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School of Engineering and Sciences

Effect of ultrasound on protein yield and fate of alkaloids during lupin alkaline extraction process

A thesis presented by

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Declaration of Authorship

I, Luis Alberto Aguilar Acosta declare that this thesis titled, “Effect of ultrasound on protein yield and fate of alkaloids during lupin alkaline extraction process” and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
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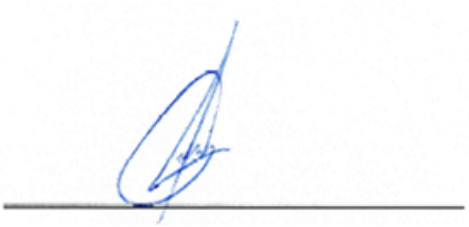

Luis Alberto Aguilar Acosta

Monterrey Nuevo León, May 14th, 2018

Dedication

This thesis is dedicated to Martha and Jesús, who with their love, compression, and example woke up in me the passion for knowledge. Thanks for being the spark that ignited the flame of my curiosity. Everything I am and what I will become is because of you, my parents. I love you.

Give me six hours to chop down a tree

and I will spend the first four

sharpening the axe.

Abraham Lincoln

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Effect of ultrasound on protein yield and fate of alkaloids during lupin alkaline extraction process

by

Luis Alberto Aguilar Acosta

Abstract

Protein isolates from legumes have attracted scientific attention because they are an efficient option to cover the daily protein requirement. One of the legumes with the highest protein content (20-50%) nowadays is *Lupinus spp.*, with a good amino acid profile and moderate protein digestibility (≈ 70%) but with the presence, in certain species, of quinolizidine alkaloids (QA), toxic to the organism. The most popular method to QA removal is one based in washing with water, but inconvenient because of the high use of water and time. With the aim to propose eventually a new process for lupin detoxification, the objective of this work was the elucidation of the fate of QA during lupin protein extraction process assisted with ultrasound and the evaluation of the nutritional and functional properties of the protein fraction. Proximal characterization, concentration of anti-nutritional compounds, amino acid profile and protein solubility profile of flours from three lupin species were assessed: *L. albus*, *L. angustifolious* and *L. mutabilis*. The result showed a significant difference ($p < 0.05$) in protein concentration, total alkaloids and particle size between the three species flours. Considering the above, the species *L. mutabilis* and *L. angustifolious* were chosen to study the behavior of the protein fraction in terms of functionality (water absorption and nitrogen solubility), composition (protein yield and percentage reduction of alkaloids during the extraction process) and resistance to thermal treatments (displacement of denaturation temperature, change in secondary structure and modification of the electrophoretic profile). The results obtained for *L. mutabilis* described the ultrasound effect as beneficial for protein yield (14% more than control with 10 min ultrasound) and QA reduction from bagasse (81% less than control with 10 min ultrasound treatment) and protein isolate (50% less than control with 10 min ultrasound treatment). In addition, the change in protein structure and composition modified the functional and thermal properties of the protein making the isolate a good candidate for food ingredient. In the other hand *L. angustifolious* was more resistant to the ultrasound effect with no significant difference between treatments (10 and 15 min) and control but with the lower toxicity (3 ppm of QA in control protein isolate) and better amino acid score (0.85 of 15 min ultrasound treatment). These results will be useful to design processes to assist in the objective for meet protein demand of the population.

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1 Introduction

Protein consumption is an essential requirement for human health maintenance. It is a functional and structural part of the human body for example, muscles, heart, intestine and a great part of the enzymes are proteins too. Regarding this, the protein intake is crucial in not only the adult life but even more important during pregnancy and child growth (Rodriguez & Garlick, 2008). Recommended Dietary Allowances (1989) of protein in adults is approximately 0.8 g/kg/day and when expressed as a percentage of the total calories consumption comes from 10 to 35%.

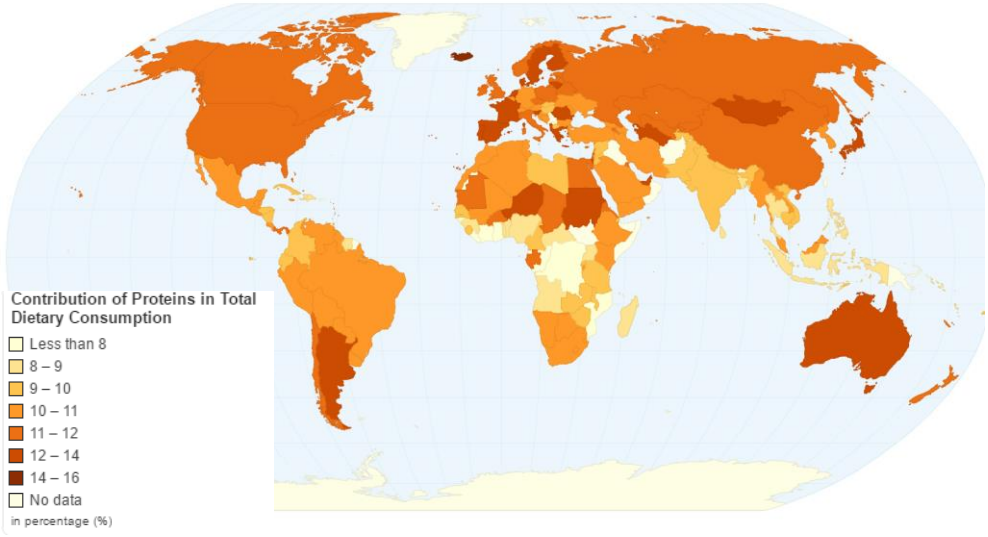


Figure 1.1 World map representing the Contribution of Proteins in Total Dietary Consumption (%) by country (FAO, 2010).

In **Figure 1.1**, the world map is presented with the countries in different orange intensities. Each orange tone represents a range of average protein intake as a percentage of the total daily caloric consumption. More than a half of the countries have lower average protein intake than the recommended, especially the ones with lower Gross Domestic Product (GDP).

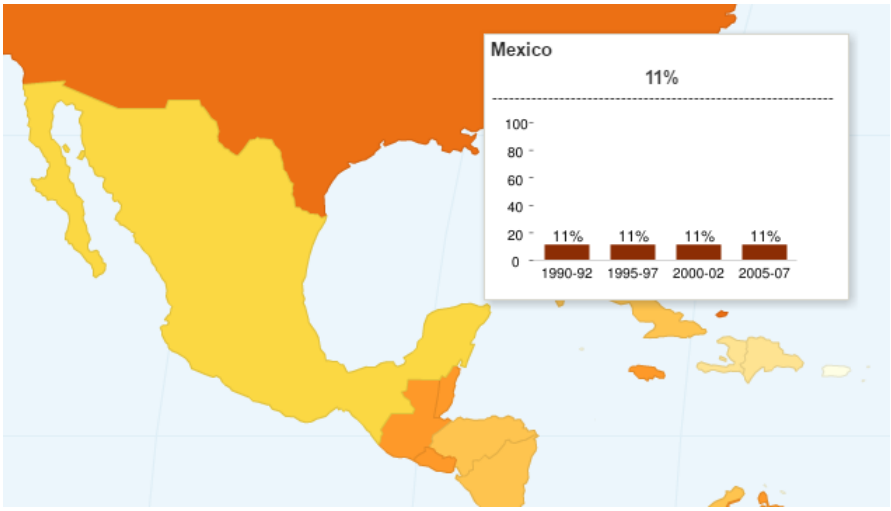


Figure 1.2 Contribution of Proteins in Total Dietary Consumption (%) in Mexico from 1990 to 2007 (FAO, 2010).

For Mexico, the average protein intake in the 17 years between 1990 and 2007 is shown in **Figure 1.2.** That flat tendency probably represents a lack of data, but despite this, a sustained 11% is near the minimum level of the recommended consumption. The low intake does not affect the same to all population being harder the health impact in children, being this population affected up to 14% with stunted growth in 2012 (more than one and a half million individuals). In **Figure 1.3a** Global Nutrition Report (2015) shows the total of children under five in 2012 and in **Figure 1.3b** a chart with the prevalence of stunting since 1989 as a percentage. The main factors correlated with the prevalence of stunting are: low total protein, low utilizable protein, low GDP per capita and low intake of total energy (Ghosh, Suri, & Uauy, 2013).

a

CHILD ANTHROPOMETRY

Number of children under 5 affected (000)		
Stunting[a]	1,551	2012
Wasting[a]	182	2012
Overweight[a]	1,026	2012
Percentage of children under 5 affected		
Wasting[a]	2	2012
Severe wasting[a]	0	2012
Overweight[a]	9	2012
Low birth weight[b]	9	2012

Sources: [a]UNICEF/WHO/WB 2015; [b]UNICEF 2015.

b

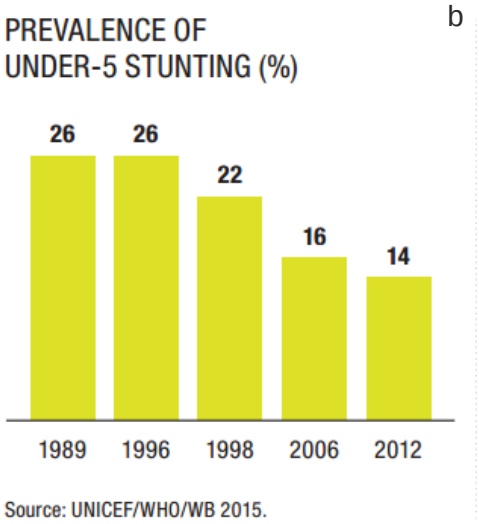


Figure 1.3 a. Children anthropometry in Mexico for under five individuals, 2012. b. Prevalence of stunting (%) in México for children under five. Period between 1989 to 2012.

A possible cause of the decreased protein intake is the classical Mexican diet. According with **Figure 1.4**, the most consumed food groups are cereals and sugar & fat, both with the poorest content of calories from protein. This non-equilibrated diet brings to the Mexican population the consequences above described but needed to say this trait is shared with other developing and undeveloped countries, being low GDP per capita the common denominator.

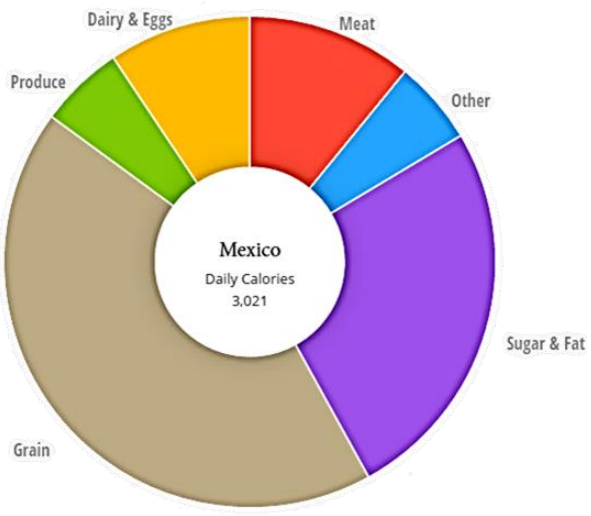


Figure 1.4 Proportion of the Daily Calories occupied by each food group in an average Mexican diet (National Geographic & FAO, 2011).

Because of low GDP per capita, a great part of the population cannot afford a high protein meal or high-quality protein sources because of the high price. In **Figure 1.5**, a comparison of the protein richness against its price is presented. Animal sources have a high protein richness and small portion size but few protein grams per unit price. In the other way, the vegetal sources have a low protein richness, big portion sizes and almost the same grams of protein per unit price. A possible solution to the lack of good and cheap protein sources could be protein isolates from legumes, like protein from soybean, with many protein grams per unit price, little portion size and a very high protein richness. The problem with this protein is its allergenicity and the presence of anti-nutritional factors that must to be inactivated before its ingestion. Besides, the amino acid profile is somehow different from the animal sources so, instead of soybean, the search for oilseeds and pulses to produce protein isolates devoid of these problems is a trend, the same that process to optimize the protein extraction from pulses.

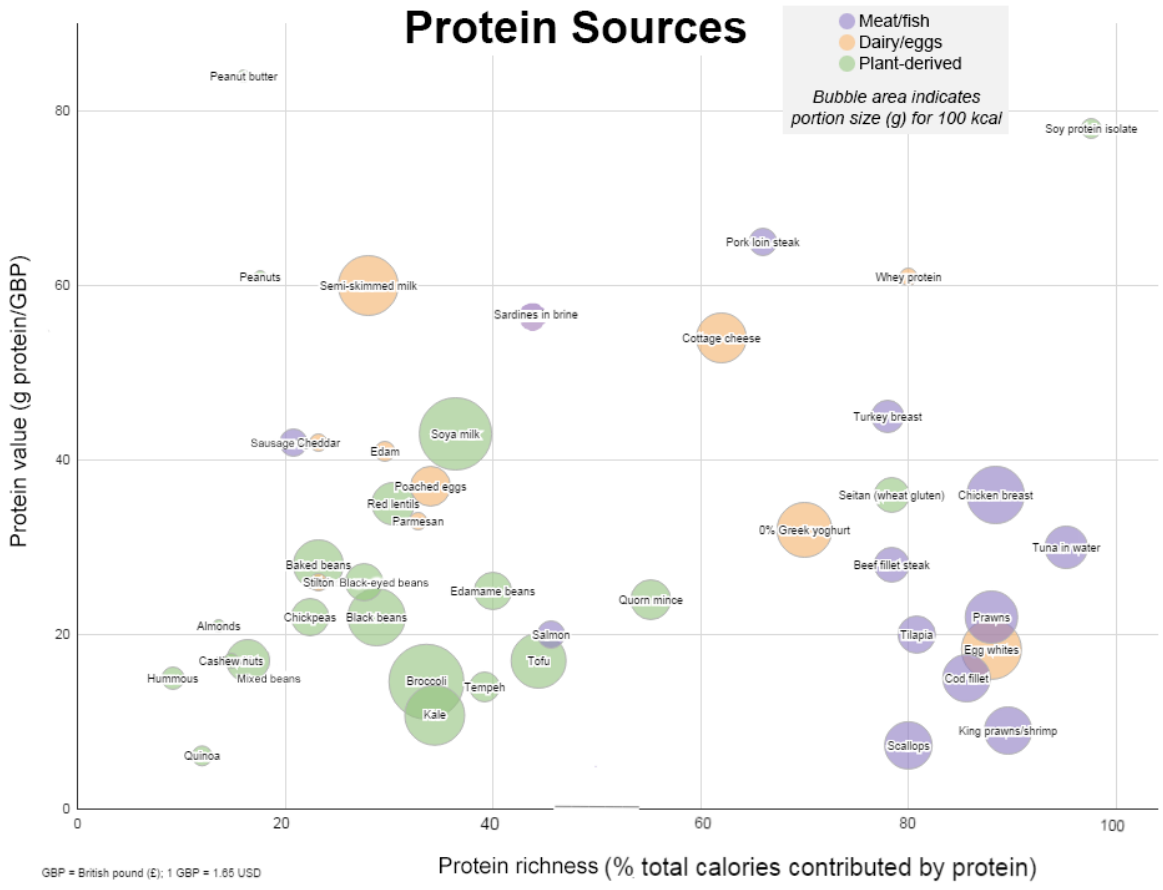


Figure 1.5 Comparison between Protein richness (%) and protein value (g protein/ British pounds –GBP-) of different protein sources.

1.1 Background

1. 1. 1 Pulses and the importance of their protein fraction.

The United Nations declared 2016 the International Year of Pulses to raise awareness about the importance of pulses in sustainable food production, healthy diets and their contribution to the nutrition of the worldwide population. Pulses also have wide benefits on soil fertility and climate change were highlighted to improve the global production and address the trade challenges (FAO, 2016).

Food and Agriculture Organization of the United Nations (FAO) was nominated to lead the implementation of the Year of Pulses objectives on Governments, relevant organizations, non-governmental organizations and all other relevant stakeholders. The action plan designed to achieve the global objectives included the knowledge compilation and the creation of a pulse database.

These organizations in the framework of the International Year of Pulses defined pulses as a subgroup of legumes, which are plants members of the *Leguminosae* family. These plants produce edible seeds used for human and animal consumption and when harvested for dry grain are classified as pulses (**Figure 1.6**). Other legumes used as vegetables (green peas, green beans, etc.), oil extraction (soybean, peanut, etc.) and for sowing proposes (clover, alfalfa, etc.) are not considered as pulses (FAO, 1994).

Figure 1.6 Some examples of pulses according FAO definition.

Some of the characteristics benefits of pulses as food are:

- Highly nutritious due its protein and besides because its cholesterol free fat content
- Economically accessible and contribute to food security because their high stability
- Health benefits through their bioactive compounds
- Contribute to climate change mitigation and sustainable agriculture with their nitrogen fixation and phosphorous-freeing properties which reduce the dependency on synthetic fertilizers
- Promote biodiversity because they can be used in different agricultural production systems.

Legume Protein

Legumes are one of the most promising alternative sources of protein for human and animal nutrition. Among the main cultivated legumes, soybean is by far the most important but other seeds are being studied as potential alternative sources of protein in order to reduce the need to import soybean (Pastor-Cavada, Juan, Pastor, Alaiz, & Vioque, 2009). Other research topic derived from legumes research as protein source is the number of both adverse and beneficial effects that they may exert on the human body, including food intolerance, allergies, inhibition of endogenous hydrolytic enzymes, and hypolipidemic, hypoglycemic, hypotensive, anti-carcinogenic and anti-obesity activities, respectively (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008). These topics must be assessed when a certain seed will be proved as potential protein source.

As the world population grows, the demand for non-animal derived products in foods with good aesthetic and organoleptic appeal too e.g., simulated meats. This market opportunity will place great emphasis on the need for proteins with, besides of nutritional benefits, multiple functional properties. Ingredient proteins should have acceptable intrinsic properties, i.e., flavor, texture and color, good nutritional value and the requisite functional properties for the variety of intended applications. Functional properties of proteins consist on those physicochemical properties, which affect the behavior of proteins in food systems during preparation, processing, storage and consumption. They are not only important in determining the quality of the final product, but also in facilitating process and development or maintaining nutritional and nutraceutical properties (Kinsella, 1979).

