

Protein Extraction and Purification of Soybean Flakes and Meals Using a Lime Treatment Followed by Ultrafiltration

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Abbreviated Title: **Soy Protein Extracted using aLime**

ABSTRACT: Protein extraction and purification by lime treatment and ultrafiltration on soybean flakes and meals is an environmentally friendly process that promises a novel alternative to conventional chemical treatment methods. Protein was extracted from soybean flakes and meals by ionic-strength of lime as alkali treatment. After centrifugation, proteins were purified by ultrafiltration. Lime treated flakes and meals showed significantly higher level of dissolved solid, protein, and carbohydrate extraction rate than conventional sodium hydroxide or water treatment. Soybean flakes represented a higher extraction rate of protein and carbohydrate than meals. This result may be because by extensive cell distortion and disruption with cracking, cooking, and flattening which allow lime solutes to easily permeate the cellular matrix. Ultrafiltration substantially purified the protein with minor loss of yields, 94.42% and 96.79% for soybean flakes and meals, respectively. Therefore, lime treatment and ultrafiltration is a viable option for extraction and purifying proteins of soybean flakes and meals.

Supplementary key words: flakes; meals; protein; extraction; purification; lime; ultrafiltration

I. Introduction

In the recent century, U.S. agriculture has been greatly impacted by the soybean industry with an annual gross income of \$15 billion dollars due to their valuable quality of oil and high protein makeup (Endres, 2001; John and Smith, 2014). Production in 2013 resulted in more than 35 million metric tons of soybean-meal products per year in the U.S. (USDA, 2014). Soybeans were mechanically processed using hydraulic techniques or expellers, which squeezed out the oil fraction from cooked soybeans in the 1930's. Since the late 1940's, solvent-extraction processes have been mainly applied to remove more oil from the soybean within the crop industry. Soybean flakes and meals are produced during this process. Soybean flakes are manufactured from heated and cracked soybean meats through a roller mill equipped with tight smooth surfaced rolls. Soybean meals are usually produced from solvent extracted flakes followed by the desolventizer-toaster. More than 99% of the U.S. soybean processing industry is using this solvent extraction process. Soybean meals can also be alternatively produced by mechanically extracting the oil and by extruding-expelling (John and Smith, 2014). However, these extraction methods might induce protein denaturation that could hinder the function of the protein makeup. Therefore, consumer demand has been increased to avoid using solvent as a harmful toxic chemical. An alternative method to soybean protein extraction, isolation, and purification from the soybean flakes and meals, using lime treatment and membrane technology, has been proposed.

In this study, we treated soybean flakes and meals with lime, and removed undesirable attributes and increased protein concentration and purification using microfiltration and ultrafiltration. Lime (calcium hydroxide and/or calcium oxide) is a calcium-containing inorganic material which is broadly used in various industries due to cheaper prices. It is also considered to enhance the flavors and increase ionic strength to extract protein. Conventionally, lime has been used as alkaline pre-treatment on lignocellulosic biomass in agricultural crops to solubilize lignin and partially remove the hemicelluloses (Beukes and Pletschke, 2010). However, few studies have been conducted to extract protein using lime from soybean products. Membrane filtration processes have lower labor requirement with a considerable shorter process time than traditional protein purification processes. It has become more appealing due to its lower energy consumption, fewer steps for production, the high efficiency of protein separation, and the improved final product quality. The greatest advantage of membrane filtration is that there is no excessive use of chemicals to produce protein concentrates and isolates compared with conventional methods.

To compare the relationship of protein extraction rates from soybean flakes and meals, contents of total dissolved solids (TDS), soluble proteins, carbohydrates in extract after centrifuge separation, and retentate with ultrafiltration were investigated.

II. Materials And Methods

2.1 Soybean flakes and meals production

Whole soybeans were donated and delivered from the Clarkson Grain Company INC., (Cerro Gerdo, IL, USA) to the Food Protein R&D Center (College Station, TX, USA) in 2013. The whole grain was stored at 0-4 °C until placed in the sealed container. The flow diagram of soybean flakes and meal production are shown in Figure 1 which follows the conventional methods (Becker, 1978), with slight modifications. The soybeans were first cleaned and selected with SWECO vibrate screen (Florence, KY, USA). Soybeans were cracked into 3-6 pieces by using a corrugated roller mill (model 10 * 12 SGL, Ferrel-Ross, Oklahoma City, OK, USA) and the hulls were separated from the meats (cotyledons) by aspirating with a multi-aspirator (Kice, Wichita, KS, USA). About 7.8 % hulls were separated from whole soybean grain. The soybean meats were cooked at 66.5°C with steam heating by using a triple-deck seed conditioner and toaster (French Oil Mill Machinery Co., Piqua, OH, USA) and flaked to approximately 0.20-0.25 mm thickness by using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA, USA). The proximate composition of soybean flakes was 7.61% moisture, 18.54% oil (dry basis; d.b.), 45.98 % protein (d.b.), 3.59 % acid detergent fiber (d.b.), 25.96 % other carbohydrate (d.b.), and 5.93 % ash (d.b.) (Table 1).

