Appendix 15

Compositional Analysis of Fractions Produced during Processing of Grain from Imidazolinone-Tolerant Soybean BPS-CV127-9 Produced in Brazil in 2006/2007 and Fate of AtAHAS in these Fractions

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d) (1) (A), (B), or (C).

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STATEMENT OF COMPLIANCE

This study was not conducted in compliance with the requirements of 40 CFR Part 160.

The data generated by ITAL on behalf of BASF Plant Science in support of product safety comply with generally accepted scientific procedures. ITAL is an ISO 9001 compliant laboratory. Record keeping is consistent with procedures used throughout the research community. This report accurately presents the raw data developed during the study.

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ABBREVIATIONS AND DEFINITIONS

ADF	Acid detergent fiber
AHAS	Acetohydroxyacid synthase
AOAC	American Organization of Analytical Chemists
AOCS	American Oil Chemists Society
CF	Crude fiber
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
FW	Fresh weight
HPLC	High pressure liquid chromatography
HU	Hemagglutinating units
ICP-OES	Inductively coupled plasma-optical emission spectroscopy
ILSI	International Life Science Institute
ITAL	Instituto de Tecnologia de Alimentos
NDF	Neutral detergent fiber
PBS	Phosphate buffered saline

Compositional Analysis of Fractions Produced during Processing of Grain from Imidazolinone-Tolerant Soybean BPS-CV127-9 Produced in Brazil in 2006/2007 and Fate of AtAHAS in these Fractions

SUMMARY

Soybean (*Glycine max* L.) plants have been developed by BASF Plant Science, L.L.C (BPS) and EMBRAPA (Empresa Brasileira de Pesquisa Agropecuaria) that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants BPS-CV127-9 (hereafter referred to as CV127) were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*csr1-2*) gene from *Arabidopsis thaliana* into the soybean plant genome. The herbicide tolerance in BPS-CV127-9 will allow growers to treat the soybean crop with imidazolinone herbicides without causing injury to the plant at normal field application rates. Therefore, introduction of CV127 offers soybean growers an additional tool for controlling weeds.

Soybean has many uses in animal and human nutrition. The grain is typically processed to two commodity products, oil and meal. The defatted toasted meal is commonly used in livestock feed. The various soybean protein fractions derived from processing untoasted defatted soybean meal are used in different human foods. Also, the soybean oil is used in different food products including cooking oil and salad dressings. A grain processing study was conducted to confirm that the grain processing characteristics as well as composition of the processed fractions from CV127 soybean are equivalent to those of the isoline control variety and other commercial conventional varieties, and thereby also confirm that processed fractions from CV127 soybean are appropriate for use in soybean-derived food and feed products. The results of these analyses of the processed soybean fractions produced from CV127 soybean, the isoline control, and two conventional control varieties support the conclusion that the nutrient and antinutrient composition of the processed fractions from CV127 soybean is comparable and within the same range as similar processed soybean fractions produced from grain of the isoline control and two conventional soybean comparator varieties. Also for most analytes the values for CV127 soybean were either within or comparable to the literature reference range of values reported for these soybean processed fractions. Therefore, composition of the toasted, defatted meal, refined oil, protein isolate and concentrate processed fractions of CV127 soybean are equivalent to composition of these same processed fractions derived from the isoline control and other conventional soybean varieties, and confirms that processed fractions from CV127 soybean are appropriate for use in human foods as well as animal feeds.

Furthermore, levels of the AHAS protein in the different grain processed fractions were measured by ELISA to determine the fate of the protein during processing. Analysis of the processed fractions for the AHAS protein demonstrated that processing reduces the levels of AHAS protein from the very low levels that are detectable but not quantifiable in soybean grain. These results further confirmed that the AtAHAS protein is present at extremely low levels in processed fractions from CV127 soybean that are used in human foods as well as animal feeds.

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants, referred to by the unique international identifier BPS-CV127-9 and hereafter as CV127 soybeans, were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase large subunit (*csr1-2*) gene from *Arabidopsis thaliana* into the soybean plant genome via biolistics. Acetohydroxyacid synthase (AHAS) is a key enzyme in plants, bacteria, and fungi that is required for the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine. Herbicides of the imidazolinone class, function by binding near the active site of the catalytic AHAS large subunit, thereby preventing normal functioning of the enzyme (Pang *et al.*, 2002). Several *ahas* genes encoding AHAS enzymes that are tolerant to imidazolinone herbicides have been discovered in plants through mutagenesis and selection and have been used to create imidazolinone-tolerant maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), oilseed rape (*Brassica napus* and *B. juncea* L.), and sunflower (*Helianthus annuus* L.). These crops were developed through mutagenesis, selection, and conventional breeding technologies and have been commercialized under the Clearfield® brand name since 1992. There are five single point mutations in *ahas* genes that have been found to result in tolerance to imidazolinones in plants (Tan *et al.*, 2005). One of these, a mutation that results in a substitution of a serine residue with an asparagine at position 653 (relative to the AHAS enzyme from *Arabidopsis thaliana*), is known to result in tolerance to imidazolinone herbicides with no cross-tolerance to other AHAS inhibitors (Lee *et al.*, 1999). The imidazolinone-tolerant AHAS large subunit *csr1-2* gene (Sathasivan *et al.*, 1990) from *Arabidopsis thaliana* that has the S653N mutation was transformed into soybean (*Glycine max* L.) plants with the native *A. thaliana* promoter, to produce soybean plants that are tolerant to imidazolinone herbicides. This has led to the development of CV127 soybeans by BASF and Embrapa.

Soybean has many uses in animal and human nutrition. The grain is typically processed to two commodity products, oil and meal. The defatted toasted meal is commonly used in livestock feed. The various soybean protein fractions derived from processing untoasted defatted soybean meal are used in different human foods. Also, the soybean oil is used in different food products including cooking oil and salad dressings. Therefore, a grain processing study was conducted to confirm that the grain processing characteristics as well as composition of the processed fractions from CV127 soybean are equivalent to

those of a null segregant control variety (isoline) and other commercial conventional varieties, and thereby also confirm that processed fractions from CV127 soybean are appropriate for use in soybean-derived food and feed products. Soybean grain was produced at four field trial locations in Brazil (Londrina, Uberaba, Sete Lagoas, and Santo Antônio de Goiás) during the 2006/2007 growing season. The different soybean treatments in these trials included CV127 soybean treated with imidazolinone herbicide for weed control and the isoline control and two conventional control varieties that are representative of commercial varieties commonly cultivated in Brazil (Monsoy 8001 and Coodetec 217) that were treated with conventional herbicide (Volt) for weed control. At each field trial location there were four replications of each treatment organized in a complete randomized block design. The grain was harvested from each replicate at each location and it was pooled to create a single grain sample of approximately 30 kg for each treatment from each location. All grain samples were properly packaged, labeled and shipped to Instituto de Technologia de Alimentos (ITAL) in Campinas, Brazil. Small grain samples from each treatment and location were submitted to GeneScan (Indaiatuba, Brazil) for analysis with a CV127 soybean specific PCR assay to confirm the identity of all grain samples. The results of the PCR analysis confirmed that only the CV127 soybean grain samples were positive by the PCR analysis. Each grain sample from each trial location was processed by ITAL into soybean meal, protein isolate and concentrate and refined oil. The composition of the meal, protein isolate and concentrate and the refined oil samples were analyzed by ITAL. The analyses included proximates (moisture, ash, fat, protein, carbohydrates, and calories), fiber (crude, acid detergent [ADF] and neutral detergent [NDF]), antinutrients (raffinose, stachyose, trypsin inhibitor, urease and phytic acid) and isoflavones for the soybean meal and proximates only for the protein isolate and concentrate fractions. The refined oil fractions were analyzed for fatty acid composition. Furthermore, levels of the AHAS protein in the different grain processed fractions were measured by validated ELISA to determine the fate of the protein during processing.