The importance of each of these properties varies with the different uses (**Table 1.1**), e.g., gelation in comminuted meats, emulsification in coffee creamers, and foaming in dessert toppings. In some applications a range of properties is required, e.g., solubility, clarity-turbidity, viscosity in beverages, while water holding, emulsion stabilization and gelation are important in meats products. Although a single protein may not possess the desired range of functional properties, proteins are heterogeneous and therefore demonstrate a variety of functional properties.

Table 1.1 Functional Properties Performed by Soy Protein Preparations in Actual Food Systems (Kinsella, 1979).

Functional property	Mode of action	Food system used
Solubility	Protein solvation, pH dependent	Beverages
Water absorption and binding	Hydrogen-bonding of water, entrapment of water	Meats, sausages, breads, cakes
Viscosity	Thickening, water binding	Soups, gravies
Gelation	Protein matrix formation and setting	Meats, curds, cheese
Cohesion-adhesion	Protein acts as adhesive material	Meats, sausages, baked goods, pasta products
Elasticity	Disulfide links in gels deformable	Meats, bakery

Fat adsorption	Binding of free fat	Meats, sausages, donuts
Flavor-binding	Adsorption, entrapment, release	Simulated meats, bakery
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes
Emulsification	Formation and stabilization of fat emulsions	Sausages, bologna, soup, cakes

The functional properties of proteins that are relevant to food production are related to their physicochemical and structural properties, such as size, shape, composition, hydrophobicity/hydrophilicity ratio, net charge, structural arrangements, and adaptability of domain structures of the whole molecule to changes in environmental conditions (Hettiarachchy & Ziegler, 1994; Kinsella, 1979). The environmental conditions are divided in interactions with food components (water, ions, proteins, lipids, carbohydrates, flavors) and the immediate environment, i.e., temperature, pH, and ionic strength. These features are directly affected by different factors during extraction and further uses and processing. The maintenance or developing of the functional properties rely on the proper isolation method and further storage conditions.

The intrinsic characteristic of the protein in which the extraction process has not effect is the composition. Protein composition of pulses protein (amino acid sequence, initial conformation and structure) depends on the species, cultivar, growth conditions and genetic modifications. The strongest association between protein and functionality has been made with the protein structure so, the maintenance of the native structure could be a challenge during processing. Nevertheless, the changes in structure could be beneficial to some other properties like digestibility or solubility (Deshpande & Damodaran, 1989). In order to obtain the protein isolate with the desired properties according to its purpose the selection of the protein extraction process cannot be taken lightly.

1. 1. 2 Traditional and Novel Protein Extraction Processes from Pulses

Vegetable protein isolates are refined as food ingredients manufactured by solubilization and extraction of protein from ground seed particles. Extraction is a complex process composed by the following phenomena (Aguilera & Garcia, 1989):

1. entry of the solvent into the particle;
2. redistribution of solvent in cell compartments and expansion of the solid matrix;
3. solubilization and/or degradation of components;
4. transport of the solute to the exterior of the particle; and,
5. migration of the extracted solute from the surface of the particle into the bulk

The rate of protein extraction is usually controlled by processes occurring in the interior of the particle, rather than by external factors (Schwartzberg & Chao, 1982), which makes microstructural modifications important. In practice, size reduction is used to obtain high extraction rates; but this result in high-energy expenditure during milling, and problems in downstream separation of the protein solution from a fine residue. Therefore, basic understanding of the effect of particle size and microstructure is desirable for designing efficient processes (Aguilera & Garcia, 1989).

There is a classification of the protein extraction processes made by Gueguen (1983), who separates them in two groups: dry and wet processes. The dry process consists in the milling of the whole seed to produce a flour and the posterior separation of its components. Pin-milling of legumes seeds with considerable starch content results in the production of two-particle systems. These two particles are different in size and density, they are separated using these differences and through air classification techniques. The light or fine fraction corresponds to a protein rich particle and the heavy or coarse fraction has the highest starch content. The result of this treatment over pea and faba bean was a protein yield on the light fraction of 35 and 41% and a protein concentration of 56 and 68 %, for both materials respectively.

In order to prepare protein isolates, the most widely used process is the wet one, which consists on the solubilization of proteins with a pH change or in a saline solution followed by the separation of insoluble material by centrifugation. Then the protein solubility is changed to precipitate or separate it from the solvent. Wet process can be at the same time subdivided by the solvent used

during extraction and the precipitation techniques (Muranyi, 2016). Extraction process can be alkaline, salt-induced and their combination, while precipitation techniques include isoelectric, dilutive precipitation and their combination. The performance of the protein isolation relays on the use of both specific extraction and precipitation techniques.

As discussed before, the protein processing could lead to the loss of certain functional and nutritional properties (Sirtori, Resta, Barambilla, Zacherl, & Arnoldi, 2010), as non-enzymatic browning (NEB), which involves reducing sugars and the free amino acids or the side chains of protein-bound lysine and arginine (D'Agostina, Antonioni, Resta, Arnoldi, Bez, Knauf, & Wäsche, 2006). Alkali solubilization causes dissociation of glycinin and subsequent unfolding as a result of disulfide bond cleavage and some disulfide bonds are changed to sulfhydryl and sulfenic acid residues. Alkali also may cause β-elimination from cysteine to form dehydroalanyl residues which in turn may interact with lysine to form lysinoalanine and result in some crosslinking. This phenomenon could lead to the formation of conglomerates which causes diminution of the protein solubility. Wet extraction of properties can also cause the losses of certain protein fractions or amino acids which represents a nutritional negative effect over the protein isolate (Kinsella, 1979).

Novel technologies for protein extraction

In the search of new technologies that do not cause the mentioned effects over nutritional and functional properties, the development of non-thermal processing methods to modify food ingredients has generated interest in the food industry recently. In particular, the use of high intensity ultrasound has attracted considerable attention due to its promise in the development of novel, gentle but targeted processes to improve the quality and safety of processed foods.

Ultrasound is an acoustic wave with a frequency greater than 20 kHz, the threshold for human auditory detection (Knorr, Zenker, Heinz, & Lee, 2004). Ultrasound can be classified in two distinct categories based on the frequency range: 1) high frequency (100 kHz to 1 MHz) low power (<1 W cm^{-2}) and 2) low frequency (20-100 kHz) high power (100-1000 W cm^{-2}) ultrasound. The first one is commonly used for the analytical evaluation of the physicochemical properties of food, whereas the second has been recently employed for the alteration of foods, either physically or chemically (O'Sullivan, Murray, Flynn, & Norton, 2016). Furthermore, the technology offers the potential to improve existing processes when used to assist traditional technologies (Ochoa-Rivas, *et al.*, 2017).

Ultrasound has been developed for several food processing applications such as degassing of liquids, extraction of enzymes and proteins, inactivation of microorganisms, ultrasound-induced product modification, extraction of gingerol from ginger, homogenization of milk, and acceleration of ageing of wines (Chandrapala, Zisu, Pamer, Kentish, & Ashokkumar, 2011). The collapse of cavitation bubbles results in the generation of high temperatures within the bubbles and mechanical, physical and chemical effects such as shockwave formation, turbulent motion of the liquid and generation of radicals (Ashokkumar, Lee, Kentish, & Grieser, 2007). The large shear forces generated can break inter and intramolecular bonds, which can help to break apart heat-induced aggregates and prevents their reformation (Ashokkumar, Lee, Zisu, Bhaskarcharya, Palmer, & Kentish, 2009). However, less work has been reported on functional protein changes as a result of sonication.

Paulsson and Dejmek (1990) stated that ultrasound treatment acts differently to temperature-based processes because it does not disrupt covalent bonds. They found that the effects of ultrasound are minimal on the secondary and tertiary structures of proteins. However, other researchers have found that sonication does result in some structural changes of individual proteins. Cavalieri et al. (2008) have observed sonochemical cross-linking between the thiol groups of protein molecules under specific experimental conditions. Taylor and Richardson (1980) found that the total thiol groups in skim milk decreased upon prolonged sonication under uncontrolled temperature conditions. In this case, it is possible that the heat generated during sonication reduced the thiol group content.

Novel technologies are used during protein extraction like the performed by Ochoa-Rivas, *et al.* (2017) where peanut protein isolates were prepared using ultrasound (US) or microwaves (MW) during alkaline extraction process. Ultrasound and microwaves resulted in an increase of protein extraction yield of 100 and 77% respectively compared with a traditional process. The first one due to the protein released from the peanut flour produced by cavitation and the second one for the temperature rising. These technologies changed the secondary structure of the protein, improving the functional properties and *in-vitro* digestibility.

Extraction of certain molecules from plant materials by ultrasound in a laboratory scale is widely published but limited number of publications have included continuous ultrasonic process development and pilot-scale applications. The range of published extraction applications include

herbal, oil, protein and bioactive compounds from plant materials (e.g. flavones, polyphenolics). The continuous high-intensity application extracted 54% and 23% more protein for aqueous and alkali extraction respectively, compared with the batch extraction using comparable processing times and volumes. During the trials, it was estimated that the continuous process used 70% less energy than the batch system to extract the same amount of protein and sonication efficiency improved with the greater load of thicker slurry, up to a 1:10 (soybean flakes to solvent) ratio (Vilkhu, Mawson, Simons, & Bates, 2008).

Ultrasound may help during alkaline protein extraction process from pulses to both, protein and anti-nutritional compounds extraction from seed tissue. The last could help reducing time, waste, solvent and water use and the separation of all the by products like bagasse with low risk of toxicity. The energy used to separate them can be also less than the used during cooking, the most common process used to remove toxic compounds.

1. 1. 3 Lupin, advantages and disadvantages when used as source of food protein.

Seed

The legume with the more similar composition to soybean is the one produce by the plant of the genus *Lupinus sp.* better known as bluebonnet. For over 3000 years, these seeds have been used as food by the Mediterranean populations (D'Agostina, *et al.,* 2006). Three species of the *Lupinus* genus native to the Mediterranean region are grown around the world. They are the white or albus (*L. albus*), narrow-leafed (*L. angustifolius*) and yellow lupin (*L. luteus*) which were selected and domesticated as sweet lupins. The pearl lupin (*L. mutabilis*) is native to South America, where it is grown but it is considered as a bitter specie. The sweet lupins (*albus, angustifolius and luteus)* were selected by breeding because their low alkaloid content (less than 200 ppm) and are used as valuable foodstuff requiring minimum prior treatment (Wäsche, Müller, & Knauf, 2001). Lupin seeds are used mainly as 1) livestock feeding rations in extensive and intensive production systems; 2) for human consumption and 3) for crop rotations for its ability to add nitrogen and increase the availability of phosphorus in soils (Industry & Investment NSW, 2011). Regarding human consumption, lupins have been used as ingredients for many products such as cakes, snacks, hamburgers, biscuits, baby foods, soups, salads and substitutes for milk and meat. Lupin protein isolates and concentrates display physical and functional properties comparable to those of soybean besides, the plant can be grown in soils and climates where

soybean cannot grow, so lupins proteins seems as potential ingredients and valuable for the food and chemical industry (Carvajal-Larenas, 2015; Ruiz & Hove, 1976).

In **Table 1.2** the macronutrient composition of the lupin (*Lupinus sp.* seeds) is shown and the most remarkable data is the high protein content (32.0 to 52.6%).

Despite all uses and advantages, the three principal difficulties when using this product as food source are: 1) presence of antinutritional factors, 2) slightly low protein quality when compared with soybean (needs to be supplemented with methionine) and the most important 3) toxicity. Antinutritional factors are mainly oligosaccharides, enzyme inhibitors, phytates, saponins and tannins, being in low concentration or absent in certain species. Low protein quality compared with an almost pure protein as casein, due to the low sulfur-containing amino acids like methionine and cysteine that together with its *in-vitro* digestibility results in a Protein Digestibility Corrected Amino Acid Score (PD-CAAS) of around 0.70 (less than the 1.0 for casein but similar to other legumes). In other way, *L. albus* and *L. angustifolius,* analyzed by Monteiro, Costa, Campos, Silva, da Silva, Martino, & Silvestre (2014), had levels similar to high protein reported for soybean protein with a digestibility superior to other legumes, such as soy and beans. Toxicity of lupine is due to the alkaloid content, in certain species (bitter) this concentration can be deathly while in sweet species, with proper pretreatment, the toxicity drops enough to be safely consumed.

Table 1.2 Macronutrient composition of Lupin seed (Carvajal-Larenas, 2015).

Nutritional Composition of the lupin in g/100g (dw)	
Protein	32.0 - 52.6
Fat	13.0 - 24.6
Carbohydrates	32.9 - 47.6
Fiber	8.2

Quinolizidine Alkaloids

The word alkaloid was mentioned in 1819 by W. Meißner who observed that these compounds appeared “like alkali”. They are biogenic, nitrogen containing and mostly N-heterocyclic with strong physiological activity. Alkaloids are often toxic and retain their own basic chemical properties, produced using lysine as precursor (that is why are called Quinolizidine). The

Quinolizidine alkaloids belong to the classification of true alkaloids derived from amino acid and sharing a heterocyclic ring with nitrogen. They are highly reactive substances with biological activity even in low doses. All true alkaloids have bitter taste and appear as a white solid, with the exception of nicotine, which is a brown liquid. True alkaloids form water-soluble salts due to their ionizable nitrogen, which at pH lower than its *pKa* tends to accept a proton forming a positive ion. This cation in presence of negative ions are capable to produce the water-soluble salts. In other words, most of them are crystalline substances that unite with acids to form salts. They may occur in plants in the free state, as salts, and as N-oxides (Aniszewski, 2015).

The toxicity of the lupin seed is due to their quinolizidine alkaloid content (**Figure 1.7**). These compounds are secondary metabolites, being their concentration dependent on the specie, cultivar and even time and place of cultivation. Almost 70 different quinolizidine alkaloids are present in the *Lupinus* species (Australia New Zealand Food Authority, 2001), most of them are in form of salt, but 28 are free bases (Ortiz & Mukherjee, 1982). They have a lethal hepatotoxicity and neurological effect on children in concentrations from 10 to 25 mg/kg of body weight per day. The bitter alkaloids, as can be named, are in concentrations from 0.07 to 4.50 mg/g (dry weight, dw) in the lupin seeds (Carvajal-Larenas, 2015). This means that a 20 kg child just need to eat 4.44 to 285.71 g of raw Lupin to reach the minimum lethal dose of total alkaloids.

Despite the high toxicity, the bitter lupin seed has been consumed for 1500 years in the Andean region of South America after a debittering process with water (around 64 times water the dry weight of the seed) followed by a day of time for cooking, soaking and washing. The traditional process to remove the toxic alkaloids result also in the loss of up to 22% of the dry weight of the seed.

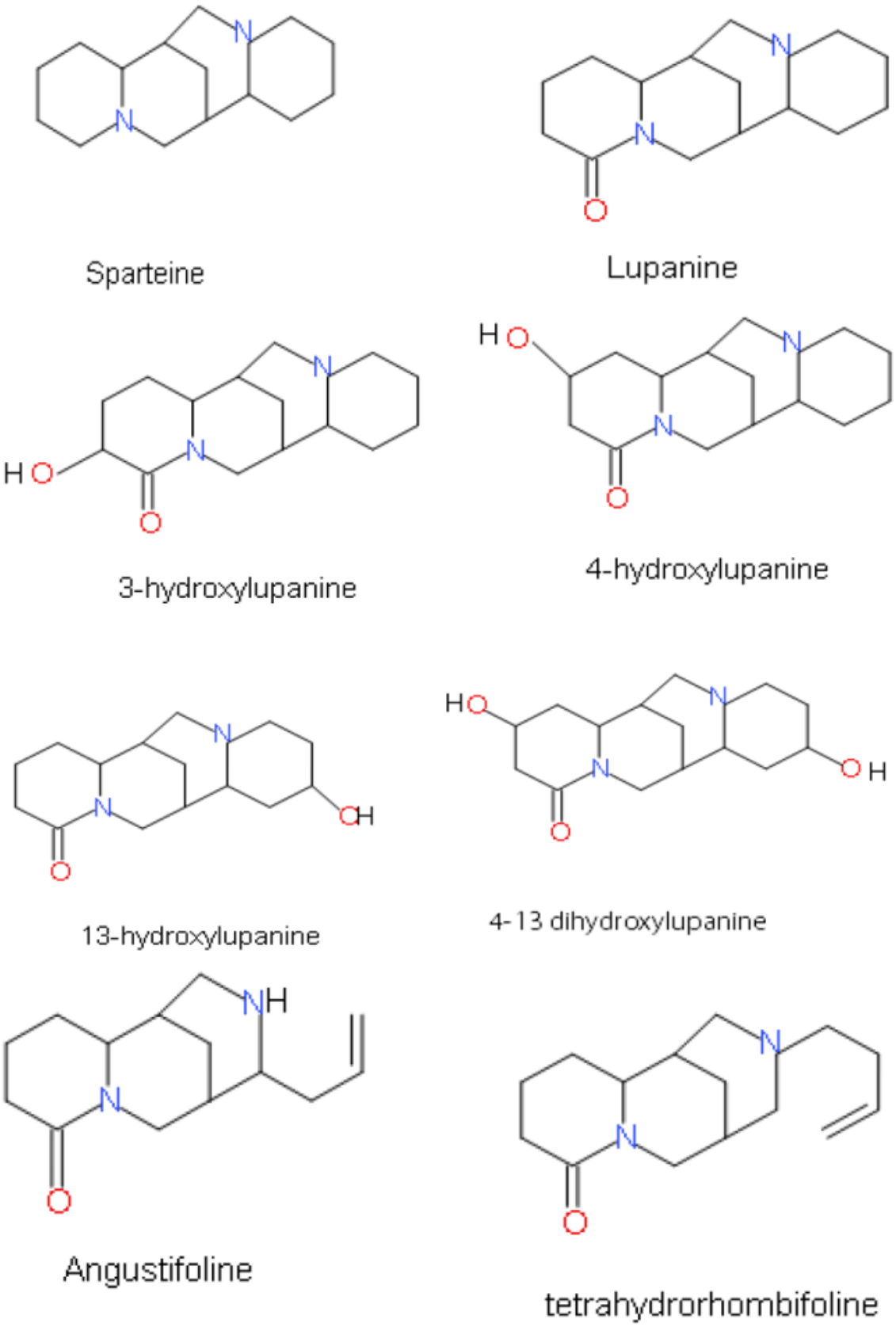


Figure 1.7 Structure of Main Quinolizidine Alkaloids from *Lupinus* spp. (Carvajal-Larenas, 2015).