Furthermore, the soybean flakes were conveyed to the solvent extractor (Crown Iron Works Company, Roseville, MN, USA) after cooling down to reach optimal room temperature. Extraction was conducted by countercurrent washes of 30 wt % hexane solvent. The extracted soybean flakes are then transferred to the desolventizer. Soybean meals were produced after desolventization from the flakes. Hexane was recovered from the soybean meals using steam heating and condensation and was further recovered from the extracted oil with vacuum distillation. The entire extraction process was conducted under an explosion-proof closed system. Meal quality was determined by residual oil content (0.48 %), moisture (10.45 %), and protein (54.86 %), (Table 1) and urease deactivation.

2.2 Extraction of protein in soybean flakes and meals using lime treatment

Soybean flakes and meals were ground by a commercial coffee grinder and all flours were passed through a 20 mesh sieve (Seedburo Equipment Company, Chicago, IL) for preparation. The entire diagram of the protein extraction and purification using a lime treatment and membrane technology is shown at Figure 1. Lime (calcium oxide) was purchased from Fisher Scientific (>96 %, w/v). At room temperature, soybean flakes and meals flour (40 g d.b.) were mixed with 360 ml of 0, 0.5, 1, and 2 mole calcium oxide (lime) solution with 1.5 hrs. of stirring. To compare the conventional extraction method with the proposed method, the pH of non-lime treated sample was adjusted to 10.2 with 5M NaOH for 30 minutes after mixing for 1 hr. All samples of slurry were agitated at 200 rpm. The lime and non-lime treated slurries were centrifuged using centrifuge (Sorvall RC-5C Plus Centrifuge and SLA-1500 rotor) to separate the supernatant from the wet cake at 4500 rpm for 20 minutes. The thickness of fat (upper) layer of centrifuged supernatant in soybean flakes was removed by glass wool filtration. Supernatant was filtered with 0.45 µm filter (Whatman #1, Sigma-Aldrich) to determine the total dissolved solid (TDS). All wet cake and supernatant were stored in the sealed container at -80 °C until used for characterization and evaluation.

2.3 Purification of protein extracts using ultrafiltration

Protein isolation and purification from 0.5 mole calcium oxide treated supernatant was conducted by using membrane filtration: a simply modified method (Doko et al., 1991). Supernatant from 0.5 mole calcium oxide treated soybean flakes and meals were filtered with stainless steel tubular composite cross flow microfiltration system (0.2 µm, SCEPTER 4-750A-5P6, Graver Stainless Steel Membrane) to remove suspended solids in the supernatant. The operating temperature was limited to a maximum of 28 ± 3 °C by a heat exchanger. The filtered (permeate) liquid was re-filtered at room temperature using an cross flow ultrafiltration (PCI tubular membrane, PU 608) which was 8,000 MWCO (molecular weight cut-off rate) and made of polysulfone. Inlet and outlet membrane operating pressures were 3 and 2 bar, respectively. Diafiltration was conducted by addition of distilled water in a feed solution tank at the rate of permeate removal such as carbohydrates while protein concentrated in the retentate.

2.4 Proximate analytical methods

Total solids, moisture, protein, oil, carbohydrate, and ash contents were determined according to the standard methods of the Association of Official Analytical Chemists (AOAC 1990). Total fats and oil contents were determined by using the Soxhlet procedure. Protein contents were measured by using high temperature

combustion process and a nitrogen to protein conversion factor of 6.25 (Texas A&M University Soil Testing Laboratory, College Station TX). Total solids and moisture contents were determined by weight after drying the samples in a dry oven at 121°C for 48 hr. Ash content was measured by using a furnace at 550 °C for 6hrs with pre-dried samples. Acid detergent fiber (ADF) was analyzed by the method of Van Soest method (Van Soest and Wine, 1968). Other carbohydrates were determined by weight differences using the data of protein, fat, ADF, ash, and moisture content. Total dissolved solids (TDS) were measured of the solid fraction of centrifuged liquid (ash free) which was passed through a Whatman #1 filter. Extracted dissolved solids rate was calculated by TDS/flour dry weight (ash free). Purity was measured by protein content/TDS in centrifuged liquid.

