QUALITY IMPROVEMENT OF SOYMILK PROCESSED FROM TWO SOYBEAN VARIETIES

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Five soymilk characteristics were investigated as affected by different grinding, heating, extraction methods and varieties. The five characteristics are (1) protein and solid recovery, (2) trypsin inhibitor activity, (3) antioxidant compounds and antioxidant capacity, (4) soy odor, and (5) isoflavone content and profile. The two varieties were Prosoy and black soybeans.

The results show that significant differences existed among the three grinding methods (ambient grinding, cold grinding, and hot grinding). Ambient grinding gave the best protein and solid recoveries. Hot grinding showed the best results for the other four parameters. Cold grinding gave the poorest performance, with the exception of the odor profile. The three heating methods (traditional stove cooking, one-phase UHT, two-phase UHT) also resulted in significant differences. In many cases, the effects of heating methods were closely related to grinding methods and varieties. The two varieties behaved differently during processing. For both varieties, extraction methods showed significant differences.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Soymilk, as a beverage extracted from soybeans, has many health-promoting functions. In 1999, the health claim of soy protein to reduce total cholesterol and low density lipoprotein (LDL) was approved by FDA. However, soymilk also has various anti-nutritional components, such as trypsin inhibitors, and lectin. The main barrier to the even greater popularity of soymilk in the Western countries is its objectionable off-flavor. As for this issue, many methods have been tried to reduce the activity of lipoxygenases. Among processing methods reported, hot grinding is regarded effective. However, hot grinding can cause protein denaturation and thus reduce protein and solid recovery. The negative effect of hot grinding has not been fully understood. In our study, we compared hot grinding, ambient grinding and a commonly used cold grinding in Japan to systematically investigate advantages and disadvantages in terms of several major soy odor compounds, protein and solid recoveries.

UHT (ultra-high temperature) is a commonly used heating approach in modern soymilk manufacturing industry. In our study, we adopted two UHT methods. One is a popular industry practice, the other was devised with consideration of its heating power. For the purpose of comparison, a traditional stove cooking method was also involved. We evaluated combinations of grinding and heating methods to achieve the best result.

Elimination of soy odor is one of the focal points in the industrial processing of soymilk. However, processing methods can also affect other components and the overall functionality. In our study, we further investigated antioxidant compounds and capacity, residual trypsin inhibitor activity, isoflavone content and profile as affected by grinding and heating methods. In these aspects, there is very little literature available.

Our objective was to give an overall picture of soymilk quality manufactured from two soybean varieties as affected by processing conditions and provide useful information to the soymilk industry.

The objectives of this study were as follows:

1. Make a comprehensive comparison of the three grinding methods and four extraction methods with regard to soymilk yield, solid yield, protein recovery, solid and protein content in soymilk.

2. Compare the effect of three grinding methods on the formation of eight major odor compounds in soymilk. Investigate the change of these odor compounds by three heating methods, especially the efficiency of vacuum chamber associated with a UHT processor. Find out proper combinations of grinding and heating methods to eliminate some undesirable off-flavor compounds. Investigate the effect of four extraction methods on the content of eight odor compounds.

3. Study the effect of grinding methods on the elimination of the two trypsin inhibitors, especially the effect of heating methods on the inactivation of these trypsin inhibitors when in conjunction with grinding methods.

4. Study the effects of three grinding methods on the extraction of antioxidants and antioxidant capacity. Study the change of antioxidants and antioxidant capacity when subjected to different heating methods.

5. Study the effect of three grinding methods, three heating methods on isoflavone content and profile. Find out if grinding has a destructive effect on total isoflavone. Compare extraction efficiency of four extraction methods.

LITERATURE REVIEW

Effect of Grinding on Protein and Solid Recovery

Soybean contains up to 40% of protein, of which 90% is water extractable. Therefore, it is a very economical protein source compared with animal protein. In addition, it has many unique properties and physiological functions. Soybean is free of lactose, and can be used to make infant formula for lactose-intolerant people. In 1999, a health claim was approved by the Food and Drug Administration (FDA) that intake of 25 g of soy protein every day in conjunction with a low-cholesterol and low saturated fat diet could prevent heart disease (FDA, 1999).

According to an investigation using ultracentrifugation (Wolf and Briggs, 1956), soybean proteins are classified into four categories: 2S, 7S, 11S, and 15S. However, this does not necessarily mean there is only one single component in each fraction. At pH 4.4-4.8, 75% of the soluble proteins would precipitate and this portion is therefore called acid-precipitated proteins or soybean globulins. Among soy globulins, 7S β-conglycinin and 11S glycinin combined comprise the most part of soy protein (Iwabuchi and Yamauchi, 1987). However, whey and other globulins, such as α-conglycinin, γ-conglycinin, basic 7S globulin also exist in soy proteins (Catsimpoolas, 1969). The proportion of 11S to 7S in total protein of soybean seeds differs considerably among varieties (Cai and Chang, 1999).

β-Conglycinin is a glycosylated trimer (Thanh and Shibasaki, 1978) composed of α' (Mw, 57,000-72,000 Daltons), α (Mw, 57,000-68,000 Daltons) and β (Mw, 42,000-52,000 Daltons) subunits (Thanh and Shibasaki, 1978). Different from α' and α subunits, which share high degree of homology, β subunits have no cysteine and methionine but have higher content of hydrophobic amino acids (Thanh and Shibasaki, 1977). β-Conglycinin is

heterogeneous with constituent subunits linked by hydrophobic and hydrogen bonding (Thanh and Shibasaki, 1978).

Glycinin is an oligomer with six acidic subunits (Mw, 35,000 Daltons) and six basic subunits (Mw, 20,000 Daltons). These subunits form two identical hexagons with one on top of the other (Badley et al., 1975). Each pair of acid and basic subunits is linked by a disulfide bond (Staswick et al., 1984). Glycinin is deficient in sulfur with 1.44 g cysteine/100 g protein and 1.84 g methionine/100 g protein, respectively (Badley et al., 1975)

During thermal denaturation, two independent phases occurs, first, breakdown of oligomeric structure and ensuing rearrangement and aggregation; second, denaturation of constituent monomers (German et al., 1982). Badley et al. (1975) stated that for 11S proteins, cleavage of the disulfide bonds occurred in response to heating, therefore, permitted dissociation of the intermediate subunits, which then allowed aggregation and precipitation of the dissociated peptides. β-Conglycinin and glycinin have different denaturation temperatures of 70°C and 90 °C respectively. Below denaturation temperature, prolonged heating time could not lead to complete denaturation of protein in soymilk (Zhang et al., 2004).

At 80°C, using one-dimensional and two dimensional SDS-PAGE, Utsumi et al. (1984) studied heat-induced interactions between purified soybean proteins in the presence of 2-mercaptoehanol (0.5% concentration for mixture and 0.25% for each protein fraction). They found heating caused dissociation of both 7S and 11S globulins, and the dissociated subunits of 7S and 11S globulins subsequently interacted with each other, forming soluble macrocomplexes with molecular weights over one million. Two-dimensional gel electrophoretic analysis revealed that the macrocomplexes contained predominantly the basic subunits of 11S globulin and the β subunit of 7S globulin. In this study, it was also indicated

that the interaction between basic subunits and β subunits is predominantly electrostatic in nature. Furthermore, disulfide bonds between the basic units were also involved in the formation of soluble macrocomplexes. In this experiment, the ratio of 11S to 7S was 1:1. Utsumi also reported that when heating alone, 11S globulin fraction readily aggregate at 80°C and attributed the precipitate to dissociation and subsequent aggregation of basic subunits of 11S glycinin. German et al. (1982) also observed the formation of soluble complex between basic subunit and 7S protein and attributed the aggregation of basic subunits to the hydrophobic interaction. In fact, thermal dissociation and association of soy proteins are influenced by ionic strength, pH, reductants (German et al., 1982). Damodaran and Kinsella (1982) attributed the differences between glycinin and β-conglycinin to their subunit composition and oligomeric nature. They further found the soluble complex between conglycinin and basic subunit was formed by electrostatic interaction which was greatly influenced by ionic strength. If for basic subunits not to precipitate, the molar ratio of conglycinin to basic subunit should be greater than 1/3.

Yamagishi et al. (1983) observed different results in the model systems of purified 7S and 11S globulins. Heating was done under 100 °C followed by gel filtration and ion exchange chromatography, as well as electrophoresis of the precipitate and supernatant. This study showed heating of 11S globulin generated precipitate, but heating 7S alone did not. While heating the mixture of 7S and 11S globulin did cause precipitate in which nearly all basic subunits and most β subunit were located in the precipitate, and accordingly, α, α' and acid subunits were mostly in the supernatant. Nevertheless, the supernatant and precipitate all showed highly heterogeneity which means every subunit can be found in both supernatant and precipitate. It was also revealed that the precipitate consisted of polymers and oligomers linked primarily through disulfide bonds, but as an exception, β subunit can only interact with other subunits via hydrophobic interaction instead of sulfhydryl-disulfide exchange due to the

lack of cysteine in β subunit (Shimada and Matsushita, 1978; Thanh and Shibasaki, 1977). The contradiction of the two research groups may arise from different heating temperatures and most importantly, different protein concentration. As suggested by German et al. (1982), the precipitation of glycinin might be repressed by β-conglycinin during thermal process at low (0.5% each) protein concentration (Damodaran and Kinsella, 1982). Zhang et al. (2004) stated protein denaturation temperature increased with concentration.

Extraction of solid is correlated with grinding temperature. Winston et al. (1968) found grinding at 55-65°C resulted in the highest solid recovery. They also found grinding at 85 °C and above lead to substantial decreases of soymilk volume and solid recovery and attributed it to gel formed at high temperatures. Using heating and mannual squeezing, Endo et al. (2004) found hot grinding at 95 °C gave significantly lower soymilk yield, protein and solid recovery compared with ambient grinding. Johnson and Snyder (1978) found hot grinding gave lower soymilk volume than ambient grinding, and contributed to the water loss during hot grinding and the soymilk residue in okara from centrifugation. Johnson and Snyder (1978) further stated that blanching and hot grinding all gave lower solid recovery than ambient grinding with the former decreasing even more. When soybean powder was used, hot grinding still gave the lowest protein recovery (Mizutani and Hashimoto, 2004). Barbosa et al. (2006) found that extraction of defatted soy flour at 25°C resulted in significantly higher protein recovery in comparison with 4°C. Yuan et al. (2008) also found 80 °C, 2 min heating of soybeans decreased solid and protein recovery.

Solid recovery increased with water-to-bean ratio and the ratio could affect the composition of soymilk (Johnson and Snyder, 1978). Johnson and Snyder (1978) established that during grinding, two processes happened concurrently: water extraction of soluble solids and breaking of large particles. Higher water-to-bean ratio favored the former process and

lower water-to-bean ratio was conducive to the latter process. And they concluded that in grinding, the solubilization of solids dominated, which showed high water-to-bean ratio could improve extraction efficiency. The extraction methods can also alter protein and solid recovery. It has been reported 7S protein and total protein could be yielded more via re-extraction (Cai and Chang, 1999). Beddows and Wong (1987a) demonstrated that 10:1 water-to-bean ratio could yield more protein and solid in soymilk compared with 8:1 ratio, but the double extraction of 8:1 plus 2:1 ratio could give 3.3% higher protein recovery than a single 10:1 ratio extraction. During extraction process, protein-water and protein-protein interaction played an important role (Beddows and Wong, 1987a). The extraction efficiency of solid and protein is also affected by the degree of hydration. Pan and Tangratanavalee (2003) found a positive relationship between hydration rate and solid recovery and suggested a lowest120% hydration ratio to separate the fiber from other component during grinding. According to Wang et al. (1979), complete hydration was achieved when soaked soybeans reached about 2.4 times the original weight of soybeans. Cai and Chang (1999) showed that higher hydration ratio rendered 11 S protein more extractable and therefore lead to the higher 11S recovery and higher 11S/7S protein ratio. According to their report, when hydration ratio was increased from 2.0-2.1 to 2.2, accordingly, the 11S/7S increased from 1.64 to 1.96 and the 11S recovery rose from 69.2% to 76.1%.

Effect of Grinding and Heating on Trypsin Inhibitors

Trypsin inhibitors have been reported to cause low protein efficiency ratio (PER) and pancreatic hypertrophy (hackler et al., 1965; Liener, 1989). According to Gandhi et al. (1984), trypsin inhibitory activity varied greatly for different varieties especially between yellow and black soybeans. In buffer solution, BBI showed higher thermal stability than KSTI (Dipietro and Liener, 1989). While, in soy flour and whole soybeans, KSTI was more thermally stable

than BBI (Dipietro and Liener, 1989; Van den Hout et al., 1998; Armour et al., 1998). Depietro and Liener (1989) found that the two inhibitors exhibited lower stability in soy extract solution than in buffer solution and attributed it to the sulfhydryl-disulfide interchange between protein and trypsin inhibitors. Instead of protein unfolding during heat denaturation, interchange of disulfide linkages between inhibitors and storage proteins such as glycinins, or the degradation of cysteine/cystine have been hypothesized to be partly responsible for the inactivation of trypsin inhibitors, and this was proven by the relatively lower activation energies of KSTI and BBI during heating process than protein unfolding which usually need an higher activation energy of several hundred kJ /mol. (Rouhana et al., 1996). Friedman et al. (1982) revealed thiols and heating could inactivate trypsin inhibitors co-operatively through thiol-disulfide interchange. Interchange lead to the alteration of the conformation of inhibitors which made it difficult to access to the active site of trypsin and chymotrypsin. Cystine-cysteine is very labile to hydroperoxides (Roubal and Tappoel, 1965) and lipid hydroperoxides can oxidize cystine-cysteine to cysteic acid and cysteinesulfinic acid (Finley et al., 1981). It has been reported that grinding at room temperature caused more SH degradation than at low temperature (about 2°C) because of different activity of lipoxygenases under different grinding temperatures (Obata et al., 1993; Obata et al., 1996). To date, no report is available about the effects of hot grinding on SH of soymilk.

The effect of heating temperatures on TI residue has been reported extensively (kow et al., 1993; Yuan et al., 2008; Johnson, et al., 1980a). However, the results of these reports were somewhat inconsistent, because a lot of factors could influence the heating effect: water-to-bean ratio, presence of protein, pH, SH content, heating apparatus, Aw, proportion of KSTI and BBI, and efficiency of heating and cooling (Kwok et al., 2002, Yuan et al., 2008). According to Johnson et al. (1980) and Kwok et al. (1993), plotting of log TI residue against heating time gave a curvilinear instead of a single linear line. In this curve, the initial

and final parts were linear lines of distinct slopes with quadratic curve in between as a transitional period. This means the two inhibitors follow different first-order kinetics because of different thermal stability

Van den Hout et al. (1998) stated that the two-phase inactivation of TIs in soy flour during heating could not be explained solely by the different thermal stability of KSTI and BBI. And the inactivation rate was determined by cysteine/sulfhydryl availability which caused sulfhydryl-disulfide interchange and heating intensity. According to Hackler and Stillings (1967), at 121°C, cystine is very vulnerable to heat treatment and damaged shortly after heating.

Until now, there has been no report regarding the effect of grinding temperatures on Kunitz and Bowman-Birk inhibitors especially the heating effect when in conjunction with different grinding temperatures. However, blanching was reported to reduce TI activity (Yuan et al., 2008).

Effect of Grinding and Heating on Antioxidant Compounds and Antioxidant Capacity

In terms of TPC, TFC, DPPH, FRAP, ORAC, black soybeans all showed significantly higher values than yellow soybeans (Xu and Chang, 2008). Xu and Chang (2007b) also revealed that phenolic contents and antioxidant capacity were highly related to seed color and attributed the higher antioxidant activity of dark colored legumes to their seed coat. In black soybeans, seed coat contributed predominantly to TPC, TFC, CTC, DPPH, but about half to ORAC. However, the total phenolics, DPPH and ORAC of dehulled black soybeans were similar to those of whole yellow soybeans (Xu and Chang, 2008c). Takahata et al. (2001) further revealed that seed coat of black soybeans contained much higher DPPH

radical scavenging activity and total phenolic content compared with that of brown and reddish-brown soybeans.

Until now, as for the heating effect on antioxidant activity, most research has been focused on vegetables, fruits, tea, and milk. To the best of our knowledge, only one report about soymilk is available (Xu and Chang, 2009). In the food system, the change of antioxidant capacity is influenced by a lot of factors. Firstly, phenolics could be oxidized enzymatically or chemically (Nicoli et al., 2000). Secondly, phenolics could be involved in the Maillard reaction as reactants (Kaanane et al., 1988). Lastly, the Maillard reaction could form new antioxidants (Manzocco et al., 2001). In the early stage of phenolic oxidation, improved antioxidant capacity was observed because of increased capability to donate hydrogen atoms (Nicoli et al., 1999; Guyot et al., 1995). Also, in the early stage in the Maillard reaction, reactive free radicals as pro-oxidants were formed prior to the Amadori rearrangement (Hofmann et al., 1999; Pischetsrieder et al., 1998). Therefore, the overall antioxidant property is closely related to heating time, heating temperature and oxygen availability. According to Calligaris et al. (2004), in milk, Maillard reaction quickly occurred at 120 °C and no decrease in chain-breaking activity was observed, while at 80 and 90 °C, it took 1.5-2 h to form browning, and the milk exhibited decrease in chain-breaking activity at early stage. When tomato juice was heated at 95°C, in the first 3 h, reduction of chain breaking activity was also observed (Anese et al., 1999). Xu and Chang (2009) reported different heating methods had different effects on phenolic compounds and antioxidant capacity. They further noted that different phenolic compounds contributed to the overall antioxidant property to different degrees. In studies of vegetables, no correlations between total phenolics and antioxidant activity were found (Ismail et al. (2004). To date, there is no report about the effect of grinding temperature on antioxidant profile during soymilk making. However, it has been demonstrated that blanched apple puree showed higher antioxidant

capacity than unbalanced apple puree and the two products showed different change pattern in antioxidant capacity during storage because of different degree of enzymatic oxidation (Nicoli et al., 2000).

Effect of Grinding and Heating on Off-Flavors

In recent years, with the FDA approved claim of health benefits for soy protein (FDA, 1999), soymilk has become more popular in the United States. However, still many Western consumers dislike it because of grassy-beany flavor (Macleod and Ames, 1988), which are volatile carbonyl compounds produced from the degradation of hydroperoxides mainly through lipoxygenases-catalyzed oxidation of polyunsaturated fatty acids (Rackis et al., 1979). Off-flavors from soymilk are represented by a mixture of many odor compounds (Yuan and Chang, 2007a; Lozano et al., 2007; Sun et al., 2010), among which hexanal has been studied the most in soy foods. The formation of odor compounds is closely related to the composition of soybeans. Yuan and Chang (2007b) revealed hexanal content in soymilk was positively correlated with protein content, lipoxygenase activity, and linoleic acid content of soybeans. Min et al. (2005) also found a high correlation between soybean protein and volatile compounds. They further demonstrated that variety and growing location had a significant effect on the formation of these compounds. To date, no reports are available regarding soymilk flavor prepared from black soybean. Heating method can affect the content and composition of odor compounds (Yuan and Chang, 2007a). Direct steam injection was effective to reduce selected odor compounds as compared to a stove cooking process (Yuan and Chang, 2007a). In addition to lipoxygenase-induced oxidation of polyunsaturated fatty acid, off-flavors could also be generated through non-enzymatic mechanisms (Frankel et al., 1981; Lee et al., 2003).

For improving soymilk quality, several treatments have been used to reduce odor content through the inactivation or inhibition of lipoxygenases, such as alkaline soaking, hot grinding, cold grinding, gallic acid-aided grinding (Nelson et al., 1976; Endo et al., 2004; Mizutani and Hashinoto, 2004; Boatright, 2002)). Soymilk manufacturing requires a series of unit operations, including soaking, grinding, heating, vacuuming, and packaging. Variation of each of these processing units may affect final quality. Traditionally, soymilk is produced by soaking soybean, followed by wet grinding at the ambient temperature, and batch heating at the boiling temperature to inactivate lipoxygenases and trypsin inhibitors (Yuan et al., 2008). In recent years, hot grinding and UHT processing have been adopted by large commercial production.

Since the discovery of the capability of hot grinding at 80 ºC to minimize soy odor in a non-quantitative report in 1967 (Wilkens, 1967), there have not been any reports on the effect of hot grinding on soymilk flavor until recent years (Sun et al., 2010; Endo et al., 2004; Mizutani and Hashimoto, 2004; Lv et al., 2011). Only one report is available on comparing the flavor profiles of soymilk processed by traditional method with selected UHT methods (Lozano et al., 2007), in which hexanal content in the UHT cooked soymilk was shown to be similar to the traditionally cooked soymilk. Blanching soaked soybeans at 80°C for 2 min before grinding makes hexanal undetectable but protein recovery is reduced (Yuan et al., 2008). Besides raw material differences, one major problem that makes comparison of the literature difficult is a lack of detailed characterization of grinding and heating devices and processing conditions that lead to a wide variation of the odor products. By comparing grinding at 25 ºC and hot water, Sun et al. (2010) conclude that hot grinding is not effective in reducing lipid derived volatiles, and it is necessary to use other processing strategies than hot grinding. Even though grinding at 80-100 ºC was able to reduce the lipid derived odor in soymilk as studied by Lv et al. (2011), the residual odor concentrations in soymilk were still

above the sensory threshold values. Comparing how different temperatures from cold (3 ºC) to hot (80 ºC) affect both odor and protein recovery in soymilk has only been reported once in literature by Mizutani and Hashimoto (2004) using coarsely ground soy powder. Under their experimental conditions, cold grinding (3 ºC) gives the highest nitrogen (protein) yield, followed by intermediate temperatures (15-55 ºC) and hot grinding (80 ºC). However, this study shows grindings at both 3 ºC and 80 °C followed by 93-94 ºC heating step are ineffective to eliminate soy odor since the finished soymilk still contained 158 ppm hexanal, which is much higher than the sensory threshold value of 4.5 ppb.

Direct steam-injection UHT processor equipped with a vacuum chamber, has been recently used by the soymilk industry. However, no reports are available about the effectiveness of vacuum chamber associated with UHT in the reduction of off-flavors. Therefore, it is desirable to investigate the effect of different grinding temperatures and heating methods, including UHT-vacuum processing on selected soy odor composition.

Effect of Grinding and Heating on Isoflavone Content and Profile

Soy isoflavones have been proved to be related to the prevention of some disease: cancer, cardiovascular diseases, osteoporosis, and postmenopausal sympotoms (Cohen et al., 2001; Chiechi LMD, 1999; Brouns, 2002). Isoflavone content and distribution in soybean are influenced by variety, location, crop year, storage condition (lee et el., 2003; Xu and Chang, 2008a; Hou and Chang, 2002). There are totally 12 forms of isoflavones in soybeans, among which malonylglucosides and β-glucosides account for about 80% and 20%, respectively. Aglycones and acetylglucosides only constituted a minor portion (Xu and Chang, 2008a). The change of isoflavone content and profile can happen in every step in soymilk making. In the soaking process, isoflavones are lost in soaking water; and interconversions also occur simutaneously (Jackson et al., 2002; Kao et al., 2004; Wang and Murphy, 1996). The loss

during grinding was also reported and the authors attributed it to the boiling water added during grinding (Jackson et al., 2002). Heating, as an indispensable step in soymilk making, could greatly alter the content and profile of isoflavones. On the one hand, isoflavones could be released from protein-isoflavone complex during heating process (Nufer et al., 2009; Malaypally, 2010). On the other hand, isoflavones can also be degraded to other non-isoflavone products (Chien et al., 2005). Phenolics could be associated with protein through hydrogen bonding, electrostatic interaction, hydrophobic interaction or even covalent bonding (Boye, 1999). As polyphenols, isoflavones might associate the globular protein of natural form in soymilk (Nufer et al, 2009). As a result, heat-induced increase of isoflavones was observed (Xu and Chang, 2009; Malaypally and Ismail, 2010). According to Nuffer et al. (2009), protein might protect isoflavones from heat-induced degradation. Various interconversions among different isoflavone forms can also occur. During heating, malonylglucosides can be readily converted to acetylglucosides and β-glucosides because of the thermal-labile nature (Chien et al., 2005). Meanwhile, acetylglucosides can also be converted to β-glucosides (Mathias et al., 2006; Chien et all, 2005). β-glucosides can also be converted to their respective aglycones. However, this does not happen at boiling temperatures (Xu et al., 2002). For degradation and conversion, the constant rates with regard to different isoflavone forms were different (Chien et al., 2005; Xu et al., 2002, Vaidya et al., 2007). Xu and Chang (2009) made a comprehensive comparison of several heating methods with regard to their effects on individual and total isoflavones and they found isoflavone content and profiles were closely related to heating methods. Prabhakaran and Perera (2006) ground dehulled soybeans at 95°C and 45°C and found hot grinding yielded higher isoflavones. This is the only report available in the literature on the effect of hot grinding on isoflavones in soymilk. However, in this study, soybeans were ground directly with water

without soaking step. So far, no reports are available regarding different heating effect when in conjunction with different grinding methods.

CHAPTER 1. YIELD, SOLID AND PROTEIN RECOVERY OF SOYMILK AS AFFECTED BY EXTRACTION AND GRINDING METHODS

Abstract

In soymilk, solid and protein are important parameters. However, their recoveries are influenced by processing conditions. In this study, two different soybean varieties (Prosoy and black) were processed with four extraction and three grinding methods (ambient, cold, and hot grinding) for soymilk making. The results showed hot grinding and cold grinding gave lower levels in terms of soymilk yield, solid and protein recovery compared with ambient grinding. The solid and protein extraction efficiency could be improved through re-extraction. Variety was also a factor to affect extraction efficiency of solid and protein.

Introduction

The effect of temperature on protein extraction in hot grinding is more complex than the model system. It is well known that cold grinding and hot grinding could reduce the formation of off-flavor through the inhibition of lipoxygenases (Endo et al., 2004; Mizutani and Hashimono, 2004). In addition, the disadvantage of hot grinding to reduce solid yield and protein recovery in comparison with ambient grinding was also reported (Johnson and Snyder, 1978; Winston et al., 1968). However, comprehensive comparison of the three grinding methods (ambient, cold and hot grinding) with regard to soymilk yield, solid yield, protein recovery, distribution of solid and protein in different fractions has not been investigated. It has been reported that re-extraction could improve solid and protein recovery (Cai and Chang, 1999; Beddows and Wong, 1987a). Therefore, it is likely the extraction efficiency can be increased through the optimization of extraction methods. This would be helpful to the industry. In addition, some other health beneficial component might be extracted more

effectively concurrently. Therefore, the objective of this study was to compare three new extraction methods for their extraction efficiency with the traditional method as a control.

Materials and Methods

Soybean materials

Two varieties of soybeans were used in this study: Prosoy (harvested in 2009) and black soybean (harvested in 2006) grown in Casselton, North Dakota. All processing methods were replicated three times.

