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Proximate Analysis and Amino Acid Profile of the Ecuadorian Andean Lupin (Fabaceae: *Lupinus mutabilis)* Protein Isolate

Disertación previa a la obtención del título de Licenciado en Ciencias Biológicas

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Certifico que la disertación de Licenciatura en Ciencias Biológicas de la candidata Daniela Guerra Freire ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Mtr. Pablo Pozo
Director de la disertación

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Proximate Analysis and Amino Acid Profile of the Ecuadorian Andean Lupin (Fabaceae: *Lupinus mutabilis)* Protein Isolate

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1. RESUMEN

El propósito de este estudio ha sido aislar contenido proteico total de los granos de chocho (Fabaceae: *Lupinus mutabilis*). El contenido de macronutrientes ha sido evaluado a través de un análisis proximal: proteína total (67.25%), carbohidratos (18.67%), grasa (5.95%), ceniza (4.12%) y humedad (4.01%). El análisis por cromatografía de capa fina ha demostrado que habían siete amino ácidos esenciales presentes en el aislado proteico: ácido glutámico, treonina, valina, isoleucina, leucina, metionina y triptófano. Además, la presencia de ácido glutámico y de amino ácidos de cadena ramificada, que son cruciales para potenciar el desempeño deportivo, sugieren que el aislado proteico de granos de chocho podría ser un posible candidato para el desarrollo de suplementos nutricionales enfocados a deportistas. Un método diferente de aislamiento debería ser probado para optimizar el aislamiento del contenido proteico. Por otra parte, otros estudios de cuantificación de amino ácidos son necesarios para tener una mejor comprensión sobre el potencial del aislado proteico como suplemento nutricional.

Palabras clave: análisis proximal, aislado proteico, deportistas, ejercicio, *Lupinus*, nutrición

2. ABSTRACT

The purpose of the present study was to isolate the total protein content of lupin beans (Fabaceae: *Lupinus mutabilis*). The macronutrient content was evaluated through a proximate analysis: total protein (67.25%), carbohydrates (18.67%), fat (5.95%), ash (4.12%), and moisture (4.01%). The thin-layer chromatography analysis showed that seven essential amino acids are present in the protein isolate: glutamic acid, threonine, valine, isoleucine, leucine, methionine, and tryptophan. Furthermore, the presence of glutamic acid and branched-chain amino acids −which are crucial for enhancing athletic performance− suggest that the lupin protein isolate could be a possible candidate for a development of nutritional supplement intended for athletes. A different protein isolation procedure should be tested in order to optimize the complete isolation of the protein content. Moreover, further studies for amino acid quantification are necessary in order to understand the protein isolate's true potential as a dietary supplement for athletes.

Keywords: athletes, exercise, *Lupinus*, nutrition, protein isolate, proximate analysis

3. INTRODUCTION

Long before the Inca civilization prospered, Andean crops have been fundamental for several other cultures. The crops great diversity has allowed the establishment and success of these societies. One of the most noticeable crops has been the Andean Lupin (*Lupinus mutabilis*: Fabaceae). The genus originated in the Ecuadorian, Peruvian and Bolivian Andean region; this area is characterized by the great variety of species of *Lupinus*. It is believed that the diversity reaches up to 83 species to date (S. Jacobsen &

Mujica, 2006) Even though lupins have been harvested for centuries, the introduction of European crops has replaced this legume (Bermejo & León, 1994); this is, probably, due to the complex processing lupins must undergo before being edible (S. Jacobsen & Mujica, 2006).

In Ecuador, both the production and consumption of lupin beans has been circumscribed to the highlands. The province with the greatest production is Cotopaxi; however, Cañar and Chimborazo are also mass producers (Horton, 2014). The *L. mutabilis* beans are grown in temperate climates and at altitudes ranging from 2,500 m to 3,600 m. This particular crop is prone to diseases and plagues, the insufficient research and lack of knowledge about lupins has not allowed the production to increase and the industry to expand. Moreover, it has been estimated that the overall lupin production is not enough to supply the national demand, it is believed that the current yield can only cover 60% of the requirement (S.-E. Jacobsen & Sherwood, 2002). However, recently, several companies have been promoting the production and consumption of lupin beans by selling ready-to-eat lupins to national supermarket chains (Horton, 2014); thus, enhancing scientific research about this bean (Peralta, E., Mazón, Á., *et al.,* 2012).

In a nutritional aspect, the lupin beans are characterized by having a high protein, moderate fatty acid, and low carbohydrate content (Bermejo & León, 1994). Furthermore, this legume contains essential micronutrients (Villacrés, Rubio, *et al*, 2006), such as: calcium 0.48%, phosphorous 0.43%, potassium 0.02%, iron 120 mg/kg, and zinc 50 mg/kg (Jaramillo Galarza, 2005). In Ecuador, lupins are one of the most consumed beans; however, only the whole bean is consumed. Moreover, its by-products, like flour, oil, or protein isolate, have not been of much interest and have not

been industrialized (S.-E. Jacobsen & Sherwood, 2002). Regardless their nutritional value and importance, this crop has not been completely exploited; this underutilization is, possibly, due to the absence of research about lupins in the food industry.

