectric precipitation at pH 4.5 ± 0.05 with 6M or 1M hydrochloric acid (HCl) and kept overnight in a cool room (5 ± 0.5 ºC). The precipitated protein (at isoelectric pH) was separated from solution by centrifugation at 3,000g for 20 min, washed 3 to 4 times with DI water, adjusted to pH 7.0 with 1M NaOH, freeze-dried and stored at 5 ºC.

4.2.3 Preparation of soy protein hydrolysate (SPH) using Alcalase

Ten gram portions of SPI were dispersed into 250mL beakers and 100mL of DI water (1:10 w/v) was added. The suspensions were stirred at room temperature for 10 min, adjusted to pH 7.0 using 1.0 M NaOH or 1.0 M HCl and pre-heated to 50 °C in a water bath. Alcalase enzyme solution at the required activity levels (0.5 AU (1.6 µL/g) and 1.0 AU (3.2 µL/g)) for each treatment was warmed to 50 °C before adding to the protein suspensions, held at 50 °C, for hydrolysis. The mixtures of enzyme and protein were incubated for 30, 45, 60, 120 and 1440 min in a water bath with shaker. The enzyme was inactivated at 90 ºC for 5 min, and then the solutions were cooled to ambient temperature and centrifuged at 3,000g for 20 min. The supernatants were separated, freeze dried, ground, passed through a 100 mesh (149 µm) standard testing sieve (VWR International, USA) and the SPH powder obtained was stored at 5 ºC. A sample of supernatants from hydrolyzed SPIs was used to determine soluble protein content (with conversion factor $N_2 \times 6.25$) and degree of hydrolysis (DH).

4.2.4 Determination of soluble protein in the hydrolysates

Soluble protein contents of the hydrolyzed SPIs' supernatants were determined using modified Biuret method (Ohnishi & Barr, 1978). Bovine serum albumin (BSA) was used to prepare protein standard by adding 500 mg of BSA into 5 ml of 0.85 % (v/v) sodium chloride

solution. Five standard solutions ranging from 0, 250, 500, 750, and 1000 µg/mL were prepared to determine the calibration curve. The hydrolysates were diluted with 0.85 % (v/v) sodium chloride solution, as needed, to give a final protein concentration range of 150-1000 µg/mL. To prepare the test sample, 0.2 mL of diluted hydrolysate solution, in triplicate, was mixed with 2.2 mL of Biuret reagent and allowed to stand for 10 min. One hundred microliters of Folin and Ciocalteau's Phenol reagent was added and mixed. The absorbance reading was measured at 750 nm wavelength after 30 min. A standard plot of BSA protein concentrations versus their absorbance was used to calculate the amount of protein in the hydrolyzed samples.

4.2.5 Determination of degree of hydrolysis (DH)

Degrees of hydrolysis was determined using a method described by Nielsen et al. (2001). The assay was conducted at ambient temperature and is based on the amount of primary α-amino groups released from hydrolysis with o-phthalaldehyde (OPA) that showed strong absorbance at 340 nm. The OPA reagent was prepared by dissolving 7.62 g of disodium tetraborate decahydrate, 200 mg of sodium dodecyl sulfate, 160 mg of o-phthalaldehyde in 4 mL of ethanol, 176 mg dithiothreitol, and DI water to a total volume of 200 mL. The hydrolysates obtained at varying incubation times were diluted with DI water as needed and a 400 µL sample was added to 3mL of OPA reagent. The absorbance was measured at 340 nm after 2 min using a spectrophotometer. Blanks were prepared using 400 µL of DI water in 3.0 mL of OPA reagent while standards were prepared using 400 µL of 0.1 % serine solution (50 mg serine in 500 mL DI water). The absorbance values were recorded and used to determine the DH using the following equations:

$$\text{Serine-NH}_2 = \frac{\text{Sample}_{OD} - \text{Blank}_{OD}}{\text{Standard}_{OD} - \text{Blank}_{OD}} \times \frac{(0.9516 \times V \times 100)}{(X \times P)} \quad (1)$$

Serine- NH_2 = meqv serine NH_2/g protein; X= protein in sample; V = sample volume; P = protein

% in sample.

$$H = \frac{(\text{Serine}-\text{NH2}) - \beta}{\alpha} \quad (2)$$

The 'α' and 'β' values for soybean are 0.970 and 0.342 respectively.

$$DH = \frac{H}{H_{tot}} \times 100 \quad (3)$$

Where, H_{tot} is known as the total number of peptide bonds per protein equivalent. For soy protein, H_{tot} is equivalent to 7.8.

4.2.6 Protein recovery of hydrolyzed SPI samples

The protein content of freeze-dried samples was determined by a combustion method that gives the total nitrogen content. The protein recoveries were calculated as the amount of protein in each hydrolysate sample relative to the initial SPI present in the reaction solution.

4.2.7 Surface hydrophobicity (S_0) determination of hydrolysates

Surface hydrophobicity of SPI and SPH was determined by using a hydrophobic fluorescence probe, 1-anilino-8-naphthalene sulfonate (ANS), binding method described by Hayakawa and Nakai (Hayakawa & Nakai, 1985). Each protein sample was prepared to 0.1 % stock solution and serially diluted to five various concentrations ranging from 0.001 % to 0.01 % (w/v, protein basis, in 0.01M phosphate buffer, pH = 7.0). Fifty microliters of 8mM ANS (in 0.01M phosphate buffer, pH = 7.0) was added to 4.0 mL of each protein solution. Fluorescence intensity of ANS-protein conjugates of each sample was measured using a spectroflurophotometer model SFM23/B (Kontron Ltd., Zurich, Switzerland) with excitation and emission wavelengths of 390 and 470 nm respectively. The coefficient of linear regression analysis of fluorescence intensity against protein concentration (%) was used as an index of the protein surface hydrophobicity (S_0).

4.2.8 Turbidity determination of hydrolysates

Solutions prepared with SPH samples were evaluated for turbidity using UV-Vis spectrophotometer (Shimadzu Corp., Kyoto, Japan) using a method described by Jiang et al. (2010) and Lee (2011). De-ionized water was used as a blank and SPI solution was used as a control. Fifty milligrams of SPI and SPH were added to 1.5 mL Eppendorf tubes (Sigma, MO) with 1 mL of DI water, and vortexed for 2 min. The samples (5% w/v) were placed in cuvettes and their absorbance was recorded at 390 nm.

4.2.9 Molecular size (MW) of soy protein hydrolysates

Molecular size of the proteins present in samples was determined by a modified method using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Each sample was dissolved in DI water (15 mg/100 µL of protein content given by Bradford test ranging from 1.1 µg/µL to 1.4 µg/µL). Loading samples were prepared by mixing 10 µL of dissolved hydrolysates with 5 µL dissociating sample buffer (10% v/w SDS, 10 mM dithiothreitol (DTT), 20% glycerol, 0.2M tris-HCl pH 6.8, and 0.05 % bromophenol blue) in 1.5 mL Eppendorf tubes (Sigma, MO), and heated in dry-bath at 100 °C for 2 min. Pre-stained protein marker (apparent MW ranging from 175kDa to 7kDa; NEB #P7708, New England Biolabs, MA), SPI control, and hydrolyzed samples (3 µL) were loaded into a 10-well hand-cast 3.5 mm vertical gel slab (12.0 × 17.0 cm) with 15 % acrylamide separating gel prepared in 1.5M Tris-HCl, pH 8.8, and 0.4 % SDS and 4 % acrylamide stacking gel prepared in 0.5M Tris-HCl, pH 6.8, and 0.4 % SDS. Electrophoresis was performed using a Bio-Rad mini-Protean II cell (Bio-Rad Laboratories, Hercules, CA, USA) with 1X tris/glycine running buffer (0.124M Tris-HCl, pH 8.3, 0.959 M glycine, and 0.1 % SDS) at a constant voltage of 200V and constant current of 120mA for approximately 60 min. Gel slabs were stained using the Coomassie blue stain solution (50 % methanol (v/v), 10 % acetic acid (v/v), and 0.25 % Coomassie blue G-250 (w/v)) for 15 min and

de-stained overnight with a de-staining solution containing 10 % acetic acid (v/v) and 5 % methanol (v/v). The gels were scanned and quantitated using UN-SCAN-IT gel- Gel Analysis Software (Silk Scientific Inc., Orem, UT, USA) which measured the pixel intensity of the bands. SPI control gel band intensity was measured by counting the number of pixels present in a box of a constant area. This constant box was moved horizontally across all the samples' gel bands at same MW range. The relative intensity of all the samples was measured and compared with the control (SPI) which was normalized to the control level (100 %).

4.2.10 Angiotensin I-converting enzyme (ACE-I) inhibitory activity assay

Angiotensin I-converting enzyme inhibitory activity was determined using the protocol described by Li et al. (2005) by direct spectrophotometric measurement of hippuric acid (HA) released from HHL by ACE-I. The sample solution including 40 µL of 4 % (w/v) SPH and 50 µL of 5 mM HHL (prepared in a 100 mM sodium borate buffer containing 300 mM sodium chloride) was vortexed and incubated at 37 °C for 5 min before adding 10 µL of ACE-I solution (250 mU/mL) to initiate the reaction. The reaction was conducted at 37 °C for 30 min and 100 µL of 1M HCl was added to terminate the reaction. Three hundred microliters of sodium borate buffer was added to bring the volume of reaction mixture to 500 µL. Subsequently, 600 µL of quinolone was added and mixed for 10 sec followed by 200 µL of benzene sulfonyl chloride (BSC) which was mixed in the dark for 20 sec. After 30 min incubation in the dark, 3.7 mL of ethanol was added and further incubated in the dark for 30 more min. Finally, absorbance of the reaction mixtures was recorded at 492 nm. ACE-I inhibitory activity (%) was calculated as follows:

$$\text{ACE-I inhibitory activity (\%)} = \frac{B - A}{B - C} \times 100 \quad (4)$$

Where, 'A' represents the absorbance of SPH sample, 'B' represents the absorbance of control

(sodium borate buffer replaced SPH), and 'C' represents the absorbance of the reaction blank (HCl was added before ACE-I).

In addition, the, the concentration of SPH required to produce 50% of the ACE-I inhibitory activity under the described condition (IC_{50} value of SPH) was also determined using regression analysis of ACE-I inhibitor (%) versus log of SPH concentration (mg/mL) as follows:

$$IC_{50} = (50 - b)/m \quad (5)$$

Where, b is the intersection and m is the slope.

4.2.11 Statistical analysis

The reported data were expressed as means of triplicate observations ± standard derivation. Data were subjected to one way analysis of variance (ANOVA) using JMP Pro 12.0 software (SAS Institute, Cary, NC, USA). Student's t test was also used to separate the means at $P \leq 0.05$.

4.3 Results and Discussion

4.3.1 Degree of hydrolysis of the hydrolyzed SPI's supernatant

The enzyme Alcalase was chosen for this study due to its broad specificity and high activity, compared to other proteases, which had been reported by Seo et al. (2008) and Segura-Campos et al. (2013). Figure 14 shows the hydrolysis profile of SPI obtained by treating with two different Alcalase concentrations, 0.5 AU (1.6 µL/g) and 1.0 AU (3.2 µL/g) respectively, at 50 °C. These levels were selected based on previous studies that used levels ranging from 0.3 AU and 1.5 AU on proteins in order to produce various sized peptides (Segura-Campos et al., 2013; Rayaprolu et al., 2013). One Anson unit (AU) is defined as that amount of enzyme which will digest (under specific conditions) urea-denatured hemoglobin to release a tricholoroacetic acid soluble product per minute and gives the same color with Folin-Ciocalteau Phenol reagent as one milli-equivalent

of tyrosine at 30 °C and pH 7.5 (Boyer, 1971). The incubation temperature, 50 °C, was chosen because it has been used in previous studies as the optimal temperature for Alcalase activity resulting in higher protein yield in comparison to an incubation temperature of 22 °C (ambient) (Vioque et al., 2000; Segura-Campos et al., 2013).

During the incubation time (0, 30, 60, 120 and 1440 min), the degree of hydrolysis increased rapidly in the initial 30 min, plateaued until 120 min and showed a sharp rise by 1440 min (24 hr). This trend is in agreement with other studies in which the reaction process curves of soy protein treated with Alcalase showed a high rate of hydrolysis within the first 60 min (Seo et al., 2008; Kong et al., 2008, Chen et al., 2011). As seen in Figure 4, the hydrolysis rate between 45 min and 60 min of incubation was low with no significant difference for both Alcalase treatments, 0.5 AU ($P = 0.06 > 0.05$) or 1.0 AU ($P = 0.82 > 0.05$). However, the degree of hydrolysis at 45 and 60 min between the two Alcalase treatments was significantly different ($P < 0.0001$). The decreased hydrolysis rate could be due to unavailability of specific peptide bonds, inhibition of active sites, or slow degradation of compact protein structure (Archer et al., 1973; Constantinides & Adu-Amankwa, 1980). There was a statistically significant difference between the samples treated with 0.5 AU and 1.0 AU Alcalase treatments at the same incubation time ($P < 0.0001$). At 1440 min, the degree of hydrolysis of 1.0 AU Alcalase reached 52.1 % (highest) while for the 0.5 AU it was 38.1 %. This was influenced by the enzyme to substrate concentration and the availability of the enzyme for catalysis. Since enzyme and substrate concentration are constant for each treatment the incubation time is the significant variable that influenced the deree of hydrolysis. Hence, the lower incubation time between 45 and 60 min, at same enzyme concentration, is the reason for the plateau observed at that time range.

Hydrolysis of SPI with Alcalase for 24 hr is considered excessive as further catalysis would have resulted in tri or dipeptides or amino acid residues, which was reported by previous researchers (Seo et al., 2008). At the same temperature and pH conditions the desired DH could be achieved with less catalysis time using a higher enzyme level. A higher enzyme concentration (1.0 AU or 3.2 µL/g) was selected for preparing the soy protein hydrolysates based on these results with 26% degree of hydrolysis.

4.3.2 Soluble protein content and yield estimation in the hydrolyzed SPI supernatants after enzymatic hydrolysis

The results shown in Figure 5 demonstrate the protein solubility between the hydrolyzed SPI obtained by treating at two different Alcalase levels, 0.5 AU and 1.0 AU. As the DH increased, the solubility increased which can be explained by the disruption of native SPI's secondary structure and cleavage of smaller peptide units from the initial protein by the protease (Adler-Nissen, 1976). As this forms (time of incubation and concentration of enzyme) the basis for all the next set of experiments, longer incubation time resulted in higher soluble protein content in the supernatant. The 1.0 AU Alcalase concentrations produced significantly higher soluble protein content in comparison to that of 0.5 AU Alcalase at all incubation times ($P < 0.0001$). Table 10 shows the freeze-dried hydrolysate yield and protein recovery of all samples relative to the initial SPI. Since the 1.0 AU Alcalase concentrations gave better yield of soluble protein, it was chosen for further testing including surface hydrophobicity, turbidity, and molecular size determination.

4.3.3 Surface hydrophobicity (S_0) and turbidity values of hydrolyzed SPI

Surface hydrophobicity and turbidity values of SPI (control) and SPH treated with 1.0 AU Alcalase at various incubation times are shown in Figure 6. As the incubation time increased, the S_0 values increased with highest after 30 min incubation. No significant increase was observed in

S_0 values between 30 and 45 min incubation ($P = 0.06 > 0.05$) and between 60 and 120 min incubation ($P = 0.45 > 0.05$). However, there was a significant increase in S_0 between 45 and 60 min incubation ($P = 0.003 < 0.05$). Hydrolysate after 1440 min incubation had the highest S_0 value, approximately four fold, in comparison to that of the control (SPI). The increase in S_0 values of hydrolyzed SPI samples could be explained by the catalysis mechanism of Alcalase. Serine endo-proteases hydrolyze peptide bonds in the core of the polypeptide due to the presence of a serine group in its active site. However, Alcalase/Subtilisin can cleave any peptide bond irrespective of the preceding and succeeding amino acids. Hence, depending on the percent of hydrolysis the hydrophobic groups are exposed to the solvent. The result is an increased exposure of non-polar/hydrophobic amino acid groups during the enzymatic hydrolysis that are unexposed in the native protein (Nielsen & Olsen, 2002). An increase in S_0 was observed in SPH samples is due to their hydrophobic groups present on the surface of protein and in contact with the polar aqueous medium (Chen et al., 2011). Presence of a large proportion of hydrophobic amino acids can be correlated to the rise of bitterness (Llano et al., 2004; FitzGerald & O'cuinn, 2006). In addition, the bitterness of hydrolyzed protein solution seemed to be more pronounced when the hydrolysis was very extensive while limited hydrolysis might prevent or minimize the formation of bitter taste peptides (Adler-Nissen, 1976). The sample incubated for 120 min had lower value of surface hydrophobicity which can be correlated to a low level of bitterness. In comparison, the SPH sample prepared for the longest incubation time (1440 min) had very high surface hydrophobicity that might potentially result in severe bitterness.