2.5 Statistical Analysis

All treatments in this study were conducted in triplicate and a 95% confidence level was applied for data analysis. Measurements were analyzed by an analysis of variance (ANOVA) using GLM (general linear model) procedure in SAS 9.1 software (SAS institute Inc., Cary, NC). Results were shown as mean values with their standard error bars. The statistical significant difference between the averages in treatments was accessed by Duncan's multiple range tests. Differences were considered significant for p-values lower than 0.05 (Park et al., 1996).

III. Results And Discussions

3.1 Soybean flakes and meals production

Figure 1 showed the soybean flakes and meals production diagram from the whole soybean grain. Flaking is the predominant method used for commercial hexane extraction process in recent years. We followed the typical process (Woerfel, 1995) and obtained approximately 1-1.5 cm across by 0.2-0.25 mm thick soy flakes. Flaking processes of soybean meats causes extensive distortion in cells and ruptures cell walls with little changes in morphology within protein and oil bodies (Bair and Snyder, 1980; Johnson and Lusas, 1983; Yiu et al., 1983). Flaking also reduced particle size and thickness which significantly affect transfer of oil into bulk solvent. The cell disruption facilitates smooth solvent passage through the cellular matrix and protein body morphology is unchanged during the oil removal. Ruptured cell easily make available large portion of oil by capillary flow transfer mechanism. However, un-ruptured cells usually contain a small portion of oil which should be transferred by osmosis to remove which can lead to a very slow oil extraction rate (Johnson and Lusas, 1983; Bair and Snyder, 1980; Campbell et al., 2011). Hexane extraction method has been predominately applied to vegetable oil processors to produce soybean meals. According to the United Soybean Board, about 45% of the soybean meals produced in the U.S.A is used by broilers, layers, and turkeys followed by 25% swine, 21% ruminants animals, and 8% other (pet foods, aqua feed, food and ingredients) (John and Smith, 2014). However, toxicological and environmental concerns, several catastrophic explosions, and fires have increased the concerning and motivating searches for alternative ways instead of hexane extraction (Johnson and Lusas, 1983).

Table 1 shows the proximate composition of dehulled soybean (meats) flour, soybean flakes, and meals. Moisture contents of meats flour, flakes, and meals were 6.51, 7.61, and 10.45%, respectively. The meats flour and flakes contained significant amounts of fats, 20.55 and 18.54 %, respectively; meals had only 0.48 % fats. The proximate contents of soybean flakes were not significantly different compared to meats flour ($P < 0.05$). The fats and oil content in soybean meals were significantly decreased (>97 %) by hexane extraction of flakes while protein and carbohydrate content increased (54.85 % and 37.41 %, respectively). These compositional results of flakes and meals were in agreement with the previous data. Feedstuff Ingredient Analysis Table (Batal et al., 2012) showed that soybean flake consists of approximately 42% protein, 20% fat, and 5.6% crude fiber on a dry weight basis. Meals contain approximately 54.3% protein, 1.1% fat, and 3.4 % crude fiber on a dry weight basis.

3.2 Extraction of protein in soybean flakes and meals using lime treatment

1. Dissolved solids extraction

Dissolved solids extraction from soybean flakes and meals are shown in Figure 2. Total dissolved solid (TDS) in centrifuged supernatant of flakes after lime and water treatments was 6-10 % higher than meals. Extensive distort cells and rupture cell walls of flakes should be allowed to easily extract the dissolved solid through the cellular matrix. The highest TDS extraction rate was provided with the 0.5mole lime treatment (40.76 % in flakes, and 32.12 % in meals, respectively) followed by 1mole and 2 mole lime treatment. Water and NaOH treated flakes and meals showed that 34.71% and 20.78 % TDS extracted which was similar result with 2mole lime treatment of flakes while non-lime treated was 22.57 % TDS in flakes, and 16.75 % TDS in meals. Lime is commercially much cheaper than NaOH (Chang et al., 1998, 2001; Kaar and Holtzapple, 2000). Calcium ions in lime, each carrying two positive charges, are considered to provide strong linkages between some functional groups including carboxyl, methoxyl, and hydroxyl groups which are negatively charged at alkaline conditions, thus contributing to prevent sudden and huge solids removal including protein

loss, which is commonly observed in NaOH treatment conditions (Torre et al., 1992; Xu et al., 2010; Xu and Cheng, 2011). However, lime has poor solubility in water (1.73 g/L at 20 °C). Even though residence time extended and temperature increased, lime has a limited functionality. According to the Kim and Holtzapfle (2005), the overall glucan conversion of corn stove pretreated using lime does not exceed 50% at 25 °C with a two week treatment period. Xu et al. (2010) also reported that the lime pretreatment of switch grass showed poor performance at ambient temperature. In this study, lime might be over loaded (>0.25 g/g) and reaction rate was increased during the process although a lower solubility of lime could be noticed.