Within the Fabaceae family, there are several beans that are fundamental to human nutrition; probably, the most notorious one is the soybean. Soy crops are grown and harvested worldwide because they are nutritious and versatile. Within the food industry, soybeans have acquired great popularity among nutritional supplements designed for athletes. Soybeans are processed into a protein isolate; this isolate is commercialized at a global scale as post-workout supplement. After an athlete completes a training session – cardiovascular or strength training – micro-traumas are created in the skeletal muscle and amino acid catabolism is promoted (Y. Shimomura et al., 2006). In order to repair these micro-injuries, proper nutrition after exercise is fundamental (Farrell, Joyner, *et al*, 2012). This means that, depending the type of exercise that is carried out; the proportion of macronutrients ingested varies. In activities that require strength or resistance, like weightlifting, it is essential to consume food rich in protein, such as protein isolate supplement. Protein intake helps to recover the amino acids that were lost during training (Farrell *et al*., 2012). The amino acids in charge of muscle repair and preservation are leucine, isoleucine, and valine (Børsheim, *et al.*, 2002). The ingestion of these is fundamental for a proper muscular recovery and growth.

The lupin bean has several advantages over the soybean. For instance, studies made with *L. mutabilis* demonstrated that this bean has high protein content; in comparison to soy, lupin beans contain 10.9% more protein (S. Jacobsen & Mujica, 2006). This means that, *L. mutabilis* beans can be a better option for nutritional supplement for athletes. Unfortunately, in Ecuador, these supplements are not produced and athletes rely solely

on imports, which makes them much more expensive. Moreover, the options are quite limited, most of the supplements come from animal sources (whey protein isolate, casein protein isolate, and egg albumin protein isolate), and the plant-based options are limited to soy. Furthermore, another advantage lupins have is that, unlike soybeans, lupin beans have not been subjected to genetic modification. Nowadays, consumers prefer to acquire non-GMO (genetically modified organisms) products, due to the health and environmental issues behind the consumption of these types of crops (FAO, 2003b); this would make lupin beans an excellent option to soybeans.

4. MATERIALS AND METHODS

5.1 Sampling

Commercially available hydrated lupin beans were acquired. All the samples came from the same manufacturer, La Verde, which processes lupin beans from the Machachi region, Ecuador.

5.2 Extraction and isolation

5.2.1 Obtaining Whole Lupin Flour

The hydrated lupin beans were dehull, dried, and milled using a cereal mill (Ika Werke) (AOAC, 2011c).

5.2.2 Obtaining Deffated Lupin Flour

The fat content was extracted with a Soxhlet extractor (Provitec) using hexane as a solvent (Lusas & Riaz, 1995)

5.2.3 Obtaining the Protein Isolate

From the defatted lupin flour, protein was solubilized with 10% NaOH (6.8-10 pH). The solution was centrifuged (MixtaSel Selecta), at 4,000 rpm during 5 minutes and solids were recovered. The protein content was precipitated with 10% HCl (4.5 pH), centrifuged at 4,000 rpm during 5 minutes, and the protein precipitated as a curd. The curd was neutralized with 10% NaOH (6.5-7 pH); distilled water was added to resuspend the solution. Finally, the solution was centrifuged at 4,000 rpm during 5 minutes and the curd was recovered. The curd was taken to an oven (Binder) and dried for 24 h (Lusas & Riaz, 1995).

5.3 Lupin Protein Isolate Proximate Analysis

5.3.1 Determination of Total Protein Content

Nitrogen content was determined by using the Kjeldahl method. Digestion was carried out in a Kjeldahl digester (Velp) using Kjeldahl tablets (Velp). Distillation of the samples was done in Velp distillator;

finally, titration was done manually with Tashiro's indicator, according to the AOAC procedure and standards (AOAC, 2011a).

5.3.2 Determination of Fat Content

Fat content was determined with a Mirco-Soxhlet extractor (Provitec), using hexane as a solvent (AOAC, 2011d).

5.3.3 Determination of Ash Content

Ash content was determined by placing the samples in porcelain crucibles in a furnace (American Snol) at 550º C during 24 h (AOAC, 2011b)

5.3.4 Determination of Moisture Content

Moisture content was determined by placing the samples in porcelain capsules in an oven (Binder) at 130º C during 3 h (AOAC, 2011c).

5.3.5 Determination of Carbohydrate Content

Carbohydrate content was determined by the mathematical difference between the sample and sum of protein, fat, ash, and moisture content (FAO, 2003a).