MATERIALS AND METHODS

Sample source. Imidazolinone treated CV127 soybean plants, together with the isoline control variety, and two other conventional soybean varieties [Monsoy 8001 (Std 1) and Coodetec 217 (Std 2), respectively] were grown at four locations in Brazil (Londrina, Uberaba, Sete Lagoas, and Santo Antônio de Goiás) during the 2006/2007 growing season. The plants were grown under standard agronomic practices in a complete randomized block design with four replicate blocks per location. With the exception of the CV127 soybean plants treated with the imidazolinone herbicide at 70 g/ai/ha, all other entries in the study were treated with Bentazon + Acifluorfen-sodium (commercial name Volt) at the rate of 1.0 liters/ha. The grain was harvested at the conclusion of the growing season, pooled to form a single sample for each treatment at each location, and was shipped to ITAL in Campinas, Brazil. ITAL processed the grain in a pilot scale processing apparatus that uses processing methodology as shown in Figure 1. The methods used to produce and process the different fractions were standard methods that

are representative of those currently used to commercially process soybean grain. The processing produced five fractions: defatted meal, toasted defatted meal, protein isolate, protein concentrate and refined oil. Each of the five processed fractions were analyzed for AHAS protein expression by ELISA, to monitor the fate of the AtAHAS protein during processing. The nutrient composition of the toasted defatted soybean meal, protein isolate and concentrate and the refined oil samples were analyzed by ITAL. Since the only difference between the toasted and untoasted defatted soybean meals is the toasting or heating treatment used to produce the former, the nutrient composition of these fractions are expected to be very similar and so only the toasted, defatted soybean meal samples were analyzed for composition. Also, the toasted soybean meal is used almost exclusively in animal feed compared to the untoasted meal, so the toasted soybean meal was the more relevant fraction for analysis to confirm nutritional value for animal feed.

Proximate analysis was conducted on the toasted defatted meal, protein isolate and protein concentrate fractions. The toasted defatted meal was also analyzed for antinutrients (raffinose, stachyose, trypsin inhibitor, urease and phytic acid); isoflavones, and fiber (crude, acid and neutral detergent fiber). Since refined oil contains very low amounts of protein and other components, it was analyzed only for its fatty acid composition.

Analytical methods. *Ash.* The method used was based on AOAC International (2000) method 945.38 C. The sample was placed in an electric furnace at 550°C and ignited to drive off all volatile organic matter. The nonvolatile matter remaining was quantitated gravimetrically and calculated to determine percent ash. The limit of quantitation for this study was 0.1% FW.

Carbohydrates. The method used was based on the USDA Agriculture Handbook No. 8 (1963) method. The total carbohydrate level was calculated by difference using the fresh weight-derived data and the following equation: % carbohydrates = 100% - (% protein + % fat + % moisture + % ash). The limit of quantitation for this study was 0.1% FW.

Fat (protein fractions). The method used was International Standard Organization (1973). The sample was weighed into a beaker and 4M HCl was added. The extract was then evaporated, dried, and weighed. The fat was extracted from the dried preparation using petroleum ether. The limit of quantitation for this study was 0.1% FW.

Fat by Butt Extraction (soymeal). The method used was based on AOAC International (2000) method 945.38 F. The sample was weighed into a cellulose thimble. Petroleum ether was dripped through the sample to remove the fat. The extract was then evaporated, dried, and weighed. The limit of quantitation for this study was 0.1% FW.

Moisture. The method used was based on AOCS International (1998) method Bc 2-49. The sample was dried in a forced draft oven at 130°C to a constant weight. The moisture weight loss was determined and converted to percent moisture. The limit of quantitation for this study was 0.1% FW.

Protein. The method used was based on AOAC International (2000) method 945.39. Nitrogenous compounds in the sample were reduced in the presence of boiling sulfuric acid and a $CuSO_4 + K_2SO_4 + Se$ mixture to form ammonia. The acid digest was made alkaline. The ammonia was distilled and then titrated with a standard acid. The percent nitrogen was calculated and converted to protein using the factor 6.25. The limit of quantitation for this study was 0.1% FW.

Crude Fiber (CF). Crude fiber was measured as the loss on ignition of dried residue remaining after digestion of the sample with 1.25% solutions of sulfuric acid and sodium hydroxide according to the method of Beythien and Diemair (1963). The limit of quantitation for this study was 0.1 g/100 g FW sample.

Acid Detergent Fiber (ADF). The method was based on AOAC International (1975) method 7.057. The sample was placed in a fritted vessel and washed with an acidic boiling detergent solution that dissolved the protein, carbohydrate, and ash. After an acetone wash to remove the fats and pigments; the lignocellulose fraction was collected on the frit and quantitated gravimetrically. The limit of quantitation for this study was 0.1% FW.

Neutral Detergent Fiber (NDF). The method used for sample preparation was the AOAC International (1995) method 920.85. Analysis for NDF was performed on the samples using the method of Van Soest et al. (1991). Samples were placed in a fritted vessel and washed with a neutral boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. After an acetone wash to remove the fats and pigments, the hemicellulose, cellulose, and lignin fractions were collected on the frit and quantitated gravimetrically. The limit of quantitation for this study was 0.1% FW.

Fatty Acids. The methods used were based on AOCS (1998) methods Ce 1-62, Ce 1f-96, Ce 1e-91, and AOAC International (2000) method 996.06 that estimate the levels of fatty acids in the samples. The lipid was extracted and saponified with 0.5 N sodium hydroxide in methanol. The mixture was methylated with a solution of NH_4Cl and H_2SO_4 in methanol based on Hartman and Lago (1973). The resulting methyl esters were extracted with hexane. The methyl esters of the fatty acids were analyzed by gas chromatography using area normalization for quantitation. The 37 Component FAME mix from Supelco (Sigma) was used as reference standards. The limit of quantitation for this study was 0.01% FW.

Isoflavones. The method used was based on Berhow (2002). The defatted sample was extracted using an aqueous solution of 70% ethanol with 0.1% acetic acid. The extract was centrifuged and filtered. The sample was analyzed on a high-performance liquid chromatography (HPLC) system with a diode array detector. Isoflavones were quantified using an external standard curve of known standards. The limit of quantification for each component was 0.3 mg/100 g FW sample.