Raw Soymilk preparation by four extraction methods

For each batch of soymilk, 100 g of soybeans were soaked in 6 times its weight (600 mL) in cold water (4°C and kept in cooler) for 16 h. The hydrated beans were drained and ground with cold water (3.5 °C) at bean-to-water ratio of 1:10 for 3 min at about 15,000 rpm with a Warring Commercial blender (model 51BL13, Connecticut, U.S.). The flow diagrams for the four extraction methods are as follows:

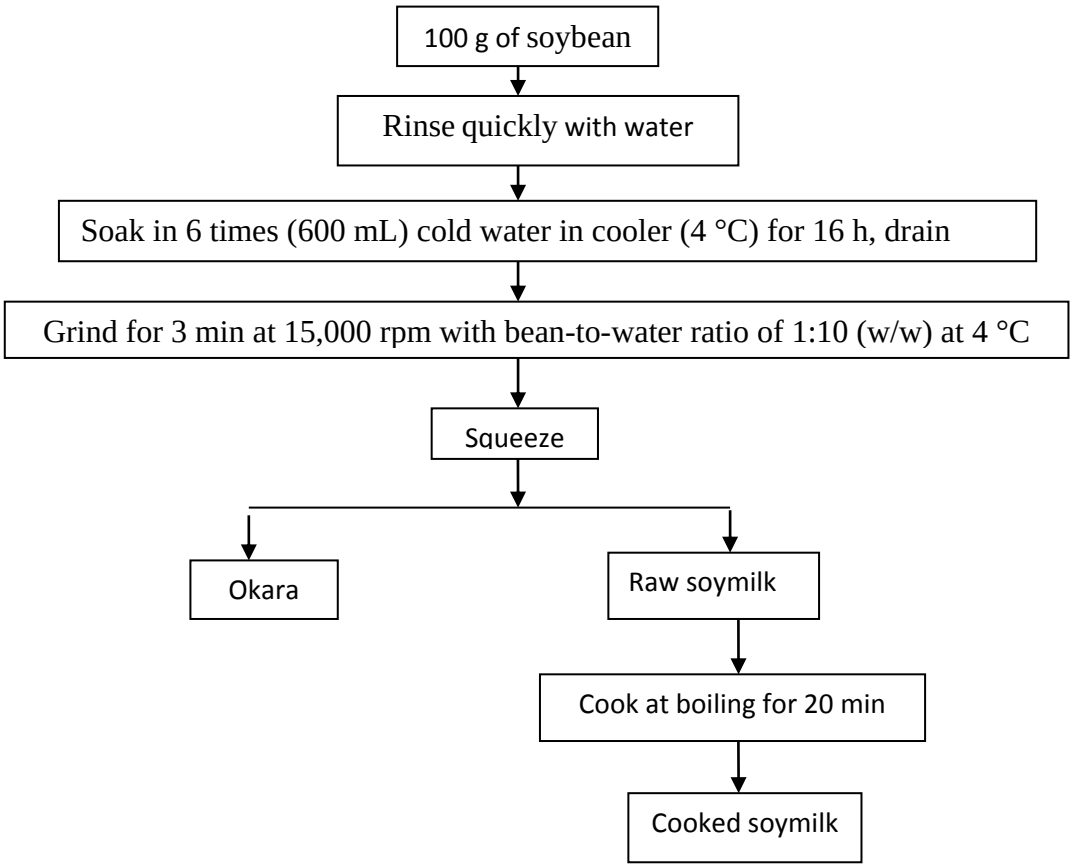

Figure 1-1. Flow diagram of extraction Method #1 (control)

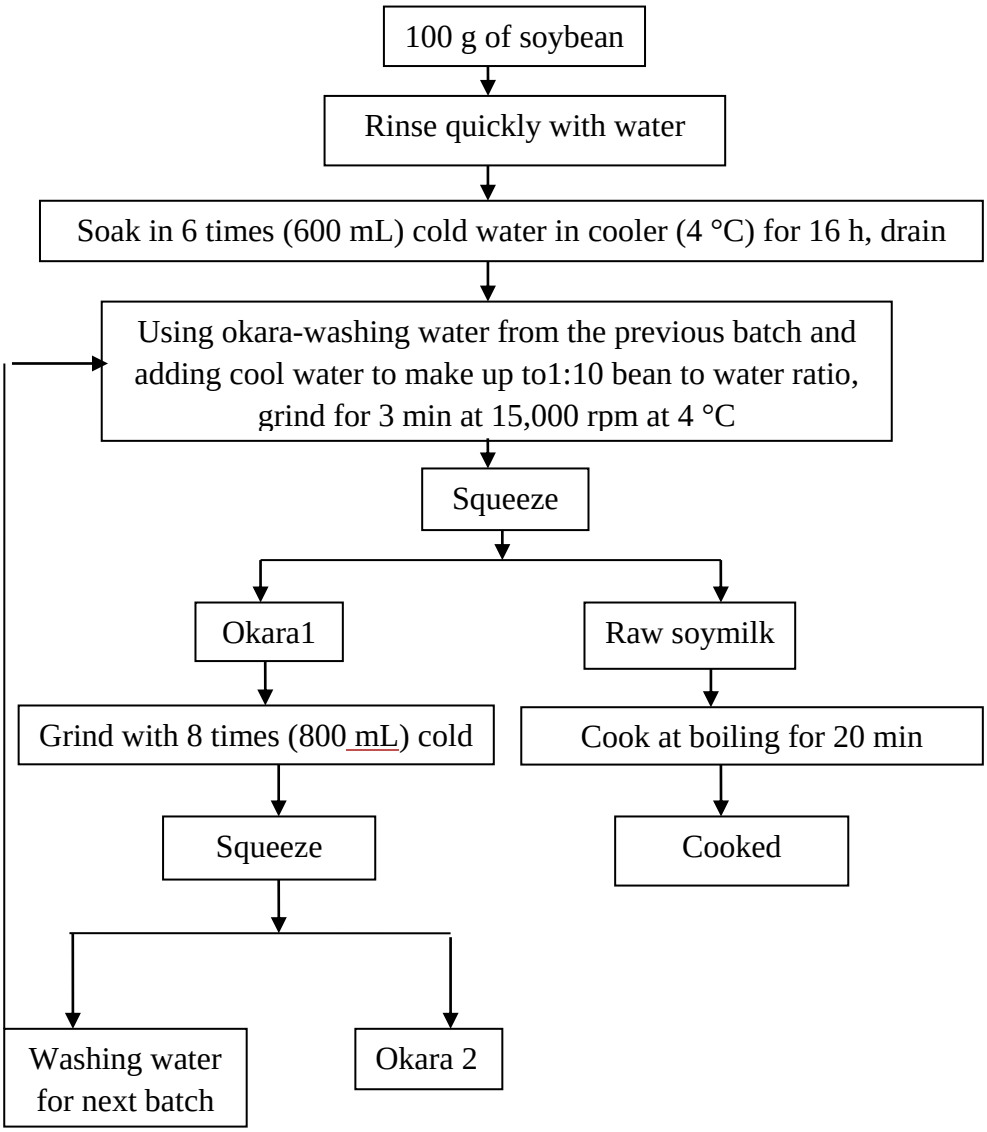

Figure 1-2. Flow diagram of extraction Method #2 (extraction with okara-washing water of last batch)

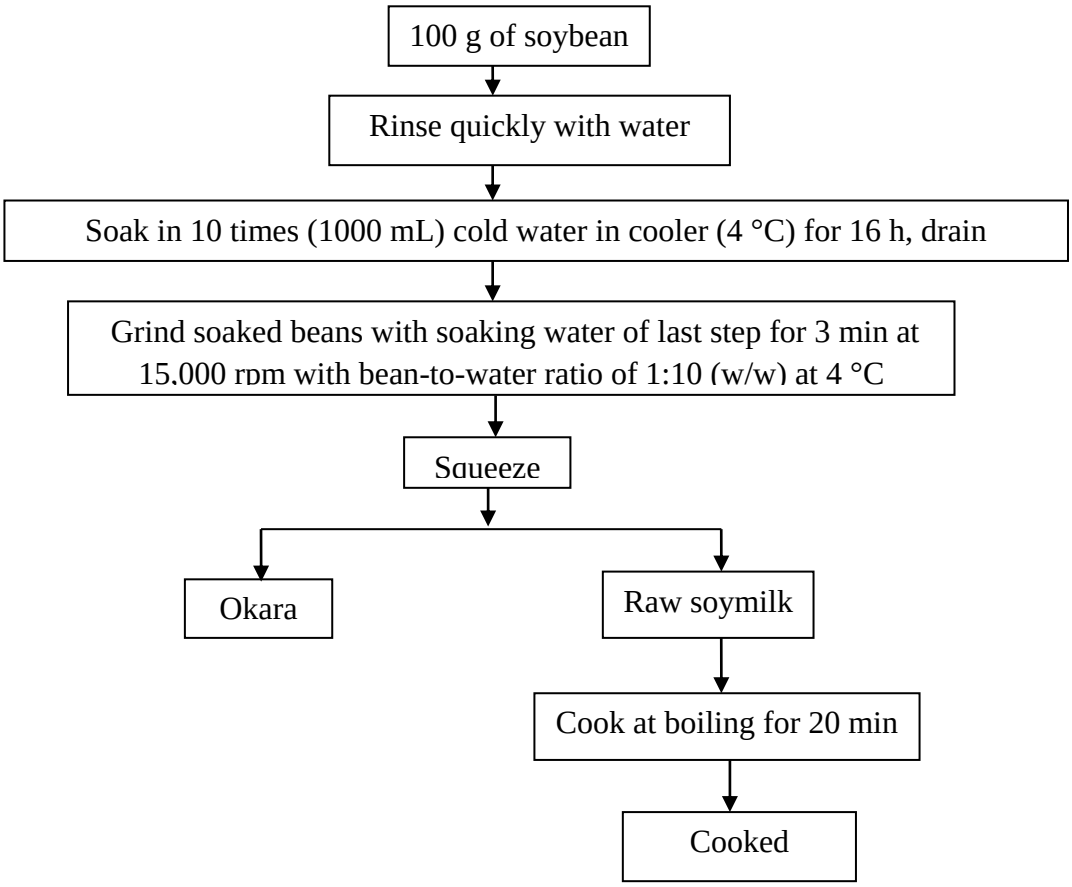

Figure 1-3. Flow diagram of extraction Method #3 (extraction with soaking water)

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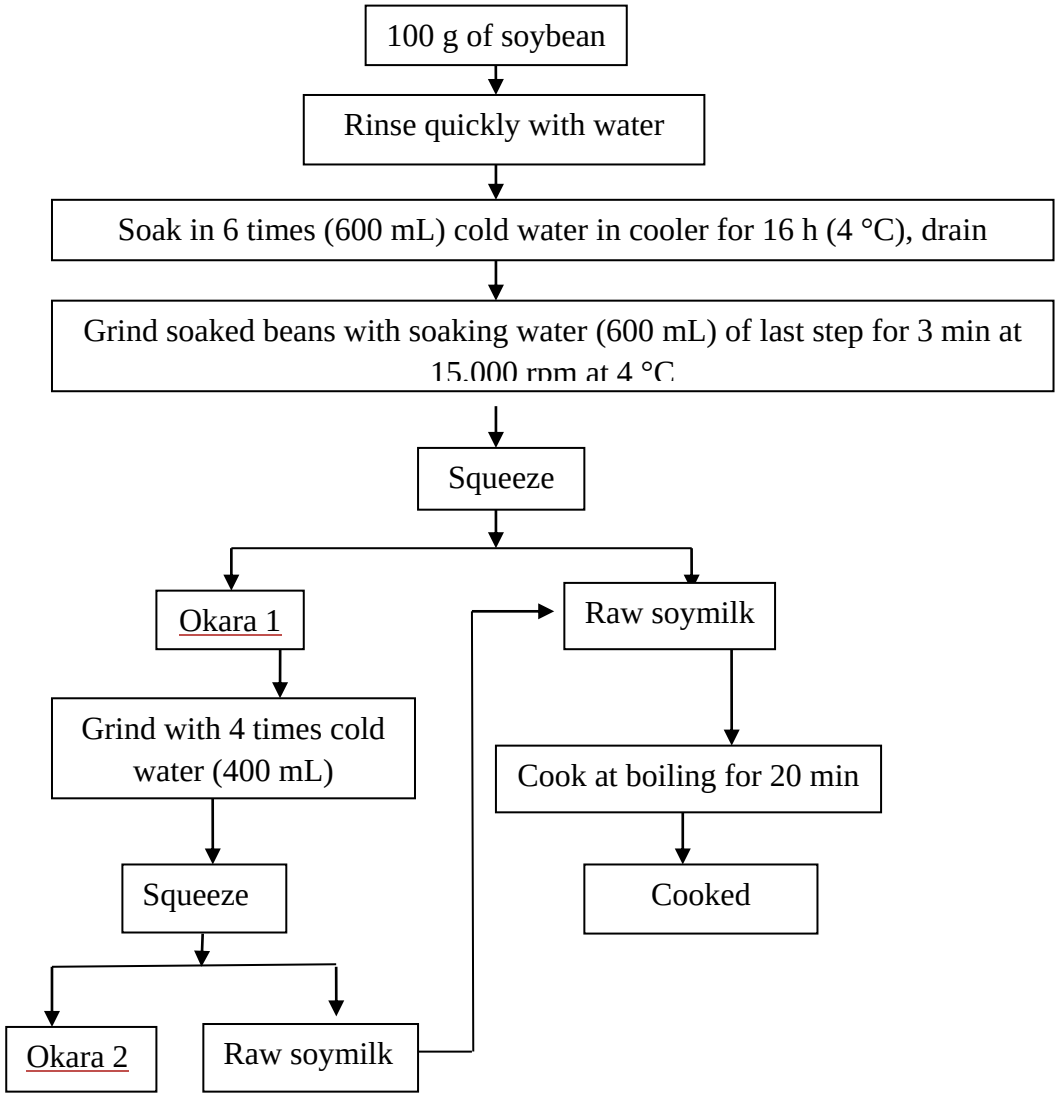

Figure 1-4. Flow diagram of extraction Method #4 (re-extraction).

Raw Soymilk preparation by three grinding methods

To prepare each batch of soymilk, 300 g of soybeans were soaked in 5 times (1500 ml) cold water (4°C and kept in cooler) or room temperature water (about 20 °C) for 16 h. The hydrated beans were drained and ground with cold water (3.5 °C), room temperature water (20 °C), and hot water (80.5 °C) with bean-to-water ratio of 1:10 (w/w). All soaked beans were ground at 10,000 rpm with a New Hartford blender (model CB-2-10, Connecticut, U.S.). The grinding temperature was recorded as the temperature 10 seconds after grinding. After grinding, the soymilk was manually pressed through muslin cloth and weighed. The pressing step was done by the same person until no soymilk was pressed out to maintain the consistency.

Moisture content and protein analysis

Moisture content of beans and freeze-dried soymilk was determined by the air-oven method (AOAC Method #945.15 2005). Crude protein was determined by the Kjeldahl method (AOAC Method #992.23, 2005).

Statistical analysis

Soymilk was prepared in triplicate, and the following analyses were completed in duplicate. Data were subject to analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant differences among variables were determined by Duncan's multiple range test (α=0.05). Data are expressed as means ± SD (n=3).

Results and Discussion

Effect of grinding methods on yield, solid and protein recovery of soymilk

Table 1-1 shows that all the three grinding method produced about 900 g of soymilk per 100 g of soy beans. However, ambient grinding gave the highest soymilk yield and hot grinding gave the lowest. This finding substantiates the report of Winston et al. (1968) who found the volume of soymilk extracted at temperatures ranging from 30-80°C had very little variations, but when temperature was increased to 90°C, the volume of soymilk would decrease significantly because of the gel formed on the filter. The result of Winston et al. (1968) is different from that of Johnson and Snyder (1978), in which ambient grinding generated almost 40% more soymilk than hot grinding. While in our study, the soymilk yields from hot grinding were only 2-3% lower than those from ambient grinding. The possible reason for the large difference may be due to different separation methods. In our study, manual pressing was used, while in their study, centrifuge was employed. The authors attributed the low soymilk yield to water lost in hot grinding and high water content held in the precipitate.

For either black soybean or Prosoy, ambient grinding achieved significantly ($p<0.05$) higher solid yield (4-12%) and protein recovery (10-15%) in comparison with the other two methods, with cold grinding giving the lowest values (Table 1-1). Deak and Johnson (2007) found that in water extraction of soy flour at pH 8.5, 20°C, 40°C, and 60°C gave very similar solid yield (42%) and protein recovery (72%), which meant heat in this temperature range influenced extraction very slightly. While at 80°C, significantly lower solid yield (39%) and protein recovery (63%) were observed. The authors attributed it to protein denaturation and subsequent lower protein solubility. Under similar temperatures, our results were higher than theirs which might be due to different extraction methods and different material. Another

study (Mizutani and Hashimoto, 2004) showed that grinding soy powder at 3°C yielded the highest protein in soymilk followed by ambient grinding, and grinding at 80°C gave the lowest protein recovery (about 30% lower than 20°C) . These researchers attributed the low protein extraction of hot grinding to the denaturation of protein prior to its solubilization. The differences between our study and the study of Mizutani and Hashimoto (2004) may be due to the material used: they used soybean powder prior to grinding, but we employed soaked whole soybeans.

Table 1-1.Summary of soymilk yield, solid yield and protein recovery as affected by three grinding methods

	Grinding methods	Soymilk yield/100g	% Solid yield	Protein recovery	% protein in solid
Prosoy	Ambient grinding	922A(4)	59.31A(0.66)	2.81A(0.04)	49.98
	Cold grinding	906B(3)	47.09C(1.31)	2.33C(0.02)	49.5
	Hot grinding	900C(6)	51.62B(0.71)	2.46B(0.02)	46.11
Black	Ambient grinding	926A(4)	56.11A(0.54)	2.97A(0.07)	49.63
	Cold grinding	908B(13)	46.79C(0.04)	2.39B(0.07)	48.17
	Hot grinding	897C(7)	51.47B(0.62)	2.48B(0.03)	45.55

Means with different capital letters in the same column are significantly different among different grinding methods for the same variety ($p<0.05$).
Values in parentheses are SD (n=3).

Ono et al. (1991) undertook a study about the changes in the composition and size distribution of soymilk protein particles by heating. They found large particles (>120 nm) from raw soymilk comprised mainly 11S globulins bonded by S-S bridges. When the slurry was heated in boiling water for 5 min, the resultant soymilk consisted mainly of medium-sized particles (40-100 nm) with the corresponding drastic decrease of large particles. As a result of size conversion, protein extractability was increased about 4% by heating the homogenate before filtration. The authors also revealed that the formation of medium-sized particles was not only from degradation from large particles, but also from combination of

small particles (<40 nm). Medium-sized particles were made primarily of β subunit and basic subunit, which was in agreement with Yamagishi et al. (1983) and Utsumi et al. (1984). The transformation from large to medium-sized particles can be attributed to the heat-induced break down of S-S bridges and the formation of complexes of β subunit and basic subunit (Ono et al., 1991). Similar results were found by Beddows and Wong (1987b) who revealed that more solid (75.6% vs 66.1%) and protein (89.8% vs 83.2%) were extracted after heating slurry at 100°C for 3 min than cold filtration, and attributed the higher solid recovery in part to other solids extracted together with protein. The difference between the report of above researchers and our study also indicates that heating during grinding and heating after grinding can result in different protein recovery. Hot grinding caused some proteins to be retained in the residue. These proteins may have interacted with other bean components or formed large aggregates which prevented dissociation during a short time of grinding. In fact, the finding of Yamagishi et al. (1987) may be a good rationale for the relatively lower protein and solid induced by hot grinding. The authors compared the heat-induced changes of glycinin in different concentrations at 100 °C for 5min. They found that after heating, 0.5% protein solution was composed of mostly monomers, dimmers and some oligomers. But when protein concentration was increased to 2-3%, acid subunits polymerized via disulfide bonds and formed gel. Meanwhile, basic subunits still precipitated as in dilute solutions. Therefore, to a large extent, gelation depends on the protein concentration. Winston et al. (1968) found when hot grinding was conducted at about 90 °C, the yield of soymilk significantly declined by about 34%, and attributed it to the gel formed on the filter, which subsequently caused much lower solid yield. Generally, soymilk contains 3.6% protein (Nik et al., 2008). In the case of our experiment, after three minutes of hot grinding, the final temperature of slurry could reach 83.5 °C. Therefore, it is very likely that the gel could be formed. Further, the soymilk volume from hot grinding (Table 1-1) was significantly ($p<0.05$) lower (by 2-3%)

than that from ambient grinding either for Prosoy or for black soybeans, which may be a proof of gel formation.

Another plausible reason for the lower protein and solid recovery may be the incomplete disruption of protein body. Johnson and Snyder (1978) found that after the blanching at 100°C for 30 min, yield of solid decreased significantly. And Yuan and Chang (2007) observed that blanching at 80°C for 1 min could cause about 10% decrease in protein recovery. In the slurry from blanched soybeans, through extensive centrifugation, Johnson and Snyder (1978) observed particles in which 80% was protein. They assumed from the microscopic observations that blanching could induced fixation of protein bodies before the destruction of cells and therefore decreasing the extractability of solid and protein. In addition, Johnson and Snyder (1978) proved that further homogenization could greatly improve solid yield for soymilk whether from blanched soybeans or from hot grinding. Therefore, grinding speed and grinding time (Beddows and Wong, 1987b) are also important to the extractability. This should be considered in order to improve the recovery of solid and protein during hot grinding.

As for the lower extractability of cold grinding compared to ambient grinding (Table 1-1), it may be because of the low solubility at low temperature. Barbosa et al. (2006) found that extraction of defatted soy flour at 25°C resulted in significantly higher protein recovery in comparison with 4°C. Another plausible reason is its incomplete hydration. According to Wang et al. (1979), complete hydration was achieved when soaked soybeans reached about 2.4 times the original weight of soybeans. Pan and Tangratanavalee (2003) found a positive relationship between hydration rate and solid recovery and suggested a lowest120% hydration rate to separate the fiber from other components during grinding. In addition, at about 20°C, it took 16-18 h to approach complete hydration. In our study, cold soaking was

conducted at 4°C for 16 h, which was very likely to influence solid and protein suspension and dispersion during grinding, because lower rate of hydration (Table 1-2) could affect the degree to which the cells are broken and therefore the release of content to the extraction solvent (Wang et al., 1979). Cai and Chang (1999) showed that higher hydration ratio rendered 11 S protein more extractable and therefore lead to the higher 11S recovery and higher 11S/7S protein ratio. According to their report, when hydration ratio was increased from 2.0-2.1 to 2.2, accordingly, the 11S/7S increased from 1.64 to 1.96 and the 11S recovery rose from 69.2% to 76.1%.

Table 1-2. Hydration ratioa in soaking

	Ambient soaking	Cold soaking
Prosoy	2.4A(0.01)	2.28B(0.00)
Black	2.31A(0.01)	2.15B(0.01)

[a]Hydration ratio is the weight ratio of soaked beans to unsoaked beans.
Means with different capital letters in the same row are significantly different between different soaking methods for the same variety ($p<0.05$).
Values in parentheses are SD (n=3).

As for the composition of solid, protein accounted for about 49% of solid in ambient and cold grinding, while for hot grinding, this proportion decreased to about 46% (Table 1-1). This is in agreement with the report of Johnson and Snyder (1978). Although the protein percentage is lower, a much higher amount of carbohydrate exists in hot grinding than ambient grinding (Johnson and Snyder, 1978). Winston et al. (1968) revealed that there was a concomitant pH increase with rising extraction temperature and attributed it to different chemical composition of proteins induced by various temperatures, because protein fractions were released differently at different temperatures. The two varieties were very similar in terms of protein proportion in solid.

Effect of grinding methods on distribution of solid in different fractions

Table 1-3 demonstrates distribution of solids in different fractions. Except cold grinding, the other two grinding methods extracted more solids in soymilk than that left in okara.The lower extractability may be due to incomplete disruption of protein body as reported by Johnson and Snyder (1978). As for the distribution of solids, black soybeans and Prosoy exhibited similar pattern. Soaking not only makes grinding easier, but also favors suspension and dispersion of the solid in the liquid. The solid loss in soaking water varied from 1.28-2.81%, which was very similar to the value of 1.6% reported by Winston et al. (1968). The above results showed soaking had very little effect on the solid and protein yield. Cold water soaking resulted in lower solid loss in relation to ambient water soaking, but the disparity was very small. However, black soybeans lost almost as twice solid as Prosoy, which may be because of their different compositions and textures. Table 1-2 shows that for both black soybean and Prosoy, cold soaking gave a lower hydration rate than ambient soaking, which could limit the release of solid and protein. Furthermore, at the same temperatures, Prosoy had a higher hydration rate than black soybeans.

Table 1-3. Percentage distribution of solid as affected by different grinding methods

Material	Grinding methods	Soaking water	Okara	Soymilk
Prosoy	Ambient grinding	1.4A(0.06)	38.16C(0.01)	59.31A(0.66)
	Cold grinding	1.28B(0.01)	49.78A(0.01)	47.07C(1.31)
	Hot grinding	1.4A(0.06)	45.29B(0.01)	51.62B(0.71)
Black	Ambient grinding	2.81A(0.04)	38.73C(0.01)	56.11A(0.54)
	Cold grinding	2.14B(0.02)	49.66A(0.00)	46.79C(0.04)
	Hot grinding	2.81A(0.04)	43.56B(0.01)	51.47B(0.62)

Means with different capital letters in the same row are significantly different among different grinding methods for the same variety ($p<0.05$).
Values in parentheses are SD (n=3)

Effect of extraction methods on yield of soymilk, solid and protein recovery

To make it easy to compare these four extraction methods. The description of them is listed as follows:

Table 1-4. Summary of four extraction methods

Extraction methods	Water to bean ratio	Water	Extraction times
Method #1 (control)	1:10	Pre-cooled tap water	1
Method #2 (extraction with okara-washing water from last batch)	1:10	Okara washing water from last batch and added pre-cooled tap water	1
Method #3 (extraction with soaking water)	1:10	Soaking water	1
Method #4 (re-extraction)	1:6 + 1:4	Soaking water + pre-cooled tap water	2

Table 1-5 demonstrates there were small significant differences ($p<0.05$) among the four extraction methods in terms of soymilk yield even though the same amount of water was used. With regard to solid and protein recovery, for Prosoy soymilk, Method #2 (extraction with okara-washing water from last batch) gave the highest value followed immediately by Method #4 (re-extraction). Method #1 (control) and Method #3 (extraction with soaking water) showed very similar values. Compared with traditional Method #1 (control), Method #2 produced 9.8% more solid and 8.3% more protein, Method #4 produced 4.8% more solid and 5.2% more protein. For black soymilk, Method #2 still produced highest solid and protein. However, Method #4 gave the lowest values in terms of both solid and protein recoveries. This proves that the solid in the okara residue can not be recovered by further extraction in black soybean (Method #4). As Method #2 showed, re-extraction of okara of the last batch with 8 volumes of water could release the remaining solid and protein and add to the next batch thus resulting in the highest solid and protein recovery among the four extraction methods.

Table 1-5. Summary of soymilk yield, solid yield and protein recovery of four extraction methods

Material	Extraction methods	Soymilk yield[a] (g)	% Solid yield	% Protein recovery
Prosoy	Method #1	968C(6)	62.78C(0.78)	72.09C(1.05)
	Method #2	979B(3)	72.60A(0.81)	80.43A(1.82)
	Method #3	968C(1)	63.78C(1.34)	72.61C(1.31)
	Method #4	992A(5)	67.57B(0.87)	77.27B(1.03)
Black	Method #1	949B(7)	62.25B(0.62)	75.26B(0.57)
	Method #2	958AB(15)	70.54A(3.30)	83.39A(5.06)
	Method #3	974A(5)	62.81B(1.09)	71.85B(1.88)
	Method #4	956AB(9)	61.50B(0.92)	70.11B(1.38)

[a]Soymilk yield is expressed as g soymilk/100 g of dry soybeans.
Means with different capital letters in the same column are significantly different among different grinding methods for the same variety ($p<0.05$).
Values in parentheses are SD (n=3)

Wolf and Briggs (1956) found the increase of water-to-soybean ratio could improve protein extractability, but re-extraction did not give additional protein. This theory was proved by Method #4 for black soybean, in which solid yield and protein recovery all showed lowest values. However, our study showed that for Prosoy, Method#4 could extract 4.5% (Table 1-5) more protein compared with Method #3. This was probably due to different composition and texture of soybeans employed in the experiments. The solid yield also showed the same trend. It has been reported that increasing water-to-bean ratio could improve solid and protein recovery (Xu et al., 2004; Johnson and Snyder, 1978). Johson and Snyder (1978) established that during grinding, two processes happened concurrently: water extraction of soluble solids and breaking of large particles. Higher water-to-bean ratio favored the former process and lower water-to-bean ratio was conducive to the latter process. And they concluded that in grinding, the solubilization of solids dominated, which showed that high water-to-bean ratio could improve extraction efficiency. Our result demonstrated that for Prosoy, extracting twice with the same amount of water could increase solid and

protein recovery compared with one-time extraction. This result is consistent with that of Beddows and Wong (1987a) which showed 10:1 water-to-bean ratio could yield more protein and solid in soymilk compared with 8:1 ratio, but the double extraction of 8:1 plus 2:1 ratio could give 3.3% higher protein recovery than a single 10:1 ratio extraction. During extraction process, protein-water and protein-protein interaction played an important role (Beddows and Wong, 1987a). In addition, higher yields of 7S protein and total protein were obtained via re-extraction (Cai and Chang, 1999). But for some varieties, the above two processes (Method #1and Method #4) gave opposite results as shown for black soybeans.