Turbidity were tested to determine the cloudiness of SPI and SPH solutions. Turbidity values of SPI (control) and hydrolyzed SPI treated with 1.0 AU Alcalase at various incubation times are shown in (Figure. 3). Turbidity values decreased significantly ($P < 0.0001$) from 2.7 to

0.9 as the hydrolysis of SPI progressed over time from 30 min to 1440 min. The SPH obtained after 30, 45 min incubation showed no significant difference in turbidity among the samples ($P = 0.14 > 0.05$). There was no statistically significant difference in turbidity between the 120 and 1440 min duration of incubation of SPH ($P = 0.95 > 0.05$), but they were both significantly lower turbidity in comparison to other incubation times and the control ($P < 0.0001$). The decrease in turbidity values with increase in incubation time could be explained by gradual hydrolysis of large molecular structure of the native protein by the enzyme. These results were in agreement with a study published by Lee (2011).

Considering the soluble protein content, turbidity, and surface hydrophobicity values the SPI treated with 1.0 AU Alcalase for 120 min is considered to be ideal, with a 43 % soluble protein recovery. It showed the lowest opacity and surface hydrophobicity (S_0), only one fold higher than the SPI control sample.

4.3.4 Molecular size of SPH

Figure 7 shows the SDS-PAGE electrophoretogram of SPI and SPHs obtained after various incubation times. The untreated SPI sample (lane numbered 1 in Figure 7) displayed major bands that could be identified as follows: the α', α, and β sub-units of β-conglycinin (7S globulin) at 80, 76, and 50 kDa respectively as well as the two acidic subunits and the two basic subunits of glycinin (11S globulin) at 35, 33, 22, and 20 kDa (Nielson, 1985; Iwabuchi & Yamauchi, 1987; Kim et al., 1990). The two subunits α'and α of 7S globulin, 80 and 76 kDa respectively, were not represented in SPH samples (lanes numbered 2 to 6 in Figure 7). In addition, the relative intensity (%) of the β sub-unit of 7S globulin significantly decreased in comparison to intensity of SPI which was normalized to a controlled level (100 %) (Figure 8). The same trend was observed in the two acidic subunits (33 and 35 kDa respectively) of 11S globulin in which they gradually decreased

throughout the length of incubation and showed a lowest relative intensity at 1440 min incubation (Figure 8). These results indicate that 7S globulin and acidic subunits of 11S globulin were unsustainable and sensitive to Alcalase cleavage even at the early stage of incubation (30 min). This can be explained by the exposed hydrophilic regions in these molecules which make them more susceptible to proteolytic attack (Shutov et al., 1996).

The basic subunits (22 and 20 kDa respectively) of 11S globulin were faint at 30 min of incubation, unnoticeable in the next 45, 60, and 120 min of incubation, but reappeared after 1440 min of incubation (Figure 7). This also confirmed that their relative intensities were not significantly different in comparison to the control, SPI (Figure 9). Therefore, the basic subunits of 11S globulin seemed to be more resistant to protease cleavage than 7S globulin and acidic subunits of 11S globulin. It can be explained by the higher compact structure with more hydrophobic groups of the basic subunits of 11S globulin (Peng et al., 1984). Figure 10 shows the relative intensity of molecular weight bands lower than 17 kDa of SPH which significantly increased with prolonged incubation time. This pattern indicated that Alcalase hydrolysis resulted in the loss of high molecular weight polypeptides ($\geq$ 60 kDa) and produced new polypeptides lower than 17 kDa. The results from the gel electrophoresis study are in agreement with the findings of other authors (Ortiz & Wagner, 2002; Surówka et al., 2004). Based on the SDS-PAGE profiles, the liberation of low molecular weight peptides by enzymatic hydrolysis contributed to the increase in soluble protein content and surface hydrophobicity, and the decreases in turbidity of SPH samples.

4.3.5 Angiotensin-I converting enzyme inhibition activity

ACE-I inhibitory activities of SPH derived at various incubation times are presented in Table 11. Soy protein isolate sample showed no ACE-I inhibitory activity while the SPH samples

showed ACE-I inhibitory activities ranging from 33.6 % (1.0 AU, 30 min) to 66.6 % (1.0 AU, 120 min). The ACE-I inhibitory activity of SPH increased during the first 120 min (66.6 %) and decreased to 40.4% after 1440 min of incubation. The significant increase in ACE-I inhibitory activity of SPH ($P < 0.0001$) might be explained by the tendency of Alcalase to produce peptides with hydrophobic residues at the C-terminus (He et al., 2012). These hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine, and proline have been reported as the most effective C-terminal residues that enhance the binding of the peptides to ACE-I (Cheung et al., 1980). However, it has been reported by He et al. (2012) that excessive protein degradation during longer incubation time resulted in significantly low ACE-I inhibitory activity which was also observed in this study. The IC_{50} values of all SPH samples ranged from 30.4 mg/mL to 76.4 mg/mL and stronger activity is representative of lower IC_{50} values among a set of samples. The best IC_{50} value was produced by SPH treated with 1.0 AU/mL 120 min. Although the ACE-I inhibitory activity of the SPH was significantly low ($P < 0.0001$) in comparison to Captopril (4.35 ng/mL), ACE inhibitor peptides derived from SPH may be an alternative choice for both treatment and prevention of hypertension due to its advantages such as no harmful side effects and lower cost (García et al., 2013)

4.4 Conclusion

This study demonstrated that potential bioactive protein hydrolysates with better solubility and higher protein quality can be obtained from this non-GM soy cultivar. The established optimal conditions to prepare SPH with high soluble protein, with significantly low opacity and surface hydrophobicity is: treatment with 1.0 AU (3.2 µL/g) Alcalase concentration at 50 ºC temperature for 120 min incubation. The limited proteolysis of SPI provided hydrolysates with highest ACE-I

inhibitory activity (66.6 %). Hence, SPH from non-GM soybeans is a protein-rich nutraceutical that can be incorporated in food products, especially beverages. Further studies can include purification of SPH for enhanced activity and product application for potential commercialization.

References

Adler-Nissen J. (1976). Enzymatic-hydrolysis of proteins for increased solubility. *Journal of Agricultural Food Chemistry*, 24(6): 1090-1093.

Archer, M.C., Ragnarsoon, J.O., Tannenaum, S.R., & Wang, D.I.C. (1973). Enzymatic solubilization of an insoluble substrate, fish protein concentrate: process kinetic consideration. *Journal of Biotechnology and Bioengineering*, 15: 181-96.

Boyer, P.D. (1971). The Enzymes. Eds. Boyer PD, Krebs EG, Sigman DS. Vol. III, 3rd ed., p. 564. *Academic Press*, Waltham, MA.

Bucci, L.R. & Unlu, L. (1999). Energy-yielding macronutrients and energy metabolism in sport nutrition. Protein and amino acid supplements in exercise and sport (1st ed). *CRC Press*, Boca Raton, FL, p 191-210.

Campbell, N.F., Shih, F.F., Hamada, J.S., Marshall, W.E. (1996). Effect of limited proteolysis on the enzymatic phosphorylation of soy protein. *Journal of Agricultural Food Chemistry*, 44: 759–762.

Chel-Guerrero, L., Domínguez-Magaña, M., Martínez-Ayala, A., Dávila-Ortiz, G., & Betancur-Ancona, D. (2012). Lima bean (Phaseolus lunatus) protein hydrolysates with ACE-I inhibitory activity. *Food and Nutrition Sciences*, 3: 511-521.

Chen, L., Chen, J., Ren, J., Zhao, M. (2011). Modifications of soy protein isolates using combined extrusion pre-treatment and controlled enzymatic hydrolysis for improved emulsifying properties. *Food Hydrocolloids*, 25: 887-897.

Cheung, H.S., Wang, F.L., Ondetti, M.A., Sabo, E.F., & Cushman, D.W. (1980). Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *Journal of Biological Chemistry*, 255: 401-407.

Chiang, W.D., Tsou, M.J., Tsai, Z.Y., Tsai, T.C. (2005). Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chemistry*, 98: 725-732.

Constantinides, A., & Adu-Amankwa, B. (1980). Enzymatic modification of vegetables protein: mechanism, kinetics, and production of soluble and partially soluble protein in batch reactor. *Journal of Biotechnology and Bioengineering*, 13: 1543-65.

Deeslie, W.D., & Cheryan, M. (1988). Functional properties of soy protein hydrolysates from a continuous ultrafiltration reactor. *Journal of Food Science*, 57: 411-413.

Iwabuchi, S., & Yamauchi, F. (1987). Determination of glycinin and β-conglycinin in soybean protein by immunological methods. *Journal of Agricultural Food Chemistry*, 35: 200-205.

FitzGerald, R.J., & O'cuinn, G. (2006). Enzymatic debittering of food protein hydrolysates. *Biotechnology advances*, 24(2): 234-237.

Jiang, J., Xiong, Y.L., & Chen, J. (2010). pH Shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of Agricultural Food Chemistry*, 58(13): 8035-8042.

Jung, S., Murphy, P.A., & Johnson, L.A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science*, 70(2): 180-187.

Gao, B., & Zhao, X. (2012). Modification of soybean protein hydrolysates by alcalase catalyzed plastein reaction and the ACE-Inhibitory activity of the modified product in vitro. *International Journal of Food Properties*, 15(5): 982-996.

García, M.C., Puchalska, P., Esteve, C., & Marina, M.L. (2013). Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrences bioactivities. *Talanta*, 106: 328-349

Hayakawa, S., & Nakai, S. (1985). Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *Journal of Food Science*, 50: 486-491.

He, R., Ma, H., Zhao, W., Zhao, J., Luo, L., & Zhu, W. (2012). Modeling the QSAR of ACE-Inhibitory peptides with ANN and its applied illustration. *International Journal of Peptides*, doi:10.1155/2012/620609.

Kim, Y., Park, S., & Rhee, C. (1990). Functional properties of proteolytic enzyme modified soy protein isolate. *Journal of Agricultural Food Chemistry*, 38: 651-656.

Kong, X., Guo, M.M., Hua, Y., Cao, D., & Zhang, C.M. (2008). Enzymatic preparation of immunomodulating hydrolysates from soy proteins. *Bioresearch Technology*, 99: 8873-8879.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, 227: 680-686.

Lassissi, T.A., Hettiarachchy, N.S., Rayaprolu, S.J., Kannan, A., & Davis, M. (2014). Functional properties and Angiotensin-I converting enzyme inhibitory activity of soy-whey proteins and fractions. *Food Research International*, 64: 598-602.

Lee, J. (2011). Soy protein hydrolysate: solubility, thermal stability, bioactivity, and sensory acceptability in a tea beverage. Master's thesis, University of Minnesota.

Li, G.H., Liu, H., Shi, Y.H., & Le, G.W. (2005). Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides. *Journal of Pharmaceutical and Biomedical Analysis*, 37(2): 219-224.

Llano, D.G., Herraiz. T., & Polo, M.C. (2004). Chapter 6: Peptides. In: Handbook of food analysis: Physical characterization and nutrient analysis (Revised ed). *CRC Press*, Boca Raton, FL, p 125-148.

Lorenzo, L.K. (2008). Improving the solubility of yellow mustard precipitated protein isolate in acidic aqueous solutions. Master's thesis, University of Toronto.

Michelfelder, A.J. (2009). Soy: a complete source of protein. *American Family Physician*, 79(1): 43-47.

Mullally, M.M., Meisel, H., & FitzGerald, R.J. (1996). Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. *Biological Chemistry Hoppe-Seyler*, 377: 259-260.

Nielsen, P.M., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science*, 57: 1223-1229.

Nielsen, P.M., & Olsen, H.S. (2002). Chapter 7: Enzymatic modification of food protein. Book: Enzymes in food technology (1st ed). *CRC Press*, Boca Raton, FL, 109-140.

Nielson, C. (1985). Structure and complexity of the 11S polypeptides in soybean. *Journal of American Oil Chemists' Society*, 62: 1680-1685.

Ohnishi, S.T., & Barr, J.K. (1978). A simplified method of quantitating protein using the biuret and phenol reagents. *Analytical Biochemistry*, 86(1): 193-200.

Ortiz, S.E.M., & Wagner, J.R. (2002). Hydrolysates of native and modified soy protein isolates: structural characteristics, solubility, and foaming properties. *Food Research International*, 35: 511-518.

Peng, I.C., Quass, D.W., Dayton, W.R., & Allen, C.E. (1984). The physicochemical and functional properties of soybean 11S glolulin. A review. *Cereal Chemistry*, 61(6): 480-490.

Qi, M., Hettiarachchy, N. S., & Kalapathy, U. (1997). Solubility and emulsifying properties of soy protein isolates modified by pancreatin. *Journal of Food Science*, 62: 1110-1115.

Ravichandran, M. & Hettiarachchy, N.S. (2013). Preparation of soy protein hydrolysates through alcalase treatment using response surface methodology with Alzheimer's β-amyloid (Aβ 1-42) peptide aggregation inhibition property. *Journal of the Federation of American Societies for Experimental Biology*, 27(1_MeetingAbstracts), 840-5.

Rayaprolu S.J., Hettiarachchy N.S., Chen, P., Kannan, A., & Mauromostakos, A. (2013) Peptides derived from high oleic acid soybean meals inhibit colon, liver and lung cancer cell growth. *Food Research International*, 50(1): 282-288.

Riaz, M. (2005). Processing of soybeans into ingredients. In: Riaz M, editor. Soy applications in food. Boca Raton, Fla.: *CRC Press*, p 41-62.

Rutherfurd, S.M. (2010). Methodology for determining degree of hydrolysis of proteins in hydrolysates: a review. *Journal of AOAC International*, 93(5): 1511-1522.

Scheide, J.D., & Brand, K.E. (1987). Process for the extraction of protein from soy flour. *U.S. Patent No. 4,704,289*. Washington, DC: U.S. Patent and Trademark Office.

Segura-Campos, M.R., Peralta-González, F., Castellanos-Ruelas, A., Chel-Guerrero, L.A., & Betancur-Ancona, D.A. (2013). Effect of Jatropha curcas peptide fraction on the angiotensin I-converting enzyme inhibitory activity. *BioMeb Research International*, doi:10.1155/2013/541947.

Seo, W.H., Lee, H.G., & Baek, H.H. (2008). Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. *Journal of Food Science*, 73(1): S41-S46.

Shutov, A.D., Kakhovskaya, I.A., Bastrygina, A.S., Bulmaga, V.P., Horstmann, C., & Muntz, K. (1996). Limited proteolysis of β-conglycinin and glycinin, the 7S and 11S storage globulins from soybean [Glycine max (L.) Merr.] Structural and evolutionary implications. *European Journal of Biochemistry*, 241: 221-228.

Sun, X.D. (2011). Enzymatic hydrolysis of soy proteins and the hydrolysates utilisation. International *Journal of Food Science and Technology*, 46: 2447-2459.

Surówka, K.D., Zmudziński, D., & Surówka, J. (2004). Enzymic modification of extruded soy protein concentrates as a method of obtaining new functional food components. *Trends in Food Science and Technology*, 15: 153-160.

Utsumi, S., & Kinsella, J. (1985). Forces involved in soy protein gelation: effects of various reagents on the formation, hardness and solubility of heat-induced gels made from 7S, 11S and soy isolate. *Journal of Food Science*, 50(5): 1278–1282.

Vioque, J., Clemente, A., Sánchez-Vioque, R., Pedroche, J., & Millán, F. (2000). Effect of Alcalase on olive pomace protein extraction. *Journal of American Oil Chemists' Society*, 77: 181-85.

Walsh, D.J., McCarthy, E., Murphy, S., & Fitzgerald, R.J. (2003). Modification of the nitrogen solubility of soy protein isolate following proteolysis and transglutaminase cross-linking. *Food Research International*, 36: 677-683.

Yang, B., Yang, H., Li, J., Li, Z., Jiang, Y. (2011). Amino acid composition, molecular weight distribution and antioxidant activity of protein hydrolysates of soy sauce lees. *Journal of Food Chemistry*, 124: 551-555.

Zmudziński, D., Surówka, K. (2003). Limited enzymic hydrolysis of extruded soy flours as a

method for obtaining new functional food components. *Polish Journal of Food Nutrition Sciences*, 12(53): 171-177.