Phytic Acid. The method used was based on Latta and Eskin (1980). The sample was extracted using 2.4% HCl. Purification and concentration was conducted using an anion

exchange column (Dowex-1 AGX-4, 100-200 mesh). Sample and standards were submitted to a color reaction with Wade reagent, with the absorbance measured at 500 nm. Inositol hexaphosporic acid was used as a standard. The limit of quantitation for this study was 0.75 mg/g FW sample.

Sugars (Raffinose and Stachyose). The method used was based on Cicek (2001) and Kennedy *et al.* (1985). Sugars were extracted from the sample with ethanol + deionized water (1:1). Proteins and lipids which co-extracted were eliminated by precipitation, followed by filtration. Raffinose and stachyose were separated and quantified by HPLC with a refractive index detector. The limit of quantification for this study was 0.2 g/100 g FW sample.

Trypsin Inhibitor. The method used was based on Rackis *et al.* (1974). Trypsin inhibitor units (TIU) were determined by photometrically measuring the inhibition of the trypsin cleavage of benzoyl-DL-arginine-p-nitroanalide hydrochloride. The sample was ground and/or defatted with petroleum ether, if necessary. A sample of matrix was extracted for 3 hours with 0.1 N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoyl-DL-arginine-p-nitroanalide hydrochloride. The sample was allowed to react for 10 minutes at 37°C. After 10 minutes, the reaction was quenched by the addition of 30% trichloroacetic acid. The solution was filtered or centrifuged and the absorbance at 410 nm was measured. The limit of quantitation for this study was 1.00 TIU/mg FW sample.

Urease Activity. The method was based on AOCS (1998) method Ba 9-58. This assay is based on an increase in pH as ammonia is released from urea by residual urease enzyme in the soy meal. The urease activity was assayed by measuring the hydrolysis of 3% urea at pH 7.0 at 37° C. A difference between the pH of the test sample and the pH of the blank is an indication and index of urease activity. The optimum pH increase has generally been considered to be 0.05–0.30.

Statistical analysis. Analysis of variance was carried out on the data using SAS Version 9.1 (SAS Institute Inc., Cary, NC) following two procedures, the General Linear Model and the Mixed Model. With the exception of moisture content, all data were expressed on a dry weight basis for statistical analyses. Differences were assessed across location. The model for across location:

y = variety + location + variety x location + block(location) + e
Random effects: location, variety x location, block(location).
Where y is the response variable (any analyte measured)

Contrasts were carried out to compare each treatment. Differences were considered statistically significant at the 0.05 confidence level.

AtAHAS quantitation. *Extraction of meal, protein isolate, protein concentrate.* A 0.1 g aliquot of each fraction was mixed with 3 ml extraction buffer (14.8 mM KH_2PO_4, 35.2 mM K_2HPO_4, 100 mM Sodium Pyruvate, 5 mM $MgCl_2$, pH 7.2). After being kept for 30

minutes on ice, the sample was extracted using a homogenizer at 24000 rpm with up and down movements for nearly 10 seconds to ensure good homogeneity. The extract was centrifuged at 3000 rpm at 5 ± 3°C for 10 minutes. The supernatant was filtered through miracloth. This filtrate was transferred to a fresh centrifuge tube and centrifuged at 14000 rpm at 5 ± 3°C for 15 minutes. The supernatant was reserved at 5 ± 3°C until analyzed as described below. Extracts were analyzed for AHAS the day prepared.

Oil extraction. Two grams of oil were extracted repeatedly (4x) with 1.2 ml of petroleum ether followed by 0.5 ml PBS (extraction buffer as above without sodium pyruvate and $MgCl_2$). The aqueous layer from the four extractions was pooled and concentrated using a Centricon™ 10 device prior to analysis by ELISA.

AHAS Analysis. Due to the high amino acid similarity between the AtAHAS enzyme encoded by the *csr1-2 ahasl* gene and the homologous endogenous soybean AHAS enzyme, the antibody used in the ELISA is not capable of distinguishing between these two enzymes but measures the total AHAS protein in the samples. Each sample was quantitatively analyzed for AHAS protein by a sandwich enzyme-linked immunosorbent assay (ELISA) [Tijssen, 1985] using immunoaffinity-purified polyclonal rabbit anti-AHAS peptide 2 antibody and Protein G-purified goat antibodies specific for AHAS. NUNC 96-well plates (VWR; West Chester, PA) were coated with rabbit anti-peptide 2 and incubated at 37°C for 1 hr. The plate was washed two times with wash buffer and then blocked with 1% BSA in Tris buffered saline (25 mM Tris-HCl, 3 mM KCl, 0.14 M NaCl, pH 7.4) with 0.05% Tween for 60 min. at 37°C. After washing twice, samples and standards were applied in triplicate. Plates were incubated overnight at 5 ± 3°C, and then washed five times prior to the addition of goat anti-*At*AHAS followed by incubation for 1 hr at 37°C. Plates were then washed three times and donkey anti-goat-horseradish peroxidase (HRP) was added. After incubation at 37°C for 1 hr, the plates were washed three times and HRP substrate was added (TMB Substrate Kit; Pierce). After 20 min at room temperature 1 M HCl was added to stop the reaction. The absorbance at 450 nm was measured using a Tecan Sunrise® multiwell plate reader. The results were analyzed using DeltaSoft PC software (Version1.71.4; Biometallics, Inc.; Princeton, NJ). The four-parameters algorithm was used to generate a curve. The AHAS protein was quantified from the standard concentration curve generated from highly purified AtAHAS protein.

RESULTS AND DISCUSSION

Toasted defatted soybean meal results. *Proximate and fiber composition.* The mean proximate and fiber values of the toasted, defatted soybean meals across all field trial locations are presented in Table 1. Except for moisture, there were no statistically significant differences in proximate or fiber composition in toasted defatted soybean meal derived from CV127 soybean compared to toasted defatted soybean meal derived from grain of the isoline control. In the case of moisture, the value obtained for the soybean meal from CV127 soybean was significantly lower than the value for meal from the

isoline control, but it was not significantly different from the same values for meal produced from one of the two conventional soybean standard varieties. Therefore, the difference in moisture content of the toasted, defatted soybean meal between CV127 and the isoline control is most likely due to slight variations in toasting conditions between treatments. In addition, there were no statistically significant differences in levels of proximates and the different fiber fractions in toasted meal between CV127 and either one or both of the two conventional standard soybean varieties in this study, except for protein, carbohydrates and ADF. These differences were attributed to germplasm differences between the varieties, since similar differences were observed between the isoline control and the two conventional standard soybean varieties. Finally, all proximate mean values for the toasted, defatted soybean meal from CV127 soybeans, except for carbohydrates, were within the ranges of those nutrients that have been reported in the literature (Table 1). In the case of the mean value for carbohydrates, the value for all four soybean treatments in this study was slightly higher than the range reported for this nutrient class in the literature, suggesting that this may be a characteristic of soybean varieties adapted for cultivation under the tropical agricultural conditions of Brazil. Collectively, these data show that proximate and fiber levels in toasted, defatted soybean meal derived from CV127 grain is equivalent to levels in the meal derived from the isoline control as well as other conventional standard soybean varieties.