5.4 Preparation and TLC Analysis of Amino Acid Content in Lupin Protein Isolate

5.4.1 Sample preparation

Approximately 0.3 g of protein isolate was placed in 150 ml of HCl 3 N. Another 0.3 g sample was placed in 150 ml of HCl 3 N with 3g of phenol. Finally, another 0.3 g sample was placed in 150 ml of NaOH 4.2 N. All samples hydrolyzed for 24h at 100º C (Kalman, 2014). After the 24 h had elapsed, all samples were filtered prior to their placement in the TLC plate. Approximately, 3 µl of sample were applied to the baseline of the plate (plastic cellulose sheets Art. 5577, 20 x 20cm, without fluorescente indicator; E.M. Reagents) (Joseph L. Staneck, 1974)

5.4.2 TLC Analysis

Ascending TLC was carried out in (Eastman Chromagram Chamber Plate Set) TLC system with n-Butanol, glacial acetic acid, and distilled water (4:1:1) solvent for approximately 4 hours. After the chromatogram

was air dried, spots were visualized by spraying ninhydrin 1% with acetone, and dried for 15 minutes in an oven (Binder) at 60º C (Haer, 1969). An amino acid mix (Amino Acid Calibration Mixture Type 1; Beckman) was used as a standard to identify the amino acids in the sample.

5.5 Statistical Analysis

IBM® SPSS® Statistics Version 21 software was used for the statistical analysis of the data. The results were expressed as average values and each experiment was conducted repeatedly until the coefficient of variation was less than 20% (IBM, 2012).

5. RESULTS AND DISCUSSION

5.1 Lupin Protein Isolate Proximate Analysis

The proximate composition of the lupin protein isolate is shown in Table 1. The results are given through the average of several replicas to ensure that the CV was less than 20% in all parameters. The analysis showed that protein was present in the highest percentage (67.25%), followed by carbohydrate (18.67%), and fat (5.95%); which means that the protein content was isolated. Furthermore, Table 2 shows a comparison between different commercially available protein isolates

(lupin, soy, pea, and whey). The table shows that both soy and whey protein isolates have the greatest percentage of protein, followed by pea protein isolate, and, finally, lupin protein isolate. The low protein content of the lupin isolate can be due to the isolation procedure; since the method followed (Lusas & Riaz, 1995) was intended and designed for processing soybeans. Even though, soy and lupin beans both belong to the Faboideae subfamily, they cannot be processed in the same manner because of their different characteristics. For instance, lupins must be debittered in order to remove the alkaloids, before human consumption. During this procedure, the composition of the raw and dry lupin beans is altered, there can be a loss of macronutrients (carbohydrates and fats) and micronutrients (minerals) (Carvajal-Larenas, Van Boekel, *et al.*, 2014); soybeans do not need to go through this procedure to be edible.

Moreover, during the precipitation phase the HCl concentration may have been too strong and may have led to the denaturation of proteins. In order to optimize the hydrolysis of proteins, a fractional extraction should be considered. Increasing gradually the concentration of the acid will allow proteins to be isolated with minimum structural damage (Sun, Sun, *et al*., 2004).

5.2 Lupin Protein Isolate Amino Acid Profile

Six TLC plates, with different solvents, were done as preliminary essays to assess the separation efficiency of the samples. Consequently, 20 more TLC plates were done and analyzed. Table 3 shows the amino acids present in the lupin protein isolate; figure 1, shows the chromatogram of the branched-chain amino

acids (BCAAs) present in the protein isolate. The samples were too diluted; hence, the spots were faint and asymmetrical, making quantification impossible. Out of the nine essential amino acids, seven were found in the protein isolate (glutamic acid, threonine, valine, isoleucine, leucine, methionine, and tryptophan). Furthermore, all of the amino acids necessary to increase the immune system capacity and muscle repair where also found: glutamic acid and branched-chain amino acids.

Glutamic acid it is not directly related to the immune system, however, its amide, glutamine plays an important role in this system's processes. Glutamine is synthesized from ammonia and glutamic acid through the action of the enzyme glutamine synthetase; the major production of glutamine takes place in the musculoskeletal system. Within the immune system, glutamine enhances T-cell proliferation and cytokine production, which help assist and regulate the immune response (Newsholme, P. Lima, *et al.*, 2003). After strenuous physical exercise, like a marathon, athletes have a decrease in glutamine levels within their bodies. This reduction may lead to mild infections, such as a cold; therefore, experts recommend a glutamine supplement to reduce the possibilities of infection (Ehrlich, 2013). Moreover, in prolonged physical exercise, ammonia levels raise in the bloodstream, which is a cause for fatigue. Exercise-induced hyperammonemia is a problem that all athletes encounter, thus, it has been proposed that supplementation of glutamine can reduce these levels, prevent fatigue, and enhance performance (Carvalho-Peixoto, Alves, & Cameron, 2007).