If we compare Method #1 and Method #3 (Table 1-5), we can find that there were no measurable differences between the two extraction methods in terms of percent solid and protein in soymilk. This can be explained by the little loss of solid in soaking water (Table 1-6). Because soaking was done at 4° C, only 1.28% and 2.14% solid remained in soaking water for Prosoy and Black soybeans, respectively, not to mention protein. This is very similar to the result of Winston et al. (1968b). In their study, when dehulled soybeans were soaked at 1 °C for 24 h, only 5% solid was lost in soaking water, in which crude protein was about 23.6%. In addition, Wang et al. (1979) also reported a 4.65% solid loss and a 0.58% protein loss in soaking water after soaking at 20°C for 20 h. Furthermore, Beddows and Wong (1987a) reported a 2.51% solid loss at 20°C for 16 h. However, Method #3 could result in the saving of water used for soymilk processing.

Table 1-6. Percentage distribution of solids as affected by different extraction methods

Material	Extraction methods	Soaking water	Okara	Soymilk
Prosoy	Method #1	1.28A(0.01)	32.06A(0.86)	62.78C(0.78)
	Method #2	1.28A(0.01)	31.48A(1.08)	72.60A(0.81)
	Method #3	1.28A(0.01)	31.48A(1.14)	63.78C(1.34)
	Method #4	1.28A(0.01)	26.95B(1.21)	67.57B(0.87)
Black	Method #1	2.14A(0.02)	32.03A(0.22)	62.25B(0.62)
	Method #2	2.14A(0.02)	32.38A(2.09)	70.55A(3.30)
	Method #3	2.14A(0.02)	32.81A(0.72)	62.82B(1.08)
	Method #4	2.14A(0.02)	32.10A(0.98)	61.80B(0.92)

Means with different capital letters in the same column are significantly different among different grinding methods for the same variety ($p<0.05$).
Values in parentheses are SD (n=3).

Conclusion

In summary, cold grinding and hot grinding could result in significantly lower solid yield and protein recovery than ambient grinding, Extraction of solid and protein in soymilk could be improved by re-extraction of proteins in okara. Different varieties showed different characteristics during processing.

CHAPTER 2. TRYPSIN INHIBITORS OF SOYMILK AS AFFECTED BY DIFFERENT GRINDING AND HEATING METHODS

Abstract

Trypsin inhibitors, as anti-nutrients, reduce digestibility of proteins and lead to pancreatic hypertrophy. Inactivation of trypsin inhibitors can be achieved by heat-induced sulfhydryl-disulfide exchange. In this study, two different soybean varieties (Prosoy and black) were processed with three grinding (ambient, cold and hot grinding) and three heating methods (traditional stove cooking, one-phase UHT, and two-phase UHT) for soymilk making. The results showed that in raw soymilk, hot grinding gave the lowest trypsin inhibitory activity (TIA) residue and ambient grinding gave the lowest Bowman Birk (BBI) residue. Kunitz (KSTI) was much more sensitive to heat than BBI; and hot grinding left TI being almost entirely BBI. For raw and cooked soymilk, in most cases, cold grinding resulted in the highest level of TI and BBI. The effect of heating was closely related to grinding methods employed. Generally, stove cooking was the most effective in the inactivation of TI, followed by two-phase UHT in the middle and one-phase UHT being the least effective. Two varieties behaved differently in response to different processing conditions. Because an array of factors could exert effect on thermal stability of TIA, it is difficult to predict TI activity using these complex factors. The actual TI and BBI retention in any specific processing methods need to be experimentally obtained.

Introduction

Adverse effects of TI on human health

It is well established that trypsin inhibitors in food could lead to poor protein digestibility and even pancreas hypertrophy, especially when food is not fully cooked (Liener, 1976; Rackis and Gumbamann, 1981). Roughly 40% decrease in PER of raw soybean in

relation to heated soybean can be attributed to the presence of trypsin inhibitory activity (Liener, 1976). Although trypsin inhibitors are more thermo-stable than major storage proteins, lectins, and lipoxygenases (Yuan et al., 2008), they can still be inactivated by heat treatment with markedly improved protein efficiency ratio and digestibility (Liener, 1976; Su and Chang, 2002).

In an attempt to retain functional properties and nutritional value of protein, most commercial soybean products contain 5-20% of the trypsin inhibitory activity of original soybeans (Rackis and Gumbmann, 1981). Because of the high presence of trypsin inhibitory activity, some population groups could be exposed to high risks. For example, infants, vegetarians, and hyperlipidemia patients who rely on soybean as the major protein source (Liener, 1986).

Molecular structures and characteristics of two major trypsin inhibitors

In soybeans, there are mainly two trypsin inhibitors: Kunitz and Bowman Birk inhibitors. The former is a protein made up of 181 amino acids and molecular weight is about 20,000 daltons. Kunitz inhibitor can depress the activity of trypsin strongly but not chymotrypsin. Bowman Birk inhibitor is composed of 71 amino acids and has a much smaller molecular weight of 8,000 daltons. Different from Kunitz inhibitor, Bowman Birk inhibitor can inhibit the activity of both chymotrypsin and trypsin in equimolar ratio at respective active sites (Dipietro and Liener, 1989a; Baintner, 1981; Wolf, 1977). The different thermal stability of these two inhibitors is related to their characteristic molecular structures. KSTI has two disulfide bonds, one of which is readily reduced. BBI has seven disulfide bonds which make it more stable in response to acid, protease and heat (Wolf, 1977). Inactivation of trypsin inhibitors could be affected by pH, heating temperature and time, water activity, and thiol concentration (Lei et al., 1981; Johnson et al., 1980). For example, in pure aqueous

system, Kunitz inhibitor and Bowman-Birk inhibitor have been reported to be much more thermal resistant than in soy extract. In soy flour, an increase in water activity can make trypsin inhibitors more susceptible to heat treatment (DiPietro and Liener, 1989a). Under alkaline conditions, trypsin inhibitors are much more vulnerable to heat treatment and therefore greatly shorten heating time (Johsnson et al., 1980a; Lei et al., 1981; Obara and Watanabe. 1971; Wallace et al., 1971). Kwok et al. (1993) found that at pH 2.0, inhibitors exhibited much higher thermal stability when heating at 93°C, but when temperature increased to 143 or 154°C, pH almost had no effect. Bowman-Birk inhibitor is primarily responsible for the residual trypsin inhibitory activity in soymilk even after heating at high temperatures (Rouhana et al., 1996). Instead of protein unfolding during heat denaturation, interchange of disulfide linkages between inhibitors and storage proteins such as glycinins, or the degradation of cysteine/cystine have been hypothesized to be partly responsible for the inactivation of trypsin inhibitors, and this was proved by the relatively lower activation energies of KSTI and BBI during heating process than protein unfolding which usually need an higher activation energy of several hundred kJ /mol. (Rouhana et al., 1996).

Objective of this study

Even though a substantial body of research has been done regarding thermal dynamics of trypsin inhibitors, there is no report dealing with effect of grinding methods on trypsin inhibitory activity, especially when in conjunction with heating methods. Yuan et al. (2008) used blanching (80 °C for 2 min) as a pretreatment to reduce trypsin inhibitory activity and this approach proved to be effective to reduce trypsin inhibitory activity. Based on the possible effect of grinding temperature on cysteine and the influence of cysteine on trypsin inhibitors during heating, we speculated that grinding methods might affect residual trypsin inhibitory activity in soymilk especially thermal stability of trypsin inhibitors during

heating. Therefore, the objective of this study was to investigate the residue of trypsin inhibitors, Kunitz inhibitors, Bowman Birk inhibitors as affected by grinding and heating methods.

Materials and Methods

Materials

A portion of soymilk prepared in Chapter 1 was subjected to traditional and UHT heating.

Traditional stove cooking processing of soymilk

One liter of soymilk was put in a small pot which was placed in a larger pot with boiling water on a stove. After the temperature of soymilk reached 90°C, the small pot was switched to the stove surface and heated to boiling, from which point the soymilk was maintained boiling with continual stirring for 20 min. Then the small pot was cooled down in an ice bath to room temperature and sampled in triplicate for GC analysis. The remaining soymilk was freeze-dried for later analysis.

UHT thermal processing of soymilk

In this study, Microthermics Direct/Indirect Steam Injection Processor (DIP, Microthermics, Inc., Raleigh, NC) was used. A combination of two batches of soymilk (about 5800 mL) was pumped into the Microthermics processor. Firstly, the soymilk was preheated quickly to 110 °C in the first stage, then the soymilk was pumped through a holding tube in which soymilk was heated according to specified times and temperatures. In this study, two sets of heating temperature and time combinations were chosen: 140 °C/5 s; 120 °C/80 s + 140 °C/4 s. In the heating tube, the heating medium (steam) was in direct contact with

soymilk. The Microthermics Processor was equipped with a vacuum chamber (50 kPa) to cool and remove volatile compounds and the added water. The soymilk was further cooled by circulating tap water in a tubular heat exchanger and the final temperature of the product was 25 °C. After sampling for GC analysis, a portion was freeze-dried for later analysis. The samples from UHT methods were not used for protein and solid analysis, but for the analysis of other parameters.

Chemicals

N-Benzoyl-DL-arginine 4-nitroanilide dydrochloride (BAPNA), N-benzoyl-L-tyrosine p-nitroanilide (BTPNA), α-chymotrypsin from bovine pancreas, trypsin from porcine pancreas were purchased from Sigma-Aldrich Inc (St. Louis, MO).

TI analysis

The method described by Kakade et al. (1974) was used with some modifications. A 2 g sample was put into plastic bottle, 50 mL 0.01N NaOH was added and the mixture was adjusted to pH 8.4-10 with HCl. The bottle was covered with screw-cap and stirred for 2.5 h. Aliquots of 0, 0.6, 1.0, 1.4, 1.8 mL of the sample extract was respectively added into a set of tubes and the volume was adjusted to 2mL. Preliminary test was done to make sure 1.0 ml sample extract could inhibitor 40-60% trypsin. Two mL of 0.002% trypsin in 0.001 N HCl were added and put in water bath at 37°C. Ten min later, 5 mL 0.04% BAPNA (N-Benzoyl-DL-arginine 4-nitroanilide dydrochloride) in tris buffer of pH 8.2 (pre-warmed at 37 °C) were added. After 10 min incubation, 1 mL of 30% (v/v) acetic acid was added and vortexed to stop the reaction. The mixture was filtered through Whatman No. 3 filter paper. The absorbance of filtrate was measured at 410 nm. In reagent blank, water was substituted for sample. In both reagent blank and sample blank, acetic acid was added before BAPNA. One

TIU is equivalent to 0.01 absorbance decrease. TIA was expressed as TIU/g of dry soymilk or mg of TI/g of dry soymilk by dividing TIU with 1900 (Kakade et al., 1969).

BBI analysis

Bowman-Birk inhibitor was assayed on the basis of method described by Bundy (1962) with some modifications. Sample extraction was the same as TI analysis mentioned above. Two mL of sample extract were mixed with 2 mL of 72 µg/mL chymotrypsin and incubated at 35°C in water bath for 10 min. Five mL of 0.06% BTPNA (N-benzoyl-L-tyrosine p-nitroanilide) in tris buffer of pH 8.0 (pre-warmed at 35 °C) was added. After 10 min of incubation, 1 mL of 30% acetic acid was added and vortexed to stop the reaction. The mixture was filtered through two-layers of Whatman No.3 filter paper and then filtered through 0.2 µm membrane filter. The absorbance of filtrate was measured at 410 nm. In reagent blank, water was substituted for sample. In both reagent blank and sample blank, acetic acid was added before BTPNA to stop reaction. A standard curve was established by plotting absorbance against chymotrypsin concentration. Linearity range of the calibration curve was 0 to 16 µg/mL (r=0.99). From the standard curve, BBI was calculated and expressed as mg chymotrypsin inhitibed/g of dry soymilk.

Estimation of Kunitz and BBI

In theory, BBI has two independent binding sites for trypsin and chymotrypsin respectively. One mole of BBI inhibits one mole of trypsin and one mole of chymotrypsin at different active sites. Kunitz has only one active site and thus one mole of Kunitz inhibits one mole of trypsin. Supposing all active sites are active in these inhibitors, we estimated the Kunitz and BBI in µmoles as follows:

Total inhibitors = Kunitz + BBI = TI (1)

BBI = CI (2)

Combining equations (1) and (2), we obtain,

Kunitz = TI - CI (3)

TI, Trypsin inhibited/g of dry soymilk; CI, chymotrypsin inhibited/g of dry soymilk. Molecular weights of trypsin and chymotrypsin are 23800 and 25000 respectively.

Statistical analysis

Soymilk was prepared in triplicate, and the following analyses were completed in duplicate. Data were subject to analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant differences among variables were determined by Duncan's multiple range test (α=0.05). Data are expressed as means ± SD (n=6).

Results and Discussion

Effect of grinding methods on trypsin and chymotrypsin inhibitory activity

As presented in Table 2-1, in raw soymilk, it is apparent that hot grinding yielded significantly ($p<0.05$) lower TIA compared with the other two grinding methods, in which ambient grinding gave significantly ($p<0.05$) lower TIA than cold grinding. In raw soymilk, with regard to BBI, cold grinding also yielded the highest activity in Prosoy soymilk (Table 2-2). However, ambient grinding and hot grinding gave similar BBI activities in the raw soymilk. This can be partly explained by different activity of lipoxygenases under different grinding temperatures. Grinding at room temperature causes more SH degradation than at low temperature (about 2°C) because of different activity of lipoxygenases (Obata et al., 1993; Obata et al., 1996). Hydroperoxide from lipoxygenase-catalyzed oxidation and their secondary products react with cysteine to form adducts (Gardner et al., 1977). Trypsin

inhibitors account for about 2.5% of soybean protein, but constitute 30-40% cystine of total (Kakade et al., 1974). Cystine-cysteine is very labile to hydroperoxides (Roubal and Tappoel, 1965) and lipid hydroperoxides can oxidize cystine-cysteine to cysteic acid and cysteinesulfinic acid (Finley et al., 1981). It is very likely that hydroperoxides generated in ambient grinding would destroy the linkage of trypsin inhibitors, therefore leading to relatively lower TIA and BBI from ambient grinding than cold grinding. In raw Prosoy soymilk, TI contents from ambient and cold grinding were 29.64 and 36.46 mg/g, respectivelly; BBI contents from ambient and cold grinding were 13.13 and 16.09, respectively. In raw black soymilk, a similar trend was observed.

Even though hydroperoxides had such a destructive effect on trypsin inhibitors, hot grinding still showed lower TIA than ambient grinding, and the residual trypsin inhibitory activity was mostly due to BBI (Table 2-2, Table 2-3). This result was mainly because of the destructive effect of heating and the differences in thermal stability of the two trypsin inhibitors.

Actually, some relatively moderate temperatures can also inactivate trypsin inhibitors to some extent. This phenomenon shows that the two trypsin inhibitors behave distinctly different because of different thermal stability. At 80°C, Lei et al. (1981) observed a 35% decline in trypsin activity after 10 min of heating of soymilk. Yuan et al. (2008) found blanching soybeans for 2 min at 80°C could reduce 43% percent of trypsin inhibitory activity of soy milk compared with soy milk from untreated soybeans. With regard to raw soymilk, as shown in Table 2-1, for trypsin inhibitor, hot grinding resulted in significantly lower level in contrast to the other two grinding methods. For raw Prosoy soymilk, residual TI were 26.94, 36.46, and 12.69 mg of TI/g for ambient grinding, cold grinding, and hot grinding, respectively. For raw black soymilk, the values were 21.43, 23.51, and 12.12, respectively.

This means that the hot grinding condition was able to inactivate TI to some extent before real heating step.

Table 2-1. Effect of variety, grinding and heating on trypsin inhititor (mg trpsin inhibited /g of dry soymilk)

Soybean material	Grinding methods	Heating methods	TIU/g	mg of TI/g	Residue%[a]
Prosoy	Ambient grinding	Raw	56320(3735)	29.64Ba1(1.97)	100
		Stove cooking	7989(298)	4.21Cd2(0.16)	14.2
		One-phase UHT	24980(696)	13.15Bb1(0.37)	44.37
		Two-phase UHT	16410(49)	8.64Cc2(0.03)	29.15
	Cold grinding	Raw	69280(512)	36.46Aa1(0.27)	100
		Stove cooking	12290(281)	6.47Ad2(0.15)	17.75
		One-phase UHT	31050(464)	16.34Ab1(0.24)	44.82
		Two-phase UHT	20840(397)	10.97Ac1(0.21)	30.09
	Hot grinding	Raw	24110(229)	12.69Ca1(0.12)	100
		Stove cooking	10590(121)	5.58Bd1(0.06)	43.97
		One-phase UHT	23410(464)	12.33Cb1(0.24)	97.16
		Two-phase UHT	17130(83)	9.02Bc2(0.04)	71.08
Black soybean	Ambient grinding	Raw	40710(1335)	21.43Ba2(0.70)	100
		Stove cooking	11060(611)	5.83Bd1(0.32)	27.2
		One-phase UHT	25790(87)	13.57Bb1(0.05)	63.32
		Two-phase UHT	20020(63)	10.54Ac1(0.03)	49.18
	Cold grinding	Raw	44670(754)	23.51Aa2(0.40)	100
		Stove cooking	13480(51)	7.10Ad1(0.03)	30.2
		One-phase UHT	28320(806)	14.91Ab2(0.42)	63.42
		Two-phase UHT	20460(611)	10.77Ac1(0.32)	45.81
	Hot grinding	Raw	23030(11)	12.12Ca2(0.01)	100
		Stove cooking	9876(110)	5.20Cd2(0.06)	42.9
		One-phase UHT	22120(356)	11.64Cb2(0.19)	96.04
		Two-phase UHT	19170(189)	10.09Bc1(0.10)	83.25

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety.
Means with different lowercase letters in the same column are significantly different among different heating methods for the same grinding methods and same variety.
Means with different numbers in the same column are significantly different between two varieties for the same grinding and heating methods.
Values in parentheses are SD (n=3)
[a]Residual TI of raw soymilk from respective grinding methods is designated as100%.

Table 2-2. Effect of variety, grinding and heating on Bowman-Birk trypsin inhibitor (mg chymotrypsin inhibited /g of dry soymilk)

Soybean material	Grinding methods	Heating methods	BBI	Residue %[a]
Prosoy	Ambient grinding	Raw	13.13Ba1(0.86)	100
		Stove cooking	4.55Cd2(0.01)	34.65
		One-phase UHT	11.54Cb2(0.10)	87.89
		Two-phase UHT	8.29Bc2(0.05)	63.14
	Cold grinding	Raw	16.09Aa1(0.67)	100
		Stove cooking	6.33Ad2(0.29)	39.34
		One-phase UHT	13.63Ab1(0.47)	84.71
		Two-phase UHT	9.03Ac1(0.14)	56.12
	Hot grinding	Raw	13.61Ba1(0.11)	100
		Stove cooking	5.84Bd2(0.02)	42.91
		One-phase UHT	12.95Bb1(0.06)	95.15
		Two-phase UHT	7.89Cc2(0.07)	57.97
Black soybean	Ambient grinding	Raw	9.87Cb2(0.34)	100
		Stove cooking	6.63Bd1(0.14)	67.17
		One-phase UHT	12.02Aa1(0.03)	121.78
		Two-phase UHT	9.02Bc1(0.07)	91.39
	Cold grinding	Raw	12.21Ba2(0.07)	100
		Stove cooking	7.53Ad1(0.05)	61.67
		One-phase UHT	11.49Bb2(0.42)	94.1
		Two-phase UHT	8.66Cc2(0.18)	70.93
	Hot grinding	Raw	13.81Aa1(0.36)	100
		Stove cooking	6.48Bd1(0.17)	46.92
		One-phase UHT	10.99Bb2(0.12)	79.58
		Two-phase UHT	10.47Ac1(0.18)	75.81

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety.
Means with different lowercase letters in the same column are significantly different among different heating methods for the same grinding methods and same variety.
Means with different numbers in the same column are significantly different between two varieties for the same grinding and heating methods.
Values in parentheses are SD (n=3)
[a]Residual BBI of raw soymilk from respective grinding methods is designated as100%.

However, for chymotrypsin inhibitor, hot grinding did not show any advantages over ambient grinding. For example, as shown in Figure 2-1 and Figure 2-2, for raw Prosoy soymilk, TIA from ambient grinding and hot grinding (Figure 2-1) were 81.29% (AR) and 34.81% (HR), respectively with TIA of raw soymilk from cold grinding being 100%. The former was 2.3 times the latter. For BBI, these values are similar, being 81.6% and 84.6%, respectively (Figure 2-2). Table 2-3 more clearly illustrates that in raw Prosoy soymilk, the residual BBI from ambient grinding and hot grinding were 0.53 and 0.54 μmol/g, respectively; the corresponding residual KSTI activities were 0.72 and 0 μmol/g, respectively. This further verified that KSTI was inactivated readily before BBI at moderate (80 °C) temperatures. Our study clearly indicated that cold grinding could prevent trypsin inhibitors from destruction by helping the enzyme stay in the native conformation and therefore limiting hydroperoxide formation. Hot grinding's effect was due to heat-induced denaturation of trypsin inhibitors. However, this denaturation of trypsin inhibitors was almost limited to KSTI, but has very little effect on BBI. This was reflected by the fact that raw soymilk from cold grinding and hot grinding contained similar BBI, especially in the case of black soybeans (Table 2-2).

Table 2-3. Estimated contents of individual inhibitors (μmol/g)

Soybean material	Grinding methods	Heating methods	TI	BBI	KSTI	BBI/TI
Prosoy	Ambient grinding	Raw	1.25Ba1(0.08)	0.53Ba1(0.03)	0.72Ba1(0.05)	0.42
		Stove cooking	0.18Cd2(0.01)	0.18Cd2(0.00)	-0.00Cc1(0.00)	1.03
		One-phase UHT	0.55Bb1(0.02)	0.46Cb2(0.00)	0.09Bb1(0.01)	0.84
		Two-phase UHT	0.36Cc2(0.00)	0.33Bc2(0.00)	0.03Cc2(0.003)	0.91
	Cold grinding	Raw	1.53Aa1(0.01)	0.64Aa1(0.03)	0.89Aa1(0.03)	0.42
		Stove cooking	0.27Ad2(0.01)	0.25Ad2(0.01)	0.02Ad1(0.02)	0.93
		One-phase UHT	0.69Ab1(0.01)	0.55Ab1(0.02)	0.14Ab1(0.03)	0.79
		Two-phase UHT	0.46Ac1(0.01)	0.36Ac1(0.01)	0.10Ac1(0.004)	0.78
	Hot grinding	Raw	0.53Ca1(0.01)	0.54Ba1(0.00)	-0.00Cab1(0.00)	1.02
		Stove cooking	0.23Bd1(0.00)	0.23Bd2(0.00)	0.001ABb1(0.00)	1.00
		One-phase UHT	0.52Cb1(0.01)	0.52Bb1(0.00)	0.00Cb2(0.00)	1.00
		Two-phase UHT	0.38Bc2(0.00)	0.32Cc2(0.00)	0.06Ba1(0.004)	0.83

Table 2-3 (continued)

Black soybean	Ambient grinding	Raw	0.9Ba2(0.03)	0.39Cb2(0.01)	0.51Aa2(0.02)	0.44
		Stove cooking	0.24Bd1(0.01)	0.27Bd1(0.01)	-0.00Ac1(0.00)	1.08
		One-phase UHT	0.57Bb1(0.00)	0.48Aa1(0.00)	0.09Bb1(0.003)	0.84
		Two-phase UHT	0.44Ac1(0.00)	0.36Bc1(0.00)	0.08Bb1(0.002)	0.81
	Cold grinding	Raw	0.99Aa2(0.02)	0.49Ba2(0.00)	0.50Aa2(0.01)	0.49
		Stove cooking	0.30Ad1(0.00)	0.30Ad1(0.00)	-0.00Ad1(0.01)	1.01
		One-phase UHT	0.63Ab2(0.02)	0.46Bb2(0.02)	0.17Ab1(0.03)	0.73
		Two-phase UHT	0.45Ac1(0.01)	0.35Cc2(0.01)	0.11Ac1(0.01)	0.77
	Hot grinding	Raw	0.51Ca2(0.00)	0.55Aa1(0.01)	-0.00Bc1(0.00)	1.08
		Stove cooking	0.22Cd2(0.00)	0.26Bd1(0.01)	-0.00Ac1(0.00)	1.19
		One-phase UHT	0.49Cb2(0.01)	0.44Bb2(0.00)	0.05Ca1(0.01)	0.90
		Two-phase UHT	0.42Bc1(0.00)	0.42Ac1(0.01)	0.01Cb2(0.001)	0.99

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety.
Means with different lowercase letters in the same column are significantly different among different heating methods for the same grinding methods and same variety.
Means with different numbers in the same column are significantly different between two varieties for the same grinding and heating methods.
Values in parentheses are SD (n=3)
Because of experimental errors, when Kunitz content was low, it was likely the calculated value of it according to equation (3) was negative. In this case, Kunitz content was expressed as 0.

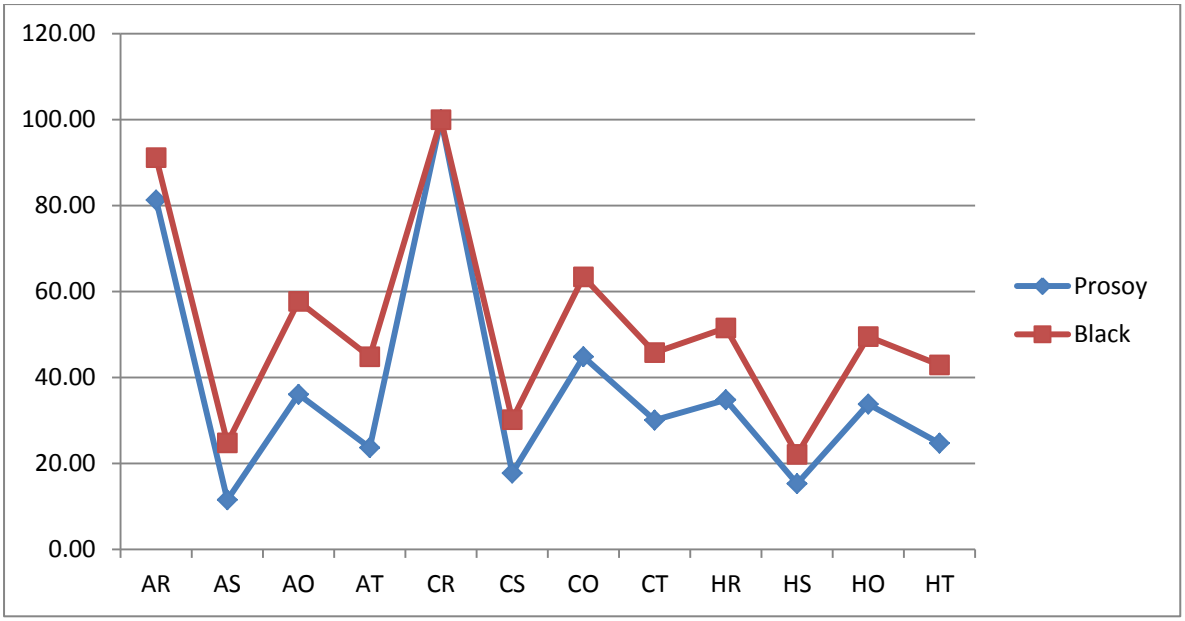

Figure 2-1. Percentage residual TI from various processing conditions with TI of raw soymilk from cold grinding being 100% (A: ambient grinding; C: cold grinding; H: hot grinding; R: raw; S: stove cooking; O: one-phase UHT; T: two-phase UHT).