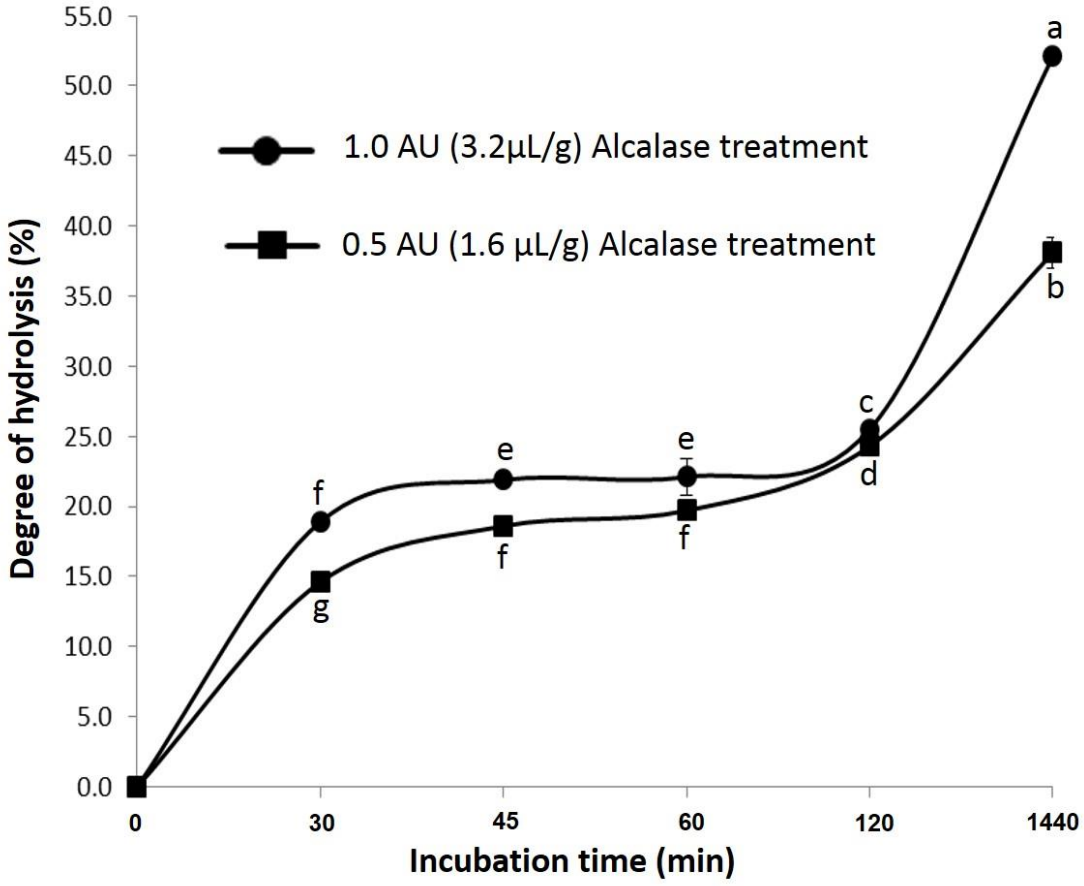


Figure 4: Degree of hydrolysis (%) of SPI treated with Alcalase (0.5 AU (1.6 µL/g) and 1.0 AU (3.2µL/g) at various incubation times.

Points on the curves are presented as means of triplicate analysis with error bars representing standard deviation and those not connected with same letters are significantly different at P value < 0.05.

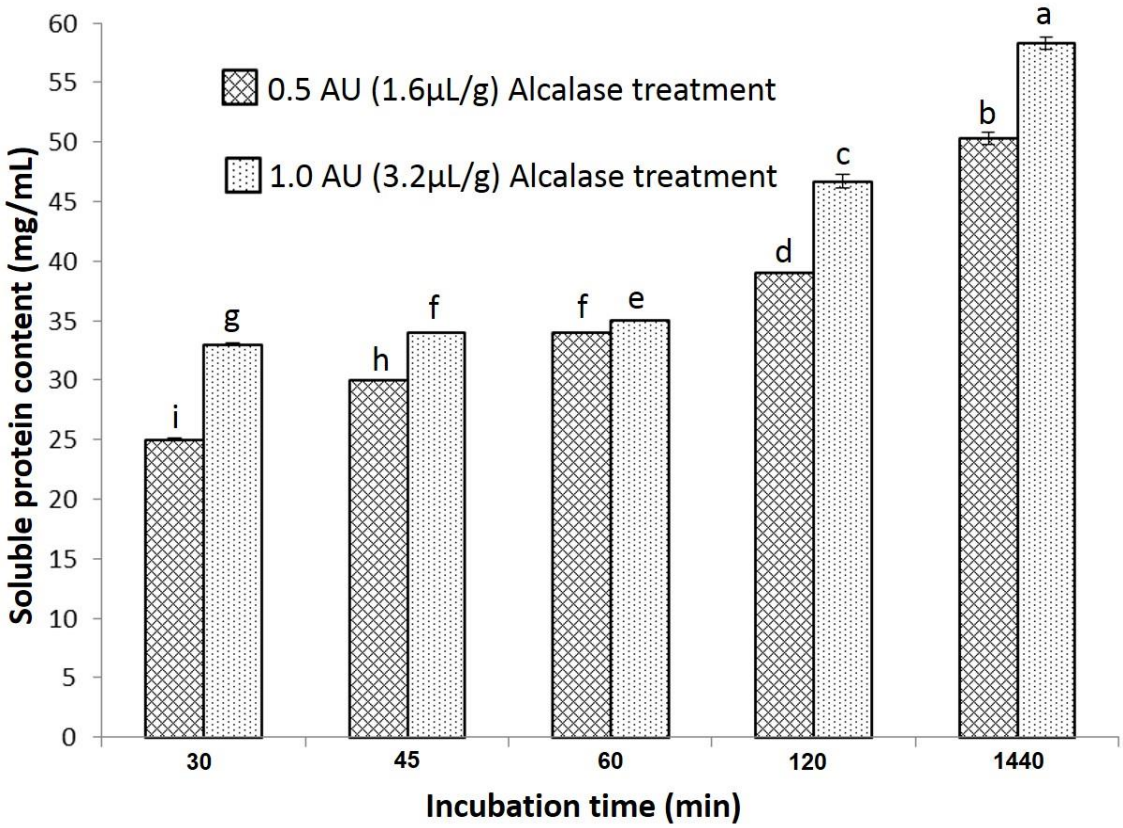


Figure 5: **Soluble protein content (mg/mL) of SPI treated with Alcalase concentrations (0.5 AU (1.6 µL/g) and 1.0 AU (3.2µL/g)) at various incubation times.**
Bars are presented as means of triplicate analysis with error bars representing standard deviation and those not connected with same letters were significantly different at P value < 0.05.

Table 10: Yield and protein recovery of SPH treated Alcalase (0.5 AU (1.6 µL/g) and 1.0 AU (3.2µL/g)) at various incubation times.

Alcalase activity	Incubation time (min)	Freeze dried SPH yield[a] (%)	Protein recovery[b] (%)
0.5 AU/mL	30	25.0 ± 2.4[d]	23.5 ± 2.5[d]
	45	31.3 ± 1.2[c]	29.0 ± 1.3[c]
	60	32.7 ± 0.9[c]	29.6 ± 0.7[c]
	120	35.3 ± 0.3[c]	31.8 ± 0.5[c]
	1440	47.7 ± 1.8[b]	43.9 ± 1.7[b]
1.0 AU/mL	30	34.3 ± 1.9[c]	31.8 ± 1.4[c]
	45	32.7 ± 0.5[c]	30.3 ± 0.6[c]
	60	33.3± 1.4[c]	32.7 ± 0.9[c]
	120	44.3 ± 0.8[b]	42.0 ± 0.7[b]
	1440	55.0 ± 2.0[a]	50.6 ± 1.9[a]

Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters in each column are significantly different at p value < 0.05.

[a] Freeze-dried SPH yield (%) was calculated as percentage of the amount of freeze dried hydrolysate derived from dry SPI after the enzymatic hydrolysis.

[b] Protein recovery (%) was calculated as the percentage of the total protein content (%N × 6.25) present in each hydrolysate after the enzymatic hydrolysis to the total protein content in SPI prior to the enzymatic hydrolysis.

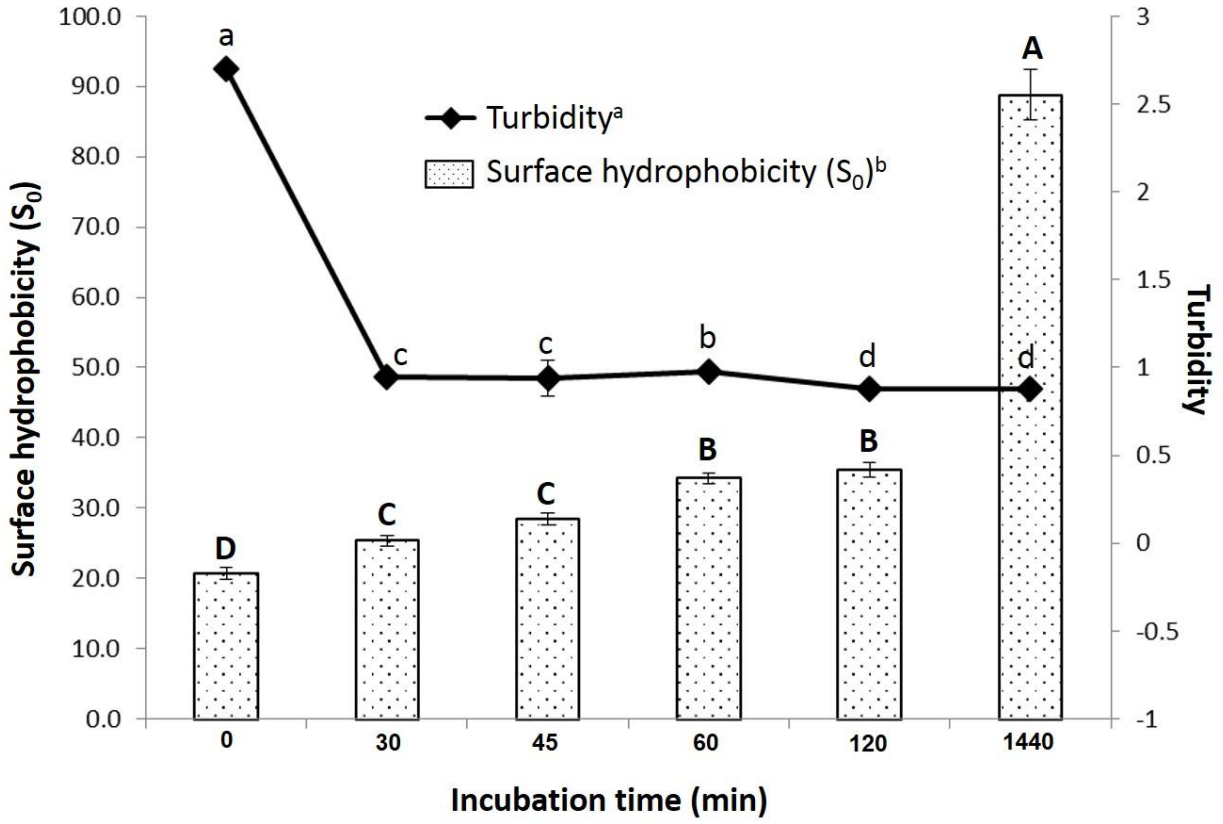


Figure 6: Turbidity and surface hydrophobicity (S_0) of SPH treated with 1.0 AU (3.2µL/g) Alcalase at various incubation times.

Values (bars and points) are means of triplicate analysis with error bars representing standard deviations and those not connected with same letters are significantly different at P value < 0.05.

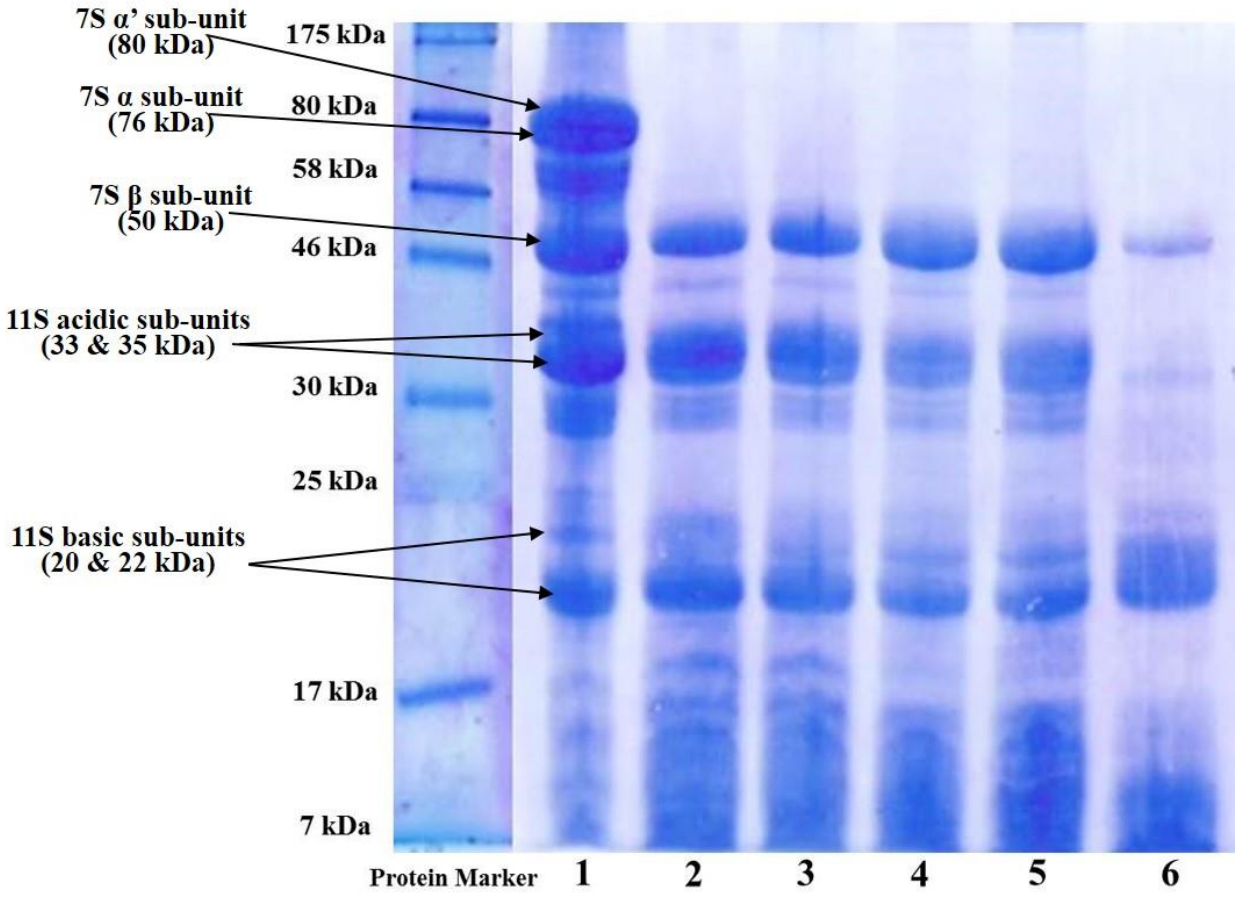


Figure 7: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of SPI (control) and SPH treated with 1.0 AU (3.2µL/g) Alcalase at various incubation times.
Sample numbers are designated as: 1 = Untreated; 2 = incubated for 30 min; 3 = incubated for 45 min; 4 = incubated for 60 min; 5 = incubated for 120 min; and 6 = incubated for 1440 min.

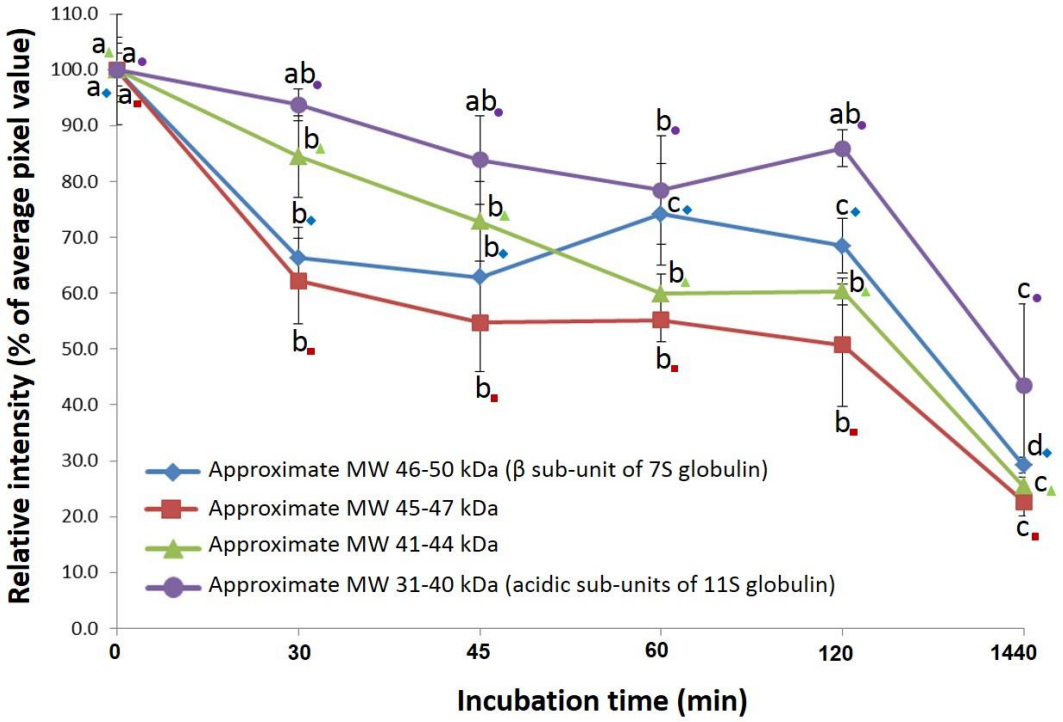


Figure 8: Relative intensity of protein segments ranging approximately from 31 to 56 kDa of SPI (control) and SPH treated with 1.0 AU (3.2µL/g) Alcalase at various incubation times. Intensity of SPI (0 min) was normalized to the control level (100%). Relative intensity values are presented as means ± standard deviation those not connected with same letters for each molecular weight cut-off hydrolysates are significantly different at P value < 0.05.