Furthermore, the chromatogram (Figure 1) shows the presence of BCAAs (leucine, isoleucine, and valine). These amino acids are present in skeletal muscle proteins (14-18%), since muscle mass in humans represents approximately 40% of the total body weight, the consumption of BCAAs is extremely important (Y. Shimomura et al., 2006). Even though, BCAAs are essential for all humans, they are more important for athletes. During and after physical exercise, the human body is exposed to constant stress and protein metabolism is altered. While exercising, protein synthesis is decreased and protein degradation is increased. Amino acids derived from musculoskeletal muscle can be used for gluconeogenesis during physical activity, which leads to muscle mass loss (Dohm, G.L., Kasperek, G.J., Tapscott, E.B., Beecher, 1980). In this muscle tissue, BCAAs are subject to oxidation due to the activation of different enzymes (Y. Shimomura et al., 2006).

Studies have suggested that the ingestion of BCAAs before and after exercising can reduce muscle soreness and fatigue (Jackman, S.R., Witard, O.C., Jeukendrup, A.E., Tipton, 2010). Furthermore, leucine promotes protein synthesis and inhibits protein degradation which helps protect muscle mass (Y. Shimomura et al., 2006). The consumption of BCAAs is beneficial for athletes since it ameliorates delayed-onset muscle soreness (Yoshiharu Shimomura et al., 2010) and prevents muscle breakdown by reducing creatine kinase (CK) efflux (Howatson et al., 2012), which may allow athletes to train at a higher intensity and improve their performance.

5.3 Possible Applications of the Protein Isolate in Sport's Food Industry

The protein, fat, and carbohydrate, ratio in the lupin protein isolate could be used for the development of foods such as meat alternatives, protein bars, or power bars. However, functional properties of the protein isolate should be done to evaluate its potential in the food industry (Lusas & Riaz, 1995).

Since the lupin protein isolate contains these essential amino acids for athletes, it could be used as a nutritional supplement. This isolate, could be used as a pre and post-exercise food when combined with other nutritional elements. For instance, for the protein isolate to work as pre-exercise food, it could be combined with a stimulant such as caffeine, essential fatty acids such as chia seeds, and carbohydrate with a low glycaemic index such as rolled oats. This combination could allow the athlete to receive essential nutrients in order to maximize performance. (Kreider, R.B., *et al.*, 2009). Caffeine will cause an ergogenic effect in the athlete; this compound affects directly the athlete's endurance while exercising (Graham, 2001). Chia seeds contain a high percentage of polyunsaturated fatty acids such as both alpha-linoleic acids (omega-3 and omega-6) which are essential for human nutrition (Peiretti & Gai, 2009). Finally, incorporating a carbohydrate with a low glycaemic index will provide constant energy throughout the exercise (Sports Dietitians Australia, 2009).

Furthermore, the protein isolate could also be used for development of a post-exercise protein bar. For instance, by combining flax seeds, peanut butter, and the protein isolate. Flax seeds will provide omega-3 fatty acids; peanut butter provides

omega-6 fatty acids (McGuire, M., Beerman, 2012). The lupin protein isolate will provide a substantial amount of protein for muscle repair and it is also a carbohydrate source to replenish glycogen stores. Athletes, usually, prefer to ingest a high protein content meal post-exercise; hence, this low carbohydrate, high protein combination will be appealing. Furthermore, by combining these elements, the protein bar could deliver an appropriate post-exercise nutrition for athletes.

6. CONCLUSIONS

The proximate analysis showed that the protein content accounted for 67.25% of the sample. However, in order to obtain greater protein content another isolation method should be tested since the one used in this study was adapted from a soy protein isolation protocol. Furthermore, according to the TLC analysis almost all essential amino acids were present in the protein isolate. Moreover, branched-chain amino acids and glutamic acid were present in the protein sample, which makes the isolate a possible candidate for developing nutritional supplements targeted for athletes. Nevertheless, amino acid quantification should be done in order to obtain a better understanding of the nutritional value of the protein isolate.