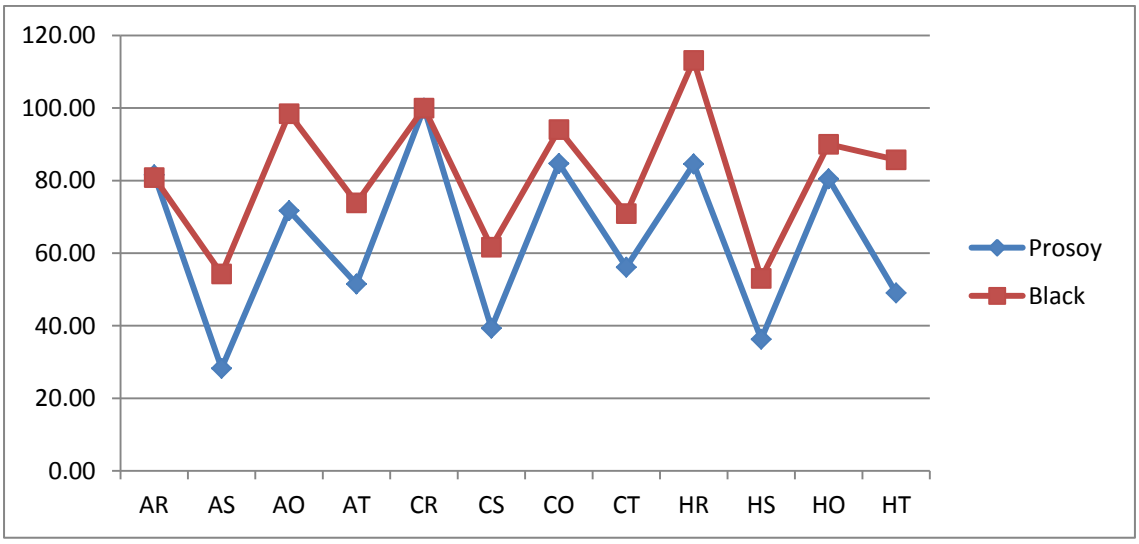

Figure 2-2. Percentage residual BBI from various processing conditions with BBI of raw soymilk from cold grinding being 100% (A: ambient grinding; C: cold grinding; H: hot grinding; R: raw; S:stove cooking; O: one-phase UHT; T: two-phase UHT).

Effect of heating methods on trypsin and chymotrypsin inhibitory activity

As for thermal stability of the two kinds of trypsin inhibitors, reports from different researchers seem somewhat contradictory, this may be due to different procedures, different temperature ranges or even different aqueous solutions employed during measurement (Obara and Watanabe, 1971; Dipietro and Liener, 1989a; Baintner, K. 1981). From Table 2-1 and Table 2-2, we can find out that the same heat treatment reduced trypsin inhibitory activity and chymotrypsin inhibitory activity to different extents for different grinding methods. For example, after stove cooking of Prosoy soymilk, percentage trypsin inhibitor residues for ambient grinding, cold grinding, and hot grinding are 14.2%, 17.75%, 43.97%, respectively, and the percentages of chymotrypsin inhibitor residues were 34.65%, 39.34%, 42.91%, respectively. The significantly different percentage of residual TI and BBI were mostly due to different compositions of soymilk resulting from different grinding methods. Dipietro and Liener (1989a) found that soy extract was more heat labile than pure KSTI and BBI, and attributed this to some possible interaction between substances in soymilk and trypsin inhibitors. Ellenrieder et al. (1980) also found that trypsin inhibitor stability decreased with concentration increase of soy flour suspension. Using chromatography, they further revealed that the substances which destabilized trypsin inhibitors were high-molecular weight, and speculated that they might be proteins. Furthermore, the authors supposed that the non-covalent interaction with other proteins could lead to the decrease of inhibitory ability. As we discussed in the Chapter one (Table 1-1, page 23), protein recovery and composition of solid from the three grinding methods varied greatly. Furthermore, cleavage of disulfide bonds is supposed to destabilize inhibitors, making them sensitive to thermal denaturation (Liu, 1977). But the main reason should be the different behavior of the two major inhibitors. According to Johnson et al. (1980), plotting of log TI residue against heating time gave a curvilinear instead of a single linear line. In this curve, the initial and final parts are linear lines of

distinct slopes with quadratic curve in between as a transitional period. This means the two inhibitors follow different first-order kinetics because of different thermal stability. Therefore, the initial composition of the two inhibitors need to be taken into consideration.

Another plausible reason for the fact that TI and BBI residues showed significantly different rates of inactivation for different grinding methods after the same heating methods was that heat facilitated sulfhydryl-disulfide exchange. Table 2-2 shows that in soymilk from black soybeans, after stove cooking or two-phase UHT process, percentage of BBI residue was lower for hot grinding (47% and 76%) and cold grinding (62% and 70%) compared with ambient grinding (67% and 91%). This can be explained by exchange between the free sulfhydryl groups in proteins and disulfide bonds of BBI (Dipietro and Liener,1989a; Friedman et al., 1982; Lei et al., 1981). Hot and cold grinding might have a higher exchange rate during heating. During heating process, disulfide bonds of inhibitors become exposed because of denaturation, which further advanced sulfhydryl-disulfide interaction (Lei et al., 1981). Interchanges among the sulfhydryl groups and disulfides from soy proteins and trypsin inhibitors could form a complex network, which made it very difficult to revert trypsin inhibitors to their original linkage and conformation, thus reducing their inhibitory ability (Friedman et al., 1982).

Grinding at room temperature could cause more SH degradation than at low temperature (about 2°C) because of different activity of lipoxygenases (Obata et al., 1993; Obata et al., 1996). Lipoxygenases were inactivated at 80°C (Wilkens et al.,1967) and as a result, SH groups of proteins were protected in hot grinding. The higher SH group from cold and hot grinding aided the inactivation of BBI during heating process. But in soymilk from Prosoy, hot grinding and cold grinding did not exhibit definite and obvious effect in the inactivation of BBI. This might be due to different compositions of lipoxygenases. Hence, in

later study, lipoxygenase activity and SH should be investigated to elucidate the effect of grinding methods on the thermal stability of TI and BBI.

Table 2-1 and Table 2-2 showed that stove cooking gave the lowest inhibitor residue, follow by two-phase UHT in the middle and one-phase UHT with highest residue. For Prosoy soymilk from ambient grinding, 20 min boiling resulted in 86% reduction of trypsin inhibitory activity. According to some researchers (Kwok et al., 1993; Hackler et al., 1965), it took 60 min to inactivate 90% TIA at 93°C. Rouhana et al. (1996) found TIA was reduced by 60%, KSTI by 97% and BBI was hardly affected after 1 min of boiling of soymilk. Kwok et al. (1993) revealed that before heating to 93°C, only 50% TIA was retained. Miyagi et al. (1997) reported 57% residual TIA when soymilk just reached boiling after 7 min heating. In our study, it took about 8 min for soymilk to reach boiling, and upon boiling, about 55.1% to 66.5% TIA residue remained depending on varieties (Yuan et al., 2008). Therefore, in studying thermal inactivation, it is necessary to take the heating before boiling into account. Using enzymatic methods and immunoelectroporesis, Diepitro and Liener (1989b) determined that the molar ratio of KSTI to BBI in unheated soy flour was 1.5. Furthermore, Rouhana et al. (1996) found the same ratio in soymilk prepared with the same procedure as ours. Their reports mean that KSTI and BBI account for roughly 60% and 40% of total TIA, respectively (Rouhana et al., 1996). As shown in Table2-3, in raw Prosoy soymilk, the BBI to TI ratio was 0.42, very close to their reports. Our results clearly showed that hot grinding could inactivate most KSTI, but had almost no denaturation effect on BBI.

Hackler et al. (1965) found when 90% of trypsin inhibitory activity was eliminated by heating, the soymilk gave the best protein efficiency ratio. They also concluded that for undercooked soymilk, trypsin inhibitor was a good index to assess the protein nutritive quality. If we take 10% TI residue as the optimal level for nutritional quality to measure the

heating effect, it seems that the heat power used in our study was marginally optimal. In Prosoy soymilk from ambient grinding, after being boiled for 20 min, about 86% was inactivated, which was very similar to the report by Yuan et al. (2008). If we take TI of raw soymilk from cold grinding as original value, there could be a 88.5% inactivation rate (Figure 2-1). As for the time required to inactivate 90% or more TIA, Johnson et al. (1980a) reported 29 min at 99°C, and Miyagi et al. (1997) found 10 min in boiling was adequate. These disagreements can be attributed to soybean employed and cooking practices, such as bean-to-water ratio (Yuan et al., 2008). Table 2-1 also shows soymilk from other grinding methods possessed even higher residual TIA. Therefore, it had been suggested that 30 min be required to achieve 90% TIA reduction (Yuan et al., 2008). At 143°C, 54 s were required to achieve 10% TI residue (Kwok et al., 1993). However, with the same direct UHT processor as ours, Yuan et al. (2008) found that only 80% TIA was inactivated at 143°C for 60 s. They also found after indirect UHT process at 140 °C for 4 s, about 24% of TIA still existed. In our study, in Prosoy soymilk from ambient grinding, about 44% TIA were present after one-phase UHT. In soymilk from cold grinding, the content was even higher. Therefore, one-phase UHT processing used in our study seemed inadequate in TI inhibition. This inadequacy also verified the conclusion of Yuan et al. (2008), that is, the addition of the second-phase heating in the two-phase UHT could not reduce TIA any further. At 121°C, it took 282 s to get 7.6% TI residue (Johnson, et al., 1980a) and Kwok et al. (1993) reported a 6 min processing time to inactivate 90% TIA. After comparison of TI inhibition curve and thermal-death-time curve of putrefactive anaerobic (PA) 3679, Kwok et al. (1993) inferred that above 125°C, more time was needed to achieve 90% TIA inhibition than sterilization. As for two-phase UHT processing, our results were very different from those of Yuan et al. (2008), who reported about 15% retention, much lower than 29.15% of soymilk from ambient grinding in our study. This large discrepancy may be derived from different varieties and the extraction

methods. In their study, an autocentrifugal separator was employed, while, in our study, slurry was pressed by hand.

After heating process, the differences among the three grinding methods became narrowed. Hot grinding, in particular did not show too much advantage over ambient grinding in stove cooking and two-phase UHT. However, in one-phase UHT, it still gave lowest retention of TIA and BBI in most cases. It seems that cold grinding still possessed significantly higher TI and BBI in comparison with the other two grinding methods.

In fact, it is unreasonable to use a unified kinetic model to predict TIA residue (Kwok, et al., 2002). For example, with a combined model, Yuan et al. (2008) and Rouhana et al. (1996) reported Ea to be 34 and 55 kJ/mol, respectively. Ea values for KSTI inactivation were reported to be 24 and 47 kJ/mol, respectively, by Rouhana et al. (1996) and Johnson et al. (1980b), for BBI, the values were 104 and 20 kJ/mol. Although it is commonly assumed that BBI is more thermal stable than KSTI, Rouhana et al. (1996) also revealed when temperature was above 137°C, the first-order reaction rate constant k of BBI became higher than that of KSTI.

The various responses of TIA to heating were not reflected in the lethality (F_0) of the process. On the basis of Z value of 28 reported by Kwok et al. (1993), The F_0 values for different heating temperature and heating time combinations of 100 °C 20 min, 120°C/ 80 s+140 °C/4s, 140°C/5s are 0.4, 1.23, 3.5, respectively. But when calculated according to Z value of 10 as reported by Guo et al. (1997), the corresponding values are 0.16, 6.35, 6.62, respectively. One-phase UHT method was selected on the basis of Z value of 10. However, the actual heat power did not conform to the calculation. As shown in Table 2-1 and Table 2-2, one-phase UHT produced the largest residue for both TI and BBI.

According to Hackler and Stillings (1967), at 121°C, cystine is very vulnerable to heat treatment and damaged shortly after heating. As one of the first-limiting amino acids in soybeans, cystine is an essential parameter to measure protein quality. In addition, at high temperatures, other essential amino acids could be destroyed (Hackler et al., 1965). Taking this into consideration and on the basis of calculated F_0 values, we designed the one-phase UHT methods in an attempt to find a good balance of retention of essential amino acids and destruction of anti-nutritional factors.

In view of the foregoing results of many researchers and our own study, it could be summarized that inactivation effect of thermal treatment on TI is influenced by an array of factors, such as water-to-bean ratio, presence of protein, pH, SH content, heating apparatus, Aw, proportion of KSTI and BBI, and efficiency of heating and cooling (Kwok et al., 2002).

Effect of varieties on trypsin and chymotrypsin inhibitory activity

If we compare the two varieties, in raw soymilk, Prosoy gave significantly ($p<0.05$) higher TI and BBI level than black soybean. However, after heating, no definite trend existed (Table2-1, Table 2-2). In addition, as Figure 2-1 and Figure 2-2 show, the percentages of TI and BBI in relation to cold raw soymilk also showed great differences with regard to the two varieties. This is very likely due to their different compositions and the responses to thermal treatment. Yuan et al. (2008) reported different varieties behaved differently in terms of TI inactivation under various heating times and heating conditions.

Conclusion

In summary, grinding methods, heating methods, and variety all had significant effects on the content and distribution of two trypsin inhibitors. Much of KSTI could be inactivated by 80 °C hot grinding. If TI and BBI from raw soymilk after cold grinding were designated

as 100%, further heating after grinding inactivated 37-89% TI and with most (28-98%) of BBI remained in the soymilk products. It is difficult to accurately predict TI residue due to a lot of factors involved. In this study, lipoxygenase activity and SH content after different grinding method should be further measured to explore the effect of grinding methods.

CHAPTER 3. PHENOLIC COMPOUNDS AND ANTIOXIDANT CAPACITY OF SOYMILK AS AFFECTED BY DIFFERENT GRINDING AND HEATING METHODS

Abstract

In soymilk, soymilk exerts its health-promoting effects mainly through its antioxidant capacity. However, antioxidant compounds and overall antioxidant property can be altered greatly during processing. In this study, two different soybean varieties (Prosoy and black) were processed with three grinding (ambient, cold and hot grinding) and three heating methods (traditional stove cooking, one-phase UHT, and two-phase UHT) for soymilk making. The results showed that hot grinding generated significantly higher ($p<0.05$) TPC, TFC, CTC, DPPH, and ORAC as compared with the other two grinding methods. Soymilk from black soybean contained significantly higher ($p<0.05$) antioxidants and antioxidant capacity. Heating effects varied greatly with regard to different grinding methods and varieties. Effect of heating on antioxidant capacity was affected by factors including heating time, heating temperature and oxygen availability.

Introduction

Phenolics are secondary metabolites generated from plant phenylalanine. Many functional properties especially antioxidant activity of food are due to the presence of phenolics. Natural phenolics exert their beneficial effects mainly through their antioxidant activity (Fang et al., 2002). Free radicals in human body cause oxidative lesion to molecules and cells, leading to a number of chronic diseases. The phenolic compounds are capable of decreasing oxygen radicals concentration, intercepting singlet oxygen, preventing 1^{st}-chain initiation by scavenging initial radicals such as hydroxyl radicals, binding metal ion catalysts, decomposing primary products of oxidation to nonradical species, and breaking chains to

prevent continuous hydrogen abstraction from substances (Shahidi and Naczk, 2004). However, these health-promoting functions vary greatly under processing conditions which can influence bioactivity, content, and bioavailability of these compounds (Nicoli et al., 1999). Flavonoid is a broad term for numerous compounds with common skeleton : C_6-C_3-C_6. According to oxidation level of the pyran ring and the groups attached at different positions of the three rings, flavonoids are divided into isoflavones, flavans, and flavones.

The effects of heating on the antioxidant profile have been studied extensively on fruits, vegetables, and tea. To the best of our knowledge, only one paper is available dealing with the effect of heating on antioxidant compounds and capacity of soymilk (Xu and Chang, 2009). However, their heating methods were different from ours. As ways to inactivate lipoxygenases, hot grinding and cold grinding have been studied only for their effect on soy odor and protein and solid recovery, but antioxidant capacity subjected to these two grinding methods has not been investigated yet. Antioxidant function, as the basis of many health benefits, should be fully studied as affected by different processing conditions. Therefore, the objectives of this study were to investigate the effects of three grinding methods on the extraction of antioxidants and antioxidant capacity and to study the change of antioxidants and antioxidant capacity when subjected to different heating methods.

Materials and Methods

Total phenolic content (TPC) analysis

Chemicals and reagents

Folin-Ciocalteu, gallic acid were purchased from Sigma-Aldrich Inc. (St. Louis, MO). $NaCO_3$ was purchased from VWR International (West Chest, PA). UV-Visible spectrophotometer (UV-160, Shimadzu, Japan) was used in this assay.

Extraction of total phenolics

Phenolic extraction was conducted according to methods described by Xu and Chang (2007). About 0.5 g of soymilk powder was put into a set of 15 mL centrifuge tubes. Five mL of extraction solvent was added. For Prosoy, the solvent was acetone/water (50:50, v/v), for black soybean, the solvent was acetone/water/acetic acid (50:49.5:0.5, v/v/v). The capped tubes were shaken for 3 h and centrifuged. The supernatant was poured into another 15 mL centrifuge tube and the precipitate was re-extracted with the same amount of solvent for about 12 h. The two extracts were combined and kept at 4°C in the dark till use.

Total phenolic content (TPC) determination

TPC was determined according to Singleton and Rossi (1965) with slight modification (Xu and Chang, 2007). In brief, 50 µl of extract, 250 µl of Folin Ciocalteu, 3 mL of deionized distilled water, 750 µl of 7% Na_2CO_3 were mixed in a test tube and incubated for 8 min at room temperature. Then 950 µl of DDW was added. The mixture was allowed to stand at room temperature for 2 h.The absorbance was measured at 765 nm against a reagent blank, in which DDW was substituted for sample abstract. A standard curve was established to calculate TPC in sample. TPC was expressed as mg of gallic acid equivalent /g of dry material (GAE/g).

Total flavonoid content (TFC) analysis

Chemicals and reagents

(+)-Catechin was purchased from Sigma-Aldrich Inc (St. Louis, MO). $NaNO_2$, $AlCl_3.H_2O$, and NaOH were purchased from VWR International (West Chest, PA). UV-Visible spectrophotometer (UV-160, Shimadzu, Japan) was used in this assay.

Extraction of samples

The same as TPC analysis mentioned on page 54.

Total flavonoid content (TFC) determination

TFC was determined according to Jia et al. (1999) as used in our lab (Xu and Chang, 2007). In brief, 1mL of extract and 75 µl of 5% $NaNO_2$ was mixed in a test tube and was allowed to stand for 6 min. 150 µl of 10% $AlCl_3.H_2O$ was added and allowed to stand for another 10 min before 0.5 mL of 1M NaOH was added. Three mL of DDW were added and mixed well. The absorbance was measured immediately at 765 nm against a reagent blank, in which DDW was substituted for sample extract. A standard curve using (+)-catechin replacing extract was established to calculate TFC in sample. TPC was expressed as mg of catechin equivalent/g of dry material (CAE/g).

Condensed tannin content (CTC) analysis

Chemicals and reagents

(+)-Catechin and Vanillin were purchased from Sigma-Aldrich Inc (St. Louis, MO). Methanol was purchased from VWR International (West Chest, PA) UV-Visible spectrophotometer (UV-160, Shimadzu, Japan) was used in this assay.

Extraction of sample

The same as TPC analysis mentioned on page 54.

Condensed tannin content (CTC) determination

CTC was determined according to Broadburst and Jones (1999) as used in our lab (Xu and Chang, 2007). In brief, 5 µl of extract, 3 mL of 4% vanillin in methanol and 1.5 mL

concentrated hydrochloric acid were mixed in a test tube which was allowed to stand at room temperature for 15 min. The absorbance was measured immediately at 500 nm against a reagent blank, in which methanol was substituted for sample extract. A standard curve using (+)-catechin replacing extract was established to calculate CTC in sample. TPC was expressed as mg of catechin equivalent/g of dry material (CAE/g).

DPPH scavenging activity analysis

Chemicals and reagents

DPPH free radical was purchased from Sigma-Aldrich Inc (St. Louis, MO). Ethanol was purchased from VWR International (West Chest, PA) UV-Visible spectrophotometer (UV-160, Shimadzu, Japan) was used in this assay.

Extraction of sample

The same as TPC analysis mentioned on page 54

DPPH scavenging activity determination

DPPH scavenging activity was determined according to Chen and Ho (1995) as used in our lab (Xu and Chang, 2007). In brief, 0.2 mL of extract, 3.8 mL of 0.1 mM DPPH in ethanol were mixed in a test tube and shaken vigorously and then allowed to stand at room temperature in the dark for 30 min. The absorbance was measured immediately at 517 nm against a reagent blank of ethanol. A control analysis was done with 0.2 mL of extraction solvent replacing sample extract. The percent discoloration of DPPH was expressed according to equation $1-(A_{sample}/A_{control})$. A standard curve using trolox in lieu of extract was established to calculate DPPH scavenging activity in samples. The DPPH scavenging activity was expressed as μmole of trolox equivalent /g of dry material.

Oxygen radical absorbance capacity (ORAC) analysis

Chemicals and reagents

Trolox, fluorescein, and AAPH were purchased from Sigma-Aldrich Inc (St. Louis, MO). Ethanol was purchased from VWR International (West Chest, PA).BMG Fluostar Optima Microplate Reader (BMG Labtech, Inc. Chicago, IL) was used in this assay.

Extraction of sample

The same as TPC analysis mentioned on page 54.

ORAC determination

ORAC was determined according to Prior et al. (2003) as used in our lab (Xu and Chang, 2007). In brief, sample extracts were diluted with phosphate buffer (0.75mM, pH7.0) to properly fit the linearity range of the standard curve. Twenty μl of sample extracts, serial standards (trolox), and blank (phosphate buffer) were filled into wells of 96-well microplate according to predesigned layout. Microplate was covered and put into the incubator for 50 min at the set temperature of 37.5°C to equilibrate the liquids in the microplate. Meanwhile, 50 mL of $4.5x10^{-7}$ g/mL fluorescein in phosphate buffer was incubated in another water bath at 38.5°C. About 50 min later, 0.216 g of AAPH was dissolved in 5 mL pre-warmed phosphate buffer at 38.5°C. Fluorescein and AAPH solutions were put into the chamber of the equipment and connected to the pumps, then the equipment was turned on to operate. The injection volume of fluorescein was set at 200 μl and AAPH injection volume was set at 20 μl. Kinetic reading was recorded for 45 cycles with 60 s each, excitation wavelength was set at 485nm and emission wavelength was set at 520nm. The area under curve was calculated with the following formula: AUC=0.5+(R2/R1+R3/R1+R3/R1F+--+0.5Rn/R1), where R1 was the first fluoresce reading and Rn was the last. The net AUC is the AUC of sample or standard

minus that of blank. ORAC was calculated through the standard curve in the range 6.25-50 μmol/mL of trolox and expressed as μmol of trolox equvilent/g of dry material (μmol of TE/g).

Statistical analysis

Soymilk was prepared in triplicate and following analyses were done in duplicate. Data were subject to analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant differences among variables were determined by Duncan's multiple range test (α=0.05). Data are expressed as means ± SD (n=6).

Results and Discussion

Effects of grinding methods, heating methods and variety on TPC

Effects of grinding methods on TPC

Table 3-1 shows that either for Porsoy or black soybeans, significant differences existed among the three grinding methods with hot grinding yielding the highest, ambient grinding in between and cold grinding the lowest TPC. However, grinding methods seemed to exert different effect on TPC values for the two varieties. For example, in the case of raw Prosoy soymilk, in comparison with ambient grinding, hot grinding produced approximately 9% more TPC, while for black soybeans, there was roughly a 50% riseThe advantage of hot grinding in the preservation of antioxidant compounds and antioxidant capacity was further supported by the subsequent analyses of CTC, ORAC, DPPH. Our results were consistent with the report of Xu and Chang (2009) who revealed that raw soymilk from lipoxygenase-null soybean variety possessed much higher phenolic compounds and antioxidant capacity than soymilk from normal variety. Therefore oxidative enzyme induced destruction of

antioxidant activity merits attention, and henceforth hot grinding is an effective way to preserve phenolics.

Table 3-1. Effect of grinding methods, cooking methods and variety on total phenolic content (TPC) (mg of GAE/g of dry material)

Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Prosoy	Cold grinding	1.11(0.02)Cb1	1.14(0.03)Cb1	1.13(0.01)Bb1	1.21(0.01)Ca1
	Ambient grinding	1.43(0.02)Bab1	1.42(0.01)Bb1	1.48(0.05)ABa1	1.44(0.01)Bab1
	Hot grinding	1.55(0.02)Ab2	1.63(0.04)Aab2	1.65(0.04)Aa2	1.63(0.05)Aab2
Black soybean	Cold grinding	1.13(0.03)Cbc1	1.10(0.02)Cc1	1.17(0.03)Cab1	1.22(0.03)Ca1
	Ambient grinding	1.26(0.02)Bb2	1.26(0.04)Bb2	1.36(0.04)Ba2	1.33(0.06)Bab2
	Hot grinding	1.87(0.02)Aab1	1.85(0.10)Ab1	1.96(0.03)Aa1	1.88(0.03)Aab1

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3)

Effects of heating methods on TPC

The three heating methods had significantly different effect on TPC level. In most cases, stove cooking reduced TPC slightly. This was similar to the report of Xu and Chang (2009) but the percentage reduction in our study was much smaller than theirs. This may be due to the different varieties used. Heating process could lead to the degradation and transformation of phenolics (Chung et al., 2011). For example, phenolics can be reactants in the Maillard reaction (Djilas and Milic, 1994).

However, conversely, an increase of TPC after two UHT treatments was observed with one-phase-UHT giving the highest value. But under UHT conditions of 143°C for 60 s, Xu and Chang (2009) observed decrease in TPC value. This tremendous effect of heating conditions on TPC and antioxidant activity had been proven by many researchers (Turkman et al., 2005; Ismail et al., 2004). In fact, the same heating method may have a distinct effect on the TPC for different products because of different composition and content of phenolics as well as other components present. During thermal treatment, some available phenolics may be decomposed and some new ones could be released (Xu and Chang, 2008b). As a major way of phenolic degradation, heat-induced decrease of phenolics include oxidation, loss of volatile compounds, decomposition of heat sensitive compounds (Georgetti et al., 2008). In view of these degradation mechanisms, the prolonged exposure to the atmosphere might in part explain why stove cooking gave the lowest TPC among the three heating methods. For example, as an important part of phenolics, isoflavones undergo intense interconversion and degradation (Ungar et al., 2003; Kao et al., 2004). In the meantime, some isoflavones are freed from the complex with proteins (Malaypally and Ismail, 2010).

It seems that the change of TPC depends on thermal conditions applied and the products under thermal treatments. For example, Xu and Chang (2008b) found that for the

same yellow soybean, different thermal conditions could lead to rise or decline of TPC. For example, the interconversion among the four forms of each isoflavone type during processing makes it even more complicated. Phenolics include a large variety of compounds, which behave differently in response to thermal processes to show complex variations.

Effects of variety on TPC

Soymilk from the two varieties followed similar pattern with regard to processing conditions. However, the difference between them changed with different grinding methods. In cold grinding, they are similar. In ambient grinding, Prosoy produced significantly higher ($p<0.05$) TPC than black soybeans, while, in hot grinding, Prosoy generated significantly lower ($p<0.05$) TPC than black soybeans.