2. Protein extraction

Figure 3 and 4 show that the protein content in wet cake and soluble carbohydrate content in centrifuged liquid (supernatants) of soybean flakes and meals after centrifuged separation. Protein content in wet cake was significantly decreased with lime treatment both on soybean flakes and meals ($P < 0.05$) while soluble carbohydrates content was slightly increased in supernatants with treatment. Protein content of flakes in wet cake was 39.89% control, 34.71% NaOH, 17.66% 0.5M lime, 15.06% 1M lime, and 12.65% 2M lime, respectively. Meals were 54.81% control, 50.77% NaOH, 35.28% 0.5M lime, 27.58% 1M lime, and 15.72% 2M lime, respectively. The 2mole lime treated flakes and meals showed the highest protein extraction rate which was 76.68% and 71.13%, respectively, compared to original flakes (45.98% protein content) and meals (54.86% protein) flour. Only 0.1% of protein extracted from soybean meals with water treatment (control) while 13.24% of protein extracted from flakes. Soybean flakes showed higher extraction rates of protein and carbohydrate than meals. This might have been caused by extensive cell distortion and disruption in flaking with cracking, cooking, and flattening which allow lime solutes to easily permeate the cellular matrix.

Protein and carbohydrate extraction rate was the highest at the 2mole lime treatment while TDS was the highest at the 0.5 mole treatment. It might be neutral substances such as soluble carbohydrates, minerals, and ash which are not significantly affected by pH and ionic strength which were highly eluted with low lime concentration while protein was selectively extracted with high concentration. The pH and ionic strength have been considered to be the most significant effect on the protein solubility (Fennema, 1985; Ali et al., 2011). Typically, plant and animal proteins show specific pH range and solubility linkage with minimum solubility in the isoelectric region, where maximum electrostatic interaction occurs. The solubility is increased at above or below the isoelectric point while proteins have a net negative or a net positive charge (Fennema, 1985). The soy proteins have been well known that the isoelectric region is near pH 4.2-4.6 (Pearson, 1994; Ali et al., 2011). Typically, alkali treatment using NaOH or KCl improves the soy protein solubility, especially when pH exceeds 10.5 (Pearson, 1994). The pH was maintained above 12.0 during the treatment using lime in this study. Therefore, lime treatment can be replaced to conventional alkali treatments such as NaOH and KCl. However, concentrated basic treatment might cause protein denaturation during the protein extraction steps which negatively impact the functional properties of the soy protein products such as soy protein concentrates (SPC) and soy protein isolates (SPI) (Petruccioli and Anon, 1994; Wagner et al., 2000). Further work would be necessary to determine the optimal pH condition using lime treatment which does not have a significant affect to functional properties.

The 12.99 % and 7.37 % protein composition of flakes and meals, respectively, at the pH 10.2 adjustments with NaOH was more extracted than the control treatment (Figure 3 and 4). However, these protein extraction rates were at least 15 % lower than lime treatments. A previous study on NaOH treatment of switch grass shows that the NaOH has a high chemical reaction rate but is expensive and hard to recycle (Xu and Cheng, 2011; Xu et al., 2010). Lime is much cheaper than NaOH (Chang et al., 1998, 2001; Kaar and Holtzapfle, 2000), and can be directly used to food without side effects, and can be easily recovered by CO₂ treatment. Therefore, lime might be used as the new commercial alternative method for protein extraction even though it has a poor solubility in water (Xu and Cheng, 2011).