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9. FIGURES

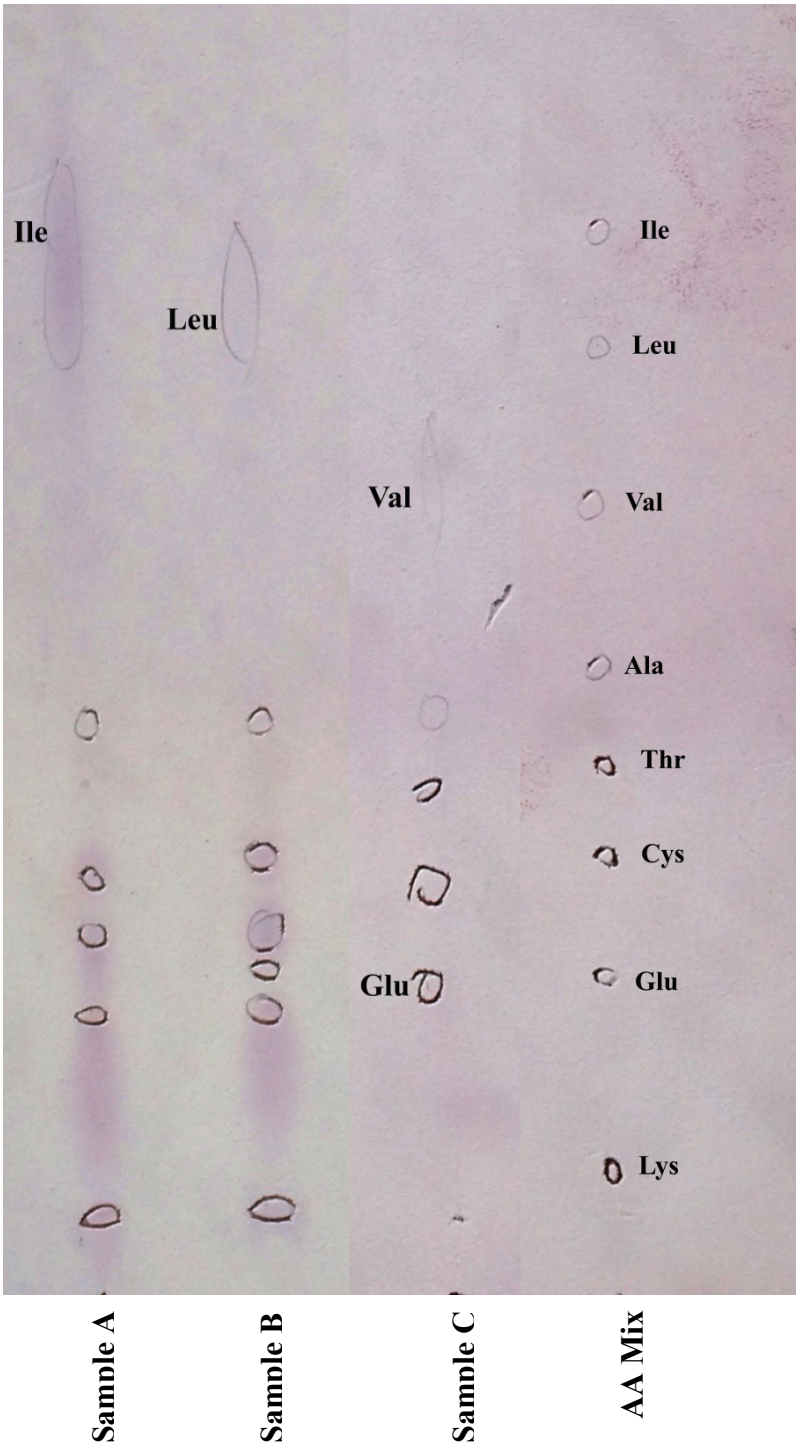


Figure 1. Amino Acid Chromatogram

In the chromatogram, branched chain amino acids and glutamic acid are shown. Three samples are displayed for a clearer view of the different amino acids

10. TABLES

Table 1. Proximate Composition of the Lupin Protein Isolate

Parameters	Lupin Protein Isolate
Protein %	67.25 ± 5.75
Fat %	5.95 ± 1.07
Ash %	4.12 ± 0.27
Moisture %	4.01 ± 0.29
Carbohydrate %	18.67

Table 2. Comparison Between Lupin Protein Isolate and Commercially Available Protein Isolates

Parameters	Lupin	Soy [a]	Pea [b]	Whey [c]
Protein %	67.25 ± 5.75	83.33	78.94	83.87
Fat %	5.95 ± 1.07	4.16	5.26	1.61
Carbohydrate %	18.67	2.08	5.26	9.68

[a] Soy Protein Isolate, NOW® Foods, Bloomingdale, IL, USA; [b] Pea Protein, Growing Naturals, Culver City, CA, USA; [c] 100% Natural Whey Protein Isolate Powder, Bluebonnet, Sugar Land, TX, USA