Effects of grinding methods, cooking methods and variety on TFC

Effects of grinding and heating methods on TFC

As Table 3-2 shows, raw Prosoy soymilk from cold and hot grinding contained significantly ($p<0.05$) lower levels of TFC compared with the ambient grinding method, which is in contrast to TPC and other antioxidant analyses. But after heating treatment, TFC of hot grinding showed an increasing trend contrary to the decreasing trend of the other two grinding methods. We do not know why hot grinding was different from other grinding methods upon heating. We think there must be a close interaction between two factors: grinding and heating. After heating, Prosoy soymilk from three grinding methods contained similar TFC to raw soymilk. However, for black soymilk, TFC from hot grinding was much higher than that from the other two grinding methods. For the same grinding method, three heating methods resulted in very similar TFC levels in black soymilks.

Effects of variety on TFC

If we compare the two varieties, it is apparent that black soymilk possessed much higher TFC than Prosoy soymilk, which is in agreement with report of Xu and Chang (2009). For example, even after heat processing, TFC in black soymilk was 2-4 times higher than Prosoy soymilk. It is noteworthy that in Black soymilk from ambient and cold grinding, heating could reduce TFC by half. This may be due to the different content of anthocyanins between the two varieties. As a kind of flavonoid, anthocyanins are rich in seed coat of black soybeans, but are not present in seed coat of yellow soybeans (Xu and Chang, 2008ab). However, anthocyanins are very thermal-labile and readily degrade during heating process (Xu and Chang, 2008b).

Effects of grinding methods, cooking methods and variety on CTC

Effects of grinding and heating methods on CTC

Table 3-3 clearly shows that significant differences existed among the three grinding methods, with hot grinding giving the highest CTC, followed by cold grinding and ambient grinding, which generated the lowest level. CTC from hot grinding was almost twice that from ambient grinding. Different from TPC and TFC, cold grinding resulted in significantly higher CTC than ambient grinding. This might be due to less leaching of tannin during cold soaking step. At the room temperature, winged beans lost more than half tannin during soaking for 24 h (Sathe and Salunkhe, 1981; de Lumen and Salamat, 1980). Further work should be done to compare the leaching of condensed tannin in the soaking water at different temperatures. It is very obvious that any heating methods could increase the content of condensed tannin, but UHT increased it more compared with stove cooking methods. Regardless of grinding methods and heating methods, black soymilk contained much higher CTC than Prosoy soymilk.Cooking had been reported to reduce CTC of beans by forming

insoluble complex with protein or other compounds (Bressania et al., 1982). However it is not always the case. As reported by Xu and Chang (2008), boiling and steaming of yellow soybeans increased CTC, but under the same thermal conditions, black soybeans showed a decreasing trend.

Effect of variety on CTC

Black soybean yielded significantly higher CTC than Prosoy soybeans in soymilk. According to Xu and Chang (2008b), black soybeans contained higher CTC than yellow soybeans .The differences may be because of the differences of seed coat color (Bressani and Elias, 1980).

Table 3-2. Effect of grinding methods, cooking methods and variety on total flavonoid content (TFC) (mg of CAE/g of dry material)

Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Prosoy	Cold grinding	0.21(0.01)Ba2	0.17(0.01)Bb2	0.19(0.02)Bab2	0.21(0.02)Aa2
	Ambient grinding	0.39(0.07)Aa2	0.22(0.02)Ab2	0.21(0.02)Ab2	0.20(0.02)Ab2
	Hot grinding	0.16(0.00)Bc2	0.22(0.01)Aa2	0.23(0.02)Aa2	0.19(0.01)Ab2
Black soybean	Cold grinding	1.12(0.01)Aa1	0.50(0.01)Bc1	0.55(0.00)Bb1	0.53(0.00)Bb1
	Ambient grinding	1.10(0.03)Aa1	0.49(0.03)Bc1	0.55(0.02)Bb1	0.50(0.01)Bbc1
	Hot grinding	0.97(0.08)Bab1	0.81(0.01)Ac1	1.03(0.06)Aa1	0.93(0.01)Ab1

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).

Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).

Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).

Values in parentheses are SD (n=3)

Table 3-3. Effect of grinding methods, cooking methods and variety on condensed tannin content (CTC) (mg of CAE/g of dry material)

Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Prosoy	Cold grinding	1.23(0.01)Bb2	1.25(0.11)Bb2	1.63(0.15)Ba2	1.39(0.01)Bb2
	Ambient grinding	0.96(0.01)Cc2	1.03(0.03)Cb2	1.09(0.06)Cb2	1.17(0.03)Ca2
	Hot grinding	2.08(0.10)Ab2	2.08(0.06)Ab2	2.18(0.05)Aab2	2.25(0.09)Aa2
Black soybean	Cold grinding	1.95(0.06)Bbc1	2.07(0.03)Bb1	1.90(0.03)Cc1	2.72(0.14)Ba1
	Ambient grinding	1.49(0.05)Cd1	1.81(0.12)Cc1	2.45(0.14)Ba1	2.25(0.07)Cb1
	Hot grinding	3.14(0.09)Ab1	3.29(0.06)Ab1	3.69(0.06)Aa1	3.73(0.10)Aa1

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3)

Effects of grinding methods, cooking methods and variety on DPPH scavenging activity

As presented in Table 3-4, hot grinding gave significantly ($p<0.05$) higher DPPH scavenging activity than the other two grinding methods. In the case of raw Prosoy soymilk DPPH from hot grinding was 5 times higher than that from ambient and cold grinding. In the black soymilk, hot grinding gave nearly two times higher DPPH compared with the other two grinding methods.

Most heating methods could substantially increase DPPH, particularly in Prosoy soymilk, in which case, there were 50 to 370% increases of DPPH after heating. However, for black soymilk, DPPH exhibited no obvious changes when subjected to the three heating methods. However, in black soybean soymilk, stove cooking method did not increase DPPH scavenging effect. The heating effect on DPPH as observed in our study was consistent with the report by Xu and Chang (2009). But if we compare Table 1 and Table 4, it is very apparent that the two tables are not comparable. This phenomenon was observed by other researchers. For example, Georgetti et al. (2008) found the coefficient of variation R^2 between TPC and DPPH was 0.67 for spray dried soybean extract, while Xu and Chang (2009) got R^2 value of 0.37 for stove cooked yellow soymilk. This suggests that DPPH change could not be explained exclusively by TPC change because of the presence of other antioxidants and the synergism of them based on their chemical structure (Georgetti et al., 2008; Djeridane et al., 2006). Antioxidant capacity undergoes complex changes depending on intensity and duration of thermal treatment. The naturally occurring antioxidants could be degraded through oxidation or heat-induced decomposition for some thermal-labile components such as anthocyanins (Georgette et al., 2008). In addition, polyphenols could also be involved in Maillard reactions as reactants (Yaylayan, 1997). In the meantime, the antioxidant activities of these naturally occurring antioxidants could be improved through

structure modification at immediate oxidation stage during heating (Kikugava et al., 1990; Nicole et al., 2000). The improved antioxidant capacity of partially oxidized polyphenols could be attributed to their increased ability to donate hydrogen atom. Chemical and oxidative oxidation of phenolics could occur at different rates influenced by time, temperature, and oxygen availability (Nicoli, 1999). Maillard reactions should be considered during soymilk processing because of the different contribution of Maillard reactions from different stages to total antioxidant capacity. Soymilk is rich in protein, especially lysine, which is very prone to react with reducing sugars such as fructose and glucose In spite of the antioxidant activity of Maillard reaction products, highly reactively radicals as pro-oxidants form during early stage before the Amadori rearrangement. Thus, the effect of Maillard reaction is greatly related to the heating intensity. According to the study of Calligaris et al. (2004), browning occurred instantly with increased chain-breaking activity when milk was heated at 120 ºC; when milk was heated at 80 and 90°C, a decrease of chain-breaking activity was observed in the first 1.5-2 hr. Anese et al. (1999) also found in tomato puree, at 95°C, in the first 3 h, oxygen uptake decreased and attributed it to the formation of pro-oxidant in the early stage. Table 3-4 clearly shows that in most cases, soymilk from UHT process contained significantly ($p<0.05$) higher (20-41%) DPPH scavenging capacity than that from traditional stove cooking. Using more severe direct-UHT method of 143°C for 60 s, Xu and Chang (2009) found the DPPH scavenging capacity after UHT processing was almost 100% higher than that from traditional stove cooking. This might be at least partly explained by the formation of brown pigment, which can be noticed though we did not conduct color test. The high positive correlation between browning and antioxidant properties have been reported in various systems extensively (Turkmen et al., 2006; Amigo-Benavent et al., 2010). Non-enzymatic browning is a very complicated process involving complex pathways forming various compounds. In addition, the compounds formed at different stages possessed

differential antioxidant properties (Turkmen et al., 2006). According to kinetic analysis, browning was highly temperature-dependent, which meant product formation and the antioxidant capacity were affected by temperature (Carabasa-Giribet and Ibarz-Ribas, 2000; Kwok et al., 1999). Therefore, processing conditions were vital for the formation of melanoidin compounds. In order to investigate how much browning contributed to the DPPH increase, the correlation analysis between them is necessary. Black soybean yielded significantly higher DPPH than Prosoy soybeans, which was in agreement with the report of Xu and Chang (2009).

Table 3-4. Effect of grinding methods, cooking methods and variety on DPPH scavenging activity (μmol of trolox/g of dry material)

Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Prosoy	Cold grinding	0.14(0.02)Bc2	0.55(0.06)Bb2	0.48(0.04)Cb2	0.66(0.03)Ba2
	Ambient grinding	0.19(0.01)Bc2	0.56(0.09)Bb2	0.79(0.03)Ba2	0.54(0.01)Bb2
	Hot grinding	0.98(0.08)Ab2	1.58(0.20)Aa2	1.49(0.23)Aa2	1.50(0.29)Aa2
Black soybean	Cold grinding	2.77(0.15)Bb1	2.73(0.05)Bb1	3.36(0.07)Ba1	3.41(0.14)Ba1
	Ambient grinding	2.32(0.35)Bc1	2.37(0.27)Cc1	3.27(0.06)Ba1	2.84(0.01)Cb1
	Hot grinding	6.60(0.27)Ab1	6.5(0.13)Ab1	7.12(0.26)Aa1	6.67(0.1)Ab1

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3)

Effects of grinding methods, cooking methods and variety on ORAC

As shown in Table 3-5, hot grinding gave rise to significantly ($p<0.05$) higher ORAC than cold and ambient grinding methods. In raw soymilk, ambient grinding and cold grinding gave similar ORAC values ($p<0.05$). As compared to raw soymilk, in most cases, all heating methods significantly reduced ORAC. It seems that stove cooking reduced ORAC the most. In some cases, for example, in cold grinding, two- phase UHT even increased ORAC. Xu and Chang (2009) also observed different changes of ORAC under different heat processing methods for different varieties. Black soybean yielded significantly higher ORAC than Prosoy soybeans, which is in agreement with the report of Xu and Chang (2009).

It should be noted that DPPH and ORAC assays did not match as much and sometimes changed in opposite directions. This phenomenon was also observed by other researchers under similar conditions (Xu and Chang, 2009). This is mainly because of different mechanism adopted for DPPH and ORAC analyses. The former uses single electron transfer (SET), the latter involves hydrogen atom transfer (HAT). The antioxidant mechanism involved for any single antioxidant varied greatly depending on the system in which antioxidants exist. In addition, SET and HAT mechanisms occurs simultaneously for all samples and pH and antioxidant structure determine the balance between the two antioxidant mechanisms (Prior et al., 2005).

Table 3-5. Effect of grinding methods, cooking methods and variety on oxygen radical absorption capacity (ORAC) (μmol of trolox/g of dry material)

Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Prosoy	Cold grinding	45.27(1.78)Bb1	38.23(1.75)Cc1	43.49(1.87)Cb1	54.15(2.88)Aa1
	Ambient grinding	47.74(3.78)Ba1	44.37(2.06)Ba1	47.71(1.95)Ba1	39.28(2.15)Bb1
	Hot grinding	76.05(1.33)Aa2	49.31(0.20)Ac2	55.88(0.81)Ab1	54.30(1.35)Ab2
Black soybean	Cold grinding	43.18(2.01)Bb1	41.16(1.74)Bb1	44.38(3.03)ABb1	55.09(1.36)Ba1
	Ambient grinding	44.22(1.56)Ba1	37.59(1.40)Bc2	40.36(0.72)Bb2	42.29(1.14)Cab1
	Hot grinding	84.98(1.44)Aa1	65.79(3.62)Abc1	57.88(12.82)Ac1	76.67(1.32)Ab1

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3)

Conclusion

In summary, hot grinding showed tremendous advantage over the other two grinding methods in the extraction of antioxidants. Heating increased or decreased antioxidants and antioxidant capacity, depending on grinding methods and variety. However, it is very difficult to obtain a definite pattern of the effect on antioxidant capacity by heating because it is affected by various factors.

CHAPTER 4. SELECTED ODOR COMPOUNDS OF SOYMILK AS AFFECTED BY DIFFERENT GRINDING AND HEATING METHODS

Abstract

Off-flavor of soymilk is a barrier to the acceptance of consumers. The objectionable soy odor can be reduced through inhibition of their formation or through removal after being formed. In this study, soymilk was prepared by three grinding methods (ambient, cold and hot grinding) from two varieties (yellow Prosoy, and a black soybean) before undergoing three heating processes: stove cooking, one-phase UHT (ultra-high temperature), and two-phase UHT process using a Microthermics Direct Injection Processor, which was equipped with a vacuuming step to remove injected water and volatiles. Eight typical soy odor compounds, generated from lipid oxidation, were extracted by solid-phase micro-extraction (SPME) method and analyzed by gas chromatography. The results showed that hot grinding and cold grinding significantly reduced off-flavor compared with ambient grinding; and hot grinding achieved the best result. The UHT methods, especially the two-phase UHT method, were effective to reduce soy odor. Different odor compounds showed distinct concentration patterns because of different formation mechanisms. The two varieties behaved differently in odor formation during the soymilk making process. Most odor compounds could be reduced to below detection limit through a combination of hot grinding and two-phase UHT processing. However, hot grinding gave lower solid and protein recoveries in soymilk.

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Introduction

As an aqueous extract of soybeans, soymilk has been consumed for a long time in Asian countries. In recent years, with the FDA approved claim of health benefits (FDA, 1999), soymilk has become more popular in the United States. However, still many Western consumers reject it because of the objectionable beany flavor (McLeod and Ames, 1988). Soy odors can be derived from proteins, carbohydrates and lipids via light, enzymes, heat treatments, and even metal catalysts (Macleod and Ames, 1988; Rackis et al., 1979). However, the major compounds responsible for the grassy-beany flavors are volatile carbonyl compounds from the degradation of hydroperoxides through oxidation of unsaturated fatty acids (Rackis et al., 1979). Many researchers have shown that normal soybean varieties can produce more off-flavor compounds than lipoxygenase-null varieties (Yuan and Chang, 2007; Endo et al., 2004). With molecular oxygen, lipoxygenases catalyze the oxidation of polyunsaturated fatty acids and esters which have a cis, cis-1,4-pendadiene structure (Gardner,1985). Wilkens et al. (1967) reported that lipoxygenase can be inactivated at 80˚C and this is the rationale of hot grinding. But in soymilk, flavor compounds can bind to protein through electrostatic interaction, hydrogen bonding, hydrophobic interaction, or even covalent bonds (Aspelund and Wilson, 1983). The flavor-protein bonding is influenced by glycinin and β-conglycinin fractions, structural state, as well as temperatures (O'Keefe et al., 1991; Damodaran and Kinsella, 1981). Damodaran and Kinsella (1981) reported 2-nonanone had much stronger affinity for soy protein at 5°C than at 25°C and 45°C. And furthermore, protein denaturation could also increase the interaction between odor compounds and soy protein (Damodaran and Kinsella, 1981; Franzen and Kinsella, 1974). Heat-denatured protein has much higher ability to bind n-hexanal than native protein (Arai et al., 1970).

Off-flavor can be reduced or eliminated by limiting its formation or removing it after its generation. Alkaline soaking (Khaleque et al., 1970; Nelson et al., 1976), hot grinding (Endo et al., 2004; Winston et al., 1968), cold grinding (Mizutani and Hashimono, 2004) and gallic acid-aided grinding (Boatright, 2002) have been used to reduce the generation of off-flavor. Direct steam injection also proved to be an effective method to decrease the content of soy odor compounds (Yuan and Chang, 2007 b). Even though hot grinding and cold grinding have been reported to be effective in improving soymilk sensory quality compared with ambient grinding through inhibition of lipoxygenase activity, no study is available to compare these two grinding methods in terms of specific odor compounds. Until now, there is no dada available with regard to the effectiveness of direct UHT on the reduction of soy odor. In this study, one-phase and two-phase direct UHT methods equipped with vacuum chamber were utilized to decrease the presence of soy odors.

According to Wilkens and Lin (1970) and Rackis et al. (1979), a mixture of odor compounds are contributable to the characteristic and most disagreeable green-beany flavor of soymilk. In addition, these compounds are mainly from the lipoxygenase-catalyzed oxidation of linoleic or linolenic acids (Endo et al., 2004). Therefore, hot grinding in combination with direct UHT processing is supposed to reduce the beany flavor to a considerably low level, especially for some odor compounds with extremely low threshold. In this study, our objective was to investigate the effect of different grinding and heating methods on the flavor profile by determining eight typical soy odor compounds quantitatively. These odor compounds have been found to contribute to the soy odor of soymilk (Kobayashi et al., 1995).

Materials and Methods

Samples

The samples were the same as described as Chapter one and Chapter two.

Chemicals

Standards of hexanal, hexanol, 2-pentylfuran, 1-octen-3-one, 1-octen-3-ol, trans-2-nonenal, trans-2, trans-4 –nonadienal, trans-2,trans-4-decadienal, and internal standard 2-methyl-3-heptanone were purchased from Sigma-Aldrich (St. Louis, MO).

Odor Extraction and Gas Chromatography

The method reported by Yuan and Chang (2007a) was used. Immediately after sample preparation, 1 mL of soymilk was put into 4 ml glass vial with Teflon-lined septum. Five μl of 50 ppm internal standard were injected into the vial by syringe. The vial was shaken to achieve equilibrium and placed into a water bath at 40°C for 4 min. Then, the vial was placed on a hot plate set at 60°C for 6 min before injecting to the gas chromatograph (GC). During this process, a SPME (solid phase microextraction) fiber was employed for headspace extraction. Before use every day, the SPME fiber was conditioned at 255°C for 15 min in the injection port. All vials, caps and septa were baked in oven at 105°C overnight to eliminate interference from other volatiles.

A HP 5890 gas chromatograph (Hewlett-Packard Product, Avondale, PA) was used. The column used was capillary column with a polar resin of DB-Wax (carbowax, 30 m x 0.25 mm i.d. x 0.25μm film thickness). Injector and detector temperatures were set at 235°C. Initial oven temperature was 35°C and was held for 2 min, then programmed at 10°C/min to 235°C and held for 5 min.

Standard curve was established for each odor compound. Cow's milk (2%) was used as food matrix for establishing standard curves because of its similarity to soymilk.

Statistical analysis

Soymilk was prepared in triplicate and following analyses were done in duplicate. The data were subject to analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant differences among variables were determined by Duncan's multiple range test (α=0.05). Data are expressed as means ± SD (n=6).

Results and Discussion

Effect of grinding methods, heating methods, and variety on hexanal in soymilk

Table 4-1 shows that significant differences existed in hexanal levels among different grinding methods, heating methods and the two varieties. Cold grinding resulted in the highest hexanal content. Hot grinding resulted in significantly ($p<0.05$) lower hexanal (0.05 ppm and 0.16 ppm for yellow and black soybean, respectively) compared with the other two grinding methods (in the range of 3-7 ppm for both soybean varieties). It is obvious the decrease in hexanal by hot grinding was mainly due to the inactivation of the oxidative enzymes at 80 ºC. Figure 1 shows that the lipoxygenase activity of raw soymilk after hot grinding was much lower than that from other two grinding methods. In fact, hot grinding inactivated approximately 99% of the lipoxygenase activities as compared to that in the cold ground soymilk. Soymilk obtained from the hot grinding method was also considered as raw soymilk since 80 ºC was not able to cook the soymilk to inactivate trypsin inhibitors. Our study is the first to report the effect of grinding at various temperatures on soy odor in raw soymilk. All other reported studies had reported the odor composition in the finished product (after both grinding and heating to inactivate antinutrients).

Compared with the raw soymilk, all further heating methods greatly reduced the hexanal content with the order of the ability to reduce hexanal content from high to low: two-phase UHT > one phase UHT > or = stove cooking. For the soymilk after hot grinding and the two-phase UHT process, the hexanal content was reduced to 0.006 and 0 mg/L for black soymilk and Prosoy soymilk, respectively, which were very close or lower than the sensory detection threshold (0.0045 mg/L) (Belitz et al., 2004). Therefore, the two-phase UHT heating method with the equipped vacuum chamber was very effective to reduce soy odor. The one-phase UHT was not more effective in reducing hexanal as compared to the stove heating process, particularly for yellow Prosoy soymilk. In our current study, the hexanal contents in the all but one heated soymilk of black soybean were all below 1 ppm, much lower than the 158 ppm in soymilk reported by Mizutani and Hashimoto processed at the

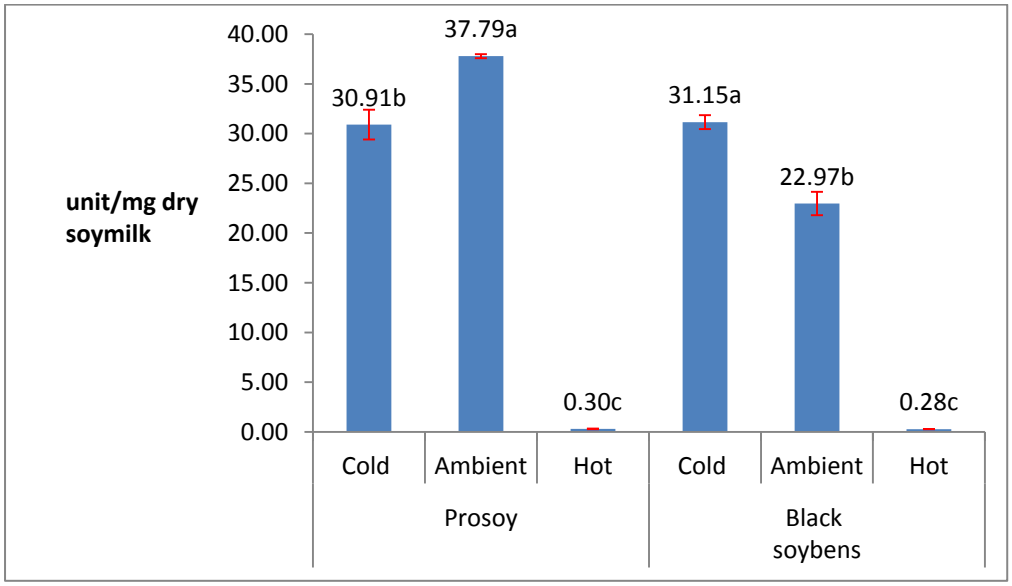

Figure 4-1. lipoxygenase activity of raw soymilk.

Means with different letters are significantly different among different grinding methods within the same variety ($p<0.05$).

Table 4-1. Effect of grinding methods, heating methods, and variety on selected odor compounds in soymilk (ppm).

Odor compounds	Soybean material	Grinding methods	Raw	Stove cooking	One-phase UHT	Two-phase UHT
Hexanal	Prosoy	Cold grinding	6.60A1(0.16)	0.27Ba2(0.04)	0.25Ba1(0.04)	0.14Ab1(0.01)
		Ambient grinding	3.23B2(0.79)	0.54Aa2(0.20)	0.34Aa2(0.01)	0.00Bb2(0.00)
		Hot grinding	0.051C2(0.004)	0.006Ca2(0.002)	0.005Ca1(0.003)	0.00Bb2(0.00)
	Black soybean	Cold grinding	7.12A1(0.47)	0.63Ba1(0.06)	0.26Bb1(0.04)	0.17Ac1(0.01)
		Ambient grinding	7.16A1(0.89)	1.19Aa1(0.34)	0.52Ab1(0.03)	0.048Bc1(0.0005)
		Hot grinding	0.16B1(0.03)	0.027Ca1(0.004)	0.012Cb1(0.005)	0.006Cb1(0.0003)
Hexanol	Prosoy	Cold grinding	0.34B1(0.03)	0.00Aa1(0.00)	0.00Ba1(0.00)	0.00Aa1(0.00)
		Ambient grinding	2.46A1(0.34)	0.00Ab1(0.00)	0.21Aa1(0.07)	0.00Ab1(0.00）
		Hot grinding	0.00B2(0.00)	0.00Aa1(0.00)	0.00Ba1(0.00)	0.00Aa1(0.00)
	Black soybean	Cold grinding	0.16B2(0.02)	0.00Aa1(0.00)	0.00Ba1(0.00)	0.00Aa1(0.00)
		Ambient grinding	1.26A2(0.26)	0.011Ab1(0.015)	0.10Aa1(0.01)	0.00Ab1(0.00)
		Hot grinding	0.038B1(0.015)	0.00Aa1(0.00)	0.00Ba1(0.00)	0.00Aa1(0.00)
2-Penty lfuran	Prosoy	Cold grinding	0.060B1(0.001)	0.24Ba1(0.01)	0.062Bb1(0.001)	0.064Bb1(0.001)
		Ambient grinding	0.064A2(0.001)	0.37Aa1(0.02)	0.074Ab1(0.004)	0.081Ab1(0.004)
		Hot grinding	0.00C1(0.00)	0.061Ca1(0.002)	0.00Cb1(0.00)	0.00Cb1(0.00)
	Black soybean	Cold grinding	0.061B1(0.001)	0.16Aa1(0.07)	0.058Bb2(0.001)	0.058Bb1(0.004)
		Ambient grinding	0.069A1(0.001)	0.23Aa2(0.05)	0.071Ab1(0.005)	0.080Ab1(0.004)
		Hot grinding	0.00C1(0.00)	0.00Ba2(0.00)	0.00Ca1(0.00)	0.00Ca(0.00)

Table 4-1 (continued)

1-Octen-3-one	Prosoy	Cold grinding	0.39A1(0.02)	0.13Ba2(0.01)	0.13Ba2(0.01)	0.11Bb2(0.004)
		Ambient grinding	0.39A2(0.06)	0.18Aa1(0.03)	0.14Ab2(0.004)	0.12Ab1(0.003)
		Hot grinding	0.00B2(0.00)	0.00Ca2(0.00)	0.00Ca2(0.00)	0.00Ca2(0.00)
	Black soybean	Cold grinding	0.40B1(0.02)	0.18Aa1(0.02)	0.17Bab1(0.01)	0.14Ab1(0.02)
		Ambient grinding	0.57A1(0.02)	0.22Aa1(0.03)	0.21Aa1(0.02)	0.084Bb2(0.004)
		Hot grinding	0.40B1(0.01)	0.19Aa1(0.02)	0.082Cb1(0.001)	0.15Aa1(0.04)
1-Octen-3-ol	Prosoy	Cold grinding	0.47A1(0.001)	0.032Aa2(0.006)	0.037Ba1(0.003)	0.019Ab1(0.002)
		Ambient grinding	0.46A1(0.09)	0.032Ab2(0.002)	0.048Aa1(0.007)	0.012Bc1(0.001)
		Hot grinding	0.039B2(0.005)	0.012Ba2(0.001)	0.013Ca2(0.001)	0.00Cb2(0.00)
	Black soybean	Cold grinding	0.22AB2(0.01）	0.047Aa1(0.004)	0.034Ab1(0.001)	0.017Ac1(0.002)
		Ambient grinding	0.25A2(0.04)	0.032Ba1(0.006)	0.031Aa2(0.001)	0.01Bb2(0.00)
		Hot grinding	0.18B1(0.01)	0.035Ba1(0.005)	0.018Bb1(0.001)	0.017Ab1(0.005)
Trans-2-nonenal	Prosoy	Cold grinding	0.032B1(0.002)	0.010Ca2(0.002)	0.00Ab1(0.00)	0.00Ab1(0.00)
		Ambient grinding	0.038A1(0.000)	0.030Ba1(0.004)	0.00Ab2(0.00)	0.00Ab1(0.00)
		Hot grinding	0.00C2(0.00)	0.039Aa2(0.005)	0.00Ab1(0.00)	0.00Ab1(0.00)
	Black soybean	Cold grinding	0.037A1(0.004)	0.037Ba1(0.005)	0.002Bb1(0.001)	0.00Ab1(0.00)
		Ambient grinding	0.044A1(0.010)	0.035Ba1(0.000)	0.007Ab1(0.002)	0.00Ac1(0.00)
		Hot grinding	0.005B1(0.002)	0.051Aa1(0.005)	0.00Bb1(0.00)	0.00Ab1(0.00)

Table 4-1 (continued)

Trans-2, trans-4-nonadienal	Prosoy	Cold grinding	0.11B1(0.002）	0.090Ba2(0.004)	0.078Bb1(0.002)	0.070Bc2(0.002)
		Ambient grinding	0.13A1(0.01)	0.13Aa1(0.02)	0.089Ab2(0.001)	0.077Ab1(0.003)
		Hot grinding	0.00C1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)
	Black soybean	Cold grinding	0.11B1(0.003)	0.10Ba1(0.002)	0.081Bb1(0.002)	0.074Bc1(0.001)
		Ambient grinding	0.13A1(0.001)	0.12Aa1(0.003)	0.10Ab1(0.004)	0.080Ac(0.001)
		Hot grinding	0.00C1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)
Trans-2, trans-4-decadienal	Prosoy	Cold grinding	0.061A2(0.017)	0.41Ba2(0.11)	0.30Bab2(0.03)	0.25Ab2(0.01)
		Ambient grinding	0.063A2(0.011)	1.08Aa1(0.18）	0.35Ab2(0.01)	0.20Ab2(0.04)
		Hot grinding	0.00B1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)	0.00Ba1(0.00)
	Black soybean	Cold grinding	0.15B1(0.04)	0.78Ba1(0.02)	0.47Bb1(0.04)	0.56Ab1(0.07)
		Ambient grinding	0.25A1(0.06)	1.17Aa1(0.04)	1.05Aa1(0.13)	0.61Ab1(0.02)
		Hot grinding	0.00C1(0.00)	0.00Ca1(0.00)	0.00Ca1(0.00)	0.00Ba1(0.00)

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same column are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3)

same hot grinding temperature and followed by a traditional heating process of 93-94 ºC for 3 min (Mizutani and Hashimoto, 2004). Particularly in the soymilk ground by ambient and hot grinding method, two-phase UHT method was very effective to totally eliminate hexanal in the yellow Prosoy soymilk (Table 4-1). Compared to the literature, Sun et al. (2010) found hot temperature grinding, followed by a 95 C and 10 min heating, gave similar hexanal content (242 ppb) compared to that (264 ppb) ground at 25 °C. Lv et al. (2011) was able to reduce the hexanal level to 20-70 ppb after hot grinding at 80-100 ºC and followed by heating at 93-95 ºC for 5 min. The study of Lozano et al. (2007) using one-phase UHT resulted in hexanal with 150-250 ppb. The above literature reports conducted by Sun et al. (2010) and Lv et al. (2011), and Mizutani and Hashimoto and (2004) Lozano et al. (2007) showed ineffectiveness of hot grinding or one-phase UHT process to reduce hexanal to a level lower than the sensory threshold value (4.5 ppb). The discrepancies between our results and others can be attributed to the accurate control of the temperature in grinding using the insulated device and the two-phase UHT equipped with a vacuuming step.