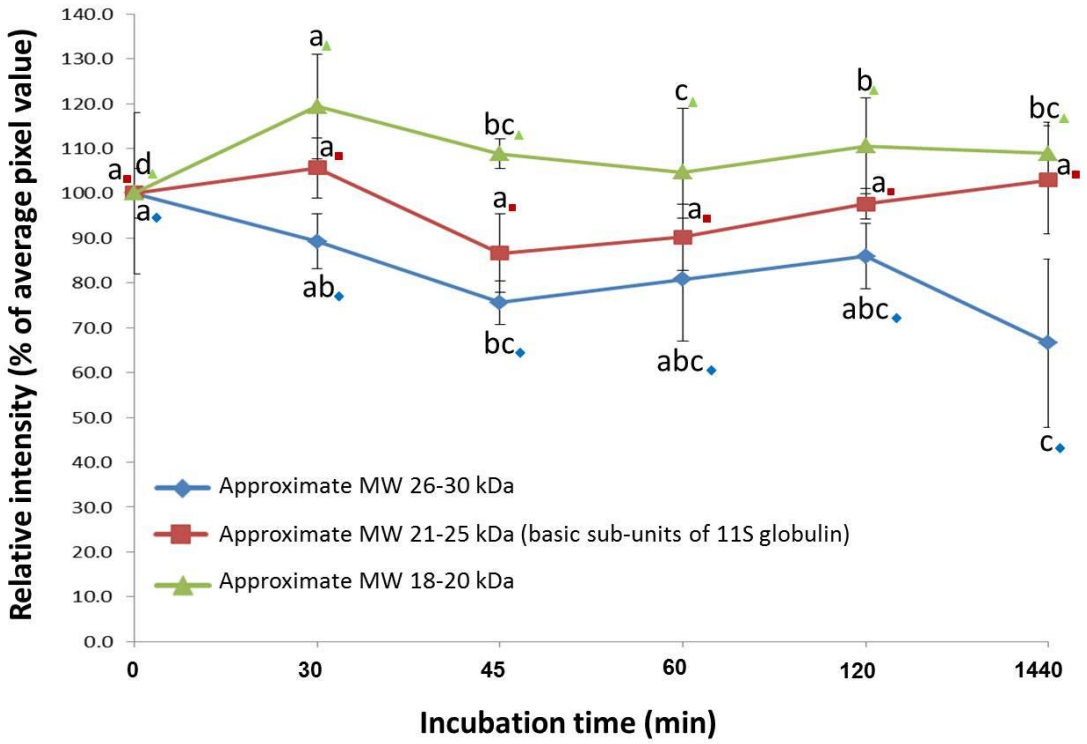


Figure 9: Relative intensity of protein segments ranging approximately from 18 to 30 kDa of SPI (control) and SPH treated with 1.0 AU (3.2µL/g) Alcalase at various incubation times. Intensity of SPI (0 min) was normalized to the control level (100%). Relative intensity values are presented as means ± standard deviation those not connected with same letters for each molecular weight cut-off hydrolysates are significantly different at P value < 0.05.

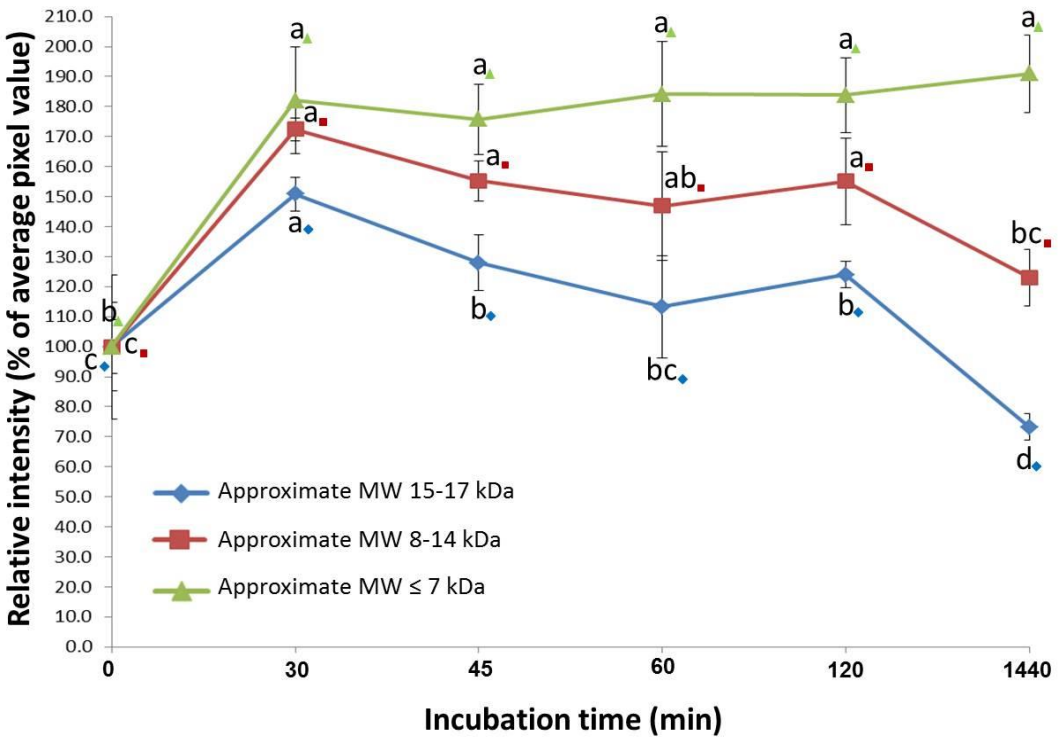


Figure 10: Relative intensity of protein segments ranging approximately lower than 17 kDa of SPI (control) and SPH treated with 1.0 AU (3.2µL/g)
Alcalase at various incubation times. Intensity of SPI (0 min) was normalized to the control level (100%). Relative intensity values are presented as means ± standard deviation those not connected with same letters in each category are significantly different at P value < 0.05.

Table 11: ACE-I inhibitory of SPI (control) and SPH samples treated with 1.0 AU (3.2µL/g) Alcalase at various incubation times[1]

Soy protein sample	ACE-Inhibition activity (%)[3]	IC_{50} value[4]
SPI R08-4004	1.0 ± 0.1^{e}	nd[5]
1.0 AU/mL 30 min	33.6 ± 3.2^{d}	76.4 ± 0.4^{a}
1.0 AU/mL 45 min	37.9 ± 0.9^{cd}	68.3 ± 1.7^{b}
1.0 AU/mL 60 min	61.9 ± 2.5^{b}	35.5 ± 3.2^{d}
1.0 AU/mL 120 min	66.6 ± 0.8^{b}	30.4 ± 1.0^{d}
1.0 AU/mL 1440 min	40.4 ± 1.7^{c}	62.3 ± 0.4^{c}
Captopril[2]	93.6 ± 0.6^{a}	4.35 ± 0.0^{f}

[1]Values were presented as means ± standard deviation. Within the same row, values connected with different letters are significantly different at p value < 0.05).
[2]Captopril was used as positive control inhibitor sample.
[3]ACE-I inhibition was determined with 4% protein concentration (w/v) for SPI and SPH samples, and with 0.1 µM Captopril solution.
[4]The unit of IC_{50} values is ng/mL for Captopril and mg/mL for SPH samples.
[5] Not determined.

Chapter 5: Development of protein-rich beverage using non-GM soybean (R08-4004) and sensory and shelf-life evaluation

Abstract

Alcalase hydrolyzed protein from a non-transgenic (non-GM) soybean line was used to develop a beverage containing 20g of protein per 500mL serving size. Three flavors: Chai tea, tangerine, and mixed berries were prepared using ingredients including bitter blocker, masking agent, and citric acid to enhance the taste and sensory appeal for acceptance by a consumer panel. Protein solubility, pH, microbial growth, instrumental color parameters, and turbidity were measured to evaluate the shelf-life stability of the beverage over a period of 42 days at refrigerated storage (5 °C). The tangerine and mixed berries flavor received overall highest score from the sensory panelists. Citric acid alone or in combination with bitter blocker or masking agent lowered the bitterness. Pasteurization (90-95 °C, 5 min) was effective in preventing microbial growth. Although pH remained constant, decrease in protein solubility and color changes were observed over the storage time in all three formulations. Turbidity (cloudiness) in the Chai tea flavor increased over the storage period while the tangerine and mixed berries flavored beverages did not show any change. Overall, the tangerine and mixed berries flavored beverages have the potential for commercial application.

5.1 Introduction

Protein based beverages have experienced a record growth of approximately 233%, due to an increase in consumer demand and expanded the market from 2008 to 2013 (Levesque, 2014). According to the market research, the protein drink segment is expected to have the highest forecasted growth rate of 90% and will reach US $4.1 billion through the year 2018 due to continuing consumer interest in healthy and nutritious eating habits (Levesque, 2014). Protein products have become more popular to general consumers, especially ready-to-drink protein beverages which are more appealing to time-crunched individuals who are also looking to improve their diets (Haderspeck, 2014). Soy is the only known plant source that contains all nine essential amino acid at levels as high as those from meat, milk, and egg (Ang *et al*., 1985; Tockman, 2002). However, native soy protein cannot be used effectively as a supplement in beverages, especially acidic beverages, since it is largely insoluble and separates on storage (Cho *et al*, 2008). Clarity is a challenge when formulating high-protein drinks since insolubility of native protein isolate is undesirable to consumers (Cho *et al*, 2008). Previous research has shown that hydrolysates prepared from soy protein have better solubility and applicability in high protein products (Wu *et al*, 1998; Lee, 2011). In addition, interest in protein hydrolysate incorporation in nutritional products has been increasing due to their effective digestion and faster gastrointestinal absorption compared to intact protein or free amino acids (Sun, 2011; Ziegler *et al*, 1990).

Another challenge faced by beverage developers when working with soy protein is lack of appealing flavor and presence of bitter note (MacLeod, 1988). Bitterness has been a major limitation in utilizing protein hydrolysates in various products, especially in beverages (Cho *et al*., 2004). Use of appropriate bitter blocker agents along with sweeteners and flavor agents can overcome the unpleasant taste of soy protein hydrolysates (Sun, 2011).

To validate the marketability of new food products, their physicochemical properties, sensory acceptability, and shelf- life aspect should be tested (Brown, 2011). Instrumental techniques can be used to analyze changes in physicochemical parameters including color, pH, and turbidity as well as microbial activity (Brown, 2011). Consumer acceptance and preference sensory test has been used as an essential tool to evaluate the sensory attributes of a new product as well as to ensure that the optimal final product formula has been achieved (Fuller, 2011; Lawless & Heymann, 2010).

Hence, with a growing interest for non-genetically modified (non-GM) soy-based protein drinks, this study aims to develop protein-rich (20g/serving) drink utilizing soy protein hydrolysate with reduced/no bitterness. Physicochemical properties, sensory acceptability, and shelf stability of the new protein drink were also determined in this study.

5.2 Material and methods

5.2.1 Preparation of soy protein hydrolysates

Protein isolate (SPI) and enzyme digested hydrolysates (SPH) were prepared from ground and defatted soybean seeds of an Arkansas grown non-genetically modified (non-GM) cultivar, R08-4004, using aqueous extraction (pH 9.0) followed by Alcalase 2.4L hydrolysis (50 °C, 1.0 AU (3.2 µL/g), 120 min incubation).

5.2.2 Protein solubility

Protein solubility as a function of pH of SPI and SPH was determined by the method developed by Bera and Mukherjee (1989). One gram of protein samples were dispersed in 25 mL of deionized water and adjusted to a pH ranging from 3.0 to 11.0 with either NaOH (0.1 N or 1.0 N) or HCl (0.1 N or 1.0 N). The dispersions were stirred at ambient temperature for 30 min and

centrifuged at 10,000 × *g* for 10 min. The soluble protein contents of supernatants were determined by the modified Biuret method (Ohnishi & Barr, 1978). The percent protein solubility was calculated as follows:

Solubility (%) = (Protein content in supernatant/Total protein content in sample) x 100 (1)

5.2.3 Bitter masking study using Box- Behnken statistical design

A Box- Behnken design (BBD) (Box & Behnken, 1960) was used to optimize surface hydrophobicity (S_0) using bitter blocker (BB), masking agent (MA), and Stevia since bitterness of proteins is directly proportional to their S_0 values (Wu *et al*, 1998). Stevia powder was purchased from Ziki's TEAki Hut LLC (East York, PA, U.S.A) and BB and MA were purchased from Blue Pacific Flavors Inc., (City of Industry, CA), The concentration of BB, MA, and Stevia in SPH solution were selected as independent variables (coded: X_1, X_2, and X_3 respectively) and incorporated in the BBD design with surface hydrophobicity as the dependent variable (Y). Surface hydrophobicity (S_0) was tested using a hydrophobic fluorescence probe, 1-anilino-8-naphthalene sulfonate (ANS), binding method described by Hayakawa & Nakai (1985). A blank sample of SPH solution (with no BB, MA, and Stevia addition) was also tested for S_0 to compare the effect of BB, MA, and Stevia.

5.2.4 Beverage formulation

Three types of protein drinks were prepared in predetermined proportions using laboratory scale trials in order to optimize the formula. Protein drinks were prepared using two different bases: distilled water for tangerine flavor (beverage T), and mixed berries flavor (beverage MB), and brewed tea for Chai tea flavor (beverage C). A control formula was also prepared with no additional flavor for comparison. Natural mixed berries flavor were provided by Blue Pacific Flavors Inc., (City of Industry, CA, U.S.A). Other natural color and flavor agents were provided

by Carmi Flavor & Fragrance Co. and D. D. Williamson & Co., Inc., (Louisville, KY, U.S.A)**.** Chai tea and citric acid was purchased from a local food store (Fayetteville, AR, U.S.A).

The four beverage formulations (including control) were prepared with the freeze dried SPH powder by slowly adding to distilled water/ or brewed tea while stirring. The sweetener, BB, MA, natural color and flavor agents, and citric acid were added as required for each formulation. The drink was mixed for 2-3 min to obtain a homogenous product. This freshly prepared drink was filled into pre-sterilized glass bottle and pasteurized at 90 to 95 ºC with a 5 min holding time. Bottles were cooled to ambient temperature and stored in a refrigerator (5 ºC).

5.2.5 Sensory evaluation

Sensory evaluation of soy protein beverages was conducted at the University of Arkansas Sensory Science Center (Fayetteville, AR, USA). A total of 61 untrained consumers (27 females and 34 males) ranging in age from 19 to 50 years (31 ± 7.8 years) participated in the sensory evaluation. All participants had neither clinical history of major diseases (e.g., diabetes, cancer, cardiovascular disease, or renal disease) nor allergy to soy products.

Four types of soy protein beverage were prepared one day before the scheduled date for sensory evaluation and kept in the refrigerator (4 ± 0.5 °C). Approximately 20 mL of each beverage was placed in a soufflé cup (60 mL), identified with a 3-digit code, and covered with a plastic transparent lid. The participants evaluated all four samples for appearance, odor, flavor, mouthfeel, and overall impression on 9-point hedonic scales ranging from 1 (“dislike extremely”) to 9 (“like extremely”). In addition, 3 sensory attributes, i.e., color, sweetness, and bitterness, were also evaluated on a 5-point “Just-About-Right” (JAR) scales (1 = “much too light/much too weak”, 3 = “JAR”, 5 = much too dark/much too strong”). Between each sample, panelists were asked to take a short 30 sec break for palate cleansing with spring water and unsalted crackers.

5.2.6 Shelf stability

The three beverage formulas, inlucding beverage T, beverage MB, and beverage C, were prepared and stored at 5 °C for 42 days shelf life test. Microbiological and physicochemical characteristics were analyzed at day 1 before and after pasteurization and throughout the shelf life period in biweekly intervals at day 14, 28, and 42. Microbiological evaluation included total plate count (TPC), and yeast and mold counts. Tryptic soy agar (TSA) (Becton Dickinson, Fraknlin Lakes, NJ) was used for TPC and Potato dextrose agar (PDA) (Becton Dickinson, Fraknlin Lakes, NJ) was used for yeast and mold count. Hundred microliters of each sample were spread plated onto both TSA and PDA plates after serial dilutions and incubated at 35 °C for 48 hours and 25 °C for 72 hours, respectively (Luvonga, 2012). The colonies were counted and recorded as colony forming units (CFU) per mL.