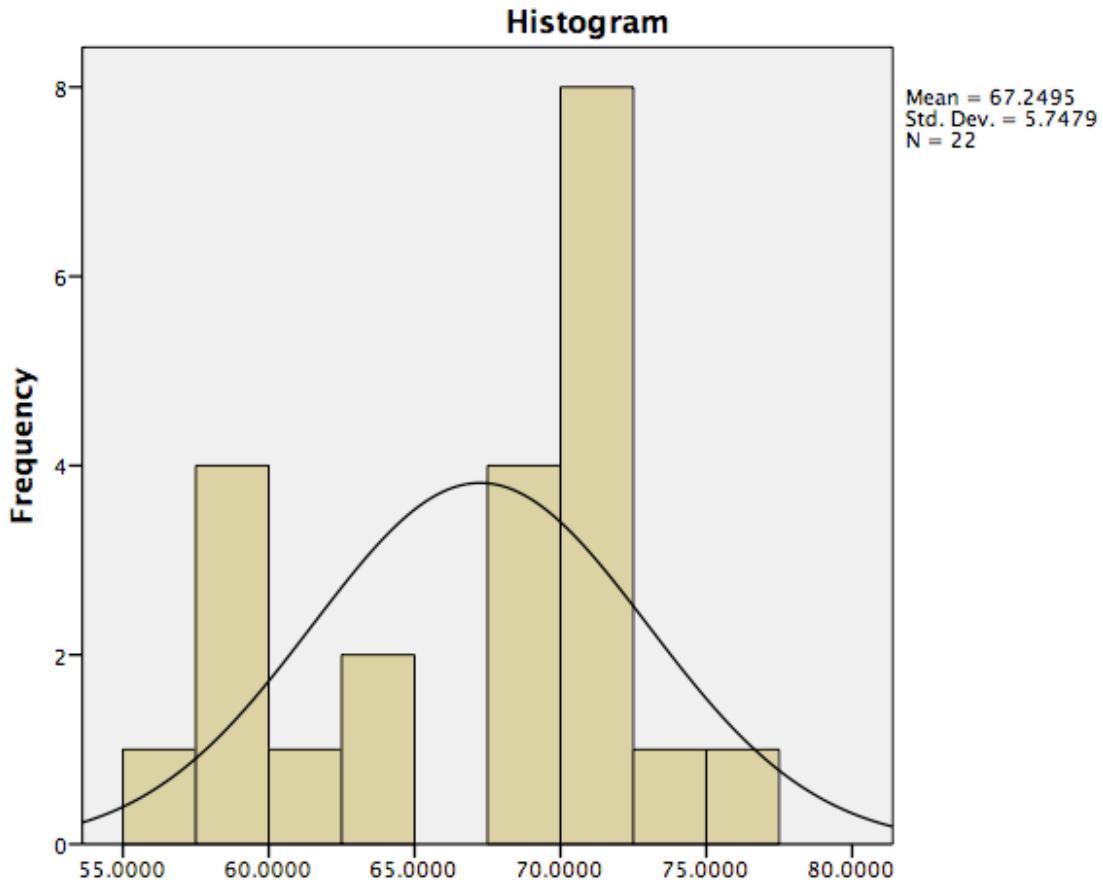
Table 3. Identified Amino Acids Found in the Lupin Protein Isolate

Amino Acid
Glutamic Acid *
Cysteine
Threonine *
Alanine
Valine * +
Isoleucine * +
Leucine * +
Methionine *
Tryptophan *

[a] Amino acids marked (*) are the essential amino acids found in the protein isolate; [b] Amino acids marked (+) are branched-chain amino acids ;[c] Underlined amino acids are involved in muscle repair and recovery