The alkaloid presence in lupin seeds follows a three steps process; biosynthesis, transport and accumulation. The first one initiates with the amino acid lysine, which is transformed to lupanine, and this one is the precursor for most other quinolizidine alkaloids. This process occurs in the leaves of the plant, specifically in the stroma of the chloroplast. The next step is the transport, where the alkaloids formed in lupin leaves are carried via the phloem to all other plant organs, including fruits. Fruits are especially rich in alkaloids, since alkaloids contribute to the 8% of nitrogen due to one of their functions are nitrogen transport. The last step is accumulation, during this process, the alkaloids are concentrated in parts important for survival and reproduction of the plant. They seem to be concentrated in peripheral cell layers, i.e. epidermis and sub-epidermal cells, implying there are specific alkaloid storage cells. The storage of quinolizidine alkaloids in lupin tissues changes during its vegetation period: from germination to flowering, all plant parts store alkaloids. During seed maturation, lupin plants senescence and alkaloids disappear from all plant parts except the fruits, which become even more "bitter" (Wink, 1985).

In other way, Lee, Pate, Harris, & Atkins, (2006) demonstrate via GC-MS that the presence of lupine quinolizidine alkaloids in fruits is due half by transport from leaves via phloem and half by synthesis *in-situ*. Pozuelo, Lucas, De Lorenzo, Fernández-Pascual, Maldonado, & De Felipe (2001) made a comparative study in protein bodies of cotyledon tissues of three species of lupins, *L. luteus, L. albus*, and *L. angustifolius*. Along with a structural investigation by histochemical methods, they carried out the subcellular localization of alkaloids by immunological methods. This was the first time that alkaloids were located in the seed tissue, where they appear mainly in the protein bodies. Their results show that a membrane surrounds the protein bodies, and that they are the main reservoirs of proteins in lupin seeds, and probably the alkaloids act as secondary defense metabolites against the predation of seeds and seedlings.

In **Figure 1.8** cotyledon tissue is shown and there, the protein bodies inside the cytoplasm can be observed. The immunolocalization of lupanine shows its close relationship with protein and cell wall. This could represent an important issue during lupins seeds processing because conventional methods for protein extraction could be less efficient due to this interaction. Therefore, the debittering or protein extraction methods that already exist take the risk of protein loss or toxicity, respectively.

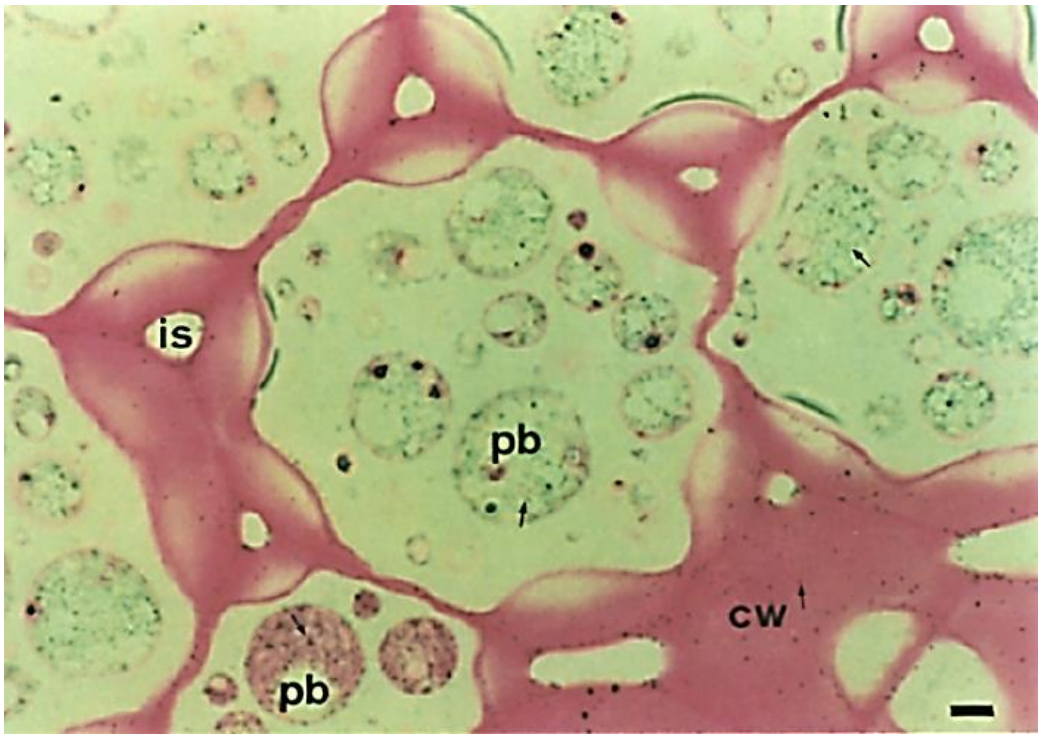


Figure 1.8 Light microscopy immunolocalization of lupanine detected by silver enhancement followed by counterstaining with fuchsine.

Colloidal gold particles can be detected in the protein bodies (pb) and cell walls (cw). Intercellular space (is). Arrows indicate immunolabeling. Bars: 300 µm (Pozuelo, et al., 2015).

Novel technologies, as ultrasound, can help to efficiently separate alkaloid from cell wall and protein bodies. Coupled with this, the increasing on protein extraction yield, functional properties and digestibility improvement (Ochoa-Rivas, *et al.,* 2017), make ultrasound a good alternative to achieve that goal.

Lupin Protein

Lupin seeds contain two groups of proteins, which according to Osborne (1897) classification: 1) albumin and 2) globulin. Their quantitative ratio is about 1/9 and other minor fractions, including prolamins, have also been detected. Albumins comprise a number of protein molecules that represent the functional proteins of a seed. Many of them are metabolic enzymes directly or indirectly related to the storage role of the cotyledon tissue. The first separation of lupin seed globulins was made in four main fractions: α-, β-, γ- and δ-conglutins (Duranti, *et al.* 2000).

Production of protein isolates can overcome the lupin toxicity problem because alkaloids are

water-soluble and would be removed during preparation of the isolates, which can be used as functional ingredients in human food (Sousa, Morgan, Mitchell, Harding, & Hill, 1996). This has already been proved by El-Adawy, Rahma, El-Bedawey, & Gafar (2001), they prepared two protein isolates from *Lupinus termis* one by isoelectric precipitation and the other by micellization that resulted with a total alkaloid concentration of 0.09 ± 0.03 and 0.15 ± 0.05 %, respectively. The alkaloid content of *L. termis* comes from 2.00 to 2.30 %, therefore, they obtain an alkaloid reduction of approximately 95.5% for isoelectric precipitation and 92.5% for micellization. Nevertheless, the waste produced due to these inefficient techniques and the possible contamination of other fractions with alkaloids makes them economically infeasible. The latter gives the chance to emerging technologies to make these processes more efficient and thus feasible.

German plant breeders produced alkaloid-free lupin (sweet lupin), which can be directly consumed by humans (Hudson, 1980). Sweet lupin could be used as a source of protein, which is especially lower in antinutritional factors and would not need to be heat-treated since trypsin inhibitors and haemagglutinins are practically absent (Chango, Villaume, Bau, Schwertz, Nicolas, & Mejean, 1998).

Similar to soybean, lupin seed protein has been studied in order to describe the preparation of protein concentrates and isolates and the functional properties of the concentrates reported. This has been carried out in different species of lupins e.g. *L. albus* cv Buttercap (Malgarini and Hudson, 1980), *L. angustifolius* (Sosulski *et al.*, 1979), *L. termis* (Morad *et al.*, 1980) and *L. mutabilis* (Sathe *et al.*, 1982). Although lupin protein derivates might have the same protein content, the functional properties are not similar because it has been shown that the ratio of the different globulin fractions is not constant for all varieties of lupin (Cerletti, 1978). Lupin's protein isolation has been studied using a diversity of methods like alkaline protein extraction or neutral extraction followed by precipitation or ultrafiltration steps (Bader, Oviedo, Pickardt, & Eisner, 2011). Other favorable features of lupin protein concentrates and isolates are related to some key functional properties, that is, gelation, emulsifying and foaming properties and the optimization of cost in comparison to most animal proteins (D'Agostina, *et al.,* 2006). In addition, the recent literature has pointed out some possible health benefits of lupin protein. A moderate daily intake of lupin protein extract led to a reduction of total and low-density lipoprotein (LDL) cholesterol (Sirtori, Lovati, Manzoni, Castiglioni, Duranti, Magni, & Arnoldi, 2004), when administered to an established animal model of hyperlipidemia. A specific protein fraction from lupin was able to

control hyperglycemia in a rat model (Magni, Sessa, Accardo, Vanoni, Morazzoni, Scarafoni, & Duranti, 2004). Moreover, recent *in vitro* studies showed positive effects of lupin protein isolates on bile-acid binding, angiotensin converting enzyme inhibition, and DPPH radical scavenging activity (Yoshie-Stark, Bez, Wada, Wäsche, 2004). In the study from Sirtori, *et al.* (2010) the results indicate that, even after harsh industrial processing, α-, β- and δ-conglutin are still able to release stable peptides, although they are completely or partially degraded. Their presence is indication of the maintenance of the health benefits mentioned before.

1. 1. 4 Lupin Debittering Processes

To solve the problem of the lupin toxicity due to quinolizidine alkaloids, many efforts have been made. The traditional debittering treatment has already discussed, but it belongs to one of the three main processes usually executed: aqueous extraction, biological degradation and chemical extraction. A list of the three debittering process figure in **Table 1.3** as well as some of their advantages and disadvantages.

Table 1.3 Advantages and disadvantages of the main debittering processes for *Lupinus sp.* (Carvajal-Larenas, 2015)

Technique	Advantages	Disadvantages
Biological Degradation	• Do not produce significant chemical residues • Might reduce anti-nutritional components of lupin seeds • Might increase some nutritional value	• Requires preliminary operations • Loses of fat, protein content and PER values. • Time consuming • Waste of water and energy. • Low percentage of alkaloids reduction.

Aqueous Extraction	• Only food-grade method known and applied at a commercial scale. • No chemical waste of residues • Not requires the recovery of chemical reagents	• Long time processing • Uses considerable amounts of water
Chemical Extraction	• Can be performed on lupin seeds with high alkaloid contents • High yields • Fastest extraction	• Decreases the methionine availability • Requires additional equipment and facilities • Production of chemical waste and residues

Biological degradation is carried out by bacterial or fungal fermentation or by germination. The seed needs to be pre-treated by dehulling, soaking, cooking, etc. After biological degradation, some anti-nutritional factors like phytates and oligosaccharides are reduced. The alkaloids reduction comes from 50 to 78 % with fermentation (Camacho *et al.*, 1991) and germination (Dagnia *et al.*,1992) respectively in at least 7 days of process (Carvajal-Larenas, 2015).

Chemical extraction is based on the common practice of isolation of alkaloids from plant material. After a particle size reduction, the conversion of the alkaloid salts in free alkaloids with alkaline treatment must be made in order to solubilize the majority of these compounds with organic solvents. Extractions with hexane and basic solutions have been made by Ortiz & Mukherjee (1982), reaching an extraction from 80.0 to 96.9 % of the original alkaloids in 3 to 24 hours of processing time. A comparison between conventional methods and ultrasound assisted solvent extraction of quinolizidine alkaloids from *Lupinus mirabilis* for leaves was made by Castañeda-González, Angarita-Pabón, Bernal, & Coy-Barrera (2014). Their study resulted in a favorable extraction time reduction when conventional method is assisted with short ultrasound periods while long time ultrasound treatment diminished the relative quantities of quinolizidine alkaloids due to extraction of cumulative proportions of other unknown nitrogen-containing compounds.

Cool and warm aqueous processing of lupin seeds can reduce the alkaloid content due to the water solubility of alkaloid salts. The alkaloid content is reduced 95.4% in 3 days when the seed is split and dehulled and 4-5 days for the whole seed. This process uses large volumes of water that must be treated before been reused. It does not produce chemical residues and does not require the recovery of chemical reagents.

It is interesting to notice that even when the alkali-based extraction step in vegetable protein isolates production can be useful as a chemical alkaloid extraction method, there are no works using the both steps in a procedure using bitter lupin species. Also, it is interesting to notice that the use of ultrasound as assistance in the production of lupin protein isolate has neither being tested, despite, as described in the theoretical background, can enhance the physical separation of alkaloids from the protein storage bodies with in lupin cotyledon.

Besides, even when some seeds as soybean have a well-studied performance as protein isolates for food industry, there is a need for protein devoid of allergenic history, genetically modifications and anti-nutritional factors. The lupin seed has a high protein content and just some reported cases of allergenicity, non-GMO species are in existence and with a negligible amount of anti-nutritional factors. Lupin's quinolizidine alkaloids has on the other hand, a possible pharmaceutical application because of their possible anti-arrhythmic, hypoglycemic and hypocholesterolemic activity (Carvajal-Larenas, 2015). The use of a byproduct can be seen as an economic improvement of protein isolation.

This thesis project was then focused in the evaluation of the fate of alkaloids and in the extraction yield from lupin when used as raw material for protein isolate production, using an alkali-based protein extraction procedure with the assistance of ultrasound as novel technique to enhance production.

2 Hypothesis

The use of ultrasound to assist lupin protein isolate production, results in a reduction of the alkaloids concentration in the final product and an increment of protein extraction yield.

3 Objectives

Evaluate the effect of ultrasound in the fate of alkaloids during the protein extraction process and yield, composition and secondary structure of protein isolates from at least two lupin species.

3.1 Specific Objectives

- To acquire and characterize the seeds of *Lupinus albus, Lupinus angustifolius and Lupinus mutabilis* in seed quality, proximate composition and native protein structure.
- To implement and execute an analytical method for the determination of lupin quinolizidine alkaloids in the three lupin species flour.
- To use different experimental levels of ultrasound time to assist protein extraction process from at least two species of lupin.
- To determinate the concentration of alkaloids from all the products and byproducts of each stage of the protein extraction process to assess the effect of the ultrasound treatments.
- To evaluate and compare the yield as well as functional, structural and nutritional properties of protein isolates obtained with the ultrasound treatments.

4 Materials and Methods

4.1 Raw Material

The lupin seeds (**Figure 4.1**) were obtained from the Centro Universitario de Ciencias Biológicas y Agropecuarias (CUCBA) of the Universidad de Guadalajara (UdeG, Guadalajara, Jalisco, México).

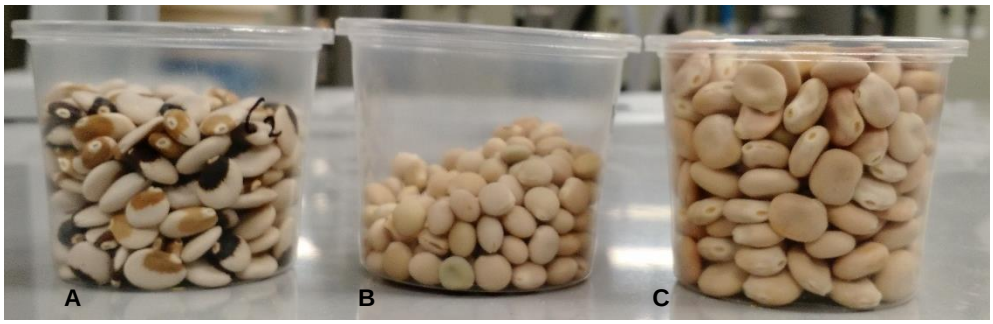


Figure 4.1 Lupin seeds. A. *Lupinus mutabilis*; B. *Lupinus angustifolius* and C. *Lupinus albus*

4. 1. 1 Seed Quality

a) Thousand seed weight was measured according to the Government of Alberta. Agricultural and Rural Development: Using 1,000 Kernel Weight for Calculating Seeding Rates and Harvest Losses.
b) Test Weight was measured using AACC 55-10 Test Weight per Bushel method.
c) Foreign material, damaged kernel and foreign seeds were measured by a gravimetric method using 100 g of lupin's seed.

4. 1. 2 Scanning Electron Microscopy (SEM)

The lupin seeds were frozen with liquid nitrogen and then cracked by half. The frozen seeds were mounted on a carbon double tape and coated with a thin gold layer (5 nm) before being observed under a Scanning Electron Microscope (Vega3, TESCAN, Czech Republic) equipped with a secondary electrons detector at X1500 resolution.

4.2 Flour Characterization

Lupin flour were obtained using two mills (**Figure 4.2**): Knife Mill (Wiley Mill, Arthur Thomas, USA) and Sample Mill (Cyclone Sample Mill, UDY Corporation, Colorado, USA).

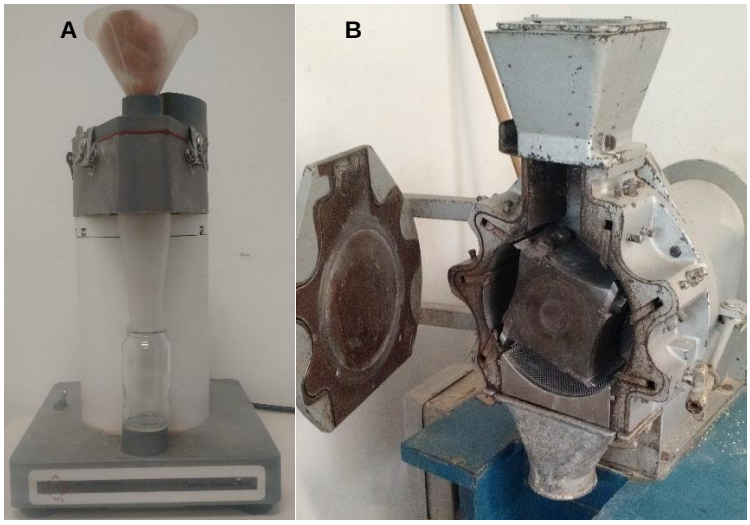


Figure 4.2 Mills: A. Cyclone Sample Mill and B. Knife Mill.