3. Purity

Purity was calculated by protein g/g TDS (Figure 5). The highest purity was showed with the lime treatment (0.5 M). The 77.25, 76.03, and 62.87 % purity were observed at 0.5, 1, and 2 mole lime treatments, respectively, in soybean flake. The 74.42, 69.66, and 68.66% were observed in meals. Water and NaOH treatment showed the 53.15 and 64.81 % purity, respectively, in flake, and 7.05 and 34.70 % in meals. Soy flakes showed slightly higher purity than meals except for 2 mole lime treatment. During the soybean flaking process, the cell should be ruptured which makes it easy for ionic strength transfer mechanism which in turn, makes for more protein extraction (Campbell et al., 2011) while meals were already once extracted with hexane solution. About 80% of the total soy protein is stored in cotyledon cell volume which is called protein bodies in the soybean (Bair, 1979). Typically, soy protein bodies range from 10 to 50 μm in diameter size. In alkaline media treatment, large cotyledon cell are ruptured more easily than smaller protein bodies (Wolf, 1970; Lee et al., 1983). Soybean flakes should be a suitable candidate of intermediate product to obtain protein if oil is not the

major product from soybean. Low fat and high protein vegetables or crops would be suitable of this approach to extract protein from flakes while it doesn't necessarily extract by solvent meals processing.

3.3 Purification of protein extracts using ultrafiltration

Figure 6 shows that the purification of protein and carbohydrate in retentate with ultrafiltration compared with permeate of microfiltration. Approximately more than 95% protein purification results from soybean flakes and meals were obtained using 8,000 MWCO ultrafiltration systems. There is no statistically significant difference between flakes and meals ($P < 0.05$). Only 23.66 and 30.68% carbohydrate of flakes and meals were recovered because ultrafiltration mainly permeated the carbohydrate. As progress of filtration, the concentration of carbohydrate in retentate might be reduced due to the washing (diafiltration) through the membrane. These results followed the previous research by Kim and Lee (2014). About 95% of soybean protein was purified with the same size of ultrafiltration (8,000 MWCO). Soy proteins are considered a source of high-quality protein because all essential amino acids except for methionine are contained (Endres and Council, 2001). The absence of methionine in the soybean is offset accompanied with the typical diet which including cereals and meats contain a sufficient amount of methionine (Endres and Council, 2001). Microfiltration with a pore size of 10 – 200 nm can selectively separate particle > 200 kDa molecular weight. Therefore, small suspended solids and microorganisms can be filtered with microfiltration system (Richard, 2004; Hua et al., 2007). Ultrafiltration with 8,000 MWCO can separate colloids like protein from small molecules such as sugars and salts (Richard, 2004). According to the Wolf (1970), soy proteins are large molecules with 8 -700 KD molecular weight range. Therefore, the protein was substantially purified with retentate of 8,000 MWCO ultrafiltration. Approximately 90% of soybean protein is globulins accompanied with trypsin inhibitor, cytochrome, and polymers constituting the rest (Kinsella, 1979). The 7S and 11S globulins are the major components of soy protein (Liu et al., 1999). The 11S protein precipitates faster and forms larger aggregates than 7S globulins which means that the 11S have a higher water holding capacity, tensile values, hardness, and expand more on heating than 7S (Saio and Watanabe, 1978).

IV. Conclusion

Soy protein extraction and purification by lime treatment and ultrafiltration on soybean flakes and meals is a suitable and novel alternative to conventional alkaline treatment methods. The protein extraction rate increased substantially with lime treated flakes and meals. Flakes showed that the higher extraction rate of protein and carbohydrate than meals due to the extensive cell distortion and disruption with cracking, cooking, and flattening which allow lime solutes to easily permeate the cellular matrix. Membrane technology, especially ultrafiltration, greatly purified the protein with minor loss of protein yields on both soybean flakes and meals. Therefore, lime treatment and membrane technology is a viable option for extraction and purifying proteins of soybean flakes and meals. Further experimentation would be necessary to determine the optimal lime treatment condition such as less dosing rate, temperature, time, etc.

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Table 1. Proximate analysis of dehulled soybean flour, soybean flakes and soybean meals.

Ingredient	Moisture (%)	Composition of dry matter (%)				
		Fats	Protein	ADF	Carbohydrate	Ash
Meats Flour	6.51±0.33 ^a	20.55±1.03 ^a	43.78±2.21 ^a	5.46±0.27 ^a	24.68±1.23 ^a	5.54±0.28 ^a
Soybean Flakes	7.61±0.36 ^b	18.54±0.93 ^{ab}	45.98±1.89 ^a	3.59±0.18 ^b	25.96±1.29 ^{ab}	5.93±0.31 ^{ab}
Soybean Meals	10.45±0.31 ^c	0.48±0.02 ^c	54.86±2.46 ^c	5.36±0.27 ^a	32.05±1.69 ^c	7.25±0.36 ^c

Value shown as mean ± standard deviation.