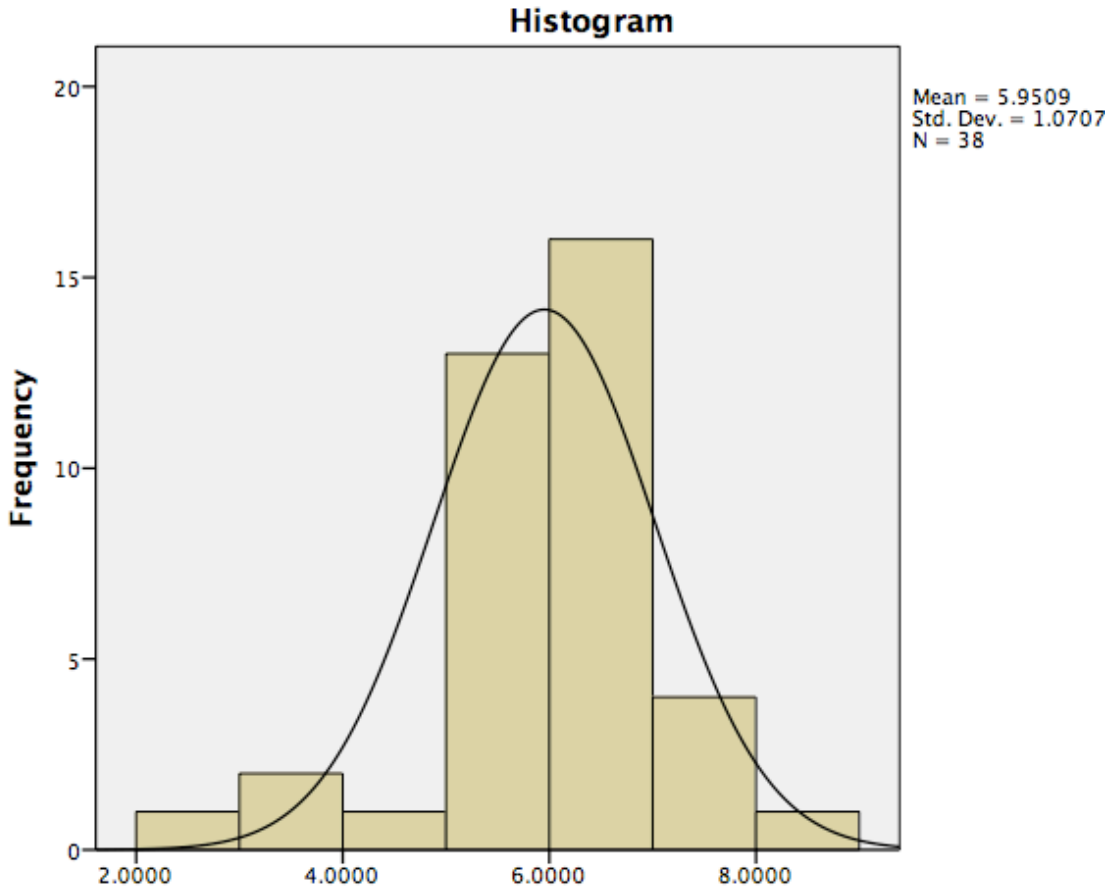
11. SUPPLEMENTARY MATERIAL

Graph 1. Histogram showing the normality curve for protein analysis



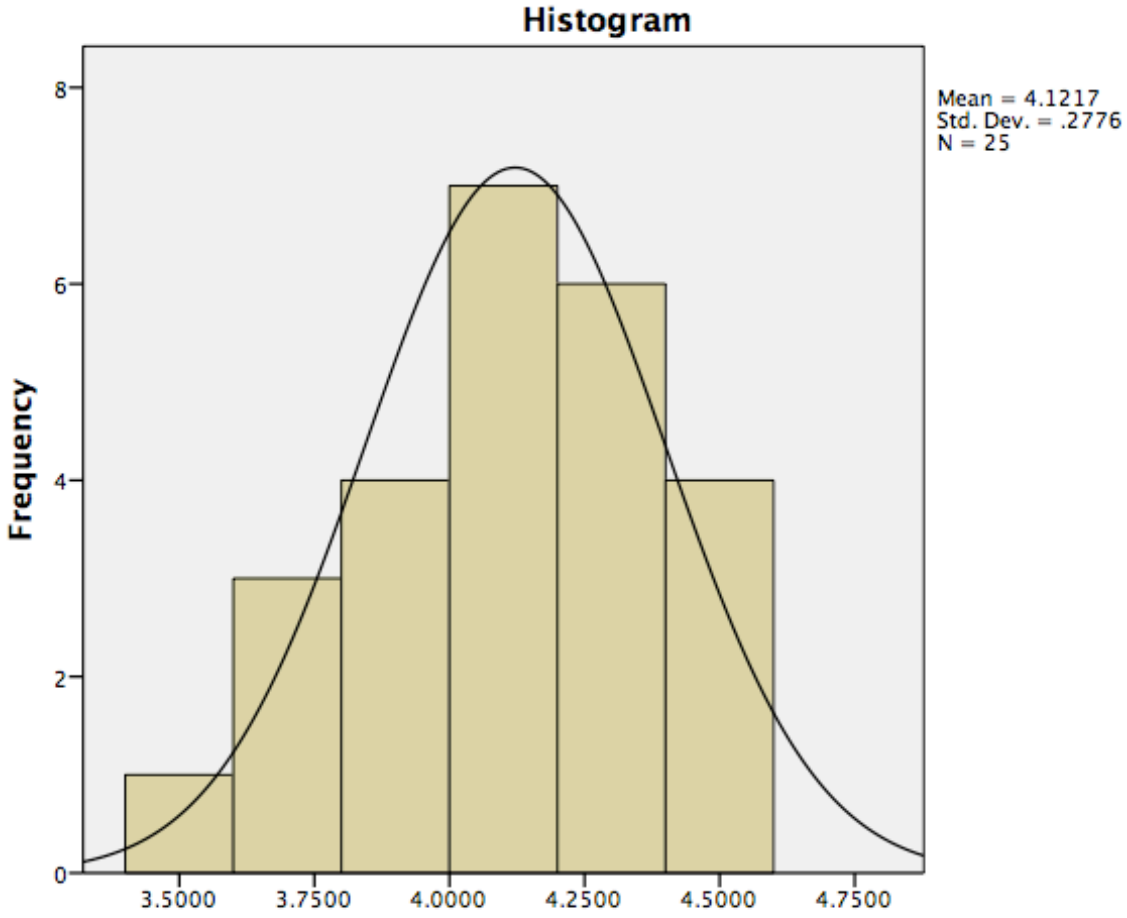
CV= 8.55%

Graph 2. Histogram showing the normality curve for fat analysis



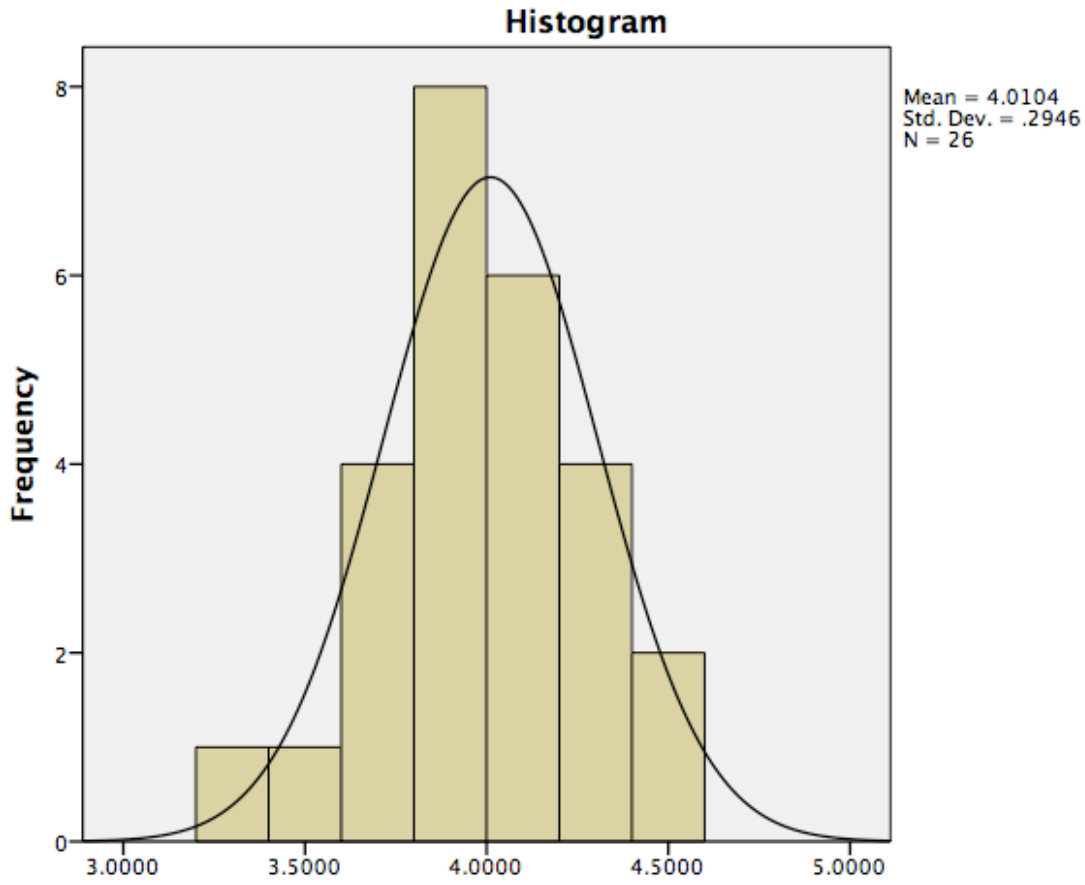
CV= 17.99%

Graph 3. Histogram showing the normality curve for ash analysis



CV= 6.73%

Graph 4. Histogram showing the normality curve for moisture analysis



CV= 7.35%

Journal of Food Chemistry and Nutrition

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Gondal, A.S. 2012. Stimulated activity of natural enemies of nematodes. Nature 233: 210-217.

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Brown, P.D. and M.J Morra. 1997. Control of soil-home plant pests using glucosinolate-containing plants. Adv. Agron. 61: 167-231.

Edwards, A.C. and M.S. Cresser. 1992. Freezing and its effect on chemical and biological properties of the soil. Adv. Soil Sci. 18: 59-79.

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Mulvaney, D.L. and L. Paul. 1984. Rotating Crops and Tillage. Crops Soils. 36: 18-19.

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Brown, J. 1996. Soils of the Okpilak River reigon, Alaska. CRREL Res. Rep. 188. U.S. Army Cold Reg. Res. Eng. Lab, Hanover, NH, USA.

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Holmes, J.W. (ed.). 1968. Trans. Int. Congr. Soil Sci., Adelaide. Elsevir, New York, NY, USA.

McGarry, D. 1992. Final report of management training workshop, Dalby. 3-4 July, 1991. Conf. and Workshop. Ser. QC92008. Dept. Primary Industries, Brisbane, QLD, Australia.

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Pyle, M.E. 1982. A comparison of determinate and indeterminate soybean lines for double cropping in Virginia. Ph.D. diss., Virginia Polytechnic Inst. & State Univ., Blacksburg, VA, USA (Diss. Abstr. Int. 438:3095).

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Moore, K. and M. Collins (eds.). 1997. Forages, CD-ROM companion [CD-ROM computer file]. 5 th Ed. Iowa State Univ. Press. Ames, IA, USA.

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