Antinutrients. The antinutrients examined included raffinose, stachyose, trypsin inhibitor, urease, and phytate. The mean values for antinutrients of the toasted, defatted soybean meals across all locations are presented in Table 2. Except for trypsin inhibitor, the antinutrient values obtained for toasted, defatted soybean meal produced from grain of CV127 soybean were not significantly different from the values obtained for the soybean meal from the isoline control soybean. In addition, there were no statistically significant differences in levels of antinutrients in the toasted, defatted meal fraction between CV127 and either one or both of the two conventional standard soybean varieties in this study, except for the trypsin inhibitor. The mean value for trypsin inhibitor from meal of CV127 soybean was statistically significantly higher than the values obtained for the meal from the isoline control and the two conventional standard soybean varieties but, it was below the lower end of the range reported for this antinutrient in the literature for other soybean varieties (Table 2). However, the mean values of the other antinutrients present in toasted, defatted soybean meal that were determined for CV127 soybean were within the ranges for these antinutrients reported in the literature.

Isoflavones. The mean values for isoflavones in toasted defatted soybean meals were determined and the values for total isoflavones were calculated by adding the values for daidzin, malonyl daidzin, acetyl daidzin and daidzein for total daidzein; glycitin, malonyl glycitin, acetyl glycitin, and glycitein for total glycitein; and genistin, malonyl genistin, acetyl genistin, and genistein for total genistein. The mean values for total isoflavones in the toasted, defatted soybean meals across all locations are presented in Table 3. A comparison of the mean total isoflavone values determined for CV127 soybean meal with

those from the isoline control show that for total glycitein, there was no significant difference between the mean values for meal from CV127 and the isoline control, but levels of this isoflavone were lower in both CV127 and the isoline control compared to the two conventional standard soybean varieties, suggesting that this difference is due to varietal differences. For total daidzein and total genistein, the values for CV127 soybean meal were significantly lower than the values obtained for the isoline control and the two conventional standard soybean varieties included in this study. These results were consistent with differences in levels of these isoflavones measured in grain of the four treatments (Privalle, 2008a). Levels of total daidzein and genistein were statistically significantly lower in grain of CV127 compared to levels in grain of the isoline control. However, mean levels of these isoflavones in CV127 were within the range of values reported for soybean varieties globally as well as for varieties cultivated in Brazil (International Life Sciences Institute, 2006, ILSI Composition Database). Therefore, it is expected that levels of these isoflavones in toasted, defatted meal of CV127 would similarly be within the range of values for toasted, defatted meal of other soybean varieties both globally and in Brazil.

Protein isolate and concentrate results. The mean proximate values for both protein isolate and concentrate fractions for all treatments across locations are presented in Tables 4 and 5, respectively. For the proximate values of the protein isolate fraction, the only analyte that was statistically significantly different between CV127 soybean and the isoline control was ash where the value for CV127 was significantly lower compared to the isoline control, but it was not different from either of the two conventional comparator varieties (Table 4). Comparison of the mean proximate values obtained for the protein isolate fraction produced from CV127 soybean with the ranges of these nutrients reported for protein isolate from soybean in the literature demonstrated that, except for the mean fat values for the protein isolate, the mean values from CV127 soybean were either within or comparable to the ranges reported for these nutrients in the literature (Table 4). For all four treatments, the mean value for fat in the protein isolate was above the range for fat that is reported for soybean protein isolates in the literature, which suggests that the higher levels of fat observed in the protein isolate from the four treatments in this study is a characteristic of soybean varieties adapted for cultivation in Brazil. In the case of the proximate values for protein concentrate, there were no statistically significant differences in any of the proximate analytes between protein concentrate prepared from grain of CV127 soybean and the isoline control variety as well as the two conventional standard soybean varieties, with the one exception of ash in Standard variety 1 (Monsoy 8001, Table 5). Furthermore, mean values for ash and protein in CV127 protein concentrate were either within or comparable to the range of values reported in the literature for protein concentrate from other soybean varieties. Levels of fat and carbohydrates in the protein concentrate fraction for all treatments were outside the range reported in the literature for this soybean protein fraction. Therefore, these differences are most likely a characteristic of soybean varieties adapted for cultivation in Brazil.

Fatty acid composition of soybean oil. The mean values for fatty acids in the refined oil fractions of the different soybean treatments across all field locations are presented in Table 6. Myristic acid (14:0) was detected in the processed oil fractiuons, but was present below the level of quantification and therefore was not included in the Table. Comparison of the mean fatty acid values of the refined oil from grain of CV127 soybean with those from grain derived from the isoline control variety showed no statistically significant differences in fatty acid content between the treatments, except for higher oleic acid content and significantly lower content for linoleic, linolenic, and behenic acids in CV127 oil compared to that of the isoline control. Furthermore, comparison of the mean fatty acid values obtained from the refined oil produced from grain of CV127 soybean with the range of values reported in the literature for soybean oil from different soybean varieties demonstrates that, with the exception of behenic acid, all mean values for fatty acids from the refined oil of CV127 soybean were within the ranges for these fatty acids in refined soybean oil that have been reported in the literature (Table 6). The mean values obtained for behenic acid from both CV127 and the isoline control soybean were slightly higher than the range reported in the literature. There were statistically significant differences in fatty acid content of the oil between the two standard comparator soybean varieties in this study compared to both CV127 and the isoline control, suggesting that these differences in fatty acid content of the oil reflected differences between soybean varieties.

AHAS levels in processed soybean fractions. The final component of the assessment of the processed fractions produced from the grain of CV127 soybean and the isoline control consisted of an analysis of the amount of AHAS protein in each of the processed fractions. Aliquots of each processed fraction sample were analyzed by the BASF Global Environmental and Consumer Safety Laboratory in Guaratinguetá, Brazil for AHAS protein by an AHAS-specific ELISA assay. The assay was validated for measurement of AHAS protein levels in each different processed fraction, including determination of limit of detection (LOD), limit of quantification (LOQ), and spike and recovery of AHAS protein (determined by adding a known amount of purified AtAHAS protein to the processed fraction followed by extraction and analysis by ELISA). The LOD, LOQ and spike and recovery validation parameters as well as the amount of AHAS detected in the processed fractions from CV127 soybean and the isoline control are presented in Table 7. The results of these analyses demonstrate that the AHAS protein, including both the AtAHAS encoded by the *csr1-2* gene and the endogenous soybean AHAS, could not be detected in any of the processed fractions produced from the grain of CV127 soybean. This result was as expected since AHAS levels were either at or below the level of quantification of the ELISA (13-14 ng/g DW.) in grain harvested from CV127 and the isoline control (Schwerz, 2008). Also, given the instability of the AHAS protein to heat treatment (Privalle, 2008b) the protein is not expected to survive the heat treatments involved in producing the processed fractions. For the untoasted defatted soybean meal produced from the grain of the isoline control variety, AHAS was detectable but not quantifiable (LOQ = 13 ng/g). In all other processed fractions produced from the isoline control, no AHAS protein was detected. It should be noted that the spike and recovery of AtAHAS from refined oil could not be determined due to an inability to recover the

AHAS protein that was spiked into the oil. However, soybean oil typically contains very little, if any, protein and this was confirmed by measurements of total protein in the refined oil fractions that gave values ranging from 1.9 – 4.0 µg/g oil. Since AHAS protein was either at or below the level of quantification of the ELISA (13-14 ng/g fresh wt.) in grain harvested from CV127 and the isoline control, it can be concluded that there are no significant amounts of AHAS protein present in the refined oil fractions.