^{a-c}Means with the same superscripts in a column are not significantly different by Duncan’s multiple range test (P<0.05).

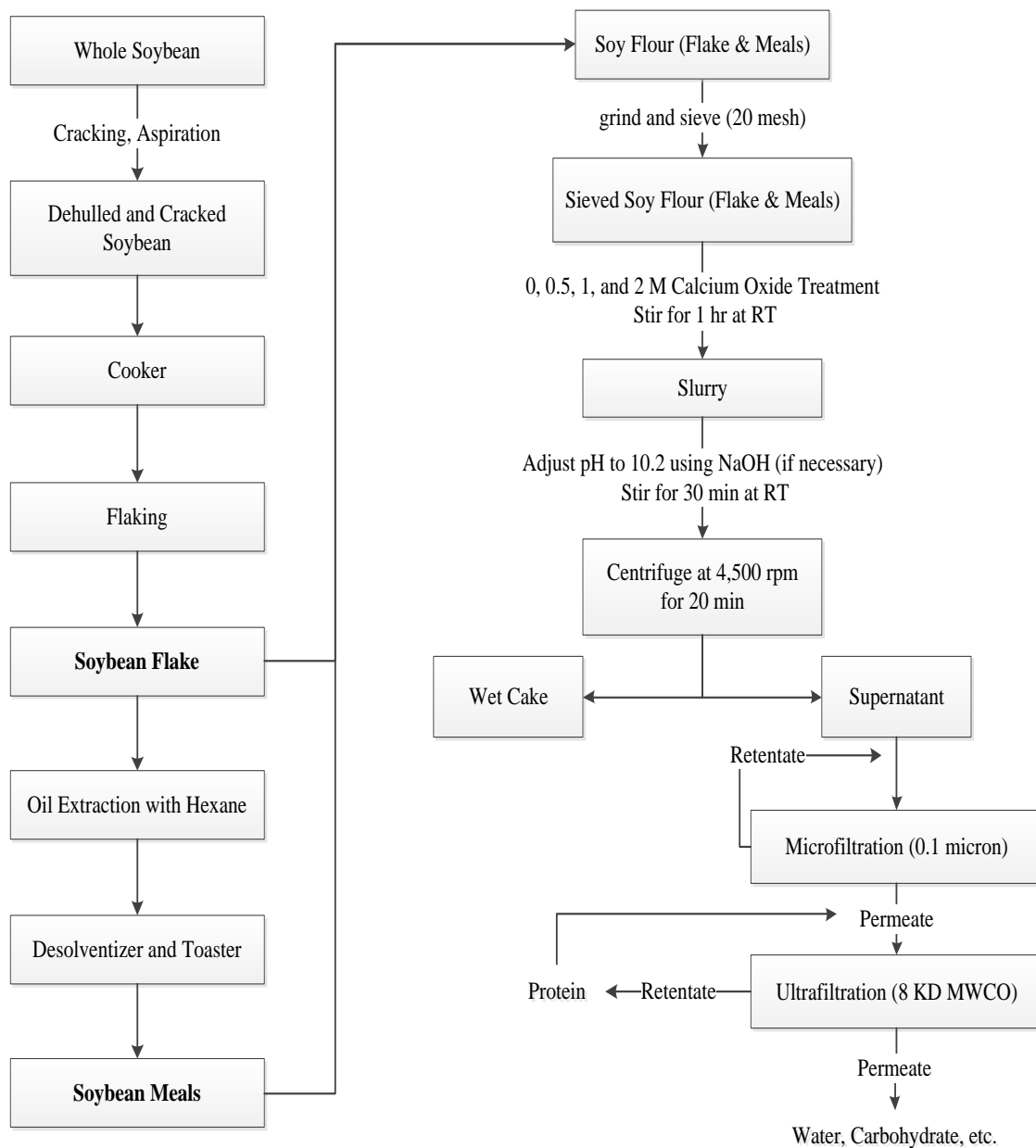


Figure 1. Flow diagram of soybean flakes and meals production, and protein extraction and purification using a lime treatment and membrane technology.