A modified Biuret test was used to determine the soluble protein content of samples during the storage (Ohnishi & Barr, 1978). The turbidity or cloudiness was determined using UV-Vis spectrophotometric method (Jiang *et al.*, 2010, Lee, 2011). All sample were vortexed before testing.

The color change in the beverage samples were evaluated using the "L^*, a^*, and b^*" Hunter Lab system. The L^* value represents the lightness (0 = black and 100 = white), a^* axis represents redness ($+a^*$) or greenness ($-a^*$), and b^* axis represents yellowness ($+b^*$) or blueness ($-b^*$). The apparatus was calibrated with a standard white tile before determining the samples were tested. The recorded values were converted into ΔE* (total color difference) according to the following equation (Calvo, 2004):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta b^*)^2 + (\Delta b^*)^2} \quad (2)$$

$\Delta L^* = L^*_{\text{sample}} - L^*_{\text{reference}}$; $\Delta a^* = a^*_{\text{sample}} - a^*_{\text{reference}}$; and $\Delta b^* = b^*_{\text{sample}} - b^*_{\text{reference}}$.

Reference value was from the fresh beverages prepared on day 1 before pasteurization (BP) and sample values were from beverages after pasteurization (AP) at various storage days (1 to 42). The values of ΔE^*, which were color difference between the samples over the storage period, were classified as "not noticeable" ($0 < \Delta E^* < 0.5$), "slightly noticeable" ($0.5 < \Delta E^* < 1.5$), "noticeable" ($1.5 < \Delta E^* < 3.0$), "well visible" ($3.0 < \Delta E^* < 6.0$), and "great" ($6.0 < \Delta E^* < 12.0$) (Cserhalmi *et al.*, 2006).

The pH of the samples (vortexed for homogeneity) was recorded using a pH meter (Orion 210A, Orion Research Inc., Boston, MA).

5.2.7 Statistical analysis

Data from all the studies were subjected to one way analysis of variance (ANOVA) using JMP Pro 12.0 software and reported as means of triplicate observations ± standard derivation. Student's t-test was also used to separate the means at $P < 0.05$.

The polynomial model equation for surface hydrophobicity (S_0) was determined as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (3)$$

Where, Y is the predicted response (S_0); β_0 is model constant (intercept); β_1, β_2, and β_3 are linear coefficients; X_1, X_2, and X_3 are the coded level of independent variables; β_{12}, β_{13}, and β_{23} are cross-product coefficients; X_1X_2, X_1X_3, and X_2X_3 are the interaction of independent variables' coded levels; and β_{11}, β_{22}, and β_{33} are the quadratic coefficients; X_1^2, X_2^2, and X_3^2 are the quadratic terms. In addition, the quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2 (Pasma *et al.*, 2013). Contour graphs were developed using the equation 2 to determine the interaction between two variables compared based on their effect on S_0. The

regression analysis and analysis of variation (ANOVA) for Box- Behnken design were conducted using JMP Pro 12.0 software (SAS Institute, Cary, NC, USA).

For sensory evaluation data, a two-way ANOVA treating sample type and panelist as a fixed effect and a random effect, respectively was used to determine the variation due to sensory appeal among the soy protein beverages. If a significant difference of means was determined, post hoc comparisons between independent variables were conducted using Tukey's honestly significant difference (HSD) tests. A statistically significant difference was defined at $P < 0.05$.

5.3 Result and discussion

5.3.1 Protein solubility

The profile of SPI and SPH's solubility in the pH range between 3.0 and 11.0 is shown in Figure 11. The SPI had lowest solubility (0.9 %) at pH 5.0 and highest (93.1 %) at pH 10.0 while SPH showed lowest solubility (48.7 %) at pH 4.0 and highest (98.3 %) at pH 7.0 which was significantly high when comparing SPI and SPH' s solubilities ($P < 0.0001$). Both had minimum solubility at pH 4.5 (isoelectric) and higher solubility values at pH 3.0, 7.0, and between 8.0 and11.0. This solubility profile of soy protein has been reported in several studies (Were *et al*., 1997; Jung *et al*., 2005; Ruiz-Henestrosa *et al*., 2009; Bae *et al*., 2012). The differences in solubility can be explained by the hydrolysis process which exposed hydrophobic portions of the protein structure. As the pH changed, the hydrophobic amino acids were exposed at the isoelectric point which allowed the protein to dissociate from water (Boyle *et al*., 2010). Solubility of both SPI and SPH improved at alkaline pH with highest at pH 7 (98.3 %) and pH 10 (93.1 %). Higher solubility of SPH was applicable in the beverage preparation.

5.3.2 Beverage formulation protocols

The aim of this study was to develop high protein beverage using SPH (88.3% protein concentration) that contains 20g of protein per 500mL serving size. However, exposure of hydrophobic amino acids during SPH preparation resulted in the development of a bitter note that must be overcome in order to make the product acceptable by consumers (Riaz, 2005; Sun, 2011). Although there are several methods, including treatment with activated charcoal or hydrophobic interaction chromatography to lower bitterness of proteins, these methods are unsuitable for food products and can potentially eliminate a fraction of peptides from the product (Neilsen, 2010; Sun, 2011). Hence, sensory acceptability was achieved by masking the bitter taste using a multifaceted approach which included addition of sweetener, flavor agent, food grade bitterness lowering compounds and organic acid compounds.

5.3.2.1 Beverage MB

Box Behnken design was used to find the optimal combination of BB (X_1), MA (X_2), and Stevia (X_3) for minimizing S_0, particularly in the mixed-berry flavored drink, which is directly proportional to lowering bitterness (Wu *et al*, 1998). Each coded factor was prescribed into three levels coded as -1, 0, and +1 represented for low, intermediate, and high value respectively. The effects of different concentrations of the three factors (BB, MA, and Stevia) on the surface hydrophobicity of SPH solution (4% w/v) as proposed by Box-Behnken design were analyzed. The experimental values of S_0 were fitted to a full quadratic second-order polynomial equation by applying multiple regression analysis. The regression coefficients were obtained to predict the polynomial model for S_0 when the values of BB, MA, and Stevia were substituted in the equation below.

$$(S_0) = 17815.3 - 8544\ BB - 2327.1\ MA + 2007.6\ Stevia + (2356.2\ BB\times MA) + (761.8\ BB\times Stevia) + (922\ MA\times Stevia) + (3767.8\ BB^2) \quad (4)$$

Significance of the polynomial model was determined using ANOVA. Bitter blocker showed significant linear and quadratic effects in reducing the S_0 of 4 % SPH solution ($P < 0.0001$), while Stevia and MA showed only linear effects ($P < 0.0001$). The combination treatments including (BB x MA), (BB x Stevia) and (MA x Stevia) were statistically significant ($P < 0.05$) according to the model. The contour plot of the response surface showed that BB was most efficient in reducing the S_0 of the SPH solution while Stevia had a minor effect. The model capability was examined using the F-test where ANOVA showed that the regression model was highly significant ($P < 0.001$). An optimal combination of BB, MA, and Stevia was obtained by solving the regression equation (4). The prediction profiler (Figure 12) showed a minimum S_0 value of 9161.8 with the corresponding optimal combination of 1.46 % BB, 1.0 % MA, and 0.02 % Stevia.

During the preliminary test, there was an increase in the bitter note of 4 % SPH solution due to the increase in Stevia higher than 0.02 % for sweeter taste. Hence, 0.02 % (w/w) pure Stevia powder was replaced with Truvia® (Cargill®, Wayzata, MN), which is commercially available with ingredients: erythritol, Stevia leaf extract, and natural flavors. This boosted the sweetness without increasing the caloric content in the three beverages (Persinger, 2014). Erythritol, an alcohol sugar (2R,3S)-butane-1,2,3,4-tetraol), naturally present in melons and peaches in small quantities, is accepted as a sugar-replacement due to its low-calories, tooth-friendly, zero toxic effects after consumption, and off-taste masking effect (Hartog *et al*, 2010, EFSA, 2015).

5.3.2.2 Beverage T

Fruit acids, particularly citrus/citric-acid- type flavor are shown to be efficient in improving consumer acceptability when combined with bitter-tasting ingredients such as proteins and peptides (Hazen, 2003). Thus, to develop the tangerine flavored beverage with lowest bitterness,

citric acid and tangerine natural flavor were used to provide a strong and favorable citrus flavor while masking the bitter note (Foster, 2007).

5.3.2.3. Beverage C

Beverage C was developed based on the hypothesis that the bitter note derived from SPH could be harmonized with the acceptable astringency and bitterness carrier such as a tea drink. In addition, tea is a low-acid drink with pH close to 7 in which SPH has high solubility. An ethnic flavor, Chai tea, that contains spices including cinnamon, cardamom, ginger, clove, and black pepper (Phillips, 2015) was used as a base to prepare the Chai tea flavored SPH drink. The bitterness reducing compounds, BB and MA, and citric acid were not needed in this formulation. Formulations for all the three beverages are shown in Table 12.

Control formulation for comparison was prepared using DI water, SPH and Truvia® without the flavor agent, BB, MA, or citric acid. All beverages were pasteurized at a temperature ranging from 90 to 95 °C for 5 minutes.

5.3.3 Sensory evaluation

The overall appearance of the four beverages, including C, MB, T, and control significantly varied according to the sensory panel evaluations ($P < 0.0001$). Participants liked the appearance of MB and T beverages among the four tested (Table 13). In addition, the panelists noted significant differences in flavor impression ($P < 0.0001$), but overall, the participants liked the flavor of beverage T.

As shown in Table 14, the participants rated color impression of the control beverage as too light, while that of MB and T were recorded as ‘just-about-right’. The color impression of beverage C was rated as too dark, which was implicit due to its formulation using prepared tea beverage. The four soy protein beverages significantly differed with respect to sweetness and

bitterness (P <0.0001). Participants rated sweetness of T and MB beverages as 'just-about-right' in comparison to the control sample ('too little'). The control sample received was rated 'too much' for bitterness. However, bitterness for tangerine or mixed berries flavor was rated as 'JAR' indicating that the added flavors decrease the bitterness intensity. The addition of citric acid for beverage T and BB, MA and citric acid combination for beverage MB successfully reduced the bitterness of the high protein beverages. Similar results were demonstrated by Lee (2011) using Alcalase-hydrolyzed soy protein formulated with lemon flavored sweet tea, indicating that citrus flavor or sour tasting ingredients might play an important role in minimizing bitter taste (Keast & Breslin, 2003). Additionally, Alder-Nissen (1986) demonstrated that citric acid could mask the bitter note of hydrolyzed proteins. The four soy protein beverages significantly differed (P <0.0001) with respect to oral tactile impression (mouth-feel) which was enhanced with the addition of flavors (Table 13), but overall, the participants liked the mouth-feel of beverage T. Among the three flavor types, Chai tea flavor was the least appreciated, which might have resulted due to its dark color and strong flavor. It may have also been due to the reason that it is not widely popular among U.S. consumers.

5.3.4 Shelf life evaluation

Apart from consumer acceptability, shelf life of a food product is a significant factor for its successful commercialization. Shelf life of the three SPH flavored beverages was evaluated for microbial growth, soluble protein content, pH, turbidity, and color over a period of 42 days under refrigerated storage (5 °C) conditions. These quality parameters were measured at day 1 (before and after pasteurization), day 14, day 28, and day 42. The unpasteurized samples served as control treatments for comparison.

5.3.4.1 Microbial survivors

No yeast or mold was observed on samples after pasteurization and during the storage period. The initial total bacterial counts in beverage C, MB, and T were 1.3, 1.3, and 1.2 CFU/mL, respectively, prior to thermal processing. Zero total bacterial counts were observed on samples after pasteurization and during the storage period. This demonstrated that the pasteurization process (90-95 °C, 5 min) effectively inhibited the growth of bacteria, yeast, and mold.

5.3.4.2 pH

The pH values of the three flavored SPH beverages over the storage period were determined and the data is presented in Figure 13, which shows that values were stable throughout 42 days of storage period. The pH among the flavored beverages varied due to the amount of citric acid added to their formulations. The beverage C without citric acid had highest pH value (pH 6.4) among the three drinks. Beverage MB that was formulated with 0.26% w/w citric acid had a pH of 5.0 and beverage T that had with 0.5% citric acid had a pH of 4.1. The low pH of the two beverages had potentially assisted in inhibiting bacterial growth.

5.3.4.3 Soluble protein content

The soluble protein content of the three SPH flavored beverages over the storage period are shown in Figure 14. The amount of SPH for preparing the three formulations was calculated as 22.7 g per 500 mL serving (based on 88.3% protein content of SPH), which was equivalent to 45.4 mg protein/mL, to ensure the claim - 20 g protein per container. At day 1 (BP), the soluble protein content of C, MB, and T beverages were 31.1 mg/mL, 22.4 mg/mL, and 17.8 mg/mL respectively. Differences in soluble protein content in among the beverages could be explained by the differences in pH. Higher pH contributed to higher soluble protein in the product based on the solubility profile as a function of pH that was shown in Figure 11.

In beverage C, the amount of initial soluble SPH measured at day 1 (BP) at pH 6.4 was 31.1 mg/mL (68% solubility) that was lower than the estimated soluble SPH at the same pH (37 mg/mL; 81 % solubility) based on the solubility profile (Figure 11). The presence of phenolic compounds in the tea base is expected to contribute to this change in solubility. The interactions between black tea tannin compounds, particularly theaflavins and thearubigins, with proteins are known to form insoluble complexes and decrease protein solubility (Ozdal *et al.*, 2013; Frazier, 2013). There was no significant difference in the soluble protein content of beverage C at day 1 (BP) and after pasteurization (AP) which was 31.1 mg/mL and 30.3 mg/mL respectively (P = 0.0970). Over the storage period, the soluble protein content in the beverage C decreased significantly (P < 0001) during day 14 to 28 storage from 29.2 mg/mL to 26.3 mg/mL and remained stable until day 42 (25.9 mg/mL).

In beverage MB, the amount of initial soluble SPH measured at day 1 BP at pH 5.0 was 22.4 mg/mL (48% solubility) that was lower than the estimated soluble SPH of at the same pH which was 25 mg/mL (56 % solubility) as observed in Figure 11. This could be due to the interactions between compounds found in BB and MA with the protein, leading to the lowered protein solubility. Similar to beverage C, there was no significant difference in the soluble protein content of beverage MB before and after pasteurization (P = 0.0998), but dropped significantly from day 14 to 28 which was 19.4 mg/mL and 16.9 mg/mL respectively, and was stable until day 42 (16.6 mg/mL).

In beverage T, the amount of soluble SPH at day 1 BP sample (pH 4.1) was 17.8 mg/mL (40% solubility) which was slightly lower than the estimated value at same pH (22 mg/mL; 49 % solubility) derived from Figure 11. This beverage has a pH value close to the isoelectronic point (pI = pH 4) of SPH that could explain the lowest soluble protein among the 3 protocols. Unlike

the other two flavors, the soluble protein content of beverage T significantly decreased to 16.6 mg/mL after pasteurization *(P* = 0.0018*).* Throughout the storage period the soluble protein content of beverage T continued to drop - 15.6 mg/mL on day 14 *(P* = 0.0030*)* and 14.3 mg/mL at day 28 *(P* = 0.0013*),* but remained stable until day 42 (14.2 mg/mL) *(P* = 0.6140*).*

Overall significance of this study is that the soluble protein content over the storage time in the three beverage formulations stabilized from day 28 of storage. The effect of pH played an important role in protein solubility during the storage period: high-acid beverages, T and MB had lower soluble protein content while low-acid beverage C had higher soluble protein content.

5.3.4.4 Turbidity

The turbidity values of the three SPH flavored beverages over the storage period are shown in Figure 15. Among the 3 formulations, the turbidity values of beverage C were lowest but increased significantly from 1.9 at day 1 (BP), 2.9 at day 14 ($P < 0.0001$) and 3.0 at day 28 ($P = 0.0133$). The turbidity of beverages T was 3.1 and MB of 3.5 did not change ($P = 0.4609$ and 0.0438 respectively) throughout the storage study. Turbidity is expected to be directly proportional to the amount of SPH that precipitated SPH and the decreasing of soluble protein content over the storage period that was in agreement with the study on SPH fortified black tea by Lee (2011).

5.3.4.5 Color

The three SPH beverages over the storage period (day 1 to day 42) are shown in Table 15. Their colors over the storage period varied as shown by differences in L^*, a^*, b^* (Table 16) and ΔE* values (Figure 16) which can be classified as “not noticeable” (0-0.5), “slightly noticeable” (0.5-1.5), “noticeable” (1.5-3.0), “well visible” (3.0-6.0), and “great” (6.0-12.0) (Cserhalmi, Sass-Kiss, Tóth-Markus, & Lechner, 2006).