CONCLUSION

Soybean has many uses in animal and human nutrition. The grain is typically processed to two commodity products, oil and meal. The defatted toasted meal is commonly used in livestock feed. The various soybean protein fractions derived from processing untoasted, defatted soybean meal are used in different human foods. Also, the refined soybean oil is used in different food products including cooking oil and salad dressings. An important component of the safety assessment of CV127 soybean is to demonstrate that the nutrient and antinutrient composition of the processed soybean fractions are equivalent to those from the isoline control variety as well as other conventional commercial soybean varieties, thereby confirming that CV127 grain is appropriate for use in soybean food and feed products. The results generated by these analyses of the processed soybean fractions produced from CV127 soybean, the isoline control, and two conventional control varieties support the conclusion that the nutrient and antinutrient composition of the processed fractions from CV127 soybean are comparable or within the same range as similar processed soybean fractions produced from grain of the isoline control and two conventional soybean comparator varieties. Also, for most analytes the values for CV127 soybean were either within or comparable to the literature reference range of values reported for these soybean processed fractions. Therefore, composition of the toasted, defatted meal, refined oil, protein isolate and concentrate processed fractions of CV127 soybean are equivalent to composition of these same processed fractions derived from the isoline control and other conventional standard soybean varieties, and confirms that processed fractions from CV127 soybean are appropriate for use in human foods as well as animal feeds.

Furthermore, analysis of the processed fractions for the AHAS protein demonstrated that processing reduces the levels of AHAS protein from the very low levels that are detectable but not quantifiable in soybean grain. These findings are consistent with the conclusions of other studies that compare CV127 soybeans with the isoline control and conventional control varieties that demonstrate that the imidazolinone-tolerance trait of CV127 soybean has no significant impact on the agronomic characteristics, nutrient composition, and other aspects related to the safe use of CV127 soybean for food, feed and other uses.

RECORDS RETENTION: Analytical reports, the original copy of this report, and other relevant records are archived at BASF, 26 Davis Drive. Research Triangle Park, NC, USA 27709.

STUDY PERSONNEL: Statistical analysis work reported herein conducted by [redacted], Ph.D., BASF Plant Science, LLC, Research Triangle Park, NC 27705.

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Table 1. Comparison of Proximate and Fiber Composition Means and Ranges for the Toasted, Defatted Meal Processed Fraction of the Isoline Control, CV127 and Conventional Standard Soybean Varieties Across Four Locations

Analyte (unit)	Isoline	CV127	Std 1	Std 2	Literature Range
	Mean (N = 4) (range)				
Proximates					
Moisture g/100 g FW	4.3a[†] (3.8 – 4.8)	3.4b (3.2 – 3.8)	3.9ab (3.6 – 4.1)	4.5a (3.6 – 5.5)	NA
Ash g/100 g DW	6.3ab (6.0 – 6.6)	6.3ab (6.1 – 6.6)	6.2b (5.8 – 6.5)	6.3ab (5.8 – 6.6)	5.5 - 6.5[1]
Fat g/100 g DW	1.2a (0.4 – 1.9)	1.2a (0.9 – 1.9)	1.0a (0.6 – 1.4)	1.2a (0.7 – 2.4)	0.5 - 2.40[2]
Protein g/100 g DW	51.1a (49.8 – 53.0)	50.6a (48.8 – 51.4)	48.2b (46.4 – 49.3)	48.1b (47.3 – 49.1)	44 – 61.4[3]
Carbohydrates[‡] g/100 g DW	41.5b (40.1 – 42.1)	41.9b (41.3 – 42.7)	44.6a (43.4 – 45.7)	44.4a (43.8 – 45.4)	32.0 – 38.0[4]
Calories kcal/100 g DW	381a (377 – 384)	381a (380 – 383)	381a (378 – 382)	381a (377 – 389)	NA*
Fiber					
Crude Fiber g/100 g DW	10.1a (9.5 – 10.7)	10a (9.7 – 10.2)	10.5a (9.9 – 11.3)	10.5a (8.9 – 12.4)	NA
ADF g/100 g DW	8.41bc (7.87 – 8.94)	7.74c (7.31 – 7.92)	9.44a (8.72 – 9.88)	9.11ab (8.41 – 9.83)	NA
NDF g/100 g DW	16.49ab (15.01 – 17.26)	14.86b (13.17 – 17.17)	16.4ab (15.43 – 17.50)	18.01a (16.50 – 20.46)	NA

[†]Values in the same row followed by the same letter are not significantly different at P<0.05.
[‡]Carbohydrates including total dietary fiber.
[1]Fulmer (1988), Orthoefer (1978); [2]Han et al. (1991), Orthoefer (1978); [3]Orthoefer (1978), Smith and Circle (1972); [4]Waggle and Kolar (1979)
*Not available

Table 2. Comparison of the Antinutrient Composition Means and Ranges for the Toasted, Defatted Meal Processed Fraction of the Isoline Control, CV127 and Conventional Standard Soybean Varieties Across Four Locations

Analyte (unit)	Isoline	CV127	Std 1	Std 2	Literature Range
	Mean (N = 4) (range)				
Raffinose g/100 g DW	1.9ab† (1.6 – 2.3)	1.9b (1.5 – 2.2)	2.1a (1.7 – 2.5)	1.8b (1.5 – 2.1)	1.0 – 2.0[1]
Stachyose g/100 g DW	5.1ab (4.4 – 5.9)	5.0ab (4.5 – 5.4)	5.4a (5.0 – 5.8)	4.8ab (4.0 – 5.5)	4.0 – 5.3[2]
Trypsin inhibitor TIU/mg	1.24b (0.84 – 1.56)	2.03a (1.69 – 2.56)	1.16b (0.52 – 1.64)	1.16b (1.01 – 1.23)	3.8 – 17.9[3]
Urease Δ pH	0.02a (0.01 – 0.04)	0.04a (0.02 – 0.06)	0.05a (0.02 – 0.09)	0.04a (0.03 – 0.05)	0.05 – 0.20[4]
Phytic Acid mg/g DW	4.32ab (3.98 – 4.78)	4.07b (2.86 – 4.96)	3.78b (3.34 – 4.65)	4.51ab (3.56 – 5.24)	1.3 – 4.1[5]

†Values in the same row followed by the same letter are not significantly different at $P<0.05$.
[1]Rackis (1974); [2]Coon et al. (1988), Kuo et al. (1988), Rackis (1974); [3]Anderson and Wolf (1995), Rackis (1974); [4]Lee and Garlich (1992); [5]Anderson and Wolf (1995), Mohamed et al. (1991).