Oil extraction was made using n-Hexane as solvent in a relation 3:1 with the lupin's flour. The extraction was carried out at 50 °C in an orbital shaker incubator (RF 1575, VWR, OR, USA) at 120 rpm for 48 h with one 24 h solvent change (**Figure 4.3**).

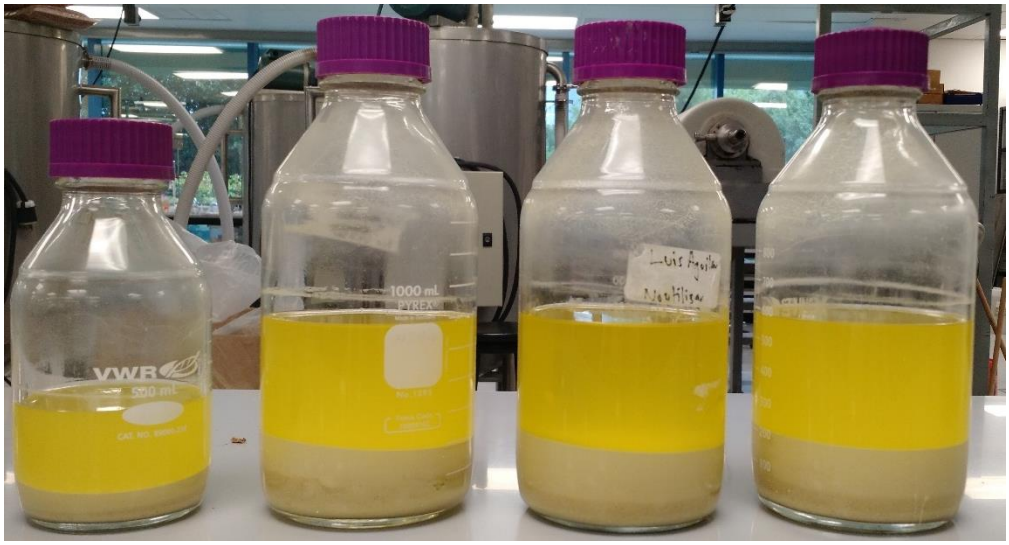


Figure 4.3 Fat Extraction of lupin's flour using Hexane.

After oil extraction, the desolvation of the flour was carried out at room temperature for 24 h. After, a mild milling process in a coffee and spice grinder (GX410011, Krups, Solingen, Germany) was performed to homogenize the flour.

4. 2. 1 Particle Size

Particle size analysis was made using a laser particle size analyzer (Mastersizer 2000E, Malvern Panalytical, Malvern, UK) coupled with a dry powder feeder (Scirocco 2000M, Malvern Panalytical, Malvern, UK). The equipment is shown in **Figure 4.4.**

Figure 4.4 Mastersizer 2000E coupled to Scirocco 2000M

4. 2. 2 Proximate Analysis

The proximate analysis over the lupin's flour was carried out by triplicate with the following methods. Crude Fiber was determinate by the AOAC official method 962.09 (1992). This method quantifies the cellulose in the sample after an acidic hydrolysis followed by an alkaline digestion using sulfuric acid (1.25% v/v) and sodium hydroxide (1.25 % w/v), respectively. The **Equation 4.1** was used to calculate the % of Crude Fiber on dry basis.

$$\%\ Crude\ Fiber\ db = \frac{(A - B)}{C} x\ 100$$

Equation 4.1 Formula for crude fiber calculation (%) in dry basis (db).

A= Crucible with residue weight (g); B= Crucible with ashes weight (g); C= Sample weight (g)

Protein analysis was performed following the micro Kjeldahl procedure under the AOAC official method 978.02 (1992). The fundament of the method is the total nitrogen determination and its conversion to protein using the conversion factor 6.25. **Equation 4.2** and **Equation 4.3** show the calculation of % N_2 and % Protein in dry basis respectively.

$$\%\ N_2 = \frac{mL\ HCl\ spent\ x\ HCl\ concentration\ (N)\ x\ 0.014\ x\ 100}{Sample\ Weight\ (g)}$$

Equation 4.2 Total Nitrogen calculation.

0.014: Nitrogen molecular weight multiplied by the L to mL conversion factor

$$\%\ Protein\ db = \%\ N_2\ x\ 6.25$$

Equation 4.3 Protein percentage using the nitrogen percentage from Equation 4.2 and a conversion factor of 6.25 (nitrogen to protein).

Result expressed in dry basis -db-

Crude fat determination over dried lupin flour was made by the Goldfish protocol, using the AACC official method 30-20.01 (1961). The organic solvent used was petroleum ether. In **Equation 4.4**, the % crude fat in dry basis calculation is presented.

$$\%\ Crude\ Fat\ db = \frac{Residue\ Weight\ (g)}{Sample\ Weight\ (g)}\ x\ 100$$

Equation 4.4 Crude fat calculation (%) in dry basis (db)

Ash determination was made using a gravimetric method following the AOAC official method 923.03 (1992) at 550 ± 3 °C temperature. The incineration time was 4 hours in a Thermo Fisher Scientific muffle furnace. The calculations were made using **Equation 4.5**.

$$\%\ Ash\ db = \frac{Ash\ Weight\ (g)}{Sample\ Weight\ (g)} x\ 100$$

Equation 4.5 Formula for ash percentage in the analyzed sample (%) in dry basis (db)

The moisture analysis was made using the gravimetric method reported by the AOAC official method 925.10 (1992). The % moisture was calculated using the **Equation 4.6.**

$$\%\ Moisture = \frac{WS - DS}{WS}\ x\ 100$$

Equation 4.6 Formula for moisture (%) determination

WS= Wet Sample Weight (g); DS= Dry Sample Weight (g)

Total Carbohydrates were obtained by difference using the following equation:

$$\%\ Total\ Carbohydrates = 100 - (\%\ moisture + \%\ Crude\ Fat + \%\ Protein + \%\ Ash)$$

Equation 4.7 Total carbohydrates (%) by difference

4. 2. 3 Osborne's Solubility Profile

Solubility profile of lupin's flour protein was carried out following the methodology proposed by Serna-Saldívar (2012). The classification was made according to Osborne (1907). In **Table 4.1**, the solvents used to the protein fractionation are presented.

Table 4.1 Solvents used to protein fractioning according to their solubility following the Osborne classification.

Protein Fraction	Solvent
Albumins	Distilled water
Globulins	NaCl 0.5 M
Prolamins	Tert-butyl alcohol (60% v/v)
Prolamin like	Tert-butyl alcohol (60% v/v) + 2-Mercaptoethanol (2% v/v)
Glutelins	SDS (2% w/v) + 2-Mercaptoethanol (5% v/v) + tris buffer pH 6.8 (0.0625 M)

Protein determination of each fraction was made following the Micro Kjeldahl method described in section 4. 2. 2.

4. 2. 4 Amino Acid Profile

The amino acid profile of the lupin's flour and protein isolates was characterized by a foreign laboratory (Experimental Station Chemical Laboratories, Missouri University) according to the AOAC official method 982.30 (1992).

4. 2. 5 Electrophoretic profile

Characterization of lupin's flour and protein isolates was performed by SDS-PAGE under reducing and non-reducing conditions using a method described by Sambrook & Russell (2006) with slight modifications. A Mini-Protean II electrophoresis cell (Bio-Rad) was used. Electrophoresis was conducted at 100 V until the tracking dye reached the bottom of the resolving gel (1 h approx.). Resolving and stacking gels had acrylamide concentrations of 12.5 % (w/v) and 5% (w/v), respectively. Protein from isolates and lupin's flour were extracted and dissolved in electrophoretic sample buffer [2% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 62.5 mM Tris-HCl pH 6.8]. For SDS-PAGE under reducing conditions, a sample buffer containing 1.4% (v/v) 2-ME (Mercaptoethanol), was employed. Samples were centrifuged (10,500 rpm, 1 min) and heated in boiling water for 3 min. Protein extracts were loaded in certain volume to reach 15 µg of protein per well. Protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the analysis of the gel images was performed using the GelAnalyzer 2010 software.

4. 2. 6 Quinolizidine Alkaloids (QA) concentration

Concentration of QA in lupin's flour was determinate by a titration method proposed by Ruiz (1977). One gram of defatted flour was incubated at room temperature with 10 mL of ammoniacal $CHCl_3$ (1% v/v concentrated NH_4OH), filtered, washed with fresh $CHCl_3$ and evaporated to dryness. Then, the residue was dissolved with 0.5 mL of $CHCl_3$, and 3 drops of indicator solution (0.1% tetrabromophenolphthalein ethyl ester in absolute ethanol) was added. The sample was titrated with 0.01 N p-toluensulphonic acid in $CHCl_3$ and the end point marked by the change of

the indicator from blue to yellow. The formula used to calculate de alkaloid concentration is presented in **Equation 4.8.**

$$\%\ Lupanine = \frac{N * AV * \left(\frac{248.37}{1000}\right)}{DSW} * 100$$

Equation 4.8 Lupanine percentage in samples, dry basis.

N= p-toluensulphonic acid concentration (N); AV= acid volume spent to reach the end point (mL); 284.37= Lupanine Molecular Weight (g/mol); DSW= Dry sample weight (g)

4. 2. 7 Trypsin Inhibitor

Trypsin inhibitor extracts were made from lupin´s flour using 1 g of sample in 50 mL of 0.01 M NaOH. The mix was stirred during 3 h (pH 8.4-10.0) according to the Hamerstrand, *et al.* (1981) methodology. The supernatant was collected and was used for the trypsin inhibitor activity (TIA) following the protocol from Shwert & Takenaka (1955) and Dabhade, *et al.* (2016). This method is based on the use of N_{α}-Benzoyl-L-arginine ethyl ester hydrochloride (BAAE) (B4500, Sigma Aldrich) as a substrate for the trypsin from porcine pancreas type IX-S (T4799, Sigma Aldrich). The BAAE degradation is measured by UV spectrophotometry at 253 nm and the absorbance change during time it is directly correlated with trypsin activity. Thus, the TIA is measured as a drop of trypsin activity.

4. 2. 8 Secondary structure of Lupin's flour for three different species using FTIR

Fourier Transform infrared spectroscopy (FTIR) was used to elucidate the protein secondary structure of the lupin's flour and isolates. Data were collected using an ATR-FTIR equipment (Perkin Elmer, Spectrum 1, Norwalk, CA, USA) in absorption mode within the region of 4000-650 cm^{-1} with a 4 cm^{-1} resolution. The Amide I region (1600-1690 cm^{-1}) correspondent to the protein secondary structure was further analyzed using the deconvolution function on the software Spectrum (v. 5.3.0) with a 1.5 gamma value and 0 % length. The number of peaks were expressed as percentage of: β-sheet (parallel and anti-parallel); α-helix; 3_{10} helix; unordered and aggregated strands according to Jackson & Mantsch (1995).

4.3 Ultrasound (US) Effect in lupin protein extraction and in the properties of isolates produced under alkaline conditions.

The protein extraction was performed by the methodology proposed by Salinas-Valdés, *et al.* (2015) which consists in an alkaline extraction followed by an acid precipitation. The complete methodology is presented **Figure 4.5**.

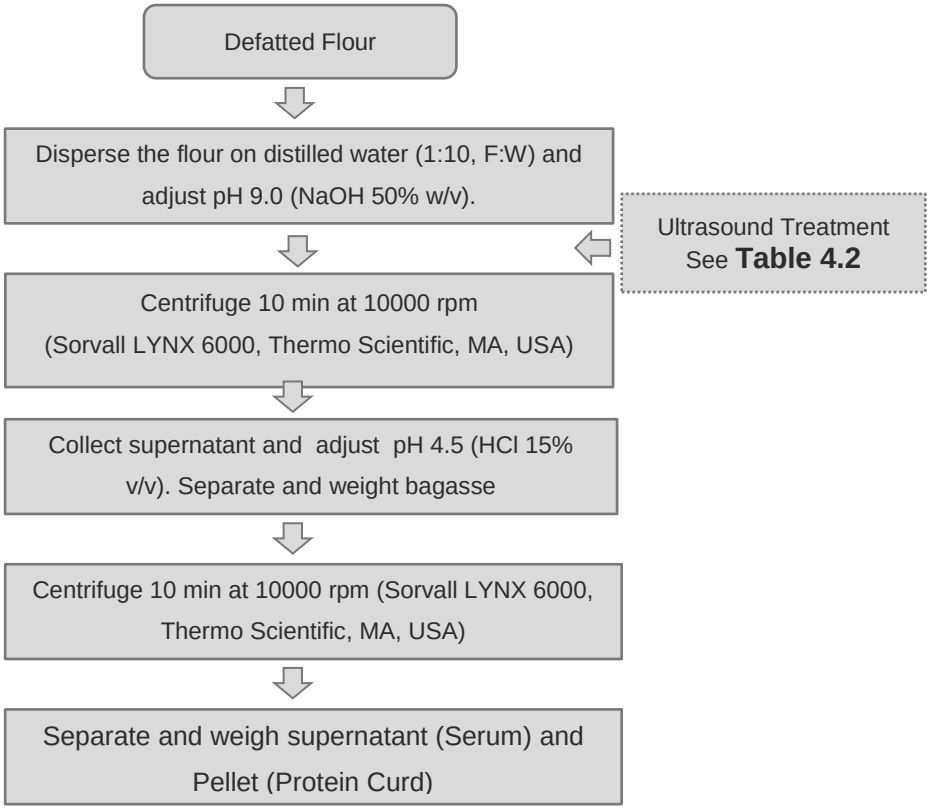


Figure 4.5 Procedure for alkaline extraction assisted with ultrasound and acid precipitation of pulse flour's protein.

Ultrasound treatments were performed in a 1000 mL beaker with approximately 550 mL of flour dispersion (1:10, ratio flour: water) in a UP400S equipment (Hielsher Inc, USA) with a 22 mm diameter cylindrical sonotrode (Horn 22, Hielsher Inc, USA) immersed 4 cm in the slurry **(Figure 4.6**). Samples were processed at a 24 kHz frequency, 100% amplitude (100 µm), acoustic power

density of 85 W/cm^2 and pH 9.0 as described in **Figure 4.5** and **Table 4.2**. The temperature was not controlled but registered using the equipment's thermocouple.

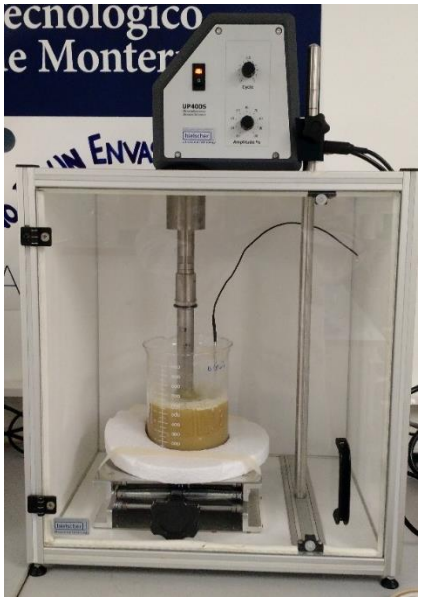


Figure 4.6 US equipment used to assist alkaline extraction of pulse flour's protein.

The US parameters used during the different treatments are summarized on **Table 4.2**.

Table 4.2 Experimental parameters used for alkaline protein extraction assisted with US using lupin flour as raw material.

Parameter	Levels
pH	9
Frequency, kHz	24
Temperature, °C	Monitored
Amplitude, µm	100
Sonotrode diameter	22 mm
Acoustic power density	85 W/cm^2
Time, min	0, 10, 15

4. 3. 1 Protein Yield of US alkaline extraction process

Once obtained and weighted the three fractions of the extraction process (bagasse, protein curd and serum) all were analyzed for moisture and protein percentages following the procedures described in section 4.2.2. Then, protein yield was calculated employing the **Equation 4.9**. This process was performed at least by triplicate.

$$Protein\ Yield = \frac{Fraction^{a}\ Protein\ Weight\ (g)}{Flour\ Protein\ Weight\ (g)} x100$$

Equation 4.9 Protein Yield Calculation based in the final protein curd weight and the protein in flour at the beginning of the process.

[a] Fraction, *i.e.* Protein Curd, Bagasse, Serum.

4. 3. 2 Functional Properties of protein isolates obtained from US assisted alkaline extraction process.

The functional properties calculated of the protein isolates were: water solubility index, water absorption index, nitrogen solubility index and *in vitro* digestibility. The procedure for each one is described in the next sections. All properties were calculated at least in triplicate.

Water Solubility Index (WSI) and Water Absorption Index (WAI)

In a single protocol both determinations were carried out, using 1 g of sample on 15 mL distilled water following the methodology described by Cheftel, *et al.* (1989). The centrifugation was performed at 5,000 rpm for 20 min. Results were calculated with the following equations:

$$WSI = \frac{Soluble\ Solids\ Weight\ (g)}{Sample\ Weight\ (g)} * 100$$

$$WAI = \frac{Absorbed\ Water\ (g)}{Sample\ Weight\ (g)} * 100$$

Equation 4.10 Water solubility Index (WSI) and Water absorption index (WAI) calculations.

Nitrogen solubility index (NSI)

NSI was realized according to the AOCS official method Ba 11-65 (2006). A sample weight of 0.4 g was dispersed over 40 mL of NaCl 0.1 M Solution (pH=7.0). Results were obtained with the following equation:

$$NSI = \frac{Supernatant\ TN}{Sample\ TN} * 100$$

Equation 4.11 Nitrogen Solubility Index (NSI) determination.