In beverage C, the ΔE^* values increased significantly throughout the storage time ($P < 0.0001$) although indicating no noticeable changes in total color during the first 14 days of storage ($\Delta E^*_{day\ 14} = 0.31 < 0.5$). However, ΔE^* value changed slightly noticeable during the last 14 days (day 14 to day 42) of storage ($0.5 < \Delta E^*_{day\ 42} = 0.87 < 1.5$). The slightly noticeable color change in beverage C could be due to the accumulation of brown-dark/reddish colored pigment compounds found in Chai tea, such as theaflavins and thearubigins (Harbowy & Balentine, 1997).

In beverage MB, the ΔE^* values increased significantly throughout the storage time ($P < 0.0001$) and indicated that noticeable changes in color were observed after pasteurization ($1.5 < \Delta E^*_{day\ 1\ (AP)} = 2.13 < 3.0$) and at day 28 ($1.5 < \Delta E^*_{day\ 28} = 1.59 < 3.0$). Slightly noticeable change in color was observed at day 14 ($0.5 < \Delta E^*_{day\ 14} = 0.89 < 1.5$) and at day 42 ($0.5 < \Delta E^*_{day\ 28} = 1.03 < 1.5$). The noticeable color change of MB beverage was expected since the stability of natural color, particularly cochineal, was generally lower than that of synthetic food colors (Downham & Collins, 2000). In addition, the precipitation of color and SPH after the heat treatment and during the storage time was due to the reaction between proteins and natural color compounds caused by the changes in their dissimilar colloidal matrix (Knehr, 2006).

As seen in the other two formulations, the ΔE^* in beverage T increased significantly throughout the storage time ($P < 0.0001$). It also indicated that visible changes in total color difference were observed right after pasteurization ($\Delta E^*_{day\ 1st\ (AP)} = 3.0 < 6.0$). Noticeable changes in color were observed at day 14 ($1.5 < \Delta E^*_{day\ 14th} = 2.85 < 3.0$) while slightly noticeable changes was observed at both day 28 ($0.5 < \Delta E^*_{day\ 28th} = 0.66 < 1.5$) and day 42 ($0.5 < \Delta E^*_{day\ 42nd} = 0.94 < 1.5$). The wide range of total color change of beverage T was expected due to the un-stabilized vegetable-based color, particularly annatto, used in this product (Foster, 2012).

5.4 Conclusions

The results of this study showed that use of citric acid alone or a combination of bitter blocking and masking agents (BB and MA) were effective in minimizing the bitter note caused by the limited enzymatic hydrolysis. The sensory analysis showed significant differences in overall acceptability for the three flavors. Among the three flavors, tangerine flavored beverage was most preferred followed by mixed berries. Since the consumer trend for soy-based products, especially from non-GM source, has been increasing in the recent years, these findings are relevant for developing formulations of protein-rich beverages. Inclusion of other consumer acceptable flavors including strawberry, blueberry, or orange with enhanced protein content, natural flavor and lowered bitterness can be part of further research.

References

Adler-Nissen, J. (1986). *Enzymatic hydrolysis of food proteins*. Elsevier Applied Science Publisher, New York.

Ang, H.G., Kwik, W.L. & Theng, C.Y. (1985). Development of soymilk-a review. *Food Chemistry*, 17, 235-250.

Bae, I.Y., Kim, J.H. & Lee, H.G. (2012). Combined effect of protease and phytase on the solubility of modified soy protein. *Journal of Food Biochemistry*. ISSN 1745-4514. doi: 10.111/jfbc.12001.

Bera, M.B & Murkherjee, R.K. (1989). Solubility, emulsifying, and foaming properties of rice bran protein concentrates. *Journal of Food Science*, 54:142-145.

Box. G.E.P. & Behnken, D.W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics*, 2, 455-475.

Botzolakis, J. E., Harris, M. R., & Nesbitt, R. U. (1990). *U.S. Patent No. 4,910,023*. Washington, DC: U.S. Patent and Trademark Office.

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*(2), 537-546.

Calvo, C. (2004). Optical properties. Hanbook of food analysis. Physical characterization and nutrient analysis. Marcel Dekker, Inc: L.M.L. Nollet, New York. Vol 1, 1-19.

Cho, M. J., Unklesbay, N., Hsieh, F. H., & Clarke, A. D. (2004). Hydrophopicity of bitter peptides from soy protein hydrolysates. *Journal of Argriculture & Food Chemistry*, *52*, 5895-5901.

Cho, M. J., Shen,R., & Mooshegian, R. (2008). Soy protein isolate. *U.S. Patent No. 7,332,192 B2*. Washington, DC: U.S. Patent and Trademark Office.

Cserhalmi, Z., Sass-Kiss, A., Tóth-Markus, M., & Lechner, N. (2006). Study of pulsed electric field treated citrus juices. Innovative Food Science & Emerging Technoligies, 7(1), 49-54.

Desh R.P, M.S Chinnan & R.D Phillips. (2008). Process development of a chocolate- flavoured peanut-soy beverage. *International Journal of Food Science and Technology*, 43: 886-894

Downham, A & Collins, P. (2000). Colouring our foods in the last and next millennium. *International Journal of Food Science and Technology*, 35, 5-22

EFSA issues positive opinion for erythritol use in beverages. (2015). *Nutraceuticals world*, 18.3, 16-18.

Foster, R.J. (2012). Vegetable-based colors. *Food Product Design*. Retrieved online at http://www.foodproductdesign.com/articles/2012/01/vegetable-based-colors.aspx?pg=2#content

Foster, R.J. (2007). Flavor mask-erade. *Food Product Design*. Retrieved online at http://www.foodproductdesign.com/articles/2007/09/flavor-mask-erade.aspx.

Frazier, R. A. (2013). Chapter 123- Interactions of tea tannins with proteins and their impact on food quality and health. Tea in health and disease prevention. Academic Press, 1479-1490.

Fuller, W.G. (2011). *New food product development: from concept to marketplace*. (3rd ed.). CRC Press, Boca Raton, FL.

Jacobsen, J. (2013). On stable footing. *Beverage Industry*, *104*(2), 10-13.

Jiang J, Xiong YL & Chen J. (2010). pH Shifting alters solubility characteristics and thermal stability of soy protein isolate and its globulin fractions in different pH, salt concentration, and temperature conditions. *Journal of Agriculture & Food Chemistry*, 58(13):8035-8042.

Jung, S., Murphy, P. A., & Johnson, L. A. (2005). Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science, 70*(2), 180-187.

Keast, R.S.J, Breslin, P.A.S. (2003). An overview of binary taste-taste interactions. *Food Quality and Preference*, 14: 111-124. doi: 10.1016/S0950-3293(02)00110-6.

Knehr, E. (2006). Maintaining color stability. *Food Product Design*. Retrieved online at http://www.foodproductdesign.com/articles/2006/08/maintaining-color-stability.aspx

Lawless, H.T, H. Heymann. (2010). *Sensory evaluation of food: principles and practices*. (2nd ed). Springer, New York.

Lee, J. (2011). Soy protein hydrolysate: solubility, thermal stability, bioactivity, and sensory acceptability in a tea beverage. *Master's thesis*, University of Minesota.

Levesque, S.D. (2014). *Performance food and drink – U.S-January 2014*. Retrieved from http://academic.mintel.com/display/679959/

Luvonga, A.W (2012). Nutritional and phytochemical composition, functional properties of Roselle (Hibiscus sabdariffa) and sensory evaluation of some beverages made from Roselle calyces. *Master's thesis*, Jomo Kenyatta University of Agriculture and Technology.

Haderspeck, J. (2014). Nondairy protein beverages have broad demographic appeal. *Dairy Foods*, *115*(2), 58.

Harbowy, M., & Baletine, D.A. (1997). Tea chemistry. *Critical reviews in plant science*, 16 (5), 415-480. doi: 10.1080/07352689709701956

Hartog, G. J. M., Boots, A. W., Adam-Perrot, A., Brouns, F., I. Verkooijen, A. R. Weseler, G. Haenen, & A. Bast. (2010). Erythritol is a sweet antioxidant. *Nutrition*, 26, 449-458.

Hayakawa, S. & Nakai, S. (1985). Relationships of hydrophobicity and net charge to the solubility of milk and soy proteins. *Journal of Food Science*, 50, 486-491.

Hazen, C. (2003). Secret of masking flavors. *Food product design*. Retrieved online at http://www.foodproductdesign.com/articles/2003/11/secrets-of-masking-flavors.aspx?pg=3#content

MacLeod, G. (1988). Soy flavor and its improvements. *CRC Critical Review Food Science & Nutrition*, 27, 219-379.

Moloughney, S. (2013). Functional beverages: Formulas for the future. *Nutraceuticals World, 16*(6), 36-46.

Neilsen, P. K. (2010). Chapter 13: *Enzymes in protein modification*. Book: Enzymes in food technology (2nd ed). Wiley-Blackwell, West Sussex, UK, 292-320.

Ohnishi, S.T., & Barr, J.K. (1978). A simplified method of quantitating protein using the biuret and phenol reagents. *Analytical Biochemistry*, 86, 193.

Ozdal, T., Capanoglu, E., & Altay, F. (2013). A review on protein-phenolic interactions and associated changes. *Food Research International*, 51, 954-970.

Pasma, S.A., Daik, R., Maskat, M.Y., & Hassan, O. (2013). Application of Box- Behnken design in optimization of glucose production from oil palm empty fruit bunch cellulose. *International Journal of Polymer Science*, article ID: 104502, 8 pages.

Peryam, D.R & Pilgrim, F.J. (1957). Hedonic scale method for measuring food preferences. *Food Technology*, 11(9), 9-14.

Persinger, P. (2014). Low calorie sugar substitute composition and methods for making the same. *U.S. Patent Application 14/201,432*.

Phillips, D. (2015). 2015 Flavor Trends: Spice heats up the grocery aisles. *Food processing*. Online retrieved at http://www.foodprocessing.com/articles/2015/flavor-trends-spice-heats-up-the-grocery-aisles/?show=all

Ruíz-Henestrosa, V.P., Sánchez, C.C., Pedroche, J.J, & Millán, F. (2008). Improving the functional properties of soy glycicin by enzymatic treatment, adsorption and foaming characteristics. *Food Hydrocolloids*, 23, 377- 386. doi:10.1016/j.foodhyd.2008.03.011

Sun, X.D. (2011). Enzymatic hydrolysis of soy proteins and the hydrolysates utilization. *International Journal of Food Science and Technology*, 46, 2447-2459. doi: 10.1111/j.1365-2621.2011.02785.x

Tockman, J. (2002). Capitalizing on increasing consumer interest in soy protein. *Cereal Foods World*, 47, 172-174.

Were, L., Hettiarachchy, N.S., & Kalapathy, U. (1997). Modified soy proteins with improved foaming and water hydration properties. *Journal of Food Science*, 62(4), 821-824. doi: 10.1111/j.1365-2621.1997.tb15463.x

Wu, W.U., Hettiarachchy, N.S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. *Journal ofAmerican Oil Chemists Society*, 75(7), 845-85.

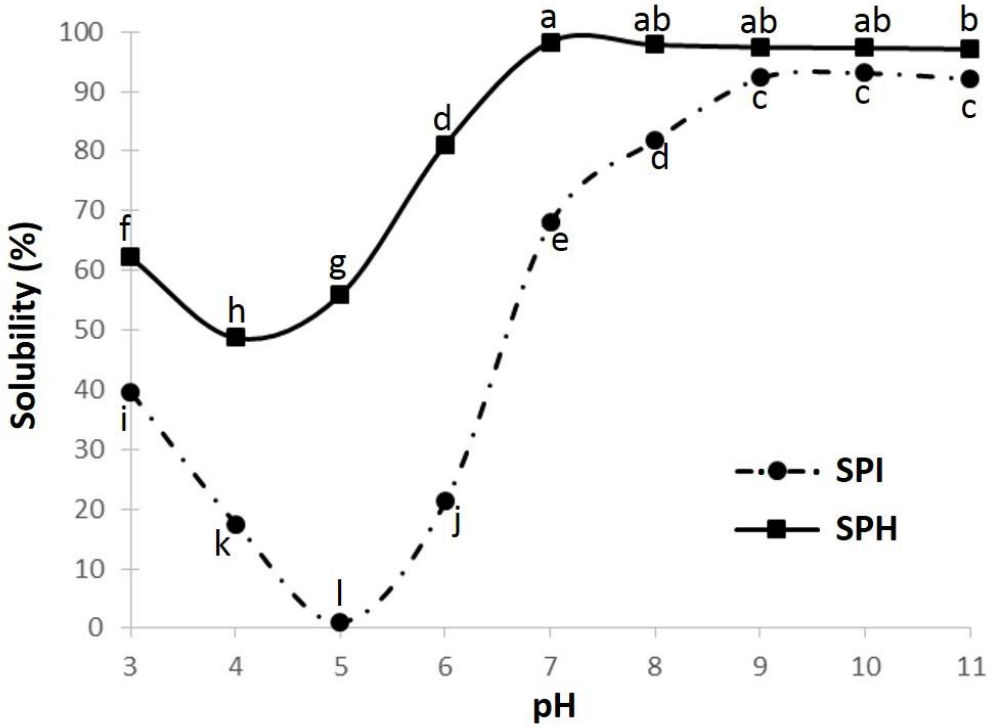


Figure 11: Solubility (%) as function of pH of SPI and SPH.
Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters are significantly different at P value < 0.05.

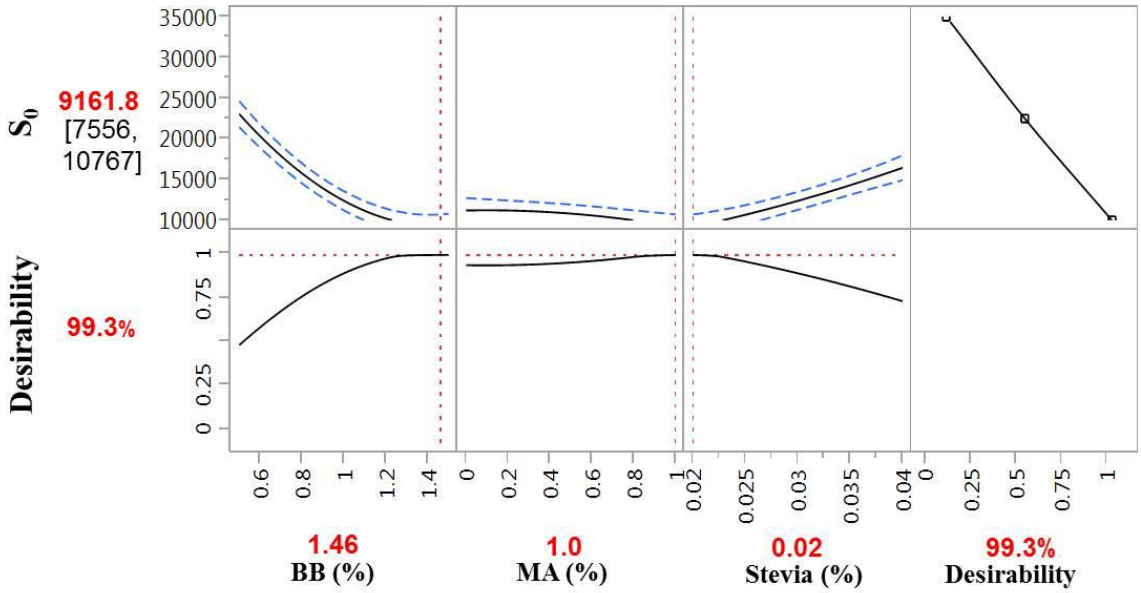


Figure 12: Prediction profiler showing the optimal combination of BB, MA, and Stevia concentration (%) to minimize the surface hydrophobicity.

Table 12: Formulas of flavored SPH beverages.

Formula	Ingredient	Weight (g)	Percentage (% w/w)
Control	Distilled water	462.3	92.46
	SPH*	22.7	4.54
	Truvia	15.0	3.00
	Total	500.00	100.00
Chai tea flavor	Brewed Chai tea	462.3	92.46
	SPH*	22.7	4.54
	Truvia	15.0	3.00
	Total	500.00	100.00
Tangerine flavor	Distilled water	452.8	90.56
	SPH*	22.7	4.54
	Truvia	15.0	3.00
	Tangerine flavor agent	4.5	0.90
	Annatto color agent	2.5	0.50
	Citric acid	2.5	0.50
	Total	500.00	100.00
Mixed berries (MB) flavor	Distilled water	440.7	88.14
	SPH*	22.7	4.54
	Truvia	15.0	3.00
	Bitter blocker	7.3	1.46
	Masking agent	5.0	1.00
	Berries flavor agent	5.0	1.00
	Cochinal color agent	3.0	0.60
	Citric acid	1.3	0.26
	Total	500.00	100.00

*Protein content of SPH = 88.3%; 22.7g SPH was added in order to have the final product that contained 20 grams of SPH per serving of 500 mL.

Table 13: Mean hedonic ratings (± standard deviation) of hedonic impression as a function of flavored soy protein beverages.