Table 3. Comparison of Isoflavone Composition Means and Ranges for the Toasted, Defatted Meal Processed Fraction of the Isoline Control, CV127 and Conventional Standard Soybean Varieties Across Four Locations

Analyte (unit)	Isoline	CV127	Std 1	Std 2
	Mean (N = 4) (range)			
Total Daidzein mg/100 g DW	99.0a† (85.1 – 123.6)	74.8b (67.4 – 82.7)	103.3a (95.2 – 118.5)	100.6a (87.3 – 123.6)
Total Glycitein mg/100 g DW	25.1b (24.1 – 26.5)	22.9b (20.9 – 27.9)	48.3a (42.8 – 52.4)	52.4a (45.9 – 57.9)
Total Genistein mg/100 g DW	133.5b (114.5 – 167.9)	115.7c (97.7 – 132.6)	166.8a (149.7 – 199.1)	161.7a (138.7 – 203.4)

†Values in the same row followed by the same letter are not significantly different at $P<0.05$

Table 4. Comparison of Proximate Composition Means and Ranges for Protein Isolate from the Isoline, CV127 and Conventional Standard Soybean Varieties Across Four Locations

Analyte (unit)	Isoline	CV127	Std 1	Std 2	Literature Range
	Mean (N = 4) (range)				
Ash g/100 g DW	3.3a† (2.9 – 4.0)	2.6b (2.2 – 3.2)	3.2ab (2.7 – 3.8)	2.6ab (1.9 – 3.1)	2.3 – 7.6[1]
Fat g/100 g DW	5.4a (4.1 – 6.6)	7.0a (5.8 – 9.0)	5.2a (3.9 – 6.1)	5.6a (4.6 – 6.9)	0.1 - 2.5[2]
Protein g/100 g DW	90.4ab (89.0 – 91.0)	90.4ab (88.1 – 92.7)	89.6b (88.4 – 91.1)	91.5a (90.6 – 92.5)	85.2 – 92.0[3]
Carbohydrates‡ g/100 g DW	0.9ab (0.1 – 1.8)	0.7ab (0.0 – 2.1)	2.1a (1.0 – 3.5)	0.8ab (0.0 – 1.7)	0.3 – 0.6[4]
Calories kcal/100 g DW	414a (407 – 417)	428a (417 – 436)	414a (408 – 418)	420a (411 – 427)	NA*

†Values in the same row followed by the same letter are not significantly different at P<0.05
‡Carbohydrates including total dietary fiber.
[1]Smith and Circle (1972), Wolf (1983); [2]Horan (1974), Wolf (1983); [3]Torun (1979), Waggle and Kolar (1979); [4]Waggle and Kolar (1979), Wolf (1983)
*Not available

Table 5. Comparison of Proximate Composition Means and Ranges for Protein Concentrate from the Isoline, CV127 and Conventional Standard Soybean Varieties Across Four Locations

Analyte (unit)	Isoline	CV127	Std 1	Std 2	Literature Range
	Mean (N = 4) (range)				
Ash g/100 g DW	4.0ab† (3.6 – 4.1)	4.4a (4.9 – 4.0)	3.7b (3.2 – 4.4)	4.0ab (3.8 – 4.2)	4.7 – 6.5[1]
Fat g/100 g DW	5.1a (3.7 – 7.4)	6.2a (4.5 – 9.2)	5.1a (3.7 – 6.8)	4.4a (3.8 – 5.7)	0.9 – 2.0[2]
Protein g/100 g DW	79.5a (76.7 – 81.1)	78.2a (70.9 – 85.6)	80.9a (77.1 – 82.8)	78.2a (77.0 – 79.3)	66.2 – 78.1[1]
Carbohydrates‡ g/100 g DW	11.5a (7.4 – 14.2)	11.4a (1.0 – 18.6)	10.4a (7.3 – 13.5)	13.3a (11.5 – 14.9)	17.1 – 25.0[3]
Calories kcal/100 g DW	410a (403 – 421)	414a (406 – 429)	411a (404 – 421)	406a (402 – 412)	NA*

†Values in the same row followed by the same letter are not significantly different at P<0.05
‡Carbohydrates including total dietary fiber.
[1]Bookwalter (1978), Smith and Circle (1972); [2]O'Dell (1979), Wolf (1983); [3]Mattil (1974), Smith and Circle (1972)
*Not available

Table 6. Comparison of Fatty Acid Composition Means and Ranges for Refined Oil from the Isoline, CV127, and Conventional Standard Soybean Varieties Across Four Locations

Analyte (g/100 g oil)	Isoline	CV127	Std 1	Std 2	Literature range[1]
	Mean (N = 4) (range)				
Palmitic 16:0	10.33ab[†] (9.66 – 10.90)	9.94b (9.61 – 10.42)	10.38a (9.66 – 10.76)	9.19c (8.94 – 9.32)	7 - 12
Stearic 18:0	3.84a (3.54 – 4.21)	3.75a (3.25 – 4.21)	3.67a (3.44 – 3.97)	3.36b (3.15 – 3.59)	2 – 5.5
Oleic 18:1	21.96c (21.03 – 22.66)	24.28a (22.56 – 25.62)	18.88d (17.78 – 19.74)	21.24c (20.65 – 21.94)	20 – 50
Linoleic 18:2	51.52b (50.95 – 52.29)	50.45c (49.43 – 51.67)	53.66a (53.20 – 54.06)	53.99a (53.58 – 54.30)	35 – 60
Linolenic 18:3	6.19b (5.93 – 6.41)	5.64c (5.26 – 6.02)	7.75a (7.27 – 8.27)	6.49b (6.31 – 6.64)	2 – 13
Arachidic 20:0	0.42a (0.38 – 0.48)	0.42a (0.38 – 0.48)	0.35b (0.29 – 0.38)	0.29 c (0.29)	0.2 – 1.0
Eicosenoic 20:1	0.23a (0.14 – 0.29)	0.22a (0.19 – 0.29)	0.18a (0.14 – 0.19)	0.19a (0.19)	< 1.0
Behenic 22:0	0.60a (0.57 – 0.67)	0.54b (0.48 – 0.57)	0.48c (0.43 – 0.53)	0.46c (0.43 – 0.48)	< 0.5
Tetracosanoic 24:0	0.19a (0.19)	0.19a (0.19)	0.13b (0.10 – 0.19)	0.19a (0.19)	NA*

[†]Values in the same row followed by the same letter are not significantly different at P<0.05
[1]Pryde (1990)
*Not available