TN= Total Nitrogen

In-vitro protein digestibility

Multienzyme methodology proposed by Hsu et al. (1977) was performed. The enzymes used were: Trypsin from porcine pancreas type IX-S (T4799, Sigma Aldrich), α-Chymotrypsin from bovine pancreas type II (C4129, Sigma Aldrich), and protease from *Streptomyces griseus* type XIV (P5147, Sigma Aldrich). The calculations were made using the following equation:

$$In\ vitro\ \text{protein digestibility}(\%) = 210.46 - 18.1\ (pH\ at\ 10\ min)$$

Equation 4.12 Percentage of *in vitro* protein digestibility.

4. 3. 3 Electrophoretic profile of isolates obtained from US assisted alkaline protein extraction process.

Protein isolates were characterized following the methodology explained in section 4. 2. 5.

4. 3. 4 Thermal properties of protein isolates obtained from US assisted alkaline extraction process.

The protein isolates (10 mg) from *L. mutabilis* were adjusted to different water activities (0.2971, 0.5379 and 0.8661) and then analyzed by Differential Scan Calorimetry (DSC) (Diamond DSC, Perkin Elmer, MA, USA). The determination was carried out with a scan from 20-130 °C at a 10 °C/min speed. The software used to the calculation of the thermal properties was PYRIS manager.

4. 3. 5 Amino acid profile of protein isolates obtained with US assisted alkaline extraction process.

Amino acid profile for protein isolates was obtained with the same procedure described in section 4.2.4

4. 3. 6 Mass balance for quinolizidine alkaloids in all fractions produced from US assisted protein extraction process

QA concentration of protein isolates from *L. mutabilis* was determinate with the same protocol used for the lupin's flour in section 4. 2. 6. QA concentration of protein isolates from *L. angustifolius* was obtained with Capillary Gas Chromatography (HP6890 Plus GC, Agilent Technologies Inc., CA, USA) coupled with a mass selective detector (5973N, Agilent Technologies Inc., CA, USA) (GC-MS). This determination was carried out with a 0.25 mm X 30 m X 0.25 µm (5%-Phenyl-methylpolysiloxane capillary column 5-HP, Agilent Technologies Inc., CA, USA). The mobile phase was helium at 1 mL/min flow rate.

The alkaloid extraction for GC-MS was carried out following the method proposed by Muzquiz, *et al.* (2000) where 0.5 g of defatted material with a solution of known concentration of strychnine (S0532, Sigma Aldrich) on dichloromethane was used. In the case of protein isolates and bagasse, they were freeze dried by 48 h and milled after that. The general process is presented on **Figure 4.7**. After evaporation, sample was resuspended with fresh dichloromethane and taken to a volumetric flask. The GC-MS was programed with an injection volume of 1 µL.

4. 3. 7 Trypsin Inhibitor Activity (TIA) for protein isolates produced with a US assisted alkaline extraction process.

The TIA determination of the protein isolates from both species was carried out following the same protocol explained on section 4. 2. 7. The only difference was the use of polyvinylpyrrolidone (PVP40, Sigma-Aldrich) at 1% w/w for TI extraction from isolates using NaOH 0.01M.

4. 3. 8. Secondary Structure of protein isolates produced with US assisted extraction procedure.

The FTIR analysis was performed following the same methodology explained in section 4. 2. 8.

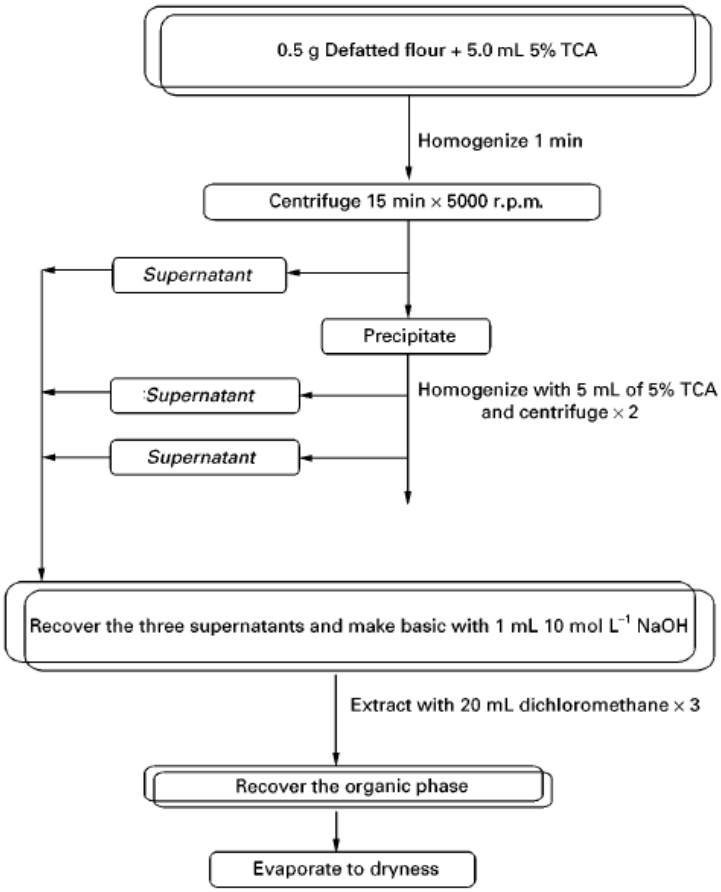


Figure 4.7 Extraction of Quinolizidine Alkaloids according to Muzquiz, et al. (2000).

TCA, Trichloroacetic acid.

4.4 Statistical analysis

The statistical analysis was performed using Minitab®18 (Analysis of Variance –ANOVA- to test differences among treatments, followed by a Tukey analysis for difference in means with 0.05 of significance).

5 Results and Discussion

5.1 Raw Material

5. 1. 1 Seed Quality

The seed cleaning and classification allowed obtaining the results presented in **Table 5.1**. The 1000 seed weight is an indicator of the size of the seed and is often used to calculate the crop densities to obtain better crop yield. This number is highly variable even between the same species (Alberta Agriculture and Food, 2007) and due to number of factors as harvest year and soil conditions. In the case of the three lupin's cultivars, results were similar to the obtained for Gdala & Buraczewska (1996).

The test weight is a commercial measure to grade grains in certain category. It is a ratio between the weight and the volume occupied by the grain. In other words, the test weight is a grain's density assessment were factors as physical damage and moisture affect directly the result, yielding changes in grain quality and thus in the final price (Lee, 1953).

In order to make a complete characterization of the seeds, foreign material and seeds as well as damaged kernels were quantified, and results are shown in **Table 5.1**. According to the Australian Pulse Standard (2017), the three species are commercial grade. These results prove the likeness of the raw material used in this study with the marketplace product.

Table 5.1 Physical characteristics of Lupin's seeds for three different species: *albus, angustifolius* and *mutabilis*.

Species	Thousand seed weight (g)	Test Weight (kg/hL)	Foreign Material (%)	Damaged Kernel (%)	Foreign seeds (%)
Lupinus albus	311.03 ± 13.64	60.23 ± 0.20	0.18	0.75	0.09
Lupinus angustifolius	140.1 ± 5.23	62.14 ± 0.42	0.95	0.36	0.35
Lupinus mutabilis	214.1 ± 0.90	59.01 ± 0.27	0.66	1.07	0.11

Besides physical characterization, the seed's anatomical fractions also give important information about the raw material, mainly about the possible yield when used as input in a protein extraction process and their association with the stability of the seeds during storage and chemical parameters as fiber or carbohydrate contents. For the different lupin species, anatomical proportions are depicted in **Table 5.2**.

Table 5.2 Anatomical mass proportion of three cultivars of Lupin (*albus, angustifolius*, *mutabilis*).

	Anatomical proportions (%)	
Species	**Cotyledon**	**Hull**
Lupinus albus	83.39 ± 1.16	16.59 ± 1.18
Lupinus angustifolius	75.05 ± 0.76	24.44 ± 1.21
Lupinus mutabilis	87.94 ± 0.76	11.97 ± 2.33

5. 1. 2 Scanning Electron Microscopy (SEM)

In order to fully understand the lupin seed's physical features, a microscopic analysis was performed, and components of the seed coat are in **Figure 5.1**. The thickness and morphology of the cell wall are relevant in the strength and seed's water absorption. There were visual differences among the species, being the more relevant the morphology of the osteosclereids layer of *Lupinus mutabilis.* This layer is responsible for two crucial processes during the seed development and germination: the water evaporation and the water absorption. The first one allows the seed to achieve the dormancy state due to dryness. The second one, besides of helping to water distribution during germination could help to the debittering processes based in aqueous extraction (Smýkal, Vernoud, Blair, Soukup, & Thompson, 2014).

The thickness of the coat is relevant for milling and cooking process. Besides, only the 1-7 % of total alkaloids are present in the coat of the seed, depending on the species. *L. angustifolius seed* had the thickest coat and this could be related to the low alkaloid content since primary functions of quinolizidine alkaloids include nitrogen transport and chemical defense against herbivores like mammals, insects and mollusks. Then, the strength of the seed as a protection mechanism against pest attack due to low alkaloid content could be the reason why the sweet lupin had a thickest coat (Wink, 1985).

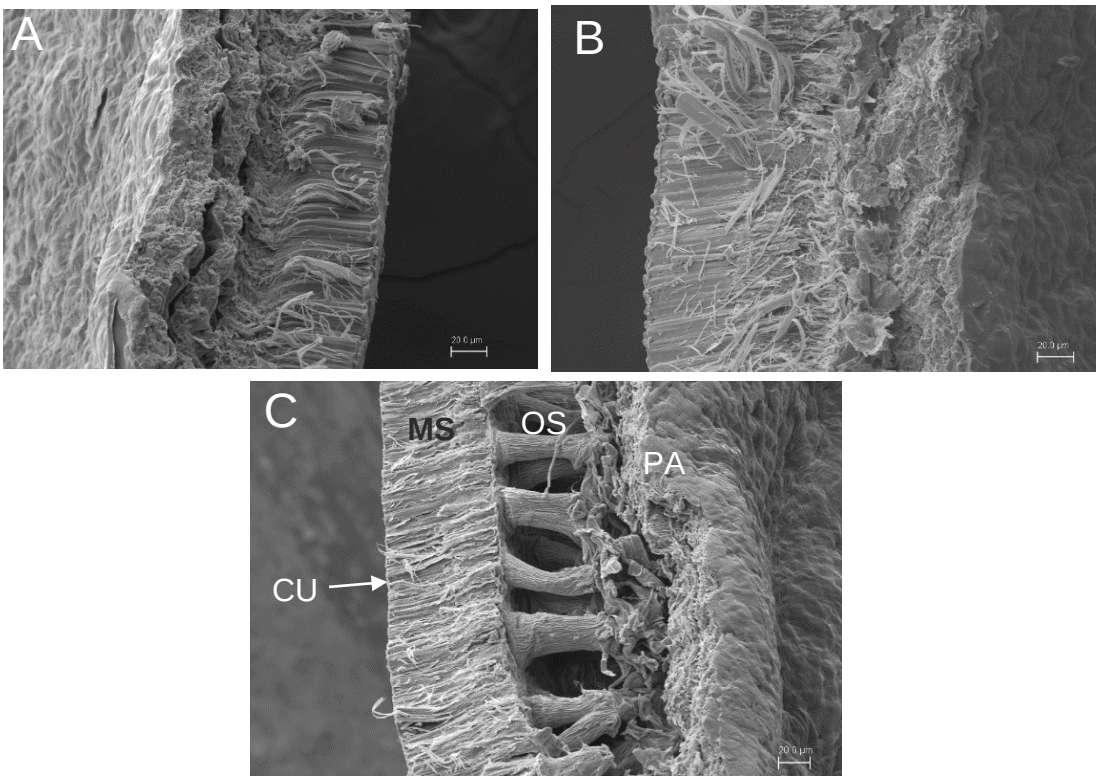


Figure 5.1 Scanning Electron Microscopy (SEM) of seed's coat of three different lupin species (*albus, angustifolius* and *mutabilis*).

A) *Lupinus albus.* B) *Lupinus angustifolius.* C) *Lupinus mutabilis* (CU – Cuticle; MS – macrosclereids layer; OS – osteosclereids layer; PA – parenchymal cells)

5.2 Flour Characterization

5. 2. 1 Particle Size

The milling protocol was designed to obtain the minimal particle size since is directly correlated with the extraction efficiency. The results obtained of the particle size are in **Table 5.3**. The value P_{50} is the highest diameter (in µm) of the 50% of the particles within the flour and this value is crucial to determinate the efficiency of the oil, protein and alkaloid extraction in terms of time and yield (Carvajal-Larenas, 2015). The milling process depends of the moisture of the seed, oil concentration and strength of the seed coat. Those differences between species results in the biggest particle size for *Lupinus angustifolius*, depicting then differences in the strength of the

seed itself and then some oil and or protein extraction differences among lupin varieties are foreseen.

Table 5.3 Particle size median (P_{50}) in microns of flours for three species: *albus, angustifolius* and *mutabilis.*

Species	Median (µm)
Lupinus albus	48.93 ± 0.31
Lupinus angustifolius	178.42 ± 0.47
Lupinus mutabilis	44.36 ± 3.26

5. 2. 2 Proximate Analysis

Strong differences among the three species were detected on the proximate analysis (**Figure 5.2)**. Compared with Carvajal-Larenas (2015), the overall results coincide with each species profile. The specie with highest protein and fat concentration was *Lupinus mutabilis,* with a similar composition to soybean. This is the specie previously reported with the highest commercial and nutritional potential.

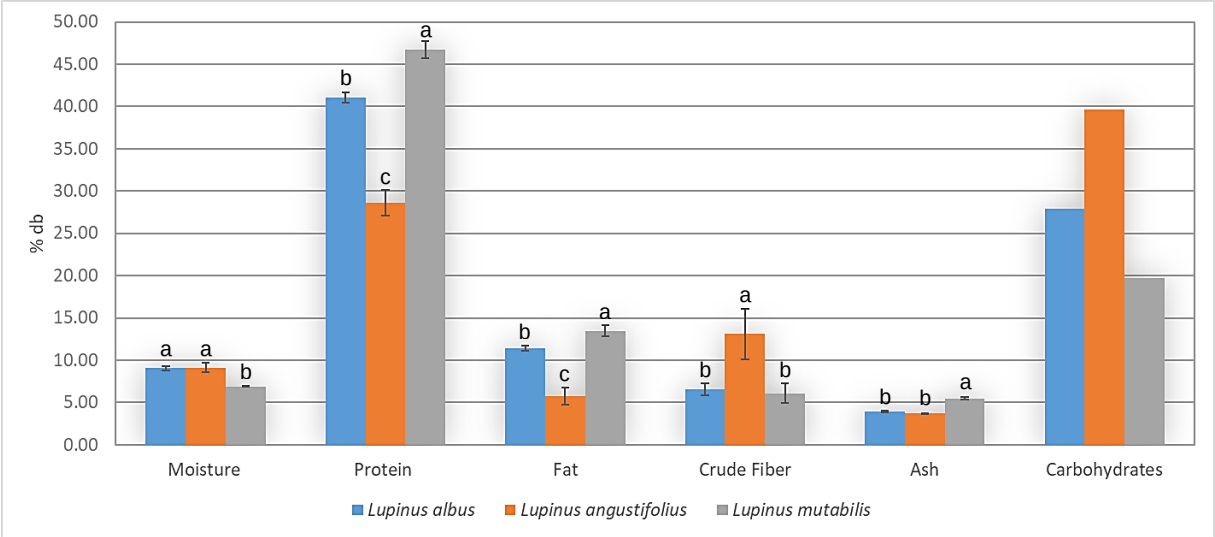


Figure 5.2 Proximate characteristics of three species of lupin: *albus*, *angustifolius* and *mutabilis*. Moisture, protein, fat, crude fiber and ash as well as carbohydrates by difference. [a, b, c] Bars with different letters within proximal parameter are statistically different ($p < 0.05$)

5. 2. 3 Osborne's Solubility Profile

The solubility profile of flour's proteins is a good indicator of the possible functional properties and the destiny of the major protein fractions. In legumes, the major protein fractions are albumins and globulins (Carvajal-Larenas, 2015). Albumins represents the functional proteins of the seed, mainly enzymes of all kind, from metabolic to indirectly related to properties of storage within cotyledon. Albumins can also play a role in the seed's defense system. On the other hand, globulins are the main protein storage in the cotyledon and have the widest amino acid diversity among all fractions (Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008).

As shown in **Figure 5.3**, around 58, 85 and 60% of total protein for *L. mutabilis, albus* and *angustifolius* respectively are associated to two Osborne's fractions: albumins and globulins, data that gives an insight about the extractability of the protein using traditional isolation techniques. From the five fractions according to Osborne, the non-extractable was in the last group in the **Figure 5.3** (residue). Is evident that *L. mutabilis,* despite its high percentage of protein, almost the 30% stayed at the end of a high and intensive protocol, so will not be available for extraction and protein isolates production using conventional techniques.

Albus and *angustifolius* species seems to be both good alternatives for industrial extraction process despite seed's physical differences and different protein conformation (**Figure 5.3**).

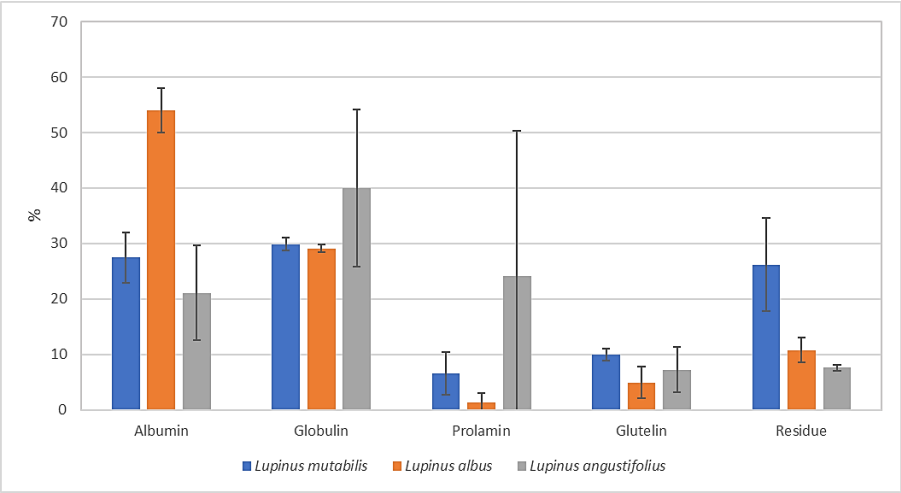


Figure 5.3 Osborne's protein solubility profile of three lupin species: *mutabilis, albus* and *angustifolius*.