Under almost all heating methods black soybean tended to produce significantly ($p<0.05$) higher hexanal than the yellow Prosoy soybean in most cases. This differential effect may be due to their different chemical compositions and oxidative enzyme activities (Yuan and Chang, 2007b).

Effect of grinding methods, heating methods, and variety on hexanol in soymilk

Hexanol is a major contributor to the green, beany flavor but has a much higher sensory detection threshold (2.5 ppm) than hexanal (Belitz et al., 2004). Hexanol is derived from 13-hydroperoxide of linoleic acid. Table 4-1 shows in soymilk, ambient grinding gave significantly ($p<0.05$) higher hexanol level than the other two grinding methods and there were no significant differences ($p<0.05$) between hot and cold grinding. However, hot

grinding showed the strongest ability to reduce hexanol and it could reduce the hexanol content in raw yellow Prosoy soymilk to the undetectable level.

All heating methods could totally eliminate hexanol or reduced it to that lower than the sensory detection threshold level. With the exception of three instances of ambient grinding plus one-stage UHT or stove heating, all soymilk processed by cold and hot grinding and followed by all heating methods were able to totally eliminate hexanol content in soymilk. Lv et al. (2011) showed after hot grinding (80-100 ºC for 2-10 min) plus 95 ºC heating for 5 min resulted in 580-80 ppb hexanol. Mizutani and Hashimoto's hot and cold grinding plus a heating process resulted in higher hexanol concentrations (5-8 ppm) (Mizutani and Hashimoto, 2004). The discrepancies between our study and others could be attributed to the accurate control of the temperature using the insulated grinding device, and heat processing equipment.

With respect to variety difference, except hot grinding, yellow soybean showed higher hexanol levels in raw soymilk as compared with black soybean under the cold and ambient grinding conditions. However, after two-phase UHT processing, there were no differences between the soymilk made from either yellow or black soybean since all hexanol was decreased to non-detectable level.

Effect of grinding methods, heating methods, and variety on 2-pentylfuran in soymilk

2-Pentylfuran is a very unique odor compound. It has a beany odor note as reported by Belitz et al. (2004) and Smouse and Chang (Smouse and Chang, 1967). The range of threshold for this odor compound is 0.25-6 ppm (Min and Boff, 2002). As shown in Table 4-1, in contrast to other odor compounds, it increased with heating. According to Bradley and Min (2003), singlet oxygen can be formed by riboflavin present in soymilk, and 2-pentylfuran was generated by singlet oxygen action on linoleic acid via a specific oxidation

mechanism (Min et al., 2005; Lee et al., 2003). Lee et al. (2003) also found only soy flour stored under light could generate 2-pentylfuran, and Bradley and Min (2003) mainly contributed it to the formation of singlet oxygen induced by chlorophyll, which can promote the reaction in a similar manner as riboflavin.

In most cases, ambient grinding resulted in significantly ($p<0.05$) higher 2-pentylfuran compared with the other two grinding methods, and hot grinding caused much lower 2-pentylfuran formation than cold grinding. These values suggests the heat inactivation of lipoxygenases could partly restrict the generation of 2-pentylfuran to some extent. Soymilk from traditional stove cooking contained significantly ($p<0.05$) higher 2-pentylfuran than that from the UHT methods. For example, for both Prosoy and black soymilk made from ambient grinding, the 2-pentylfuran content after stove cooking was almost three to four times higher than that after UHT processing. In stove cooking, continuous stirring and longtime exposure to light and air could lead to the extensive formation of singlet oxygen. Therefore, in storage and processing, soymilk products need to be protected from light in order to minimize the involvement of singlet oxygen (2003). Sun et al. (2010) showed hot grinding plus a 95 ºC heating did not have any advantages in reducing 2-pentylfuran when compared to an ambient grinding (25 ºC). However, their contents were below the threshold levels. Among our processing methods, the hot grinding and the UHT processing were particularly effective in reducing 2-pentylfuran to that below sensory threshold values.

When the two varieties are compared, yellow soybean showed higher value after cooking, but slightly lower in raw soymilk, and this is very likely due to their specific chemical and enzyme compositions.

Effect of grinding methods, heating methods and variety on 1-octen-3-one in soymilk

1-Octen-3-one has a mushroom odor note and an extremely low threshold of 0.005 ppb in water as reported by Buttery et al. (1978). Generally, hot grinding generated significantly ($p<0.05$) lower levels of 1-octen-3-one as compared with ambient grinding (Table 4-1). However, in the case of raw black soymilk, there was almost no significant differences ($p<0.05$) between hot grinding and cold grinding. In general, the contents of the 1-octen-3-one from three grinding methods followed the same trend: ambient grinding>cold grinding>hot grinding. All heating methods greatly reduced 1-octen-3-one levels, but UHT methods were more effective, and the two-phase UHT method was the most effective in total elimination of 1-octen-3-one. However the contents in the yellow Prosoy soymilk from cold and ambient grinding after the two-phase UHT process were still much higher than the threshold values. The effect of hot grinding and heat processing on 1-octen-3-one has not been reported by others in the soymilk in the literature.

In most cases, black soybean-made soymilk possessed more 1-octen-3-one than yellow soymilk. What is striking is that hot grinding could totally eliminate 1-octen-3-one in soymilk made from yellow Prosoy soybean, but not from black soybean. We do not know why the two varieties in our study exhibited such a large disparity under hot grinding. More studies should be conducted in the future for improving black soymilk quality since the sensory detection threshold of this odor is very low.

Effect of grinding methods, heating methods and variety on 1-octen-3-ol in soymilk

1-Octen-3-ol also has a mushroom odor note with extremely low threshold of 0.005 ppb (Buttery, 1989). Yuan and Chang (2007a) and Kobayashi et al. (1995) reported that lipoxygenase-deficient varieties had no advantages over normal varieties in terms of 1-octen-3-ol content. It is very likely that some 1-octen-3-ol is formed during soaking phase via

biologically (enzyme)-controlled mechanism during soaking and is affected by hydration rate, pH, temperature (*26*). Kobayashi et al. (1995) suggested that 10-hydroperoxide was formed by other forms of hydroperoxidation other than lipoxygenase-activated oxidation. While Frankel et al. (1981) found that 1-octen-3-ol was derived from 10-hydroperoxide, which was formed by photosensitized oxidation from linoleic acid. Lee et al. (2003) detected higher levels of 1-octen-3-ol in soy flour stored under light compared with soy flour stored in the dark. Our results (Table 4-1) showed hot grinding reduced 1-octen-3-ol to less than 10% of that in cold and ambient ground raw yellow Prosoy soymilk. Cold grinding is not effective in reducing this odor compound when compared to ambient grinding. These results at least demonstrated that inactivating lipoxygenases by hot grinding at 80 ºC for 3 min could partially inhibit the formation of 1-octen-3-ol in the yellow Prosoy soybean.

In general, the two-phase UHT gave significantly ($p<0.05$) lower odor note in comparison with the other two heating methods, and hot grinding plus two-phase UHT was effective to eliminate all 1-octen-3-ol in the yellow Prosoy soybean. Without using hot grinding, Lozano et al. (2007) reported high levels (dilution factors ranged from 27 to 729) of 1-octen-3-ol in traditionally cooked and one-phase UHT processed soymilk, whereas using hot grinding at 80-100 ºC and a traditional cooking method, Lv et al. (2011) reported 40-10 ppb of 1-octen-3-ol in soymilk.

Hot grinding of black soybean resulted only in a slightly lower level of 1-octen-3-ol than ambient ground soymilk, showing the inactivation of lipoxygenases in black soybean was not effective in the inhibition of the formation of 1-octen-3-ol. Therefore, yellow and black soybean behaved differently. This may be due to the unique oxidation mechanism of 1-octen-3-ol and the differences in the lipid composition of the two varieties.

Effect of grinding methods, heating methods and variety on trans-2-nonenal in soymilk

Trans-2-nonenal has a cooked carrot odor note with a low threshold of 0.08 ppb in water (Belitz et al., 2004; Buttery, 1989). Sun et al. (2010) reported this odor has a cucumber or hay note with a 0.15 ppb threshold. For both our varieties, in raw soymilk, hot grinding produced significantly ($p<0.05$) lower levels of trans-2-nonenal than the other two grinding methods (Table 4-1). Yuan and Chang (2007b) and Kobayashi et al. *(*1995) also reported that lipoxygenase-null varieties produced much lower trans-2-nonenal in comparison with the normal varieties. These results implied lipoxygenases were involved in the formation of this compound.

Stove cooking after hot grinding could greatly increase trans-2-nonenal but not after the other two grinding methods. Yuan and Chang (2007b) found a similar trend in comparing the effect of stove cooking on soymilk made from normal and lipoxygenase-null varieties. In addition, Lv et al. (2011) found that after hot grinding at 80-100 ºC for two min and heating for 5 min at 93-95°C, hot grinding did not show much advantage over ambient grinding. However, after hot grinding for 10 min, this compound and three other odor compounds were totally eliminated (Lv et al., 2011). The study of Lv et al. (2011) has a weakness that the reported grinding temperature fluctuated widely (80-100 ºC), not well controlled, hence the findings would limit its industrial applications.

The results from stove heating following hot grinding suggested that trans-2-nonenal could also be formed non-enzymatically during heating processes. According to Frankel et al. (1981), trans-2-nonenal could be derived from 9-/10 –OOH via autooxidation and photosensitized oxidation of linoleic acid. As shown in Table 4-1, it was very likely some polyunsaturated fatty acids were oxidized in stove cooking in the presence of enough light and oxygen even if the lipoxygenase had been inactivated during hot grinding. Both one-

phase and two-phase UHT methods could effectively reduce trans-2-nonenal level to be below sensory threshold values and after two-phase UHT treatment, it was totally eliminated.

In most cases, soymilk from black soybeans contained more trans-2-nonenal as compared to that from yellow soybeans when soymilk was prepared by stove cooking and one-phase UHT methods. However, there were no differences between the two varieties after two-phase UHT processing, showing the vacuum chamber was very effective to eliminate this odor compound.

Effect of grinding methods, heating methods and variety on trans-2, trans-4-nonadienal in soymilk

Trans-2, trans-4-nonadienal has a beany note and a low threshold value of 0.09 ppb (Sun et al., 2010). Table 4-1 clearly shows the order of trans-2, trans-4 -nonadienal concentrations from low to high is: hot grinding<cold grinding<ambient grinding. In particular, hot grinding could achieve zero value, which implied that lipoxygenases played a vital role in the oxidation of lipid to form this compound. Kobayashi et al. (1995) also found that in raw soymilk from lipoxygenase-null variety, no trans-2, trans-4-nonadienal was detected. Following hot grinding, all three cooking treatments also kept this odor compounds undetectable. For soymilk from cold grinding and ambient grinding, two-phase UHT produced the lowest level of trans-2, trans-4-nonadienal, followed by one-phase UHT, and stove cooking. Different from other odor compounds, all cooking methods following the cold and ambient grinding only reduced trans-2, trans-4-nonadienal slightly, even after two-phase UHT process. In some cases, the percentage reduction after heat process was about 10%. This may be due to strong association of this odor compound with other components, such as proteins in the soymilk (Suppavorasatit and Cadwallader, 2010). Sun et al. (2010)

reported a reduction of this odor compound by hot grinding from ambient grinding of about 70 ppb to 13 ppb, which is still much higher than the low threshold value of 0.09 ppb.

In most cases, there were no significant differences between the two varieties with respect to trans-2, trans-4-nonadienal content. Hot grinding also was very effective in elimination of this odor in black soybean.

Effect of grinding methods, heating methods, and variety on trans-2, trans-4-decadienal in soymilk

Table 4-1 shows significant ($p<0.05$) differences existed among the three grinding methods with hot grinding producing the lowest trans-2, trans-4-decadienal, followed by cold grinding and ambient grinding. However, after UHT processing, especially the two-phase UHT treatments, the disparity between cold grinding and ambient grinding was very small. We had observed this same trend for hexanol, and this was mostly because of the evaporation of the soymilk volatiles in the vacuum chamber. Trans-2, trans-4-decadienal has a fried fatty sensory note and the sensory detection threshold of it is 180 ppb in water or 0.07 ppb in palm oil as reported by Belitz et al. (2004) and Buttery et al. (1989), respectively. In view of such a low threshold, hot grinding is a very efficient way to reduce it to undetectable level. According to Frankel et al. (1981), trans-2, trans-4-decadienal could be formed from linoleate through autoxidation or photosensitized oxidation. However, Kobayashi et al. (1995) found much lower levels of trans-2, trans-4-decadienal from lipoxygenase-null variety compared with the lipoxygenase-normal variety, and Yuan and Chang (2007b) detected it only in one normal variety. In view of their reports and our results, we can conclude that lipoxygenases contribute greatly to the presence of trans-2, trans-4-decadienal. After heating, this odor compound increased dramatically for cold grinding and ambient grinding, but not for hot grinding. Yuan and Chang (2007b) also observed the same phenomenon. We do not know

why this happened and we postulate that may be due to thermal decomposition. Trans-2, trans-4-decadienal is derived from 9-hydroxyl linoleic acid (1981) and hydroperoxide lyase from soybean only specifically catalyzes 13-OOH (Gardner,1989). It is very likely that hydroperoxides from cold and ambient grinding decomposed during heating. Hot grinding inactivated the enzyme instantly without forming the hydroperoxide. Therefore, the following heat treatments did not form trans-2, trans-4-decadienal. However, if we compared the effects of three heating methods for the cold and ambient grinding methods, the order of trans-2, trans-4-decadienal content from high to low concentration is: stove cooking>one-phase UHT>two-phase UHT. Sun et al. (2010) reported a reduction of this odor compound by hot grinding from ambient grinding of about 225 ppb to 102 ppb, which was still much higher than the threshold value of 0.09 ppb, indicating the ineffectiveness of their hot grinding method. The report of Lv et al. (2011) also showed 10 ppb of this compound when soybean was ground at 80-100 ºC for 2-4 min. Therefore, our hot grinding method is more effective than the processing methods reported by these researchers. Again, this can be attributed to the temperature control in grinding soybean using the insulated device in our laboratory. Both raw and heated soymilk from yellow soybeans possessed significantly ($p<0.05$) lower trans-2, trans-4-decadienal content in comparison with that from black soybeans when the soybean were ground at cold or ambient temperature. We do not know the reason why black soybean had higher trans-2, trans-4-decadienal content except their differences in raw material composition. Black soybean contains more polyphenolics and antioxidant capacities (Xu and Chang , 2007). However, these compounds did not seem to inhibit the oxidative process of lipoxygenases when compared to the effectiveness of the use of gallic acid to reduce beany odor (Boatright, 2002). Therefore, other factors may be important in influencing the quality of soymilk.

Effect of extraction methods, and variety on hexanal in traditionally cooked soymilk

Table 4-2 shows that significant ($p<0.05$) differences in hexanal were found in soymilk produced by four extraction methods followed by traditional stove cooking methods. Either for raw or stove cooked soymilk, in most cases, Method #3 and Method #4 gave the lowest level and Method#2 gave the highest level. As mentioned above (page 77), hexanal is mainly generated by hydroperoxide lyase-catalyzed cleavage of 13-L-c,t-HPO, which is predominantly derived from the reaction of L-2 isozyme and linoleic acid. Therefore, the Prolonged exposure to air and light may be attributable to the higher hexanal content in soymilk from Method#2 than other methods. As for the reason why Method #4 yielded the lowest content of hexanal, this may be because of the higher antioxidant activity in the soymilk that this method possessed. Antioxidant compounds and antioxidant capacity of these samples were assayed by another researcher in our lab (Tan, 2011) who found that soymilk from Method #4 contained significantly higher TPC, TFC,CTC, FRAP, ORAC and DPPH scavenging activity compared with the other three methods. Antioxidants are capable of scavenging free radicals and breaking chain reactions and thus inhibit the formation of oxidative products. It seems no definite trend exists between the two varieties, which means there is an interaction between the two factors: extraction method and variety.

Table 4-2. Effect of extraction methods, traditional stove cooking, and variety on selected odor compounds in soymilk (ppm)

		Prosoy		Black soybean	
Odor compounds	Extraction methods	Raw	Cooked	Raw	Cooked
Hexanal	Method #1	4.84Ba2(0.06)	0.24Bb2(0.01)	5.85Aa1(0.04)	0.30Bb1(0.01)
	Method #2	7.46Aa1(1.01))	0.34Ab1(0.01)	6.02Aa1(0.36)	0.37Ab1(0.01)
	Method #3	5.91ABa1(1.00)	0.19Cb1(0.01)	5.00Aa1(1.08)	0.23Cb1(0.29)
	Method #4	4.26Ba2(0.18)	0.17Db2(0.01)	4.90Aa1(0.06)	0.22Cb1(0.01)
Hexanol	Method #1	0.61Ba1(0.19)	0Ab1(0.00)	0.30Ca1(0.00)	0Ab1(0.00)
	Method #2	1.09Aa1(0.00)	0Ab1(0.00)	0.95Aa1(0.131)	0Ab1(0.00)
	Method #3	0.79ABa1(0.21)	0Ab1(0.00)	0.73ABa1(0.12)	0Ab1(0.00)
	Method #4	0.97ABa1(0.02)	0Ab1(0.00)	0.60Ba2(0.01)	0Ab1(0.00)
2-Pentyl furan	Method #1	0.00Bb1(0.00)	0.26Ba2(0.01)	0.00Ab1(0.00)	0.36Ba1(0.02)
	Method #2	0.06Ab1(0.00)	0.41Aa1(0.01)	0.00Ab2(0.00)	0.45Aa1(0.02)
	Method #3	0.00Bb1(0)	0.27Ba1(0.01)	0.00Ab1(0.00)	0.35Ba1(0.03)
	Method #4	0.00Bb1(0)	0.25Ba1(0.02)	0.00Ab1(0.00)	0.29Ca1(0.01)
1-Octen -3-one	Method #1	0.24Ca2(0.01)	0.10Bb2(0.00)	0.28Ca1(0.00)	0.12ABb1(0.01)
	Method #2	0.38Ba1(0.01)	0.12Ab1(0.01)	0.39Aa1(0.02)	0.13Ab1(0.00)
	Method #3	0.45Aa1(0.04)	0.11ABb1(0.00)	0.31Ba2(0.01)	0.11BCb1(0.00)
	Method #4	0.29Ca2(0.00)	0.11ABb1(0.00)	0.32Ba1(0.00)	0.11Cb1(0.00)
1-Octen -3-ol	Method #1	0.35Aa1(0.14)	0.00Ab1(0.00)	0.18Ba1(0.02)	0.00Ab1(0.00)
	Method #2	0.39Aa1(0.06)	0.00Ab1(0.00)	0.22ABa2(0.03)	0.00Ab1(0.00)
	Method #3	0.46Aa1(0.23)	0.00Ab1(0.00)	0.24Aa1(0.02)	0.00Ab1(0.00)
	Method #4	0.47Aa1(0.06)	0.00Ab1(0.00)	0.25Aa2(0.02)	0.00Ab1(0.00)

Table 4-2 (continued)

Trans-2, -nonenal	Method #1	0.027ABa1(0.005)	0.000Bb1(0.000)	0.025Ba1(0.009)	0.000Bb1(0.000)
	Method #2	0.016Ba2(0.000)	0.009Ab2(0.000)	0.026Ba1(0.004)	0.0108A1(0.002)
	Method #3	0.020ABa1(0.005)	0.000Bb1(0.000)	0.048Aa1(0.013)	0.000Bb1(0.000)
	Method #4	0.036Aa1(0.010)	0.000Bb1(0.000)	0.047Aa1(0.008)	0.000Bb1(0.000)
Trans-2, trans-4-nonadienal	Method #1	0.000Ba1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)
	Method #2	0.092Aa1(0.005)	0.000Ab1(0.000)	0.000Aa2(0.000)	0.000Aa1(0.000)
	Method #3	0.000Ba1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)
	Method #4	0.000Ba1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)	0.000Aa1(0.000)
Trans-2, trans-4-decadienal	Method #1	0.00Ba1(0.00)	0.00Ca2(0.00)	0.00Ab1(0.00)	0.16Ca1(0.01)
	Method #2	0.15Ab1(0.03)	0.53Aa1(0.24)	0.00Ab2(0.00)	0.48Aa1(0.04)
	Method #3	0.00Ba1(0.00)	0.00Ca2(0.00)	0.00Ab1(0.00)	0.33Ba1(0.02)
	Method #4	0.03Bb1(0.02)	0.33Ba1(0.05)	0.00Ab1(0.00)	0.17Ca2(0.00)

Means with different capital letters in the same column are significantly different among different extraction methods for the same heat treatment and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different between raw and cooked soymilk for the same extraction methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same extraction and heat treatment ($p<0.05$).
Values in parentheses are SD (n=3)

Prolonged exposure to air and light may be attributable to the higher hexanal content in soymilk from Method#2 than other methods. As for the reason why Method #4 yielded the lowest content of hexanal, this may be because of the higher antioxidant activity in the soymilk that this method possessed. Antioxidant compounds and antioxidant capacity of these samples were assayed by another researcher in our lab (Tan, 2011) who found that soymilk from Method #4 contained significantly higher TPC, TFC,CTC, FRAP, ORAC and DPPH scavenging activity compared with the other three methods. Antioxidants are capable of scavenging free radicals and breaking chain reactions and thus inhibit the formation of oxidative products. It seems no definite trend exists between the two varieties, which means there is an interaction between the two factors: extraction method and variety.

Effect of extraction methods, and variety on hexanol in traditionally cooked soymilk

Table 4-2 shows that for both Prosoy and black soybeans, Method #1 generated significantly ($p<0.05$) lower hexanol in comparison with other three extraction methods. Method #2 gave the highest level of hexanol. Traditional stove cooking could reduce hexanol to undetectable levels.

Effect of extraction methods, and variety on 2-pentylfuran in traditionally cooked soymilk

As Table 4-2 presents, in most cases, 2-pentylfuran could not be detected in raw soymilk, but traditional stove cooking made it detectable, and significant differences ($p<0.05$) among the four extraction methods were also observed. Cooked soymilk from Method #4 contained the lowest ($p<0.05$) level of 2-pentylfuran, and soymilk from Method#2 possessed the most ($p<0.05$). Cooked black soymilk contained little higher 2-pentylfuran than cooked Prosoy soymilk, which may be due to the higher level of pigment of black soybeans.

Effect of extraction methods, and variety on 1-octen-3-one in traditionally cooked soymilk

As showed in Table 4-2, significant ($p<0.05$) differences existed among the four extraction methods. Like other odor compounds, Method #2 produced significantly ($p<0.05$) higher1-octen-one than other methods. Traditional stove cooking could significantly reduce 1-octen-one content. The differences between the two varieties were very small, particularly for cooked soymilk.

Effect of extraction methods, and variety on 1-octen-3-ol in traditionally cooked soymilk

Table 4-2 shows that in raw soymilk, Method #1 generated the lowest ($p<0.05$) level of 1-octen-3-ol. According to Badenhop et al. (1968), 1-octen-3-ol is formed during soaking phase via biologically (enzyme)-controlled mechanism during soaking. In Method #3 and Method #4, soaking water was used in grinding. Therefore, it was very likely 1-octen-3-ol formed during soaking process was retained in the soymilk. Because 1-octen-3-ol was also derived from 10-hydroperoxide, which was formed by photosensitized oxidation from linoleic acid (Frankel et al., 1981), Method #2 contained significantly ($p<0.05$) higher 1-octen-3-ol than Method #1 due to longer exposure to light and air during process (over 2 h). No significant differences ($p<0.05$) among the other three extraction methods existed. Traditional stove cooking could reduce the 1-octen-3-ol level to undectable level. This large reduction might be related to its association with protein. Raw Prosoy milk contained significantly ($p<0.05$) higher1-octen-3-ol than black soymilk.

Effect of extraction methods, and variety on trans-2-nonenal in traditionally cooked soymilk

Table 4-2 shows in raw soymilk, Method #3 and Method #4 generated significantly ($p<0.05$) higher trans-2-nonenal than the other two methods. However, after traditional stove cooking, except for soymilk from Method #2, trans-2-nonenal was reduced beyond detection. For raw soymilk, in most cases, black soymilk contained more trans-2-nonenal than prosoy soymilk.

Effect of extraction methods, and variety on trans-2, trans-4 -nonadienal in trationally soymilk

Table 4-2 shows clearly that except raw Prosoy soymilk from Method #2, trans-2, trans-4-nonadienal from all samples was not detected.