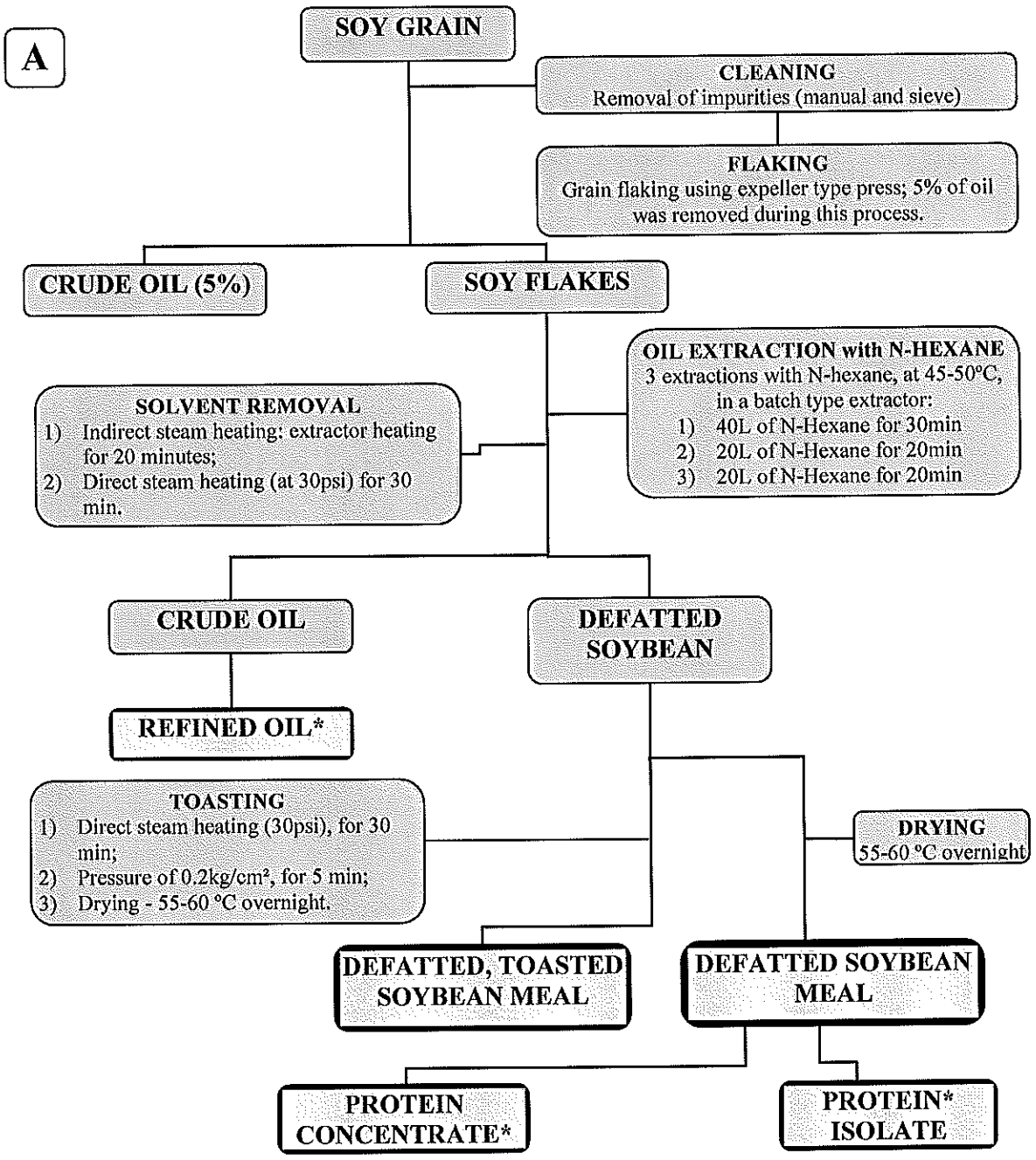
Table 7. Assay Validation Parameters and AHAS Levels in Processed Soybean Fractions Produced from CV127 Soybean and the Isoline Control as Determined by ELISA

Fraction	LOD ng AHAS /g DW	LOQ ng AHAS /g DW	Spike and Recovery Efficiency % ± C.V.	AHAS ng/g DW	
				CV127 (N=4)	Isoline Control (N=4)
Defatted Toasted Meal	6	8	75 ± 5	ND†	ND
Defatted Untoasted Meal	6	13	88 ± 15	ND	ND - < 13
Protein Isolate	6	24	79 ± 9	ND	ND
Protein Concentrate	9	24	79 ± 8	ND	ND
Oil	*	*	*	ND	ND
Grain	3	15	88 ± 7	<14	<14

†Not detected
*Extraction efficiency experiments using AtAHAS protein were unsuccessful, however, recovery of bovine serum albumin (BSA) protein was possible with an extraction efficiency ranging between 2 - 24% for 10 ppm and 37 – 62% for 100 ppm.

Figure 1. Schematic Diagram Depicting the Methods Employed in the Grain Processing Study

The processes used to produce the different processed fractions derived from the soybean grain are presented on the following pages. The general details of the production of refined oil, toasted and untoasted defatted soybean meal and protein isolate and concentrate are presented in panel A and further details of the production of refined oil and the protein isolate and concentrate are presented in panels B and C, respectively.