5. 2. 4 Amino Acid Profile

Both animal and plant proteins are made up of about 20 common amino acids. The proportion of these amino acids varies as a characteristic of a given protein, but all food proteins hold some of each. Amino acids are required for the synthesis of body protein and other important nitrogen-containing compounds, such as creatine, peptide hormones, and some neurotransmitters (Recommended Dietary Allowances, 1989).

The amino acid profile is key for the assessment of nutritional potential of vegetal proteins. The results of the three species are in **Table 5.4**. The essential amino acid with the lower concentration was methionine and there were no significant differences between the species, but if these results are compared with the Carvajal-Larenas (2015), there are a few different values. For example, the concentration of valine reported for *L. albus, L. angustifolius* and *L. mutabilis were* 3.8, 3.7 and 3.8 g/100g protein, respectively. Meanwhile, the results obtained herein were 4.01, 4.48 and 4.05 % for the three species. Protein percentage as well as amino acid profile (specially methionine) are two of the compositional characteristics more affected by cultivar, cultivation year and season as well as the soil and nutrients in it (Sujak, Kotlarz, & Strobel, 2006).

The concentration of the sulfur-containing amino acids was low. The recommended dietary allowance for both cysteine and methionine is: 58, 27, 22 and 13 mg/kg per day depending on the age group (infants <6 months old, 2-year old children, 10-12 years old and adults respectively). Considering a 4 months old, 7 kg infant will have a requirement of 0.406 g of cysteine plus methionine per day. The results obtained for *L. albus, L. angustifolius* and *L. mutabilis* were 2.30, 2.53 and 2.06 g/100g protein respectively. Taking that in account, the infant must consume around 50 g of *L. mutabilis* seed (Protein: 40% dw) in a day to achieve the dietary requirements (Recommended Dietary Allowances, 1989).

Table 5.4 Amino Acid Profile of lupin flour from three different species (*albus*, *angustifolius* and *mutabilis*).

Amino Acid	g/100 g Crude Protein		
	L. albus	*L. angustifolius*	*L. mutabilis*
Aspartic Acid	10.52	10.35	10.36

Threonine	3.65	3.76	3.61
Serine	4.63	4.05	4.04
Glutamic Acid	21.66	21.20	22.45
Proline	4.38	4.43	4.23
Glycine	4.07	4.65	4.25
Alanine	3.45	3.89	3.73
Cysteine	1.74	1.77	1.46
Valine	4.35	4.56	4.32
Methionine + Cys	2.50	2.58	2.20
Methionine	0.76	0.80	0.73
Isoleucine	4.71	4.56	4.82
Leucine	7.74	7.52	6.87
Tyrosine	4.32	3.59	4.20
Phenylalanine + Tyr	8.39	7.81	8.17
Phenylalanine	4.07	4.22	3.97
Lysine	5.02	5.62	5.93
Histidine	2.38	2.91	2.95
Arginine	10.86	10.56	10.67
Tryptophan	0.76	1.06	0.97
Amino acid score[1]	0.96	0.99	0.84

[1] Amino acid score based on cysteine + methionine as limiting amino acid.

5. 2. 5 Electrophoretic profile

The globulins are the main fraction of lupin's seed storage proteins since that they hold the most nutritional, technological, nutraceutical and allergenic potential. Due to this, this fraction has been exhaustively studied and its maintenance during processing is crucial to ensure the final protein quality of the product.

Globulins are the salt-soluble storage proteins and can be divided in 4 groups, depending on its electrophoretic mobility, α-, β-, γ- and δ-conglutins. In **Figure 5.4** the SDS-PAGE of raw lupin's flour is presented. As can be seen, the profile depicts differences among samples, being the *L. mutabilis* the cultivar with the highest number of bands in the upper part of the gel (high molecular

weight), whereas *L. albus* seems to be the variety with a slight deviation down the ladder. In non-reducing conditions, a 20 kDa band in *L. angustifolius* seems to make the difference among all samples. This particular band is preceded by a 25 kDa in all three cultivars and followed by several between 10 and 20 kDa.

There are few reports regarding electrophoretic profile for lupine and this information is very useful, more than just for characterization, to create strategies for protein separation and fractioning. The size and weight of each fraction give us an idea of the centrifugal force needed to separate the protein curd from the serum. Also, the isoelectric point is a little bit different among fractions and species. This could cause the loss of certain fractions crucial to nutritional and/or functional properties.

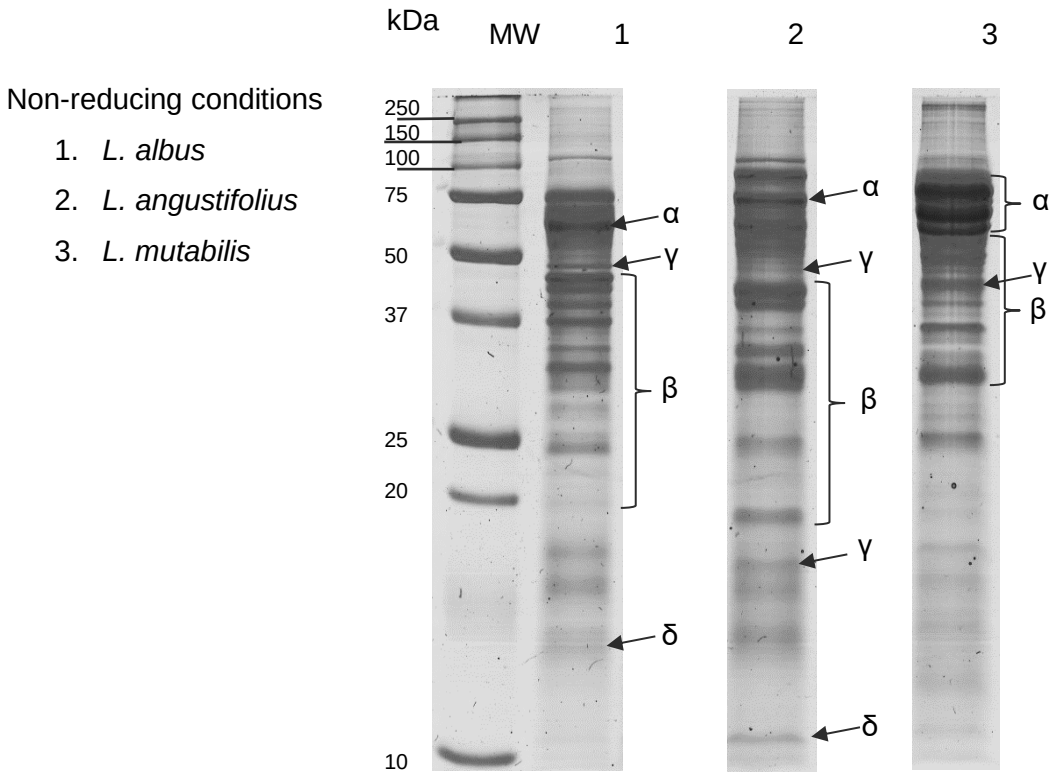


Figure 5.4 SDS-PAGE of three lupin species: *albus*, *angustifolius* and *mutabilis* respectively, showing the groups of globulins (α-, β-, γ- and δ-conglutins).

MW stands for molecular weight, line used for standard weight and the rest of lines for samples as described in the figure.

Molecular weights of the lupin's conglutins are presented in **Table 5.5**. The different conglutins can be observed in the SDS-PAGE of the flour.

Table 5.5 Reported molecular weight of Lupin's conglutins from the three-different species under non-reducing conditions.

	Conglutin	Molecular Weight (kDa)	Reference
Lupinus albus	α	58-78	Duranti *et al*., 2008
	β	60, 50, 40, 20	
	γ	48	
	δ	10-15	
Lupinus angustifolius	α	62-74	Muranyi *et al*., 2016
	β	54-72, 38-48, 27-31, 15-21	
	γ	17, 47	
	δ	13	
Lupinus mutabilis	α	50, 45, 40, 36, 30	Santos, Ferreira, & Teixeira, 1997
	β	50, 66, 36, 29,	
	γ	36-45	
	δ	-	

5. 2. 6 Quinolizidine Alkaloids (QA) concentration

QA are the most toxic anti-nutritional factors of Lupin's seeds. The lupin's flour QA concentration obtained by titration is presented in **Figure 5.5**. Considering a maximal QA daily dose for adults of 0.35 mg/kg body weight, a 75 kg grown man can consume 26.25 mg of QA per day (Australia New Zealand Food Authority, 2001). The lupin's flour needed to reach the daily dose is 16.4, 65.6 and 1.5 g of *albus, angustifolius* and *mutabilis,* respectively. According to these results, *L. albus* and *angustifolius* are consider sweet species due to the low concentration of QA compared with others like *mutabilis* which is considered a bitter one.

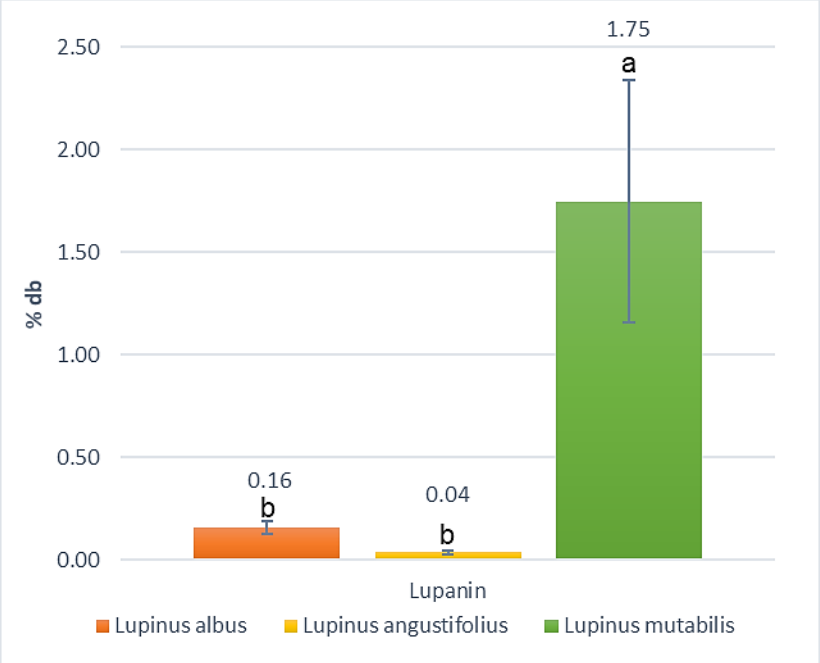


Figure 5.5 Quinolizidine Alkaloids concentration in three lupin species (*albus, angustifolius* and mutabilis) expressed as grams of lupanine per 100 g flour in dry basis.
Bars with different letters are statistically different ($p < 0.05$)

5. 2. 7 Trypsin Inhibitor

The analyzed lupin flours did not have detectable trypsin inhibitor activity. Nevertheless, the protein extraction process can concentrate this factor due to their protein nature. Meanwhile, these results offer an advantage over other legumes like soybean which has a 30.1 Trypsin Units Inhibited (TUI)/mL, according to Schoeneberger *et al*., (1982). In this study (Schoeneberger *et al*., 1982), five ecotypes of *L. mutabilis* where analyzed and four of them did not show any trypsin inhibitor activity. Only one ecotype resulted to have just 1.16 TUI/mL, 30 times less than soybean. According to Erbas, Certel, & Uslu (2005), the overall lupins inhibitor activity is very low, and the reported concentration is in the range of 0.1 to 0.2 mg/g.

5. 2. 8 Secondary structure of Lupin's flour for three different species using FTIR

Fourier Transform Infrared (FTIR) Spectroscopy is an established tool for the characterization of protein secondary structure. Examples of proteins that can be analyzed by this method include membrane-associated proteins, small proteins and peptides that exist in many rapidly

interconverting states like ribosomes and large water-soluble proteins. In the last category are the albumins and globulins, the main component of legumes proteins.

Protein secondary structure of protein from lupin's flour are summarized in **Table 5.6**. They are presented in percentage of total Amide I peaks. Native protein of legumes has a secondary structure dominated by β-sheets and β-turn structures. Secondary structures are responsible of protein functional, nutritional and nutraceutical properties and there is evidence that revealed the effect of thermal processing over the secondary structure (Carbonaro, Maselli, & Nucara, 2015).

Czubinski, Barciszewski, Gilski, Szpotkowski, Debski, Lampart-Szczapaa, & Jaskolski, (2015) have described the complete structure of a 7S basic globulin. This is a two subunits monomer present in *L. angustifolius* called γ-Conglutin (45.4 kDa). The secondary structure of this protein has a rare conformation formed by a 3_{10} Helix/α-helix complex. In the FTIR deconvolution a non-common percentage of 3_{10} Helix was found, a possible indicator of the presence of high concentrations of γ-Conglutin. Several studies show the ability of this monomer to bind to insulin and other growth factor. Also, there is experimental evidence that relate the uptake of γ-conglutin with the blood glucose reduction.

Table 5.6 Protein Secondary Structures (%) of Lupin's Flour for three different species (*albus, angustifolius* and *mutabilis*).

Lupin's species	*β-sheet*[a]	3_{10} Helix	α-helix	Unordered	*β-sheet*	Aggregated strands
L. albus	21.4	7.1	28.6	21.4	21.4	0.0
L. angustifolius	50.0	6.3	25.0	18.8	0.0	0.0
L. mutabilis	34.8	13.0	17.4	8.7	26.1	0.0

[a] antiparallel /aggregated strands

5.3 Ultrasound (US) Effect in lupin protein extraction and in the properties of isolates produced under alkaline conditions.

During the flour characterization described above, the three Lupin species showed significant differences mainly in protein content, secondary structure, solubilization profile, electrophoretic pattern and composition (amino acid profile). Due to this, was expected a distinct protein

extraction performance especially when the process was assisted with ultrasound. Results of the effect of processing over the protein isolate from lupin's flour is shown and discussed on the next sections.

Based on the preliminary results, two species of lupin were selected in order to obtain more evidence of the effect of the treatment in a wide range of alkaloid and protein content within this seed. Henceforth, only *Lupinus angustifolius* and *Lupinus mutabilis* flours were used as raw material of the protein concentration procedure. The work was then done with the so called sweet and bitter lupins (low and high alkaloid percentage).

5. 3. 1 *Lupinus mutabilis*

5. 3. 1. 1 Protein yield of US alkaline extraction process using *L. mutabilis* as raw material.

For industrial and commercial proposes this parameter is extremely important. The yield of the process is influenced by three principal factors: flour particle size, temperature and protein composition. In Aguilera & Garcia (1989), a mathematical model was proposed to correlate the particle size and the protein concentration on the isolate. In **Figure 5.6** the protein yield of every fraction (protein curd, bagasse and serum) derived from the three protein extraction processes (control, US assisted 10 min and US assisted 15 min) over *L. mutabilis* is presented. The significantly differences were on protein curd and bagasse.

The 10 min ultrasound treatment result in the highest protein yield extraction on protein curd. Karki *et al*. (2010) found a direct correlation between ultrasound time and protein concentration on isolates made from soybean flakes. They attribute this phenomenon to the cell rupture caused by the cavitation that releases sugars and protein from the seed tissue. Nevertheless, the longest time that they prove were 120 s, where they obtained a 78 ± 0.40 % of protein yield. For lupin, 10 min was the maximal ultrasound time treatment to enhance de protein release. The 15 min treatment is counterproductive, as can be seeing in the **Figure 5.6**, the protein yield drops. This could be due to extreme protein structural damage which produces aggregates that do not solubilize with the pH rising and can end in the bagasse (centrifugation residue) as can be seen in **Figure 5.6** where the 15 min Bagasse fraction is represented.

Ultrasound amplitude is an important parameter that can be regulated to optimize the energy applied to the raw material and combined with the frequency, produces the total energy input from the equipment. Thus, the commercial profit of the extraction process is directly correlated with the efficient use of energy. In the other hand, Ochoa-Rivas, Nava-Valdez, Serna-Saldívar, & Chuck-Hernández (2017) found that the 100% amplitude had the strongest effect over the protein yield obtained from peanut flour, reaching more than 100% more yield when compared with a non-treated protein extraction control. Taking this in account, the ultrasound time is the main variable to be discussed in this project. Their interaction with the amplitude and energy input for this specific food matrix must be optimized to reach the better yield at the lower cost.