Effect of extraction methods, and variety on trans-2, trans-4 -decadienal in traditionally cooked soymilk

Table 4-2 demonstrates that very little trans-2, trans-4-decadienal was generated in raw soymilk, except raw Prosoy soymilk from both Method #2 and Method #4. But in most cases, the traditional stove cooking could significantly ($p<0.05$) increase trans-2, trans-4-decadienal. This might be due to thermal decomposition. As mentioned above, trans-2, trans-4-decadienal is derived from 9-hydroxyl linoleic acid (1981) and hydroperoxide lyase from soybean only specifically catalyzes 13-OOH (Gardner, 1989). As a result, the hydroperoxides formed were decomposed during heating process.

Conclusion

This study clearly shows that hot grinding could achieve the lowest off-flavor, and cold grinding also exhibited some advantages over ambient grinding. In addition, UHT process, in particular, two-phase UHT could effectively remove the selected volatiles to a large extent. As expected, proper combination of grinding methods and heating methods is a desirable way to tackle the off-flavor problem. For eight selected odor compounds, four extraction methods showed different effects with regard to their specific formation mechanisms. Method 2# gave highest level of odor compounds due to its longer exposure to light and air. This study only provided a quantitative analysis of selected odor compounds as affected by processing methods. Therefore, it is necessary to employ a sensory evaluation panel to test its consumer acceptance. Nevertheless, it should be noted that in the meantime, cold and hot grinding could reduce the protein recovery, and solid yield. Other properties may also be influenced, i.e., antioxidant capacity, isoflavone profile, trypsin inhibition

activity. Further studies should continue to investigate the effects of extraction, grinding, heating on the overall sensory and food functional (physical) quality of soymilk.

CHAPTER 5. ISOFLAVONE PROFILE AND CONTENT OF SOYMILK AS AFFECTED BY DIFFERENT GRINDING AND HEATING METHODS

Abstract

Isoflavones have a lot of health benefits. However, isoflavone content and profile are greatly altered by each step during processing. In this study, two different soybean varieties (Prosoy and black) were processed with three grinding (ambient, cold and hot grinding) and three heating methods (traditional stove cooking, one-phase UHT, and two-phase UHT) for soymilk making. Also, four different extraction methods were investigated. The results showed hot grinding could significantly increase isoflavone extraction. However, grinding process had a destructive effect on isoflavones and this effect varied with grinding temperature. Different heating methods had different effects on different isoflavone forms. Two soybean varieties showed distinct patterns during processing. Isoflavone extraction efficiency could be increased through the improvement of extraction methods.

Introduction

Isoflavones, referred to as phytoestrogens, are present abundantly in soybean and soybean based foods. The health benefits to reduce risks of cancers, cardiovascular diseases, and bone loss have been studied extensively (Adlercreutz et al., 1992; Anderson et al., 1995; Cohen et al., 2000; Zhang et al., 2003). In soybeans, there are three isoflavone types and each with four structural (Figure. 5-1, Figure. 5-2, Table 5-1). The bioavailability of isoflavones in foods is affected by their stability and chemical forms (Kao and Chen, 2002). The content and distribution of isoflavones are affected by variety, processing conditions, storage conditions, and the addition of other ingredients (Wang and Murphy, 1994; Hou and Chang, 2002). The change of isoflavone content and profile during soymilk processing can take place in each step of processing (Jackson et al., 2002, Wang and Murphy, 1996). Furthermore, all

isoflavone forms impart objectionable tastes with different thresholds, such as astringency and bitterness (Kudou et al., 1991). Therefore, it is important to characterize the change of every isoflavone form during the process in order to achieve desired isoflavone profile. In addition, kinetic models regarding reaction rate constants and activation energy of some isoflavone forms have been established to describe the degradation and conversion during heating process (Chien et al., 2004; Vaidya et al., 2007; Eisen et al., 2003). However, all the studies were conducted either in buffer system or on the laboratory scale. The heating step of our current study was undertaken on a pilot-plant scale and could better mimic the commercial soymilk processing. Hot grinding and cold grinding have been studied for their inhibition of off-flavor during soymilk manufacturing. However, the effect of grinding on isoflavone content and distribution is not fully understood. Prabhakaran and Perera (2006) made a comparison of hot and cold grinding. However, in their study, hot grinding and cold grinding temperatures were 95 °C and 45 °C, respectively, and grinding was conducted without soaking. The objective of our study was to determine the isoflavone recovery and compositions as affected by three grinding methods and four soymilk extraction methods.

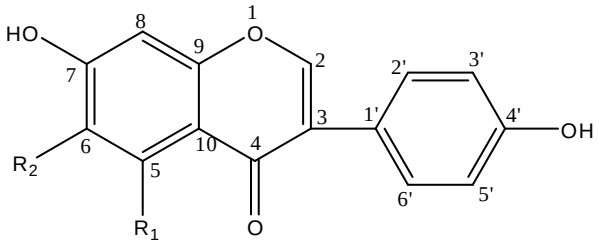

Figure 5-1. Chemical structure of aglycones

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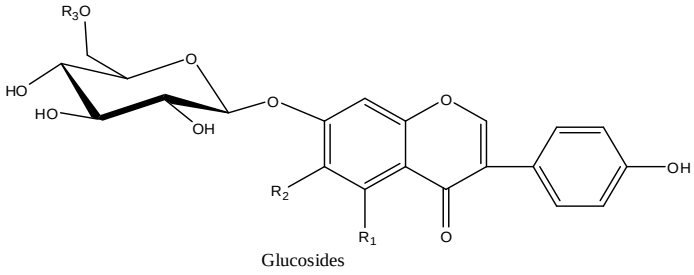

Figure 5-2. Chemical structure of glucosides

Table 5-1. Classification of 12 isoflavones

Compounds	R_1	R_2	R_3	Isoflavones
	H	H	-	Daidzein
Aglycones	OH	H	-	Genistein
	H	OCH_3	-	Glycitein
	H	H	H	Daidzin
β-glucosides	OH	H	H	Genistin
	H	OCH_3	H	Glycitin
	H	H	$COCH_3$	Acetyldaizin
Acetylglucosides	OH	H	$COCH_3$	Acetylgenistin
	H	OCH_3	$COCH_3$	Acetylglycitin
	H	H	$COCH_2COOH$	Malonyldaizin
Malonylglucosides	OH	H	$COCH_2COOH$	Malonylgenistin
	H	OCH_3	$COCH_2COOH$	Malonlglycitin

Materials and Methods

Chemicals

Daidzein, malonyldaidzin, malonylgenistin, malonylglycitin, acetylglycitin were purchased from Nacalai USA (San Diago, CA). Glycitin, daidzein, genistein, genistin were purchased from LC Laboratories (Woburn, MA). Glycitein was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and acetonitrile were purchased from VWR international (West Chester, PA)

Extraction of isoflavones

Extraction of isoflavones was conducted according to method of Xu and Chang (2009) with a slight modification. In brief, freeze-dried soymilk and soaking water samples were pulverized with pestle and mortar to fine powder. Freeze-dried soybeans and okara were ground with coffee blender and passed through 60 mesh screen. About 1g of sample powder was accurately weighed into a 15 mL centrifuge tube. Five mL of acetonitrile, 4.75 mL of DDW, 0.25 mL of 0.5 mg/g 6-hydroxyflavone (in 80% methanol) was added and vortexed. The mixture was shaken in a shaker for 2 h at room temperature. The centrifuge tube was centrifuged at 5500 rpm (5073xg) for 20 min in an Allegra 21R Centrifuge (Beckman Coulter Led., Palo Alto, CA). The supernatant was transformed to a 125 mL flat bottom flask and evaporated at 35°C until dryness in a rotary evaporator. The residue was dissolved in 5 mL of 80% methanol and filtered through 0.2 μm syringe filter into a vial. All vials were kept at -20°C until HPLC analysis.

HPLC analysis of isoflavones

HPLC analysis was run according to method of Xu and Chang (2009) with some modification. An Agilent Technologies 1200 series system equipped with an YMC –pack

ODS-AM-303 C18 reversed phase column (250mm x 4.6mm i.d., 5μm) was used. The UV detector was set at 262 nm and column temperature was set at 35 °C. Mobile phase A was 0.1% glacial acetic acid in water, mobile phase B was 0.1% glacial acetic acid in acetonitrile. Initially, B was set 15% for 5 min. Then B was increased to 29% until 36 min, increased to 35% till 44 min, then increased to 50% till 46 min and held till 56 min. Then B was recycled to 15% till 58 min and held till 60 min. Peaks of isoflavones were identified by retention times of the authentic standards. And isoflavones were quantified by calibration curve established on the basis of standards in a series of dilutions. Individual isoflavones were expressed as μg/g of dry material, and for the purposes of comparison, total isoflavone and total individuals were expressed as nmol/g of dry material on the basis of their individual molecular weights.

Statistics analysis

Soymilk was produced in triplicate and the following chemical analyses were conducted in duplicate. Data were subject to analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant difference among variables were determined by Duncan's multiple range test (α=0.05). Data are expressed as means ± SD (n=6).

Results and Discussion

Effect of grinding methods on isoflavone profile and content in soymilk

As presented in Table 5-2 and Table 5-3, it is very clear, significant differences ($p<0.05$) were found in individual isoflavones, total individuals and total insoflavones among the three grinding methods. For Prosoy soymilk, in most cases, except aglycones, hot grinding generated significantly higher ($p<0.05$) isoflavones than ambient grinding, and cold grinding gave the least value. In raw Prosoy soymilk, the total isoflavone content from

ambient, cold, and hot grinding methods were 5013, 3917, 5949 nmol/g respectively. If we look at the distribution of isoflavones in okara and soymilk (Table 5-4, Table 5-5), isoflavone content in soymilk is negatively related to isoflavone retention in okara. Aglycones exhibited distinct pattern in responding to grinding methods, that is, hot grinding resulted in significantly ($p<0.05$) lower level than ambient grinding method. This can be explained by the instant inactivation of β-glucosidase during hot grinding and this phenomenon was also observed by another study (Prabhakaran et al., 2006). Until now, we only found one paper comparing hot grinding (95 °C) and cold grinding (45°C) with regard to isoflavone extractability (Prabhakaran et al., 2006). In this study, soaking step was omitted, but hot grinding still showed much higher isoflavone extraction efficiency compared with cold grinding. Wang and Murphy (1996) found that heating slurry at 95°C for 7 min prior to pressing could result in about 12% loss on okara with almost 90% isoflavone present in soymilk. A 90% isoflavone recovery was very high compared with results of ours and others. Using soy protein isolate, Malaypally and Ismail (2010) also found heating could significantly facilitate extraction of isoflavones. Therefore, on the basis the results combined, we suppose that heating before separation between insoluble okara and soymilk might advance the extraction of isoflavones. And this is perhaps the main reason for the relatively higher extractability of hot grinding as observed in our study. In fact, different recovery resulted mainly from different solubility of different forms responding to different grinding temperatures. Jackson et al. (2002) found in ambient grinding, percentage contents of aglycones, β-glucosides, and acetylglucosides in okara were higher than those in soymilk, but malonylglucosides showed an opposite trend. As shown in Table-4 and Table-5, our results showed that the distributions of each single isoflavone form in okara and soymilk are greatly different for different grinding methods. For example, in raw Prosoy soymilk, as the most abundant isoflavone form, the combined content of malonylglucosides in soymilk and okara

from cold grinding was 5100 nmol/g, much higher than that from hot grinding of 4777 nmol/g (Firgure 5-3). However, only 56% remained in soymilk from cold grinding in comparison to 82% from hot grinding. Apart from temperature-induced effect on isoflavone extractability, Wang and Murphy (1996) attributed isoflavone content to the association between isoflavones and soluble proteins in soymilk. However, if we refer to protein recovery in our study as presented in Chapter one, protein content alone at least did not account for the tremendous variance induced by different grinding methods. Even within the same grinding methods, individual isoflavones also showed distinct extractability.

Table 5-2. Effect of grinding, heating methods and variety on isoflavone content and profile in Prosoy soymilk (μg or nmol/g of dry material)

Grinding methods	Heating methods	Din	Gly	Gin	MDin	MGly
Cold grinding	Raw	193.6Bc1(1.88)	33.33Ba2(1.87)	175.6Bc2(7.51)	570.2Ca1(31.89)	69.91Ca2(4.92)
	Stove cooking	290.2Ca2(16.60)	33.34Ca2(2.39)	268.8Ca2(15.85)	397.7Cc1(10.79)	49.79Cb2(1.56)
	One-phase UHT	163.5Cd2(8.29)	29.85Ba2(3.53)	146.2Bd2(4.55)	591.9Ca1(10.30)	69.27Ba2(1.00)
	Two-phase UHT	223.6Cb1(11.10)	32.06Ba2(2.55)	200.7Cb2(9.11)	512.1Bb1(15.24)	65.01Ba2(2.66)
Ambient grinding	Raw	175.7Bd1(4.19)	36.29ABa2(1.47)	172.8Bd2(2.83)	700.9Ba1(19.84)	79.88Ba2(2.80)
	Stove cooking	414.2Ba1(13.09)	42.80Ba2(2.56)	403.3Ba1(11.12)	515.7Bb2(14.06)	58.76Bc2(2.49)
	One-phase UHT	246.4Bc1(16.33)	36.73ABa2(4.79)	231.7Ac1(12.67)	673.4Ba1(14.98)	71.99Bab2(3.95)
	Two-phase UHT	272.9Bb1(18.05)	35.03ABa2(6.86)	275.5Bb1(13.86)	560.2Bb1(46.25)	64.22Bbc2(7.72)
Hot grinding	Raw	278.2Ac1(19.39)	38.11Ab2(1.32)	241.9Ac1(14.84)	893.4Aa1(30.66)	106.9Aa2(3.93)
	Stove cooking	606.1Aa1(18.35)	64.83Aa2(3.99)	550.3Aa1(11.22)	629.0Ac1(16.99)	81.14Abc2(2.55)
	One-phase UHT	296.7Ac1(19.73)	39.39Ab2(4.51)	257.6Ac1(17.97)	750.8Ab1(22.91)	86.59Ab2(5.33)
	Two-phase UHT	365.9Ab1(9.87)	43.27Ab2(2.01d)	332.4Ab1(7.92)	682.9Ac1(11.11)	78.82Ac2(3.33)

Table 5-2 (continued)

Grinding methods	Heating methods	MGin	Agly	Dein	Glein	Gein
Cold grinding	Raw	826.6Ca1(23.42)	3.39Cd2(0.33)	15.88Ba2(0.89)	0.00Ba2(0.00)	11.16Bb2(0.82)
	Stove cooking	584.1Cc1(17.75)	22.29Ca1(0.91)	16.60Ba2(1.67)	0.00Aa2(0.00)	13.72Ba2(1.74)
	One-phase UHT	797.1Ba1(13.24)	8.70Cc2(0.99)	11.54Cb2(0.52)	0.00Aa1(0.00)	8.46Cc2(0.19)
	Two-phase UHT	732.8Bb1(21.24)	18.17Bb1(1.74)	11.71Cb2(1.29)	0.00Aa1(0.00)	8.62Cc2(0.76)
Ambient grinding	Raw	1118Ba1(26.65)	5.40Bd1(0.34)	50.30Aa1(18.06)	3.49Aa2(0.99)	50.16Aa2(18.36)
	Stove cooking	817.0Bc1(23.29)	30.71Ba1(2.11)	42.37Aa2(16.78)	0.00Ab2(0.00)	43.51Aa2(17.13)
	One-phase UHT	1055Aa1(30.69)	11.14Bc1(0.97)	36.11Aa2(2.89)	0.00Ab2(0.00)	36.34Aa2(1.81)
	Two-phase UHT	922.7Ab1(71.52)	20.51Bb1(2.74)	52.71Aa2(2.81)	0.00Ab2(0.00)	59.33Aa2(4.02)
Hot grinding	Raw	1266.1Aa1(46.47)	11.83Ad1(1.56)	23.59Ba2(2.19)	0.00Ba1(0.00)	26.38Bb2(2.37)
	Stove cooking	918.4Ac1(23.14)	43.47Aa1(1.27)	24.35ABa2(2.58)	0.00Aa2(0.00)	26.35ABb2(2.80)
	One-phase UHT	1056Ab1(51.94)	16.78Ac1(1.54)	21.52Ba2(2.41)	0.00Aa2(0.00)	23.27Bb2(1.79)
	Two-phase UHT	958.2Ac1(6.70)	28.26Ab1(1.16)	25.31Ba2(1.26)	0.00Aa2(0.00)	32.44Ba2(1.42)

Table 5-2 (continued)

Grinding methods	Heating methods	Total daidzein	Total glycitein	Total genistein	Total isoflavones
Cold grinding	Raw	1662Ca1(57.84)	212.9Ca2(13.81)	2041Ca(24.90)	3917Ca1(95.7)
	Stove cooking	1553Cb1(63.16)	213.9Ca2(9.70)	1799Cc(72.92)	3567Cb2(145.7)
	One-phase UHT	1556Cb1(28.51)	214.8Ba2(8.11)	1907Bb(27.83)	3678Bb1(54.5)
	Two-phase UHT	1614Cab1(58.65)	231.1Ba2(13.73)	1910Bb(61.70)	3755Cab1(133.9)
Ambient grinding	Raw	2014Bb1(63.24)	254.6Ba1(9.34)	2743Ba(67.56)	5013Ba1(135.4)
	Stove cooking	2187Ba1(38.60)	269.1Ba2(4.69)	2670Ba(64.07)	5127Ba1(100.8)
	One-phase UHT	2074Bab1(49.12)	240.3Ba2(18.53)	2705Aa(72.51)	5020Aa1(138.8)
	Two-phase UHT	1977Bb1(123.1)	241.1Ba2(34.85)	2637Aa(153.9)	4855Ba1(310.5)
Hot grinding	Raw	2538Ab1(71.37)	310.3Ab2(8.54)	3099Aa(82.46)	5949Ab1(156.3)
	Stove cooking	2803Aa1(62.55)	386.6Aa2(11.65)	3142Aa(52.98)	6332Aa1(116.3)
	One-phase UHT	2291Ac1(89.32)	285.2Ab2(22.84)	2720Aa(140.2)	5296Ac1(251.7)
	Two-phase UHT	2277Ac1(33.57)	302.8Ab2(12.80)	2737Aa(6.84)	5318Ac1(44.5)

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$)
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3).

Table 5-3. Effect of grinding, heating methods and variety on isoflavone content and profile in black soybean soymilk (μg or nmol/g of dry material)

Grinding methods	Heating methods	Din	Gly	Gin	MDin	MGly
Cold grinding	Raw	199.5Ac1(10.20)	68.13Ab1(5.15)	231.2Ac1(10.72)	442.5Aa2(22.70)	143.0Aa1(9.53)
	Stove cooking	324.3Ba1(0.96)	82.52Ba1(1.67)	366.6Aa1(6.29)	296.3Bd2(3.04)	97.31Bc1(2.45)
	One-phase UHT	229.1Ab1(11.51)	66.73Bb1(3.35)	263.9A1(18.03)	402.6ABb2(8.05)	133.6Aab1(2.76)
	Two-phase UHT	218.4Bb1(8.88)	63.61Bb1(2.68)	251.1Abc1(9.07)	367.5Ac2(11.30)	123.7Ab1(4.66)
Ambient grinding	Raw	178.5Ac1(7.31)	58.63Ab1(3.86)	209.1Bc1(7.92)	357.7Ba2(10.44)	104.0Ba1(3.72)
	Stove cooking	258.2Ca2(9.38)	67.96Ca1(2.05)	303.4Ba2(11.54)	271.9Cc2(6.01)	77.11Cc1(1.50)
	One-phase UHT	172.7Bc2(5.72)	55.92Cb1(3.11)	198.1Cc2(8.58)	378.1Ba2(7.09)	109.3Ba1(3.99)
	Two-phase UHT	202.1Bb2(16.09)	60.25Bab1(6.91)	233.6Ab2(19.38)	330.4Bb2(17.18)	94.57Bb1(7.05)
Hot grinding	Raw	197.3Ad2(14.81)	71.22Ac1(9.76)	205.5Bd2(8.69)	445.2Aa2(6.25)	147.0Aa1(4.05)
	Stove cooking	355.7Aa2(3.68)	109.6Aa1(3.15)	376.0Aa2(3.11)	331.8Ad2(6.45)	111.4Ab1(3.88)
	One-phase UHT	228.4Ac2(7.71)	82.23Ab1(4.18)	226.1Bc2(7.04)	418.1Ab2(19.63)	137.0Aa1(7.80)
	Two-phase UHT	254.8Ab2(11.79)	83.31Ab1(2.59)	259.9Ab2(14.60)	370.3Ac2(16.23)	119.2Ab1(5.72)

Table 5-3 (continued)

Grinding methods	Heating methods	MGin	Agly	Dein	Glein	Gein
Cold rinding	Raw	774.8Aa1(43.35)	11.46Ac1(0.71)	28.79Ca1(0.51)	2.93Bb1(0.10)	31.18Ca1(0.98)
	Stove cooking	521.0Bd2(11.86)	23.72Aa1(0.35)	28.26Ca1(0.69)	3.25Ba1(0.16)	33.25Ca1(0.72)
	One-phase UHT	704.0Ab2(22.06)	14.91Ab1(0.45)	21.31Cb1(1.11)	0.00Cc1(0.00)	23.56Cb1(1.66)
	Two-phase UHT	638.0Ac2(20.13)	21.67Aa1(2.27)	19.10Cc1(1.16)	0.00Cc1(0.00)	21.56Cb1(1.17)
Ambient grinding	Raw	680.4Ba2(21.25)	6.36Cd1(0.51)	74.20Aa1(0.98)	5.61Aa1(0.32)	102.23Aa11.17)
	Stove cooking	530.0Bc2(13.73)	19.78Ba2(0.44)	72.96Aa1(6.14)	5.78AAa1(1.47)	98.35Aab1(7.05)
	One-phase UHT	714.4Aa2(17.59)	10.15Cc1(0.72)	67.75Aab1(2.16)	4.63Aa1(0.65)	94.54Aab1(3.02)
	Two-phase UHT	633.6Ab2(33.82)	14.13Bb2(1.32)	64.11Ab1(2.79)	4.52Aa1(0.48)	91.24Ab1(4.40)
Hot grinding	Raw	780.2Aa2(17.17)	10.14Bc1(0.65)	52.21Ba1(0.84)	0.00Cc1(0.00)	76.31Ba1(1.23)
	Stove cooking	582.7Ac2(18.16)	26.17Aa2(3.24)	53.45Ba1(1.72)	2.90Ba1(0.42)	74.93Ba1(2.23)
	One-phase UHT	709.0Ab2(42.23)	11.80Bc2(0.76)	49.46Bb1(0.70)	2.21Bb1(0.11)	74.10Ba1(1.90)
	Two-phase UHT	632.9Ac2(30.87)	18.59Ab2(0.49)	43.93Bc1(1.14)	2.38Bb1(0.05)	64.83Bb1(2.76)

Table 5-3 (continued)

Grinding methods	Heating methods	Total daidzein	Total glycitein	Total genistein	Total isoflavones
Cold grinding	Raw	1473ABa2(69.07)	454.9Aa1(30.15)	2145Aa(11.18)	4073ABa1(206.1)
	Stove cooking	1480Ba1(7.45)	427.6Ba1(7.66)	1976Cbc(35.23)	3883Bab1(46.29)
	One-phase UHT	1435Ba2(43.49)	430.9Ba1(13.18)	2055Aab(89.67)	3922Aa1(145.0)
	Two-phase UHT	1331Bb2(43.80)	419.2Aa1(19.33)	1891Bc(56.89)	3641Bb1(114.6)
Ambient grinding	Raw	1433Ba2(33.79)	316.8Ba1(84.71)	2174Aa(57.38)	3966Ba2(107.8)
	Stove cooking	1448Ba2(19.79)	357.9Ca1(6.38)	2088Ba(39.32)	3894Ba2(64.92)
	One-phase UHT	1434Ba2(28.46)	367.6Ca1(17.07)	2186Aa(60.48)	3988Aa2(99.21)
	Two-phase UHT	1395Ba2(71.53)	357.4Ba1(31.25)	2100Aa(114.3)	3853ABa2(216.3)
Hot grinding	Raw	1565Ab2(44.58)	456.5Ab1(29.10)	2262Aa(48.01)	4284Aab2(105.7)
	Stove cooking	1725Aa2(23.10)	518.5Aa1(21.28)	2271Aa(46.88)	4515Aa2(91.24)
	One-phase UHT	1575Ab2(57.09)	475.5Aab1(23.95)	2165Aab(101.0)	4216Ab2(177.2)
	Two-phase UHT	1522Ab2(63.39)	457.7Ab1(14.84)	2062ABb(102.3)	4041Ab2(181.5)

Means with different capital letters in the same column are significantly different among different grinding methods for the same heating methods and same variety ($p<0.05$).
Means with different lowercase letters in the same row are significantly different among different heating methods for the same grinding methods and same variety ($p<0.05$).
Means with different numbers in the same row are significantly different between two varieties for the same grinding and heating methods ($p<0.05$).
Values in parentheses are SD (n=3).

Table 5-4. Distribution of isoflavones in different fractions of Prosoy soymilk (μg or nmol/g of dry material)

Grinding methods	Fractions	Din	Gly	Gin	MDin	MGly	MGin
Cold grinding	Soybeans	598.4A (1.71)	79.33A(6.20)	640.9A(9.77)	1242A(14.50)	160.8A(13.65)	2238A(20.09)
	Soaked beans	525.1A(2.50)	76.40A(1.25)	594.2A(7.54)	1215B(13.50)	103.1B(1.34)	2236B(8.10)
	Soymilk	193.6B(1.88)	33.33B(1.87)	175.6B(7.51)	570.2C(31.89)	69.91C(4.92)	826.6B(23.42)
	Okara	109.21A(0.44)	15.40A(0.96)	126.7A(1.23)	417.6A(0.75)	45.97A(3.74)	686.2A(0.76)
	Soymilk+okara	302.8B(2.13)	48.72A(2.46)	302.3B(6.58)	987.8B(32.48)	115.9A(6.96)	1513A(24.99)
	Soaking water	6.35B(0.10)	0.76B(0.05)	4.18B(0.12)	4.12B(0.46)	0.52A(0.04)	4.50B(0.68)
Ambient grinding	Soybeans	598.4A (1.71)	79.33A(6.20)	640.9A(9.77)	1242A(14.50)	160.8A(13.65)	2238A(20.09)
	Soaked beans	500.3B(1.20)	67.63B(2.30)	563.8B(3.56)	1262A(14.89)	119.4A(6.78)	2372A(14.12)
	Soymilk	175.7B(4.19)	36.29A(1.47)	172.8B(2.83)	700.9B(19.84)	79.88B(2.80)	1119B(26.65)
	Okara	54.36C(0.34)	12.60B(1.59)	53.13C(0.04)	298.9B(16.43)	46.69A(3.24)	404.7B(21.14)
	Soymilk+okara	230.1C(4.53)	48.89A(0.39)	225.8C(2.80)	999.8B(14.16)	126.6A(4.73)	1523A(16.11)
	Soaking water	8.51A(0.63)	1.06A(0.14)	6.39A(0.56)	6.06A(0.38)	0.90A(0.12)	8.05A(0.70)
Hot grinding	Soybeans	598.4A (1.71)	79.33A(6.20)	640.9A(9.77)	1242A(14.50)	160.8A(13.65)	2238A(20.09)
	Soaked beans	500.3B(1.20)	67.63B(2.30)	563.8B(3.56)	1262A(14.89)	119.4A(6.78)	2372A(14.12)
	Soymilk	278.2A(19.39)	38.11A(1.32)	241.9A(14.84)	893.4A(30.66)	106.9A(3.93)	1266A(46.47)
	Okara	98.68B(5.51)	10.96B(0.73)	107.6B(5.82)	159.0C(6.45)	18.01B(1.02)	275.9C(10.45)
	Soymilk+okara	376.8A(19.94)	49.06A(0.90)	349.4A(16.60)	1052A(24.51)	124.9A(3.07)	1542A(38.76)
	Soaking water	8.51A(0.63)	1.06A(0.14)	6.39A(0.56)	6.06A(0.38)	0.90A(0.12)	8.05A(0.70)

Table 5-4 (continued)

Grinding methods	Fractions	Agly	Dein	Glein	Gein	Total isoflavones
Cold grinding	Soybeans	19.30A(0.47)	28.22A(0.93)	0.00A(0.00)	21.48A(0.10)	10425A(7.92)
	Soaked beans	0.00A(0.00)	39.25B(1.45)	0.00A(0.00)	32.13B(1.93)	10005B(23.89)
	Soymilk	3.39C(0.33)	15.88B(0.89)	0.00B(0.00)	11.16B(0.82)	3916C(95.74)
	Okara	0.00B(0.00)	42.25B(5.26)	3.16B(0.03)	38.36B(0.09)	3150A(14.98)
	Soymilk+okara	3.39C(0.33)	58.12B(5.70)	3.16B(0.030	49.52B(0.88)	7067B(97.70)
	Soaking water	0.00A(0.00)	0.55B(0.01)	0.00B(0.00)	0.22B(0.02)	47.45B(2.84)
Ambient rinding	Soybeans	19.30A(0.47)	28.22A(0.93)	0.00A(0.00)	21.48A(0.10)	10425A(7.92)
	Soaked beans	0.00A(0.00)	91.91A(2.34)	0.00A(0.00)	101.1A(3.67)	10702A(50.34)
	Soymilk	5.40B(0.34)	50.30A(18.06)	3.49A(0.99)	50.16A(18.36)	5013B(135.4)
	Okara	0.00B(0.00)	64.07A(7.830	4.96A(1.05)	72.89A(9.31)	2284B(98.13)
	Soymilk+okara	5.40B(0.34)	114.4A(14.63)	8.45A(0.11)	123.1A(15.76)	7297A(123.0)
	Soaking water	0.00A(0.00)	3.28A(0.14)	0.24A(0.03)	1.42A(0.03)	85.93A(6.20)
Hot grinding	Soybeans	19.30A(0.47)	28.22A(0.93)	0.00A(0.00)	21.48A(0.10)	10425A(7.92)
	Soaked beans	0.00A(0.00)	91.91A(2.34)	0.00A(0.00)	101.1A(3.67)	10702A(50.34)
	Soymilk	11.83A(1.56)	23.59B(2.19)	0.00B(0.00)	26.38B(2.37)	5949A(156.3)
	Okara	3.63A(0.33)	11.27C(1.28)	0.00C(0.00)	16.13C(2.02)	1504.C(73.84)
	Soymilk+okara	15.46A(1.73)	34.86C(3.46)	0.00C(0.00)	42.51B(4.39)	7453A(83.58)
	Soaking water	0.00A(0.00)	3.28A(0.14)	0.24A(0.03)	1.42A(0.03)	85.93A(6.20)

Means with different capital letters in the same column are significantly different among different grinding methods for the same fraction ($p<0.05$).
Values in parentheses are SD (n=3).