	Mixed berries flavor	Tangerine flavor	Chai tea flavor	Control
Overall appearance	7.1 ± 0.2^{a}	7.2 ± 0.1^{a}	3.9 ± 0.2^{c}	6.3 ± 0.2^{b}
Odor impression	7.0 ± 0.2^{a}	7.3 ± 0.1^{a}	5.6 ± 0.3^{b}	4.5 ± 0.2^{c}
Flavor impression	5.2 ± 0.3^{b}	6.4 ± 0.2^{a}	3.8 ± 0.3^{c}	2.9 ± 0.2^{d}
Oral tactile	5.9 ± 0.2^{b}	6.3 ± 0.2^{a}	5.0 ± 0.2^{c}	4.1 ± 0.2^{d}
Overall impression	5.4 ± 0.2^{b}	6.5 ± 0.2^{a}	3.8 ± 0.2^{c}	3.1 ± 0.2^{d}

Mean ratings with different letters within a row are significantly different at $P < 0.05$.

Table 14: Mean Just-About-Right ratings (± standard deviation) of sensory attributes as a function of flavored soy protein beverages.

	Mixed berries flavor	Tangerine flavor	Chai tea flavor	Control
Color impression	3.0 ± 0.1^{b}	3.1 ± 0.0^{b}	4.1 ± 0.1^{a}	2.5 ± 0.1^{c}
Sweetness	2.9 ± 0.1^{a}	2.9 ± 0.1^{a}	2.1 ± 0.1^{b}	1.4 ± 0.1^{c}
Bitterness	3.4 ± 0.1^{b}	3.1 ± 0.1^{b}	3.9 ± 0.1^{a}	4.0 ± 0.1^{a}

Mean ratings with different letters within a row are significantly different at $P < 0.05$.

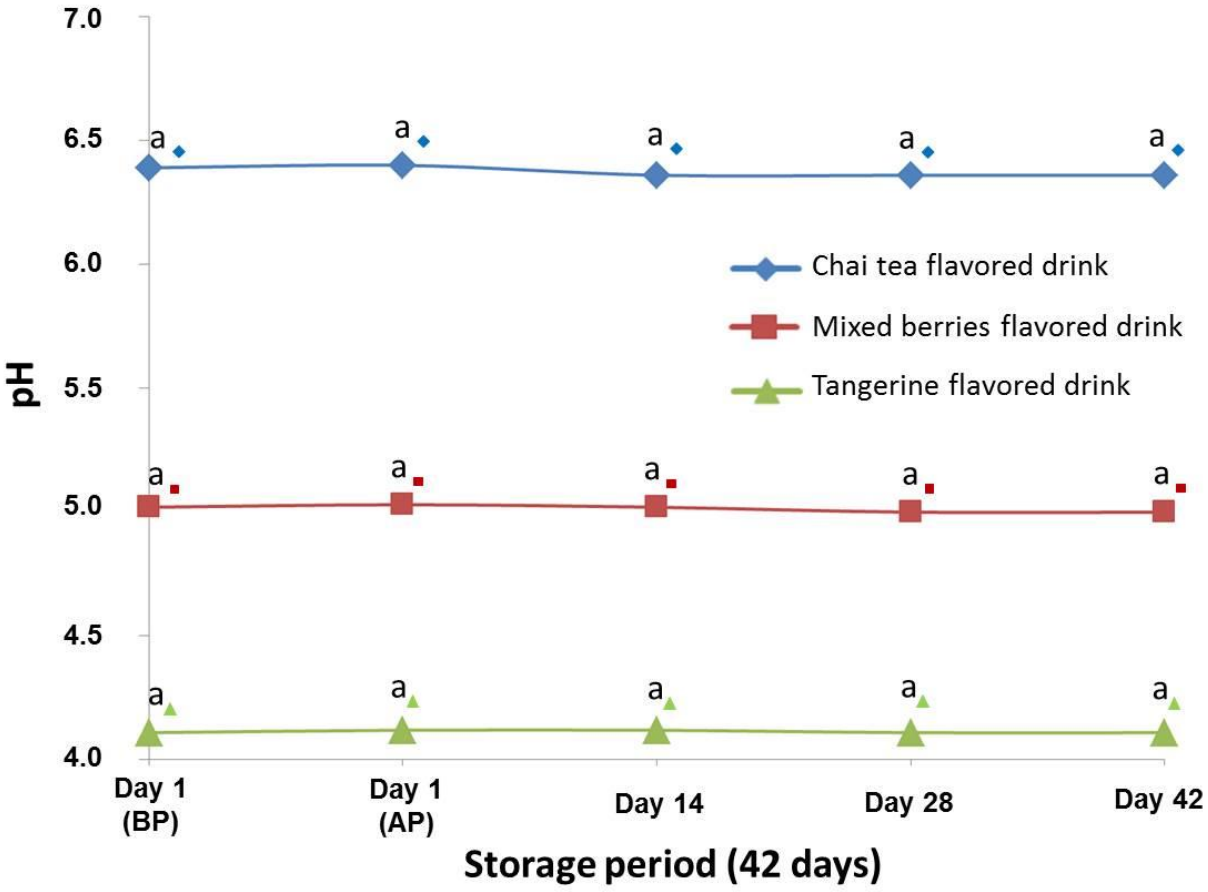


Figure 13: The pH values of the three flavored SPH beverages over the storage period.
*BP = before pasteurization; **AP = after pasteurization.
Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters of each drink are significantly different at P value < 0.05.

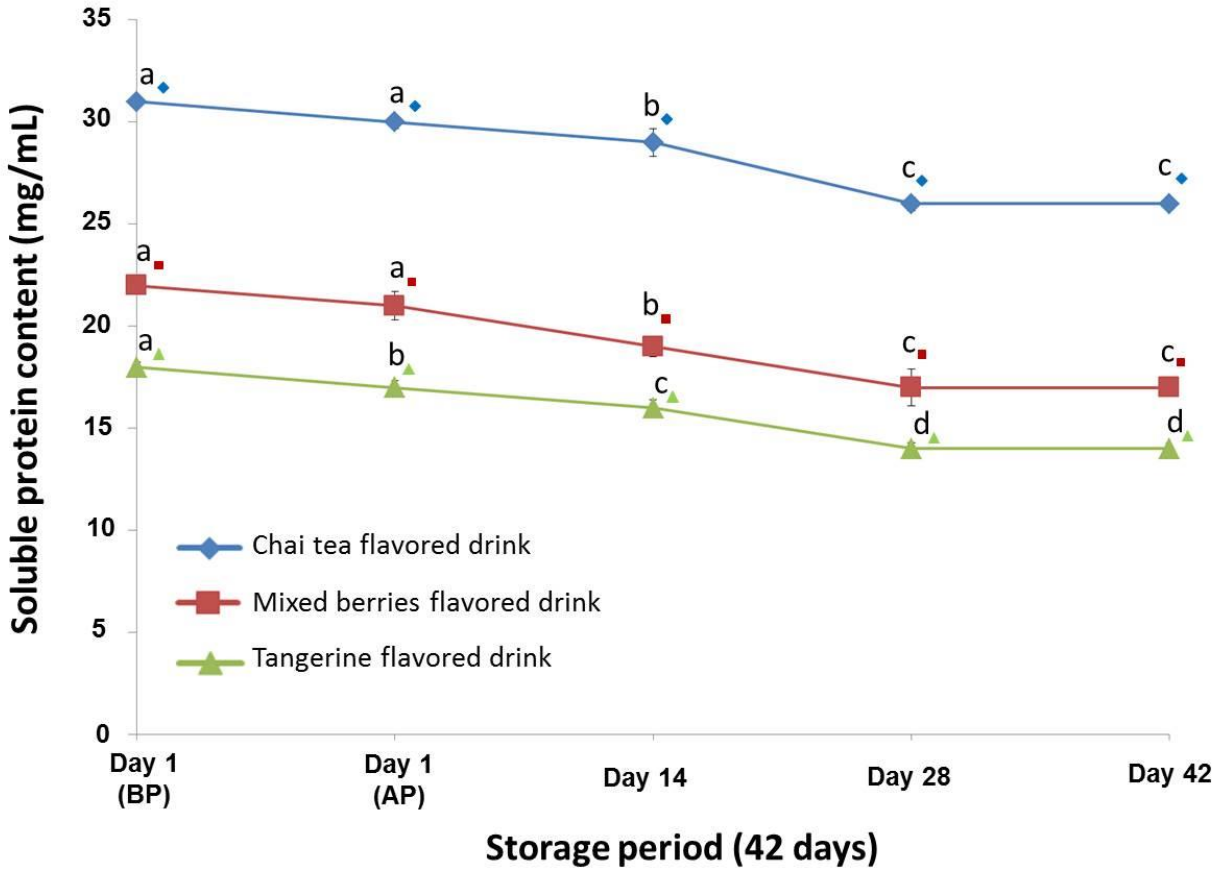


Figure 14: Soluble protein content (mg/mL) of the three flavored SPH beverages over the storage period.

*BP = before pasteurization; **AP = after pasteurization.

Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters of each drink are significantly different at P value < 0.05.

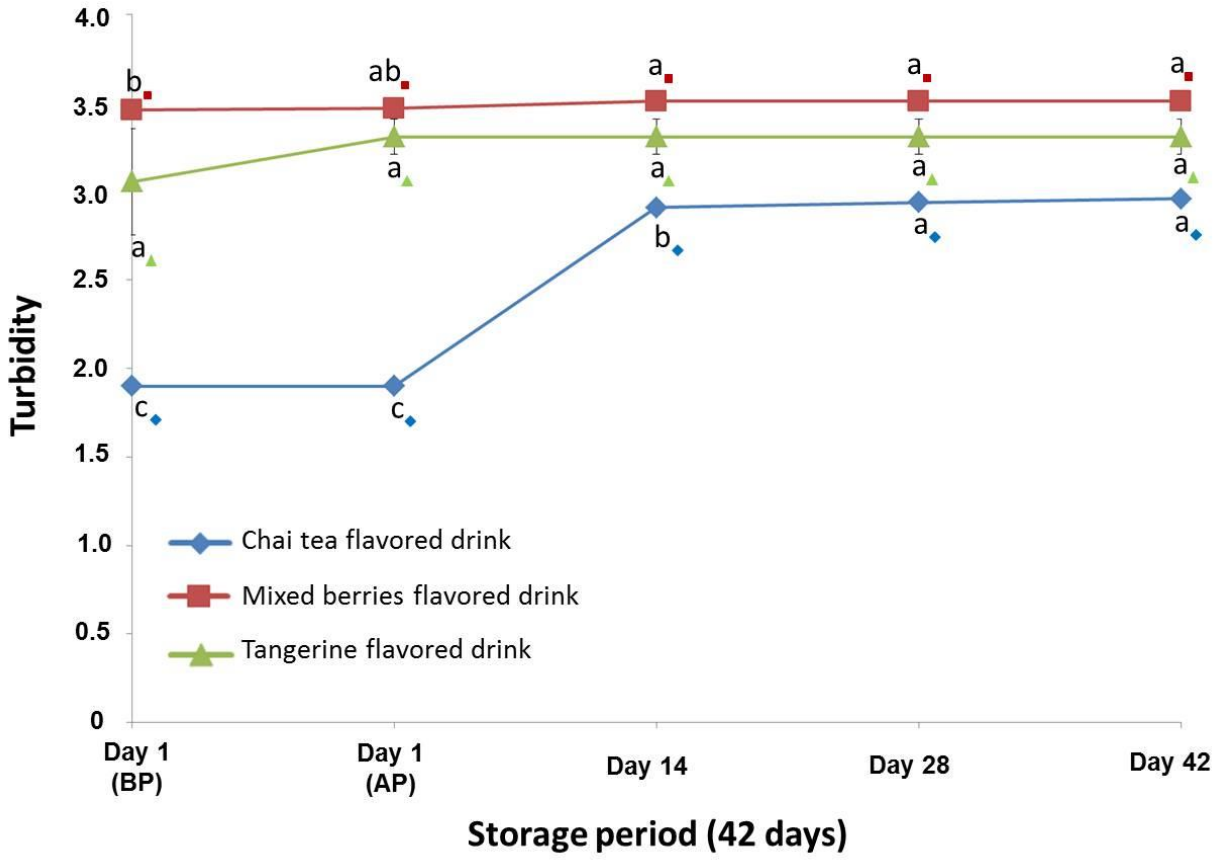


Figure 15: Turbidity values of the three flavored SPH beverages over the storage period.
*BP = before pasteurization; **AP = after pasteurization.
Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters of each drink are significantly different at P value < 0.05.

Table 15: The three SPH beverages over the storage period (0 to 42 days).

	Day 1	Day 14	Day 28	Day 42
Chai tea flavor				
	Day 1	**Day 14**	**Day 28**	**Day 42**
Tangerine Flavor				
	Day 1	**Day 14**	**Day 28**	**Day 42**
Mixed berries Flavor				

Table 16: The color parameters (L^*, a^*, b^*) of the three SPH beverages over the storage period.

Chai flavor			
	a^*	L^*	b^*
Day 1 (BP)*	0.57 ± 0.01^c	37.42 ± 0.02^a	0.37 ± 0.01^a
Day 1 (AP)**	0.78 ± 0.01^b	37.05 ± 0.02^b	0.39 ± 0.00^a
Day 14	0.79 ± 0.00^b	37.04 ± 0.02^b	0.08 ± 0.00^b
Day 28	0.90 ± 0.01^a	36.55 ± 0.07^c	0.07 ± 0.00^b
Day 42	0.77 ± 0.01^b	36.25 ± 0.02^d	0.06 ± 0.02^b
Mixed berries flavor			
	a^*	L^*	b^*
Day 1 (BP)*	6.34 ± 0.02^c	37.03 ± 0.02^e	-2.93 ± 0.02^a
Day 1 (AP)**	6.75 ± 0.02^b	38.41 ± 0.01^c	-1.36 ± 0.00^c
Day 14	7.48 ± 0.00^a	38.04 ± 0.00^d	-1.01 ± 0.00^e
Day 28	7.43 ± 0.01^a	39.83 ± 0.00^a	-1.62 ± 0.00^b
Day 42	6.73 ± 0.02^b	39.43 ± 0.02^b	-1.21 ± 0.02^d
Tangerine flavor			
	a^*	L^*	b^*
Day 1 (BP)*	5.42 ± 0.02^e	55.98 ± 0.00^a	26.95 ± 0.01^d
Day 1 (AP)**	7.22 ± 0.00^c	54.48 ± 0.01^b	28.82 ± 0.00^c
Day 14	6.98 ± 0.01^d	53.43 ± 0.02^e	26.18 ± 0.02^e
Day 28	7.27 ± 0.02^b	54.26 ± 0.00^c	29.45 ± 0.01^b
Day 42	7.72 ± 0.01^a	54.21 ± 0.00^d	29.57 ± 0.00^a

*BP = before pasteurization; **AP = after pasteurization.

Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters in the same column are significantly different at P value < 0.05.

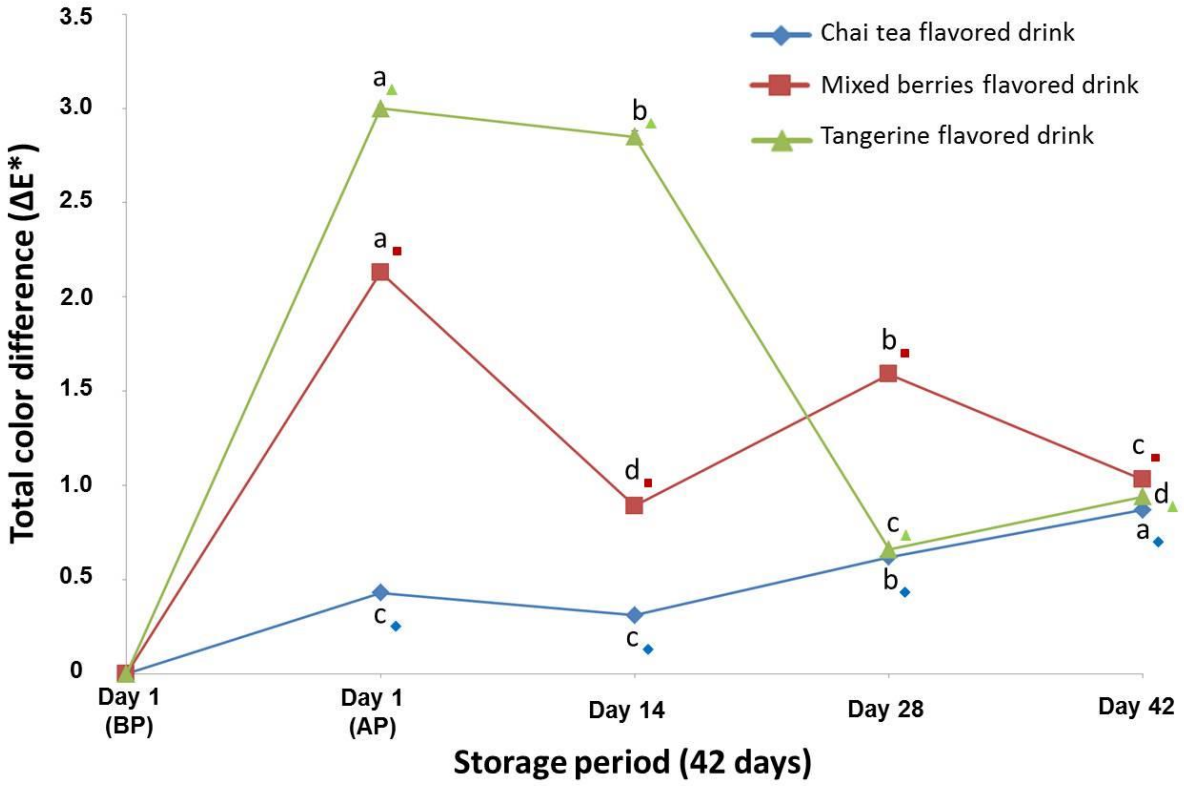


Figure 16: Total color difference (ΔE* values) of the three flavored SPH beverages' over the storage period.