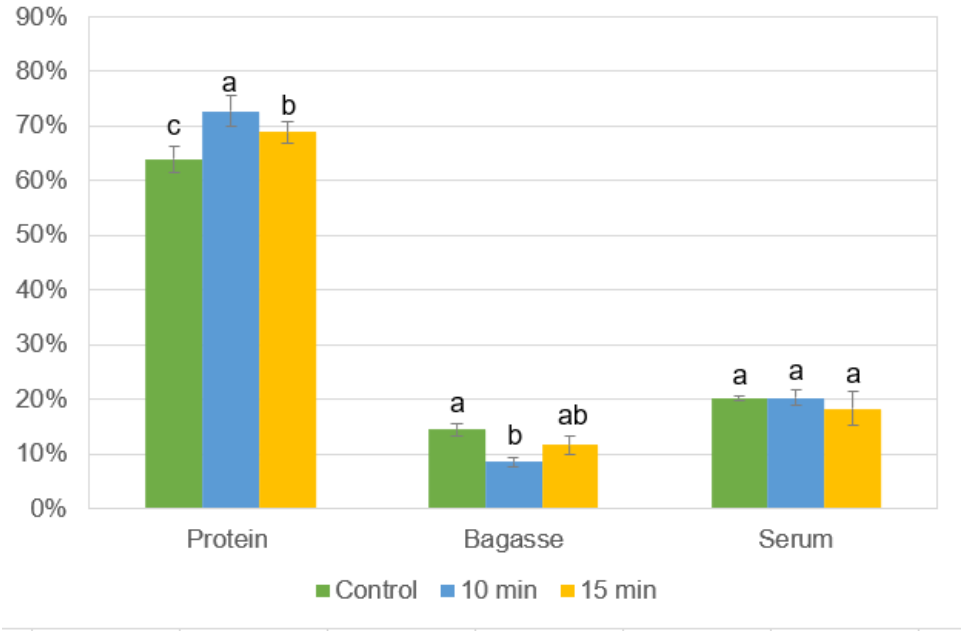


Figure 5.6 *Lupinus mutabilis* protein extraction yield for each fraction (protein curd, bagasse and serum) obtained during alkaline process assisted with ultrasound followed by isoelectric precipitation

[a, b, c] Bars with different letters within evaluated fraction (protein curd, bagasse and serum) are statistically different ($p < 0.05$)

5. 3. 1. 2 Functional Properties of protein isolates from *L. mutabilis* obtained from US assisted alkaline extraction process.

The nutritional and technological value of the proteins may be negatively influenced by the industrial processing. Non-enzymatic browning and secondary structure change due to heat may be the main causes of this problem. The first is produced by the reaction of reducing sugars and the free amino acids or the side chains of protein-bound lysine and arginine and the second by the dissociation of the hydrogen bonds that forms the specific secondary structure (D' Agostina *et al*., 2006). In **Figure 5.7** the evaluation of the technological and functional properties of protein fractions from *L. mutabilis* are summarized.

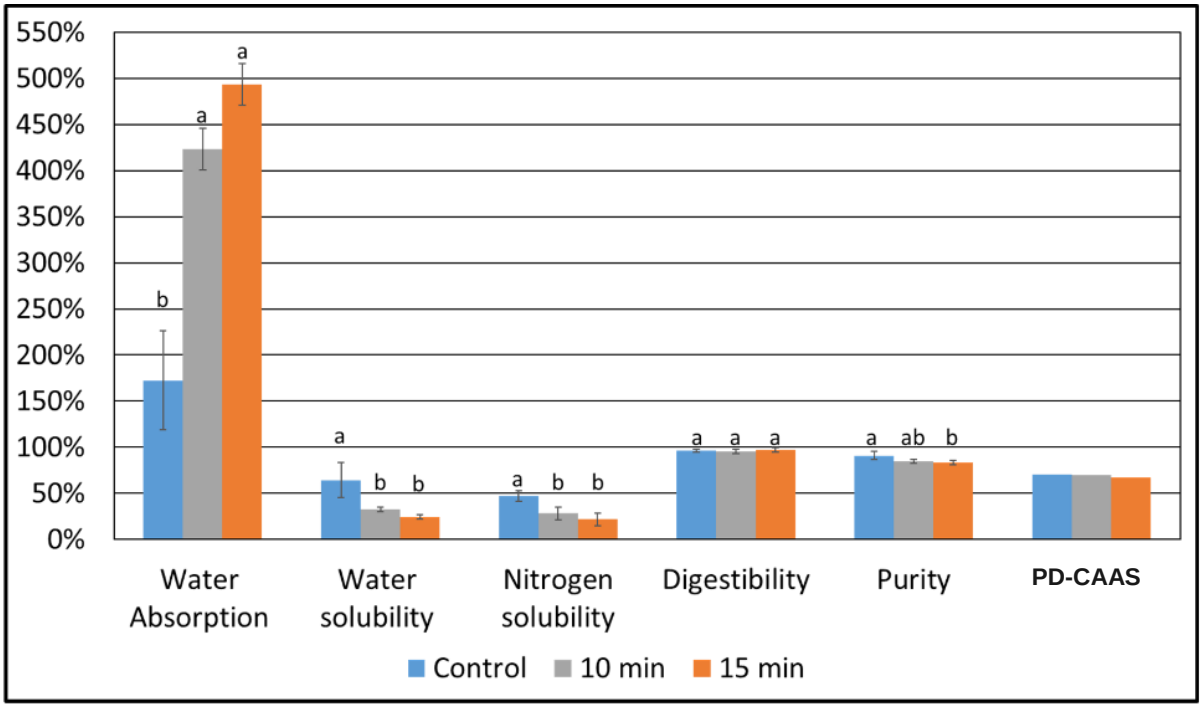


Figure 5.7 Functional properties for protein isolates from *Lupinus mutabilis* obtained with alkaline process assisted with ultrasound.

[a, b, c] Bars with different letters within each functionality differ significantly ($p < 0.05$)

Lupin proteins offer excellent functional properties such as water absorption, foaming and emulsification properties (Muranyi *et al.*, 2016). Water absorption (WAI) is closely related to the protein amino acid profile, secondary structure, hydrophobicity, pH, thermal treatment, ionic strength, and fat and protein concentrations. Even with the same protein amount, two protein isolates can have different water absorption. This could be because of the different globulin fractions between species and even between cultivars (Carvajal-Larenas, 2015).

The reported lupin flour water absorption (*L. albus* and *L.* mutabilis) are 2.3 and 1.2 g water/g flour dw, respectively. The reason why the *L. mutabilis* lowers WAI is due to the fat content and the probable absence of δ-conglutin, rich in polar amino acids (Carvajal-Larenas, 2015).

Water absorption of soybean protein isolate is 8.0 g water/ g isolate dw. In comparison, the lupin protein concentrates, and isolates can vary among species but generally are in the range of 0.5-6.0 g water /g protein dw. WAI of protein isolate obtained without ultrasound (control) was 1.5 g water/g isolate similar to results reported by Carvajal-Larenas, (2015). Most important is the effect of the ultrasound during the alkaline extraction from *L. mutabilis* process: the 15 min treatment resulted in more than four times higher WAI than the control. This could be due to the denaturalization of the protein during the treatment, which causes the exposition of polar amino acids to the medium. The polar amino acids have the ability to form hydrogen bonds that enhance the interaction with polar solvents like water (El-Adawy, Rahma, El-Bedawey, & Gafar, 2001).

Water solubility index (WSI) is a parameter that can be used as an indicator for the degradation of molecular compounds, it depends on the availability of hydrophilic groups that bind water and enhance gel formation of macromolecules. The augment on protein concentration could lead to the diminution of WSI due to the ligation to other soluble molecules (Morsy *et al.*, 2015). The protein concentrates have a little concentration of oligosaccharides which can play an important role on the water solubility of the product (Lampart-Szczapa *et al.*, 2006). Other proteins like albumins can increase the WSI due to their affinity with water. Nevertheless, legumes have low concentration of albumins and the protein isolates obtained by isoelectric precipitation does not contain albumin and the amount of protein-polysaccharide complex is very low (Carvajal-Larenas, 2015).

The WSI results of protein isolates compared with the control were lower, in fact both treatments (10 and 15min) showed half the control solubility. The ultrasound treatment could lead to the protein denaturalization and to the possible formation of non-soluble structures capable of interact with other macromolecules present in the protein isolate. Besides, the ultrasound treatment can affect the flour carbohydrates too i.e. gelatinizing the starch leading to gel formations and insolubilization of previously soluble fiber.

Nitrogen solubility index (NSI) is the third functional property depicted in **Figure 5.7** and it describes the ratio between soluble nitrogen and total nitrogen. This parameter is affected by pH, temperature and ionic strength of the protein solubilization media. The NSI for control was near 50%, being higher compared with US treatments (10 and 15 min), where a loss of half NSI was observed. This could be due to the thermal treatment caused by ultrasound in a pH higher than the isoelectric point. The temperature in the beaker reached the 63 and 77 °C for the 10 and 15 min treatment, respectively. The protein isolate was not neutralized after being freeze-dried, this also can drop the solubility of the proteins, as more time is stored in their isoelectric point (King *et al*., 1985). The explanation of this could be the irreversible formation of protein conglomerates and solid conformation with low solubility where the protein is trapped.

In-vitro protein digestibility was also assessed, and results are depicted in **Figure 5.7**. All samples (control, 10 and 15 min treatments) reached almost a 100% digestibility with no differences from the statistical point of view. These results from *L. mutabilis* were significantly higher than the 71.07 and 75.05% reported by Sathe, Desphanade, & Salunkhe (1982) when assessed *L. mutabilis* flour and protein concentrate, respectively. The same authors described the effect of a 30 min boiling step in flour and of protein extraction of *L. mutabilis*, yielding an increase of 4.63 and 3.81% in this parameter, respectively. This could be due to the protein denaturation which facilitated the enzymatic degradation.

The protein nutritional value not only depends on the amino acid composition and protein structure. The essential amino acid content, the human essential amino acid requirements and the bioavailability of those amino acids, together they are the best indicator of protein quality. Protein digestibility corrected amino acid score (PDCAAS) is then the most useful tool to determine the ability of certain protein to proportionate essential amino acids to the human diet. It reflects the essential amino acid content, true protein digestibility and the bioavailability of the amino acids on the food (Rozan *et al*., 1997). The reported PDCAAS from *L. mutabilis* is around 0.7 (Carvajal-Larenas, 2015; Pastor-Cavada *et al.*, 2009). This data corresponds with the obtained from the ultrasound assisted alkaline extraction: 0.67 for the control and 10 min treatment isolates and 0.65 for the 15 min treatment. In this case is very close to the reported but apparently the higher the ultrasound time, the lower is the PDCAAS not because of a low digestibility but by a reduction of cysteine (0.03 g/ 100 g of protein). Also, a slightly decrease of cysteine could be seen on 10 min ultrasound treatment, but this is compensated with a little bit

higher digestibility and that is why this treatment did not present significant difference in the PDCAAS when compared with the control.

5. 3. 1. 3 Electrophoretic profile of *L. mutabilis* isolates obtained from US assisted alkaline extraction process.

Changes in composition and secondary structure may be expected after protein isolation process due to physical and chemical damage as well as a different solubility of fractions. Filtration, dilutive and isoelectric precipitation are the main techniques used to separate the protein from the aqueous solution in order from the less to the more severe. Nevertheless, according to Muranyi *et al*. (2016), the most efficient is the last one. The different solubility of the protein fractions could lead to a concentration of certain proteins like globulins and albumins and the discard of other lipophilic fractions. The result is a protein isolate with different functional, nutritional and nutraceutical properties. Physical damage due to heat and shear stress can denaturalize the protein resulting in certain conformational behavior and interaction with other seed components. These changes can directly influence the separation during the production of protein concentrates or isolates. Other food quality aspects could be influenced by the protein modification like water activity, lipid and protein oxidation (Berghout *et al*., 2015).

The changes in protein fraction proportions can be elucidated by SDS-PAGE. The results of the electrophoresis pattern of the protein isolate obtained from *L. mutabilis* are illustrated in **Figure 5.8.** Protein structure obtained from control treatment of *L. mutabilis* flour did not have significant changes compared with the native seed protein (Line 2 and 1 from **Figure 5.4** respectively). Nevertheless, the 10 and 15 min shows a bunch of higher molecular weight bands between 250 and 50 kDa. In addition, two bands one of 37 and other from 31 kDa appeared. The high molecular weight bands can be produced by new protein aggregations and coagulation attributed to physical damaged due to ultrasound treatment. The low molecular weight new bands can be attributed to protein denaturalization because of the inherent heat produced during ultrasound treatment. In this case, as described above, the temperature in treatment of 15 min US assisted was 77°C.

Non-reducing conditions

1. Defatted flour
2. Protein Isolate 1 (Control)
3. Protein Isolate 2 (10 min US Treatment)
4. Protein isolated (15 min US Treatment)

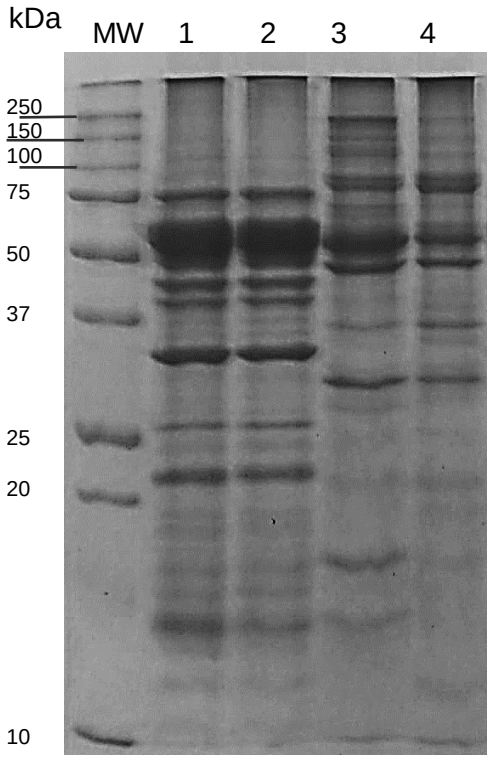


Figure 5.8 Electrophoretic profile for *Lupinus mutabilis* protein extracts in non- reducing conditions.

Line 1 is defatted flour, Line 2 is protein isolate, Line 3 protein isolate using 10 min US assisted extraction and Line 4 protein isolate using a 15 min US assisted extraction. MW is molecular weight standard.

5. 3. 1. 4 Thermal properties of protein isolates from *L. mutabilis* obtained from US assisted alkaline extraction process.

Differential scanning calorimetry (DSC) is a thermo-analytical technique for determination of physical or chemical properties modifications of food materials as a function of temperature by measuring the heat changes associated to processing. DSC technique compare the rate of heat flow to the sample with an inert material, which are heated or cooled at the same rate. Changes in the material that are associated with variation of heat cause a change in the differential heat flow which is then recorded as a peak. The area under the peak is directly proportional to a change in enthalpy and its direction indicates whether the thermal event is endothermic or exothermic (Biliaderis, 1983).

In **Table 5.7,** the DSC parameters measured over the protein isolates from *L. mutabilis* are depicted: T_{max}, as a peak temperature of the denaturation event; T_{onset} as the initial temperature of the curve denoting a change in the tested material as well as *Δ*H (enthalpy of denaturalization). Arntfield & Murray (1981) ran an experiment where compared denaturation temperature (Td, Tmax) and thermal transitions of alkaline extracted proteins from faba bean adjusted to different pH values near and far from its isoelectric point. They demonstrate that the farthest the pH from the isoelectric point, the lower the temperature of denaturation. Also, the denaturation temperature of certain globulins is dependent of water content. This statement proposed by Sousa, *et al.* (1995) is corroborated because the higher the water activity, the lower the denaturation temperature (see **Table 5.8**).

Table 5.7 Differential scanning calorimetry parameters from *L. mutabilis* protein isolates obtained by different ultrasound treatments and adjusted to 0.5379 Aw.

	T_{max} (°C)	T_{onset} (°C)	ΔH (J/g)
Control	58.39 ± 1.36[a]	51.19 ± 0.61[a]	2.41 ± 0.16[b]
10 min	60.54 ± 0.11[a]	53.26 ± 0.14[a]	2.87 ± 0.01[b]
15 min	59.92 ± 1.18[a]	52.73 ± 1.02[a]	3.64 ± 0.24[a]

T_{max} is the peak temperature associated with a denaturation of the tested material; T_{onset} is the initial temperature on the enthalpy curve and ΔH denaturation enthalpy. [a, b, c] Columns with different letters differ significantly ($p < 0.05$)

Denaturation temperature among treatments did not differ significantly (**Table 5.7**). This demonstrate that ultrasound treatment does not have effect over heat stability of protein isolate despite some reports affirm that industrial processing of legumes can alter the denaturation temperature and solubility of protein isolates (Sirtori, *et al*., 2010). In their research, they obtain a DSC thermogram with two peaks that correspond to vicilin (lower Td) and legumin (higher Td). After a heat treatment, the vicilin peak diminish until it disappears (71.49 °C) and this could be the reason why only one peak (legumin fraction) was found. Probably the vicilin fraction was denaturalized due the heat generated under ultrasound treatment (77 °C), as already commented in last sections.

Enthalpy of denaturalization (ΔH) did not change with 10 min treatment but it was significantly increased with 15 min ultrasound application. These results agree with Chandrapala, et al. (2011) who proved that more than five min ultrasound treatment over whey protein concentrate increase

the ΔH due to protein aggregation. Taking this in account the protein stability of 15 min treatment was increased because the energy needed to denaturalize the protein aggregates was higher. The protein aggregation was already discussed in the section for electrophoretic profile, and despite the tendency of both 10 and 15 min treatments to increase the number of bands above 75 kDa, the change in thermal properties only was observed with a statistical difference between control and 15min US assisted protein isolate (**Table 5.7**).

Table 5.8 Differential scanning calorimetry parameters from *L. mutabilis* protein isolate without ultrasound treatment adjusted at different water activities (Aw).

Aw	T_{max} (°C)	T_{onset} (°C)	ΔH (J/g)
0.2971	62.02 ± 0.36[a]	51.48 ± 0.50[a]	1.93 ± 0.072[a]
0.5379	58.39 ± 1.36[a]	51.19 ± 0.61[a]	2.41 ± 0.158[a]

T_{max} is the peak temperature associated with a denaturation of the tested material; T_{onset} is the initial temperature on the enthalpy curve and ΔH denaturation enthalpy. [a, b, c] Columns with different letters differ significantly ($p < 0.05$)

5. 3. 1. 5 Amino Acid Profile of protein isolates from *L. mutabilis* obtained with US assisted alkaline extraction process.

The main interest in lupin for foods is related to its high content of protein which is considered a good source of lysine (especially *L. mutabilis*), but generally poor in the sulfur-containing amino acids (El-Adawy, Rahma, El-Bedawey, & Gafar, 2001). In **Table 5.9** are shown the amino acid profile and amino acid score for *L. mutabilis* protein isolates obtained with control, 10 and 15 min of US assisted extraction.

The most remarkable increase (10% of difference compared with control) were for valine, methionine, tyrosine and phenylalanine, whereas serine and cysteine were reduced. Loss of polar amino acids and concentration of non-polar amino acids are expected when aqueous processing is carried out and also because protein agglomeration leads the under exposure of hydrophobic amino acids to the medium and additional protection for physical and chemical damage exists.