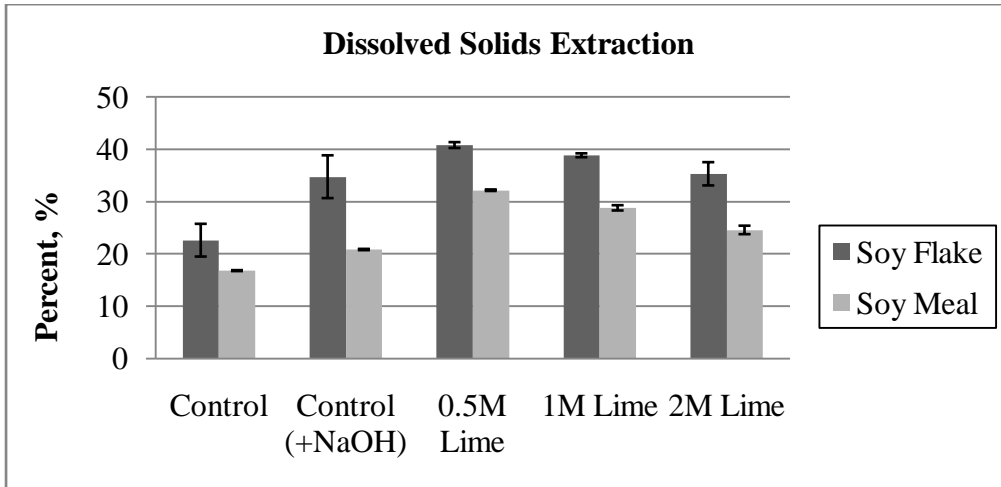


Figure 2. Dissolved solid extraction from soybean flakes and meals with different lime treatments. Pooled standard errors are shown.

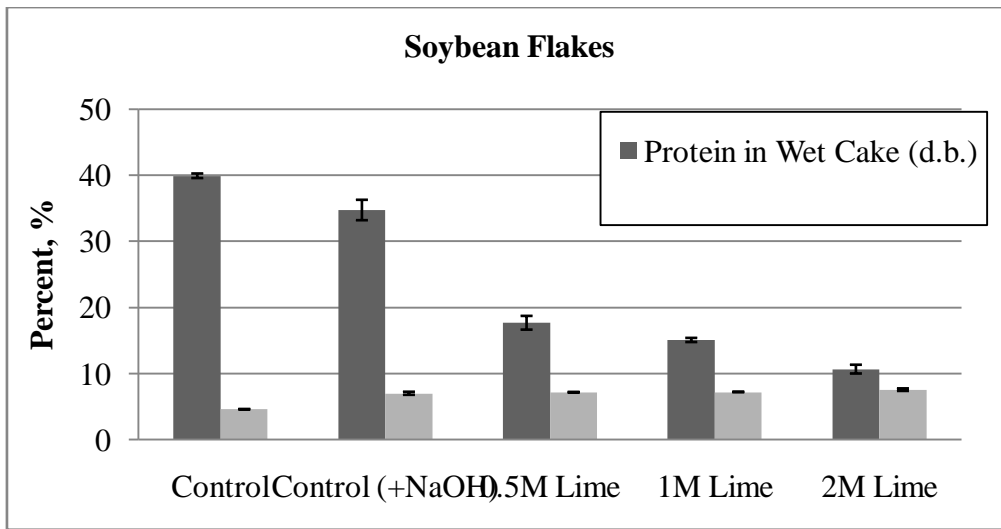


Figure 3. Protein content in wet cake and soluble carbohydrate content in centrifuged liquid of soybean flakes after centrifugation. Pooled standard errors are shown.

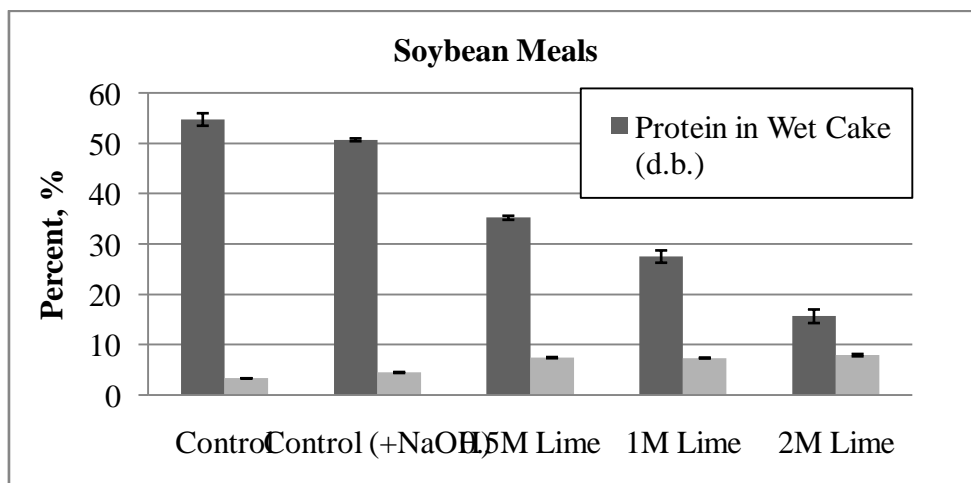


Figure 4. Protein in wet cake and soluble carbohydrate in centrifuged liquid of soybean meals. Pooled standard errors are shown.