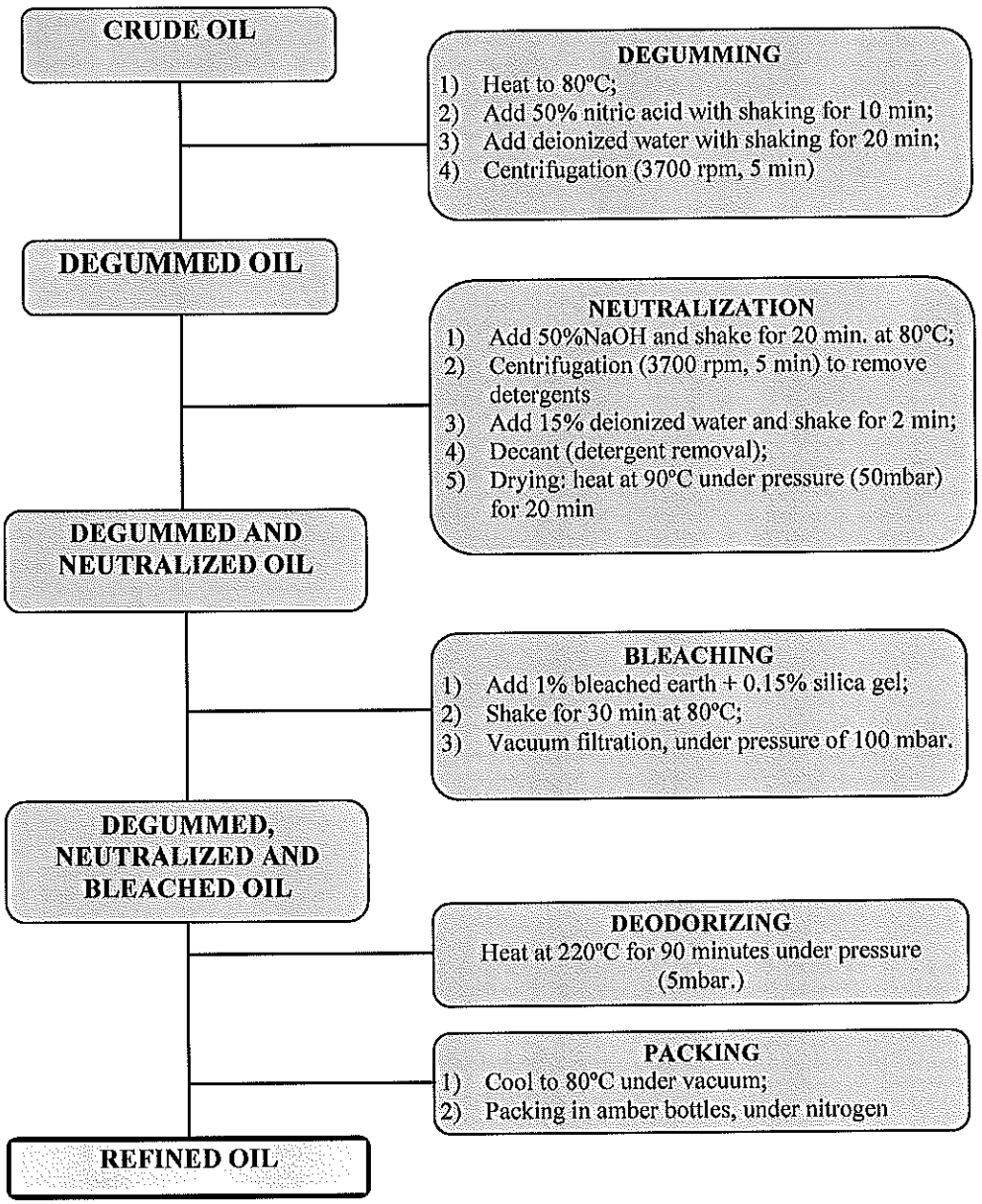


Equipment cleaning → All materials in contact with soybeans were cleaned before every new processing step. All sanitary connections were disassembled and the pipes washed. Direct steam was used through the piping.

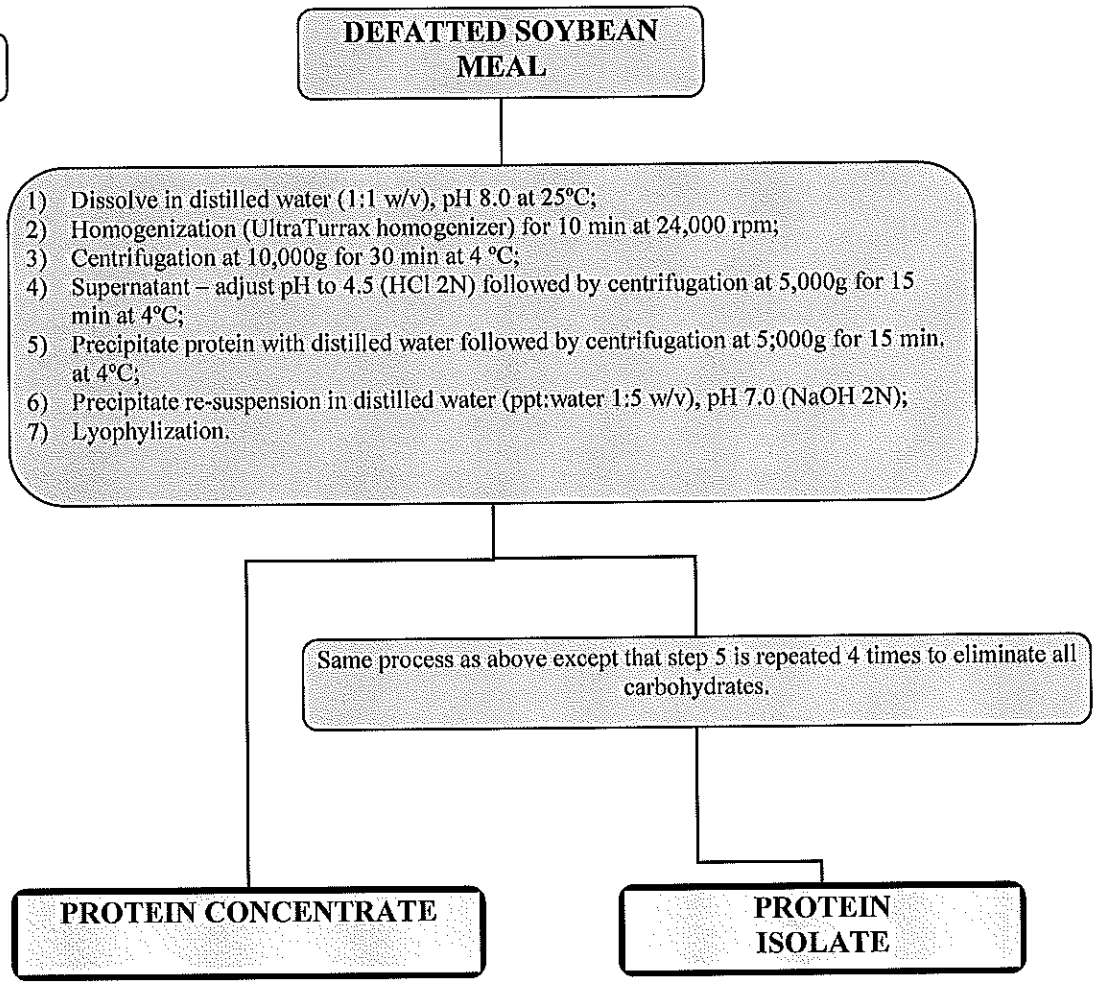
* The steps to obtain refined oil, protein concentrate and isolated protein are presented in the following pages

B. **ANALYTICAL STEPS:**

1) Free fatty acids analysis→ Determine the amount of NaOH to be added for neutralization.
2) Amount of Phosphorous present→ Determine the amount of water and acid to be added for phospholipid removal.



C


DEFATTED SOYBEAN MEAL
1) Dissolve in distilled water (1:1 w/v), pH 8.0 at 25°C;
2) Homogenization (UltraTurrax homogenizer) for 10 min at 24,000 rpm;
3) Centrifugation at 10,000g for 30 min at 4 °C;
4) Supernatant – adjust pH to 4.5 (HCl 2N) followed by centrifugation at 5,000g for 15 min at 4°C;
5) Precipitate protein with distilled water followed by centrifugation at 5;000g for 15 min, at 4°C;
6) Precipitate re-suspension in distilled water (ppt:water 1:5 w/v), pH 7.0 (NaOH 2N);
7) Lyophylization.
Same process as above except that step 5 is repeated 4 times to eliminate all carbohydrates.
PROTEIN CONCENTRATE
PROTEIN ISOLATE