Table 5-5. Distribution of isoflavones in different fractions of black soymilk (μg or nmol/g of dry material)

Grinding methods	Fractions	Din	Gly	Gin	MDin	MGly	MGin
Cold grinding	Soybeans	675.8A(3.55)	145.8A(4.65)	875.5A(13.61)	715.7A(35.18)	204.5A(4.79)	1750A(14.20)
	Soaked beans	568.9A(7.63)	114.9A(4.43)	739.1A(12.45)	768.9A(8.95)	156.0A(1.56)	1676.89A(22.56)
	Soymilk	199.5A(10.2)	68.13A(5.15)	231.2A(10.72)	442.5A(20.70)	143.0A(9.53)	774.8A(43.35)
	Okara	151.85A(8.16)	51.63A(1.71)	211.5A(13.71)	287.8A(6.24)	90.56A(2.92)	570.4A(17.61)
	Soymilk+okara	351.4A(18.28)	119.8A(4.70)	442.7A(23.43)	730.3A(28.85)	233.6A(10.50)	1345A(59.54)
	Soaking water	8.88B(0.03)	8.41A(1.07)	5.94B(0.21)	11.84B(0.43)	1.84A(0.20)	14.35B(0.05)
Ambient grinding	Soybeans	675.8A(3.55)	145.8A(4.65)	875.5A(13.61)	715.7A(35.18)	204.5A(4.79)	1703A(20.68)
	Soaked beans	469.0B(12.12)	97.25B(2.43)	665.9B(5.32)	667.1B(20.29)	148.8B(2.45)	1735A(23.56)
	Soymilk	178.53A(7.31)	58.63A(3.86)	209.1B(7.92)	357.7B(10.44)	104.0B(3.72)	680.4B(21.25)
	Okara	52.78C(2.70)	33.24B(2.35)	62.12C(4.38)	155.4B(14.16)	76.48B(4.23)	289.0B(28.32)
	Soymilk+okara	231.3C(7.28)	91.87B(6.21)	271.2C(6.26)	513.1B(22.75)	180.5B(7.79)	969.4B(43.98)
	Soaking water	11.24A(0.36)	6.55A(1.52)	11.03A90.35)	20.45A(1.42)	2.44A(0.05)	32.25A(1.43)
Hot grinding	Soybeans	675.8A(3.55)	145.8A(4.65)	875.5A(13.61)	715.7A(35.18)	204.5A(4.79)	1703A(20.68)
	Soaked beans	469.0B(12.12)	97.25B(2.43)	665.9B(5.32)	667.1B(20.29)	148.8B(2.45)	1735A(23.56)
	Soymilk	197.3A(14.81)	71.22A(9.76)	205.5B(8.69)	445.2A(6.25)	147.0A(4.05)	780.2A(17.17)
	Okara	83.10B(0.61)	25.24C(0.63)	112.3B(0.63)	90.81C(2.83)	29.02C(1.31)	197.4C(7.13)
	Soymilk+okara	280.4B(14.90)	96.46B(9.54)	317.8B(9.36)	536.1B(4.66)	176.1B(3.16)	997.5B(21.24)
	Soaking water	11.24A(0.36)	6.55A(1.52)	11.03A90.35)	20.45A(1.42)	2.44A(0.05)	32.25A(1.43)

Table 5-5 (continued)

Grinding methods	Fractions	Agly	Dein	Glein	Gein	Total isoflavones
Cold grinding	Soybeans	48.25A(1.54)	34.85A(0.87)	0.00A(0.00)	33.18A(0.45)	9506A(111.1)
	Soaked beans	0.00A(0.00)	56.30B(3.94)	0.00A(0.00)	53.40B(2.61)	8780A(23.85)
	Soymilk	11.46A(0.71)	28.79C(0.57)	2.93B(0.10)	31.18C(0.98)	4073B(206.1)
	Okara	9.50A(0.19)	52.91B(3.46)	6.24B(0.38)	55.00B(8.41)	3265A(61.12)
	Soymilk+okara	20.96A(0.64)	71.70B(4.98)	9.17B(0.36)	86.18C(7.46)	7338A(253.7)
	Soaking water	0.00A(0.00)	1.21B(0.06)	0.00A(0.00)	0.31B(0.02)	114.6B(3.72)
Ambient grinding	Soybeans	48.25A(1.54)	34.85A(0.87)	0.00A(0.00)	33.18A(0.45)	9506A(111.1)
	Soaked beans	0.00A(0.00)	125.1A(5.67)	0.00A(0.00)	75.00A(4.21)	8608B(43.62)
	Soymilk	6.36C(0.51)	74.20A(0.98)	5.61A(0.32)	102.23A(1.17)	3966B(107.8)
	Okara	0.00C(0.00)	95.40A(2.14)	16.81A(0.47)	140.6A(4.140	2309B(118.9)
	Soymilk+okara	6.36C(0.51)	169.6AA(3.02)	22.42A(0.50)	242.3A(5.31)	6276B(266.7)
	Soaking water	0.00A(0.00)	4.49A(0.08)	0.00A(0.00)	3.15A(0.02)	204.0A(9.35)
Hot grinding	Soybeans	48.25A(1.54)	34.85A(0.87)	0.00A(0.00)	33.18A(0.45)	9506A(111.1)
	Soaked beans	0.00A(0.00)	125.1A(5.67)	0.00A(0.00)	75.00A(4.21)	8608B(43.62)
	Soymilk	10.14B(0.65)	52.21B(0.89)	0.00C(0.00)	76.31B(1.23)	4284A(105.7)
	Okara	4.27B(0.09)	29.59C(0.44)	0.00C(0.00)	52.09B(1.83)	1450C(19.70)
	Soymilk+okara	14.42B(0.720	81.80B(0.77)	0.00C(0.00)	128.4B(0.91)	5734C(108.1)
	Soaking water	0.00A(0.00)	4.49A(0.08)	0.00A(0.00)	3.15A(0.02)	204.0A(9.35)

Means with different capital letters in the same column are significantly different among different grinding methods for the same fraction ($p<0.05$).
Values in parentheses are SD (n=3).

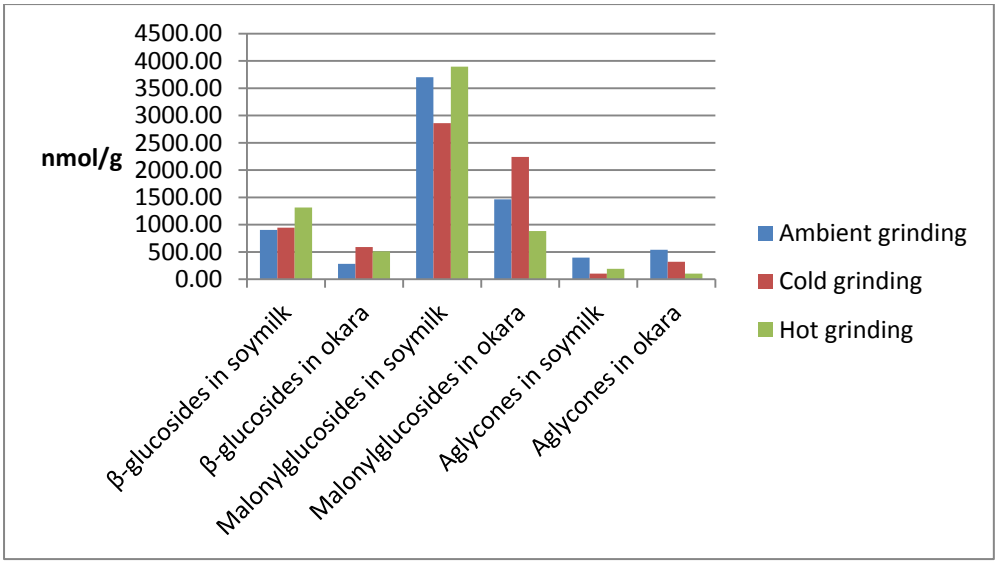

Figure 5-3. Distribution of β-glucosides, malonlyglucosides,and aglycones in raw Prosoy soymilk and okara as affected by three grinding methods

. In contrast to our results, Barbosa et al. (2006) found that extraction of defatted soy flour at 4°C, 25°C and even 50°C for 1 h did not show any significant differences. Therefore, we believe if we grind the slurry with longer time and higher speed, it is very likely the differences between cold grinding and ambient grinding could be narrowed.

Nevertheless in raw black soymilk, isoflavone profile followed a different pattern as a result of grinding methods applied (Table 5-3). Except for aglycones and total genistein, hot grinding and cold grinding generated significantly ($p<0.05$) higher values than ambient grinding methods, and hot grinding was higher than cold grinding, though the differences between these two were not significant ($p<0.05$). For example, the total isoflavone contents from ambient, cold, and hot grinding methods were 3966, 4073, 4284 nmol/g, respectively. However, for aglycones, the order from high to low was ambient grinding >hot grinding>cold grinding. While for total genistein, no significant differences ($p<0.05$) were found.

The fact that the two varieties behaved differently in response to different grinding methods demonstrated that there was an interaction between the varieties and grinding methods.

Effect of heating methods on the isoflavone profile and content

Effect of heating methods on malonylglucosides

Except for one-phase UHT, heating methods significantly reduced the contents of malonyglucosides with stove cooking method reducing the most (Table 5-2, Table 5-3). Our result was in agreement with report of Xu and Chang (2009) who also found stove cooking reduced malonylglucosides even more than direct and indirect UHT at 143°C for 60 s. Malonylglucosides are very thermal labile and are prone to convert to β-glucosides and acetylglucosides under moist heat (Xu and Chang, 2009; Chien et al., 2005). After one-phase UHT, three types of malonylglucosides decreased slightly, or even increased in some cases.

Effect of heating methods on β-glucosides and acetylglucosides

Concomitantly, corresponding increases in β-glucosides were observed with stove cooking increasing the most, and one-phase UHT increasing slightly or decreasing. As for acetylglycitin, all heating methods significantly increased its content as compared with raw soymilk (Table 5-2, Table 5-3). This is consistent with other studies (Wang and Murphy, 1996; Xu and Chang, 2009). Therefore wet heating can also convert some malonylglucosides to acetylglucosides (Kudou et al., 1991). The observed low level of acetylglucosides was mainly due to quick concurrent degradation (Chien et al., 2005).

Effect of heating methods on aglycones

As shown in Table 5-2 and Table-3, heating methods also had a great impact on aglycones. Heating methods did not follow definite trend with regard to grinding methods

and varieties. As the end products in the interconversion chain, aglycones can be formed and degraded simultaneously. β-glucosidase has optimal temperature of 45°C and remains active in the pH range of 4.3-7.0 (Matsuura and Obata, 1993). In the stove cooking, because it took about 8 min to reach boiling, some aglycones could be formed by β-glucosidase-induced hydrolysis. In soymilk from hot grinding, this hydrolysis was unlikely to occur, because β-glucosidase can be inactivated at 60°C (Matsuura and Obata, 1993). On the other hand, heating can also convert β-glucosides to aglycones, but this can only happen above 135°C (Xu et al., 2002). In fact, daidzin, glycitin and genistin showed different thermal stability. Meanwhile, degradation of aglycones can also occur during heating process. As a consequence, three aglycones (daidzein, glycitein, genistein) did not follow definite patterns as affected by different heating methods, grinding methods and varieties. However, heating had no noticeable impact on the content of aglycones, which is in agreement with report of Prabhakaran and Perera (2006). However, Huang et al. (2006) reported that genistein was more thermal stable than daidzein, and under similar heating process as ours, daidzein decreased dramatically and genistein remained almost constant. It is no surprise to find these conflicts in various studies because of the complexity of the degradation mechanisms especially in the food matrix. Using model system, Ungar et al. (2003) proved that genistein was more thermal stable than daidzein and their stability were highly pH and temperature dependent. Based on the results, they further proposed that several different degradation mechanisms may exist depending on reaction conditions, which further affect the antioxidant activity of degradation products. As reported by Davis et al. (1998), aglycones can be involved in Maillard reaction in particular with lysine, or react through autodegradation. And the resultant products from Maillard reaction may be carcinogenic (Gallaher et al., 1996). Heating-induced interconversion and degradation can alter the bioactivity of isoflavones (Singletary et al., 2000). Notwithstanding numerous reactions involved in the formation and

degradation of aglycones, in most cases, the three heating methods applied in our studies did not show any significant differences. As aglycone forms may be absorbed faster and in higher amounts by human body than their corresponding glucosides, heating conditions should be optimized to achieve better health results.

Effect of heating methods on total isoflavone and total individuals

In the case of total isoflavones, significant differences ($p<0.05$) can be found among different heating methods, but they imparted different effect in relation to different grinding methods (Table 5-2, Table-3). All heating methods could reduce total isoflavone content of soymilk from cold grinding. However, for the soymilk from ambient and hot grinding methods, stove cooking increased total isoflavone, while the other two UHT methods decreased total isoflavone. The increase of total isoflavone after stove cooking did not mean that some new isoflavones were formed during thermal process. It was because of the release of bonded isoflavone from isoflavone-protein complex. Heating causes denaturation and unfolding of protein, thus disrupting the association between them (Nufer et al., 2009). Enzyme-aided extraction has proved such isoflavone-protein interaction and showed that for raw soymilk, the measured isoflavone is somewhat lower than its real value, while for heated soymilk, it is very close to the real value (Nufer et al., 2009). Hence, in our study, we can only make a comparison of different treatments, but could not measure the loss during thermal process accurately. This phenomenon was also observed by other researchers and it seemed that the retention of isoflavones was largely dependent on the specific heating methods applied (Xu and Chang, 2009). From our results and others (Prabhakaran and Perera, 2006), the commonly used two-phase UHT could decrease total isoflavone to some extent suggesting its degradating effect. The different effects of stove cooking and UHT methods on the total isoflavone may be attributed to the protective effect imparted by associated protein. The thermal stability of isoflavone in response to conversion and degradation was affected by

protein content and denaturation state (Malaypally and Ismail, 2010). For Prosoy soymilk from ambient grinding, two-phase UHT reduced total isoflavne from 5013 to 4855 nmol/g (Table 5-2), while, for Prosoy soymilk from hot grinding, two-phase UHT reduced total isoflavone from 5948 to 5317 nmol/g. This represents 3.1% and 10.6% decrease, respectively. The larger loss from hot grinding can be due to its lower protein content and the denaturation state of protein upon heating. The protein contents from ambient and hot grinding were 2.81 and 2.46 g/100 of soymilk respectively (not shown). In our study, total individuals exhibited similar change pattern as total isoflavones.

Effects of soaking on isoflavone content and isoflavone profile

Soaking process had almost no effect on the total isoflavone content, but it greatly altered the isoflavone profile. As shown in Table 5-4 and Table 5-5, concentrations of aglycones increased with a concomitant decrease of their corresponding β-glycosides. This is mainly because of the activity of β-glucosidase. The conversion from β-glycosides to aglycones increased with soaking time and soaking temperature (Kao et al., 2004). For example, in ambient and cold soaking of Prosoy, daidzin content declined from 598.4 μg/g to 500.3 μg/g and 525.1 μg/g, respectively (Table 5-4). Under the soaking conditions of our study, malonylgenistin and malonyldaidzin did not change significantly, However, malonylglycitin decreased significantly. Kao et al. (2004) attributed the decrease of malonylglucosides to the conversion to β-glucosides or aglycones and leaching into water. However, the sum of conversion and leaching did not match the loss of malonylgluosides, therefore, there must be some degradation in some glycitein forms during soaking. In the case of acetylglucosides, soaking made them undetectable, which was in agreement with the results of Kao et al. (2004).

Isoflavone loss in soaking water

For Prosoy, the losses at room temperature and cold water soaking were 1% and 0.5%, respectively (Table 5-4). For black soybeans, the losses were greater being 2.5% and 1.4%, respectively (Table 5-5). Our report was similar to that of Wang and Murphy (1996) who reported a 0.5% loss but less than that of Jackon et al. (2002) who reported a 4% loss. However, from our results, isoflavone loss seemed quite related to soaking temperature.

Loss during grinding

If total isoflavones of raw soymilk and okara are summed, the value is obviously much lower than the total isoflavone of soaked beans. This clearly means that there must be some destruction of isoflavones during grinding process. For Prosoy, the losses after ambient grinding, cold grinding, and hot grinding are 31.8%, 29.4%, 30.4% (data not shown) respectively; for black soybeans, the losses are 27.1%, 16.4%, 33.4% (data not shown), respectively. However, not all forms of isoflavones followed the decreasing trend during grinding, in which β-glucosides, acetylglycitin, malonylgenistin, and malonyldaidzin decreased but malonlyglycitin increased. Aglycones, except for in hot grinding, also exhibited increasing pattern. The different change patterns of individual isoflavones demonstrated during grinding, not only destruction occurred, conversion also took place simultaneously. The above results (Table 5-4, Table 5-5) also demonstrated that although cold grinding yielded the lowest total isoflavone content in soymilk, it destroyed total isoflavone the least. As for the loss of isoflavones during grinding, to the best of our knowledge, only one paper mentioned it and attributed it to grinding in boiling water added during grinding (Jackson et al., 2002). However, from the results of our study, grinding at lower temperature could also cause loss and the loss was related to the temperature employed during grinding. The extent of losses differed for different soybean varieties.

Effect of four extraction methods on isoflavone profile and content

As presented in Table 5-6, for total isoflavones, Method #2 (extraction with okara-washing water from last batch) and Method #4 (re-extraction) yielded significantly ($p<0.05$) higher isoflavones than the other two extraction methods. The total isoflavone contents from Method #2 and Method #4 were very close. The total isoflavone content from Method #3 (extraction with soaking water) was slightly higher than that from Method #1 (control), which was due to isoflavone loss in soaking water which was added before grinding. Most individual isoflavones also followed similar extractability order with some variations. One exception was daidzin from Method #2 which showed significantly ($p<0.05$) lower level than other three extraction methods. The extractability discrepancy can be partly attributed to different protein recovery from the four extraction methods. According to Achouri et al. (2005), isoflavone extraction efficiency was related to the protein content of the material because of the interaction between them. The positive correlation between protein content and isoflavone content in soymilk and soybeans was also reported by Malaypally and Ismail (2010). Polyphenols and protein would form complex through ionic interaction, hydrogen bonding, and most importantly, hydrophobic interaction (Boye, 1999). It is very likely that isoflavones were extracted into soymilk together with protein because of the association between them. For the two most abundant isoflavone forms: malonylgenistin and manlonyldaidzin, a high correlation between their level and protein contents exists. The correlation coefficients are 0.95 and 0.94, respectively. Another possible reason is the higher extraction efficiency of the re-extraction process. Extraction efficiency of isoflavones increased with increasing solvent-to-sample ratio and decreasing protein-isoflavone interaction (Malaypally and Ismail, 2010). Using 80% methanol, 80% acetonitrile, and 80% ethanol as extraction solvent, Achouri et al. (2005) found that multiple extraction could substantially increase extractability, especially for protein-rich products.

Table 5-6. Effects of extraction methods and cooking on isoflavone content and profile of Prosoy soymilk (ug or nmol/g of dry material)

Extraction methods	Heating methods	Din	Gly	Gin	MDin	MGly
Method #1	Raw	194.57Bb(22.70)	24.26Bb(0.13)	224.32Cb(25.70)	465.37Ba(2.24)	56.19BCa(1.53)
	Stove cooking	434.25Aa(4.54)	43.57Aa(0.47)	505.83BCa(22.56)	439.91Ab(2.00)	41.29ABb(7.13)
Method #2	Raw	115.17Cb(5.73)	51.28Aa(18.16)	313.65Ab(6.19)	523.31Aa(20.37)	61.86ABa(2.02)
	Stove cooking	212.18Ca(16.43)	42.96Aa(0.22)	596.57Aa(42.74)	350.74Bb(5.45)	44.43Ab(0.92)
Method #3	Raw	209.38B(5.71)	23.61Bb(0.75)	269.57Bb(9.72)	442.10Ba(28.69)	54.92Ca(2.00)
	Stove cooking	379.00B(7.19)	34.50Ca(1.83)	464.95Ca(23.79)	269.13Db(2.15)	35.81Bb(0.59)
Method #4	Raw	243.89Ab(24.68)	26.79Bb(0.68)	307.38Ab(8.15)	507.73Aa(0.03)	63.51Aa(5.53)
	Stove cooking	438.86Aa(12.55)	40.10Ba(1.26)	531.83Ba(9.75)	308.73Cb(0.11)	40.66ABb(0.32)

Mgin	Din	Gly	Gin	MDin	MGly	Mgin
927.87Ba(27.11)	194.57Bb(22.70)	24.26Bb(0.13)	224.32Cb(25.70)	465.37Ba(2.24)	56.19BCa(1.53)	927.87Ba(27.11)
857.21Ab(7.75)	434.25Aa(4.54)	43.57Aa(0.47)	505.83BCa(22.56)	439.91Ab(2.00)	41.29ABb(7.13)	857.21Ab(7.75)
1099.42Aa(48.40)	115.17Cb(5.73)	51.28Aa(18.16)	313.65Ab(6.19)	523.31Aa(20.37)	61.86ABa(2.02)	1099.42Aa(48.40)
797.45Bb(1.08)	212.18Ca(16.43)	42.96Aa(0.22)	596.57Aa(42.74)	350.74Bb(5.45)	44.43Ab(0.92)	797.45Bb(1.08)
988.58Ba(0.58)	209.38B(5.71)	23.61Bb(0.75)	269.57Bb(9.72)	442.10Ba(28.69)	54.92Ca(2.00)	988.58Ba(0.58)
640.80Db(32.88)	379.00B(7.19)	34.50Ca(1.83)	464.95Ca(23.79)	269.13Db(2.15)	35.81Bb(0.59)	640.80Db(32.88)
1068.89Aa(57.88)	243.89Ab(24.68)	26.79Bb(0.68)	307.38Ab(8.15)	507.73Aa(0.03)	63.51Aa(5.53)	1068.89Aa(57.88)
732.20Cb1.94)	438.86Aa(12.55)	40.10Ba(1.26)	531.83Ba(9.75)	308.73Cb(0.11)	40.66ABb(0.32)	732.20Cb1.94)

Means with different capital letters in the same column are significantly different among different extraction methods for the same heating methods ($p<0.05$).

Means with different lowercase letters in the same column are significantly different between raw and cooked for the same grinding methods ($p<0.05$).

Values in parentheses are SD (n=3).

Conclusion

In summary, the content of each isoflavone form was greatly influenced by grinding, heating, extraction methods and variety. Interconversion, degradation, leaching, and heat-induced release were all involved in the whole process. This study provided a foundation for the soymilk industry to optimize the processing conditions.

OVERALL CONCLUSIONS

1. Hot grinding and cold grinding produced lower soymilk yield, solid yield, and protein recovery compared with traditional ambient grinding for both yellow (Prosoy)and black soybeans. Extraction Method #2, which uses the okara-washing water of last batch as grinding water, achieved the best extraction results (70% in solid recovery and 80% in protein recovery) among the four extraction methods.
2. In raw soymilk, KSTI was almost inactivated by hot grinding, but hot grinding had nearly no effect on BBI. However, hot grinding did not show any advantages over ambient grinding after traditional stove cooking and UHT methods. Cold grinding generated the highest TI and BBI in raw and cooked soymilk. The order of effectiveness to inactivate TI from high to low is stove cooking > two-phase UHT > one-phase UHT.
3. Hot grinding resulted in higher antioxidant content and antioxidant capacity. Heating methods had different effects in relation to variety and grinding methods. In most cases, black soymilk product possessed higher antioxidants and antioxidant capacity. It is not known if the higher retention of phenolics and isoflavones would affect the astringency taste of the product.
4. Cold grinding and hot grinding in particular were more effective than ambient grinding to reduce the presence of odor compounds in soymilk. UHT methods especially two-phase UHT were effective to reduce off-flavor. The elimination of some odor compounds could be achieved by proper combination of grinding and heating methods.
5. Hot grinding had the highest extraction efficiency in total isoflavones. Different heating methods had different effect on content and distribution of isoflavones. Isoflavone content was decreased by all grinding methods. Method #2 achieved the

highest extraction efficiency (16% higher than traditional Method #1 in terms of total isoflavones).

FUTURE RESEARCH

1. For hot and cold grinding, heating before filtration, increased grinding time and speed should be tested to figure out if these could increase solid, protein, antioxidant , isoflavone, and phenolic recovery.

2. In current study, the effect of grinding methods on SH was based on previous literature findings and hypothesis. SH and lipoxygenase activity should be quantitively measured to confirm whether different grinding methods could result in different lipoxygenase activity and SH which further influences TI thermal stability.

3. Browning should be measured to see the relationship between browning and antioxidant capacity change.

4. Because the ultimate goal of this study is the acceptance of consumers, sensory evaluation should be done to see whether the GC measured values really reflect the sensory score. Except the odor compounds measured in this study, there are other components which might be influenced by grinding and heating method and contribute to the overall flavor of soymilk.

5. The destructive effect of grinding on isoflavones should be investigated to find out what is the reason.

6. Sensory and functional evaluation also should be carried out to determine the effect of hot grinding and heating methods on quality of the products.

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