*BP = before pasteurization; **AP = after pasteurization

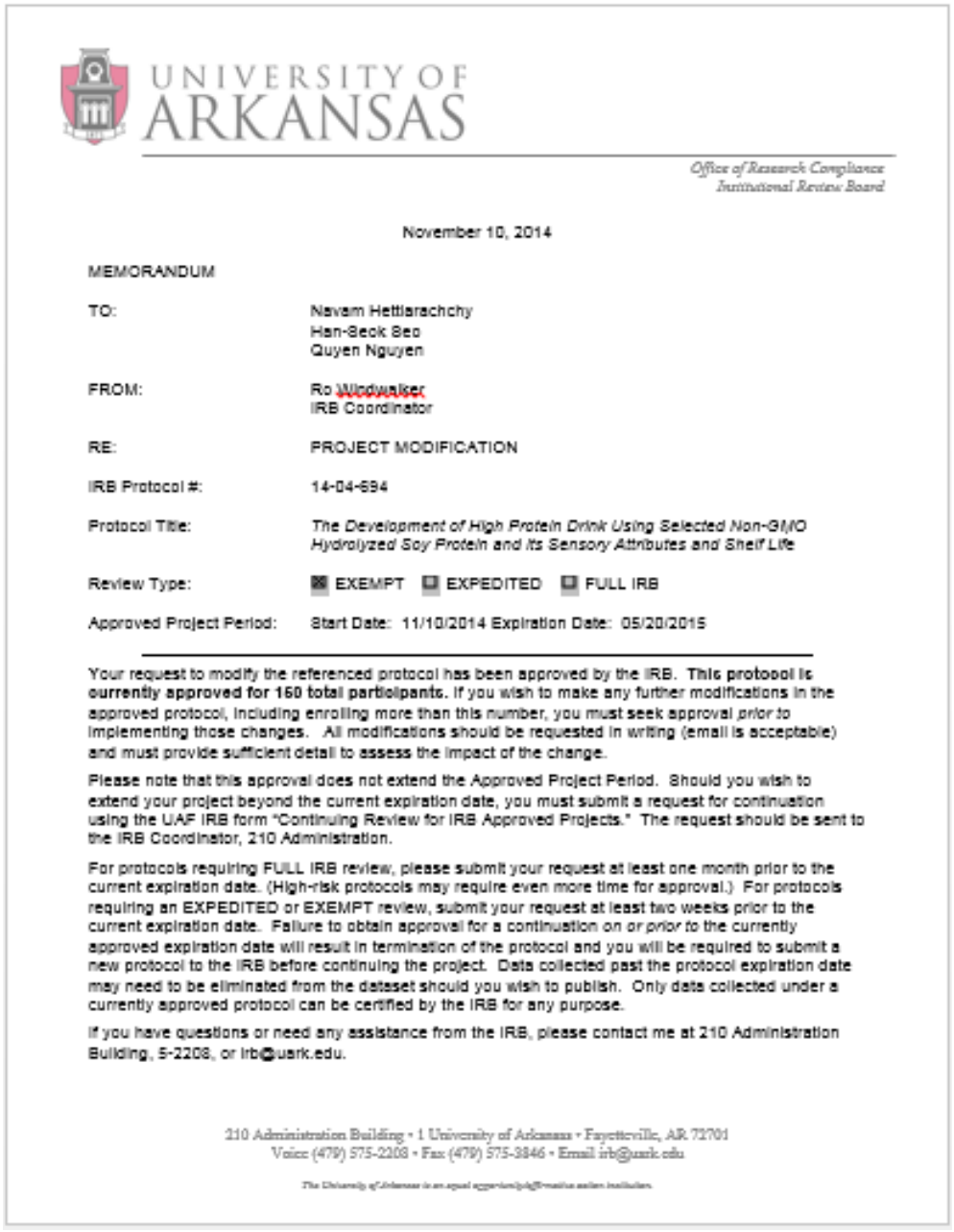
Values are presented as means ± standard deviation of triplicate analysis and those not connected with same letters of each drink are significantly different at P value < 0.05.

Overall conclusions, implications, and recommendation

This work provided, for the first time, the development of novel flavored high protein beverages (20g protein/500mL serving size) prepared with soy protein hydrolysates from Arkansas-grown non-GM source along with sensory and shelf life data. The limited enzymatic hydrolysis using optimal condition of alcalase concentration 1.0 AU (3.2 µL/g), 50 ºC, and 120 min incubation produced SPH with relatively good solubility (98% at pH 7), low turbidity (0.9) at 4% protein concentration as well as its potential antihypertension property (ACE-I inhibition activity 66.6%). The result of sensory evaluation indicated that the addition of citric acid alone or combined with bitter blocker and masking agent successfully reduced the bitterness of SPH and improved the consumer acceptability to soy-based high protein beverages. Therefore, these two tangerine and mixed berries flavored high protein beverages have potential to support healthy eating habit and wellness lifestyle.

However, more studies are needed to modify the hydrolysis conditions such as using different food grade enzyme or pools of various enzymes to enhance the SPH protein yield and solubility at acid pH range for high acid beverage application. In addition, since fruity flavors including mixed berries and tangerine were found to be preferable in this study, formulation with consumers' trend with exotic fruit flavors such as pomegranate, passion fruit, lychee, mango, pink guava, kaffir lime, or tropical fruit mix is recommended in order to create various flavor profiles and provide unique drink experiences with non-GMO soy based high protein beverages to consumers.

Appendix 1: Sensory study approval form by University Institutional Review Board & consent forms.



UNIVERSITY OF ARKANSAS

Office of Research Compliance
Institutional Review Board

November 10, 2014

MEMORANDUM

TO: Navam Hettiarachchy
Han-Seok Seo
Quyen Nguyen

FROM: Ro Windwalker
IRB Coordinator

RE: PROJECT MODIFICATION

IRB Protocol #: 14-04-694

Protocol Title: *The Development of High Protein Drink Using Selected Non-GMO Hydrolyzed Soy Protein and its Sensory Attributes and Shelf Life*

Review Type: ☒ EXEMPT ☐ EXPEDITED ☐ FULL IRB

Approved Project Period: Start Date: 11/10/2014 Expiration Date: 05/20/2015

Your request to modify the referenced protocol has been approved by the IRB. **This protocol is currently approved for 160 total participants.** If you wish to make any further modifications in the approved protocol, including enrolling more than this number, you must seek approval *prior to* implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

Please note that this approval does not extend the Approved Project Period. Should you wish to extend your project beyond the current expiration date, you must submit a request for continuation using the UAF IRB form "Continuing Review for IRB Approved Projects." The request should be sent to the IRB Coordinator, 210 Administration.

For protocols requiring FULL IRB review, please submit your request at least one month prior to the current expiration date. (High-risk protocols may require even more time for approval.) For protocols requiring an EXPEDITED or EXEMPT review, submit your request at least two weeks prior to the current expiration date. Failure to obtain approval for a continuation *on or prior to* the currently approved expiration date will result in termination of the protocol and you will be required to submit a new protocol to the IRB before continuing the project. Data collected past the protocol expiration date may need to be eliminated from the dataset should you wish to publish. Only data collected under a currently approved protocol can be certified by the IRB for any purpose.

If you have questions or need any assistance from the IRB, please contact me at 210 Administration Building, 5-2208, or irb@uark.edu.

210 Administration Building • 1 University of Arkansas • Fayetteville, AR 72701
Voice (479) 575-2208 • Fax (479) 575-3846 • Email irb@uark.edu

The development of high protein drink using selected non-GMO hydrolyzed soy protein and its sensory attributes and shelf life

Consent to Participate in a Research Study

Principal Researcher: Navam S. Hettiarachchy
Co-Reseacher: Han-Seok Seo
Co-Reseacher: Quyen Nguyen

INVITATION TO PARTICIPATE

You are invited to participate in a research study about protein drink made with Arkansas grown non-GMO soy protein hydrolysate. You are being asked to participate in this study because you are interested in non-GMO, vegan-friendly protein drink and have no allergy with soybean.

WHAT YOU SHOULD KNOW ABOUT THE RESEARCH STUDY

Who is the Principal Researcher?

Navam S. Hettiarachchy, Ph.D., Faculty Department of Food Science
Email: nhettiar@uark.edu
Campus phone: 479-575-4779

What is the purpose of this research study?

The purpose of this study is is to development a high protein drink (500mL), which contains 20 gram of non-GMO Arkansas grown soy protein hydrolysate, targeting for all consumers. Protein drinks will be prepared at three flavors include chai tea, mixed berries, and tangerine due to their increasing consumers' popularity.

Who will participate in this study?

Maximum hundred fifty (150) random untrained participants will be selected. All participants are expected to be over 18 year olds, interesting in non-GMO, vegan-friendly protein drink, and should have no allergy with soybean.

What am I being asked to do?

Your participation will require the following:
Each panelist will have 4 samples to evaluate. All panelists will be instructed to start the test with visual observation, smell, taste, and evaluate one by one sample. The participants will be administered a paper ballots accompanied with all sample cups to express their evaluation on samples' sensory attributes including appearance, aroma, flavor, sweetness, mouthfeel, aftertaste, and overall acceptability.

What are the possible risks or discomforts?

Product contents soybean which is recognized as one of the eight common food allergies. Therefore, all panelists will be informed before participate. They should have no allergy with soybean to be selected for this study.

What are the possible benefits of this study?

The outcome of this research will be helpful to understand the consumer acceptability to new protein drink made with Arkansas grown non-GMO hydrolyzed soy protein. This will be the first time that Arkansas-grown non-GMO soybean cultivars are being used for preparing novel food

products. We expect that the result of this study will potentially provide commercial interest in utilizing the protein from non GMO soybean lines in healthy products which is economically advantageous to both Arkansas soybean growers and the soybean processing industry.

How long will the study last?
The study will last 15-minute survey for each panelist.

Will I receive compensation for my time and inconvenience if I choose to participate in this study?
Participants will receive a candy bar. There will be many scheduled time for participants to select according to your best convenience.

Will I have to pay for anything?
No, there will be no cost associated with your participation.

What are the options if I do not want to be in the study?
Your participation in the research is completely voluntary. Therefore, if you do not want to be in this study, you may withdraw from this study at any time. Also, you may refuse to participate at any time during the study. Your job, your grade, your relationship with the University, etc. will not be affected in any way if you refuse to participate.

How will my confidentiality be protected?
All information will be kept confidential to the extent allowed by applicable State and Federal law. In addition, you will be assigned a code number and all information will be recorded anonymously. Only the principal and co- researchers will know your name, but will not divulge it or identify your answers to anyone. All information will be held in the strictest of confidence. Results from the research will be reported as aggregate data.

Will I know the results of the study?
At the conclusion of the study you will have the right to request feedback about the results. You may contact the Principal Researcher, Navam S. Hettiarachchy. You can reach her through her email nhettiar@uark.edu or her phone number (479)-575-4779. You will receive a copy of this form for your files.

What do I do if I have questions about the research study?
You have the right to contact the Principal Researcher or Faculty Advisor as listed below for any concerns that you may have.

Principal Researcher: Navam S. Hettiarachchy Food Science nhettiar@uark.edu 479-575-4779

Co-Researcher: Han-Seok Seo Food Science hanseok@uark.edu 479-575-4778

Co-Researcher: Quyen Nguyen Food Science qtnguyen@email.uark.edu 479-575-4799

You may also contact the University of Arkansas Research Compliance office listed below if you have questions about your rights as a participant, or to discuss any concerns about, or problems with the research.

Ro Windwalker, CIP
Institutional Review Board Coordinator
Research Compliance
University of Arkansas
210 Administration
Fayetteville, AR 72701-1201
479-575-2208
irb@uark.edu

I have read the above statement and have been able to ask questions and express concerns, which have been satisfactorily responded to by the investigator. I understand the purpose of the study as well as the potential benefits and risks that are involved. I understand that participation is voluntary. I understand that significant new findings developed during this research will be shared with the participant. I understand that no rights have been waived by signing the consent form. I have been given a copy of the consent form.

Signature **Date**

Appendix 2: Sensory ballot.

Ballot

Place a check mark (✓) in the blank space provided below the statement that best describes your impression. Check only one response per question and be sure that you respond to each question.

SAMPLE # _____

1. ***Please LOOK AT the sample*** *in the cup.* All things considered, which of the statements below best describes your impression for the *OVERALL APPEARANCE* of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

2. Considering the ***COLOR*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too light	Too light	Just about right	Too dark	Much too dark
☐	☐	☐	☐	☐

3. ***Please SMELL the sample*** and considering the ***ODOR*** of this sample, which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

4. ***Please DRINK the sample*** and considering the ***FLAVOR*** of this ***sample,*** which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

5. Considering the intensity of ***SWEETNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

6. Considering the intensity of ***BITTERNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

7. Considering the ***ORAL TACTILE ASPECT*** of this sample, which of the statements below best describes your impression of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

8. All things considered, which of the statements below best describes your ***OVERALL IMPRESSION*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

page 1

SAMPLE # _____

1. ***Please LOOK AT the sample*** *in the cup.* All things considered, which of the statements below best describes your impression for the *OVERALL APPEARANCE* of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

2. Considering the ***COLOR*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too light	Too light	Just about right	Too dark	Much too dark
☐	☐	☐	☐	☐

3. ***Please SMELL the sample*** and considering the ***ODOR*** of this sample, which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

4. ***Please DRINK the sample*** and considering the ***FLAVOR*** of this ***sample,*** which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

5. Considering the intensity of ***SWEETNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

6. Considering the intensity of ***BITTERNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

7. Considering the ***ORAL TACTILE ASPECT*** of this sample, which of the statements below best describes your impression of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

8. All things considered, which of the statements below best describes your ***OVERALL IMPRESSION*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

page 2

SAMPLE # _____

1. ***Please LOOK AT the sample** in the cup.* All things considered, which of the statements below best describes your impression for the *OVERALL APPEARANCE* of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

2. Considering the ***COLOR*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too light	Too light	Just about right	Too dark	Much too dark
☐	☐	☐	☐	☐

3. ***Please SMELL the sample*** and considering the ***ODOR*** of this sample, which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

4. ***Please DRINK the sample*** and considering the ***FLAVOR*** of this ***sample,*** which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

5. Considering the intensity of ***SWEETNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

6. Considering the intensity of ***BITTERNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

7. Considering the ***ORAL TACTILE ASPECT*** of this sample, which of the statements below best describes your impression of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

8. All things considered, which of the statements below best describes your ***OVERALL IMPRESSION*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

SAMPLE # _____

1. ***Please LOOK AT the sample** in the cup.* All things considered, which of the statements below best describes your impression for the *OVERALL APPEARANCE* of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

2. Considering the ***COLOR*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too light	Too light	Just about right	Too dark	Much too dark
☐	☐	☐	☐	☐

3. ***Please SMELL the sample*** and considering the ***ODOR*** of this sample, which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

4. ***Please DRINK the sample*** and considering the ***FLAVOR*** of this ***sample,*** which of the statements below best describes your ***impression*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

5. Considering the intensity of ***SWEETNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

6. Considering the intensity of ***BITTERNESS*** of this sample, which of the statement below best describe your ***impression*** of this product?

Much too weak	Too weak	Just about right	Too strong	Much too strong
☐	☐	☐	☐	☐

7. Considering the ***ORAL TACTILE ASPECT*** of this sample, which of the statements below best describes your impression of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

8. All things considered, which of the statements below best describes your ***OVERALL IMPRESSION*** of this product?

Dislike extremely	Dislike very much	Dislike moderately	Dislike slightly	Neither like nor dislike	Like slightly	Like moderately	Like very much	Like extremely
☐	☐	☐	☐	☐	☐	☐	☐	☐

9. **Among these 4 samples, which one you like the most?**

469	513	261	702
☐	☐	☐	☐

DEMOGRAPHIC QUESTIONS

1. What is your gender:

Male	Female
☐	☐

2. How old are your: _____

3. Ethnicity:

White/Caucasian	African American	Asian	Hispanic	Native American	Others
☐	☐	☐	☐	☐	☐

Thank you so much for your participation!
Have a great day!