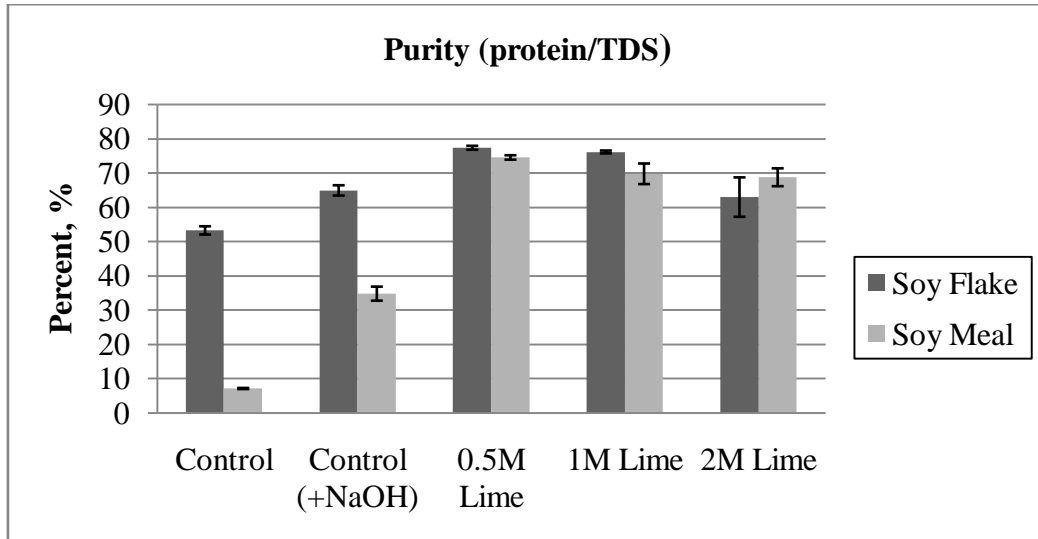


Figure 5. Purity test (protein g/g TDS). Pooled standard errors are shown.

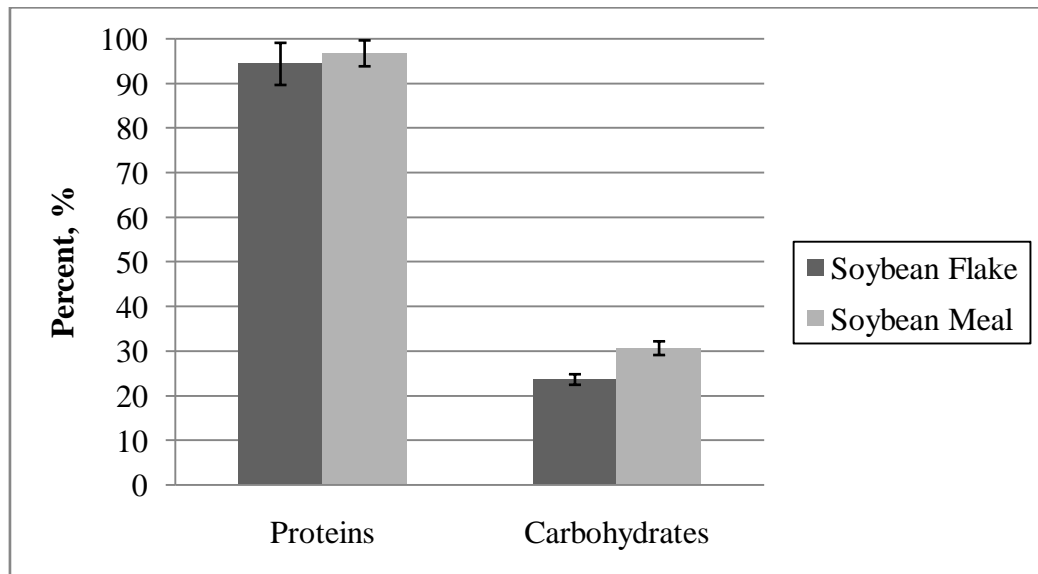


Figure 6. Purification of protein and carbohydrate in retentate with ultrafiltration compared with permeate after microfiltration. Pooled standard errors are shown.