Table 5.9 Amino acid profile of *L. mutabilis* protein isolates (control and obtained with US assisted alkaline extraction during 10 and 15 min).

Amino Acid	g/100 g Crude Protein		
	Control	10 min Treatment	15 min Treatment
Aspartic Acid	10.27	10.34	10.39
Threonine	3.24	3.43	3.43
Serine	5.05	4.22	4.20
Glutamic Acid	24.08	21.78	22.03
Proline	4.17	4.37	4.37
Glycine	3.87	4.04	4.03
Alanine	3.25	3.53	3.52
Cysteine	1.25	1.14	1.11
Valine	4.09	4.59	4.53
Methionine + Cys	1.82	1.82	1.74
Methionine	0.57	0.68	0.63
Isoleucine	5.01	5.25	5.22
Leucine	7.08	7.38	7.31
Tyrosine	3.81	4.26	4.24
Phenylalanine + Tyr	7.75	8.57	8.51
Phenylalanine	3.94	4.31	4.28
Lysine	5.34	5.59	5.59
Histidine	2.69	2.72	2.72
Arginine	11.28	11.14	11.19
Tryptophan	0.96	0.98	1.00
Amino acid Score[1]	**0.70**	**0.70**	**0.67**

[1] Amino acid score based on cysteine + methionine as limiting amino acid.

5. 3. 1. 6 Mass balance for Quinolizidine alkaloid in all fractions produced from US assisted protein extraction process for *L. mutabilis* (Fate of alkaloids during a *L. mutabilis* process for protein extraction).

The final concentration of alkaloids in every fraction obtained from the *L. mutabilis* protein extraction is presented in **Figure 5.9**. As can be seen, the blue fraction represents the percentage

of alkaloids obtained in the protein curd of control, 10 and 15 min US assisted treatments with 0.06, 0.03 and 0.04 % dw of alkaloids expressed as lupanine, respectively. The 10 min treatment had the lower alkaloid content with 30 mg Lupanine/ 100 g protein isolate, *i.e*. 58 times less alkaloid concentration than the raw flour (1750 mg/ 100 g *L. mutabilis* flour) and half the concentration compared with the traditional (control) protein curd. This means that an adult of 70 kg can eat 8 kg of protein isolate, which is equivalent to almost 7 kg of crude protein, per day with no adverse effects (Carvajal-Larenas, 2015). In other words, the ultrasound treatment (10 min) reduced 50 % alkaloids concentration from this fraction when compared with no ultrasound alkaloid extraction (control).

The bagasse fractions for the control (green section in **Figure 5.9**.), 10 and 15 min US treatment where 0.49, 0.09 and 0.08 % lupanine dw, respectively, depicting also a reduction in the toxicity compared to the control due to the US effect. The 15 min treatment had 6 times less alkaloids than control. The 80mg Lupanine/100g of bagasse fraction is more than 21 times less toxic than *Lupinus mutabilis* flour.

The cavitation produced by US over seed cells is the responsible for the liberation of alkaloids in the solution and this phenomenon together with the pH changes lead the migration of the majority of alkaloids to the aqueous phase (serum fraction, **Figure 5.9**). Serum resulted to have the highest alkaloid concentration and compared to control, treatments had up to 41% more of these compounds.

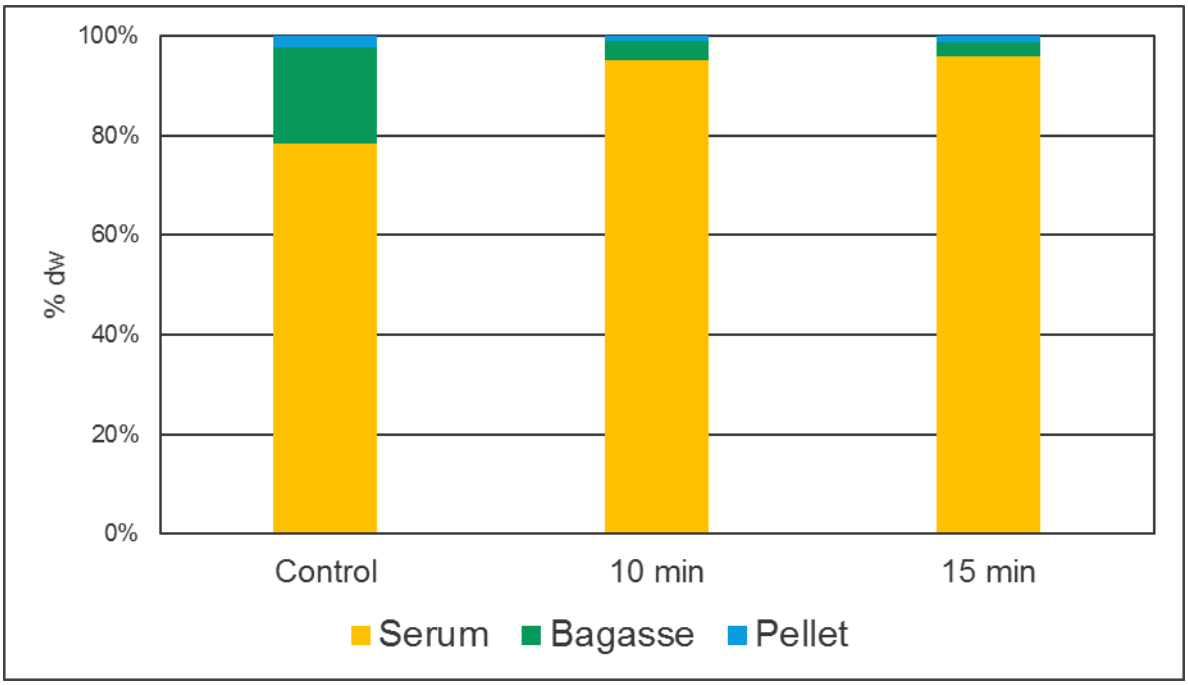


Figure 5.9 Quinolizidine alkaloids (QA) distribution in each fraction obtained during US assisted alkaline extraction and isoelectric precipitation from *L. mutabilis*.

5. 3. 1. 7 Trypsin Inhibitor Activity (TIA) for *L. mutabilis* protein isolates produced with a US assisted alkaline extraction process.

Agree with the results presented before for *Lupinus* flour, the protein fractions did not have trypsin inhibitor activity.

5. 3. 1. 8 Secondary Structure of protein isolates from *L. mutabilis* produced with US assisted extraction procedure.

In this section, the secondary structure of the lupin's protein from the isolates produced with and without US assisted extraction is presented (**Table 5.10).**

Table 5.10 Protein secondary structures of *Lupinus mutabilis* protein isolates obtained during US assisted alkaline extraction and isoelectric precipitation (%).

Protein isolate	*β-sheet*[a]	3_{10} Helix	α-helix	Unordered	*β-sheet*	Aggregated strands
Control	40.0	20.0	20.0	0.0	10.0	10.0
10 min treatment	50.0	0.0	12.5	12.5	12.5	12.5
15 min treatment	57.1	0.0	14.3	14.3	0.0	14.3

[a] antiparallel /aggregated strands

The main difference among treatments and the native protein (section 5.2.8) was the presence of aggregated strands that increased according with the ultrasound time. As discussed before, the alkaline solubilization could lead to changes in secondary structure, therefore the control protein isolate also had aggregated strands. The ultrasound time enhanced this phenomenon and the 15 min treatment had the highest aggregated strands percentage.

Unordered structures just appeared on ultrasound treatment, this finding might be the explanation of the changes in functional and electrophoretic properties of protein isolates. The unordered structures are low soluble and easily degradable by enzymes. The disappearance of 3_{10} helix could lead to the formation of that unordered structures due to the low intermolecular forces. The 3_{10} helix loss decreases the nutraceutical potential of the protein isolates due to the possible denaturalization of γ-conglutin and the consequent loss of hypoglycemic activity.

The unordered structures and aggregated strands are directly correlated with the disappearance of certain protein conformations that results in different thermal properties that can be correlated with the differential calorimetry scan thermogram. The lack of the vicilin denaturalization peak corresponds with the loss of this protein structure since it is formed by weak and heat sensitive bonds mainly hydrogen bonds.

5. 3. 2 *Lupinus angustifolius*

After the analysis of the effect of US in the yield and properties of the protein isolates obtained from *L. mutabilis*, the same was made for *L. angustifolius*, the specie with the lowest concentration

of alkaloids and the flour with the highest particle size. The results are described in the following sections.

5. 3. 2. 1 Protein Yield of US alkaline extraction process using *L. angustifolius* as raw material.

Lupinus angustifolius was processed and results of the protein yield are shown in **Figure 5.10.** There were no significant differences between treatments on protein yield of the fractions. Nevertheless, the highest mean protein yield for the protein curd was achieved with the strongest treatment (15 min). This result showed a different tendency when compared with the *L. mutabilis* extraction, where the highest yield was reached with the 10 min US assisted process and when contrasted to Karki *et al*. (2010), results where more ultrasound time was used over soybean flakes resulted on a high protein yield. Comparing the protein yield results with Muranyi *et al.* (2016), who combine several methods like isoelectric point precipitation, salt induced extraction, alkaline extraction, dilutive precipitation and their combination over lupin flour the results obtained with ultrasound are higher. The most comparable result presented by them was a 31.7 ± 0.8 % yield using alkaline extraction and a combination of dilutive and isoelectric precipitation. With US alkaline extraction, in this research, more than 50% yield was obtained compared with conventional alkaline extraction in combination with isoelectric precipitation.

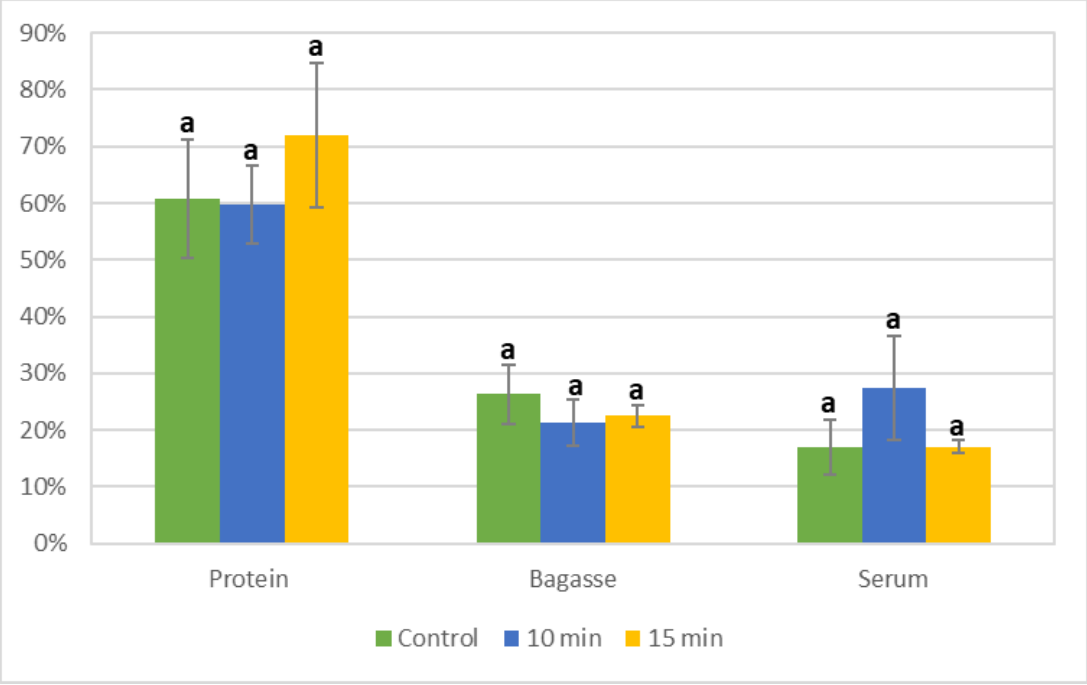


Figure 5.10 *Lupinus angustifolius* protein extraction yield for each fraction (protein curd, bagasse and serum) obtained during alkaline process assisted with ultrasound followed by isoelectric precipitation.

[a, b, c] Bars with different letters differ significantly ($p < 0.05$)

The lower yield of *L. angustifolius* protein fraction (compared to *L. mutabilis*) may be explained by the differences on the flour particle size (**Table 5.3**) and/or the composition and structure of storage proteins (**section 5.2**, Flour Characterization). In the case of proximal composition, the only difference worth to be enhanced is the *L. angustifolius* fiber content, higher than *mutabilis* (around 14 vs 6% respectively). Osborne, electrophoretic and amino acid profile were similar in both species, exposing the fact that the yield difference must be related to the particle size.

5. 3. 2. 2 Functional Properties of protein isolates from *L. angustifolius* obtained from US assisted alkaline extraction process.

In **Figure 5.11** the evaluation of the technological and nutritional functional properties of protein fractions from *L. angustifolius* are summarized.

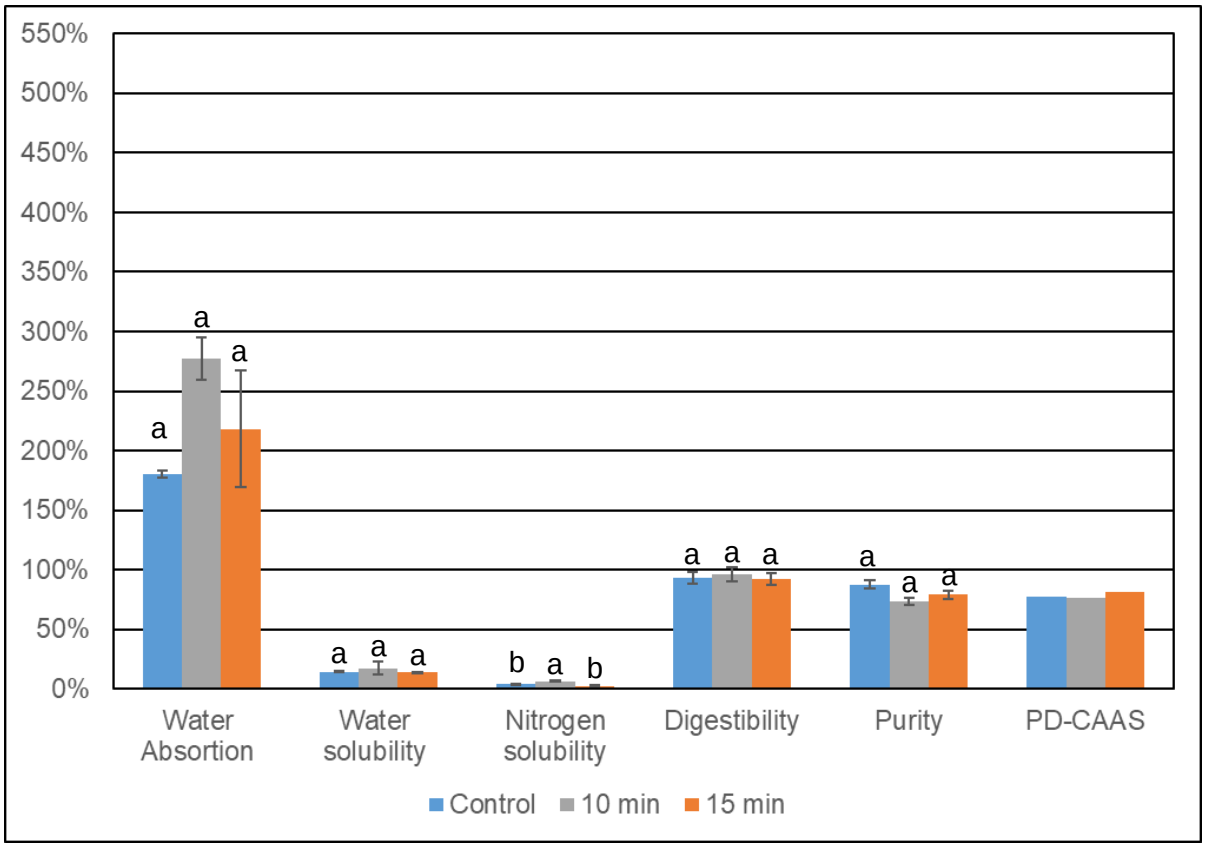


Figure 5.11 Functional properties for protein isolates obtained during US assisted alkaline extraction and isoelectric precipitation from *Lupinus angustifolius.*

[a, b, c] Bars with different letters differ significantly ($p < 0.05$)

The reported flour water absorption for *L. angustifolius* is 2.4 water/g flour dw (Carvajal-Larenas, 2015), this result did not correspond with the depicted in **Figure 5.11** and could be related to a high carbohydrate concentration in flour. Despite there was no a significant difference among the ultrasound treatments and control regarding water absorption, the 10 min ultrasound treatment had the highest mean. Compared with *L. mutabilis,* the *L. angustifolius* protein isolates had lower water absorption. This could be due to composition differences, glycosylation pattern and protein concentration (Eaton-Mordas & Moore, 1978). In the case of results depicted in **Figure 5.11** and **Figure 5.7**, the difference in protein concentration for *mutabilis* and *angustifolius* isolates can indeed affect the water absorption, mainly because in the case of *L. angustifolius,* the protein concentration was lower, indicating the presence of other compounds that affect directly to the protein swelling process (Kinsella, 1979). In other words, to have a higher water absorption, the

protein needs to change their structure and make space for the water molecules but when other compounds as fiber are in the system, this absorption process can be altered.

Water solubility index (WSI) of *L.* angustifolius was lower than *L. mutabilis* protein isolates because of the high insoluble fiber present in the raw seed. There were no significant differences between ultrasound treatments and control.

The same reduction when compared with *L. mutabilis* isolates, was observed for NSI (**Figure 5.11** and **Figure 5.7**). Only one report was found regarding NSI for *L. angustifolius:* 13.1% NSI for flour and 19.2 to 33.8% for its isolates obtained with different pHs through alkaline extraction (King *et al*., 1985).

In-vitro protein digestibility for *L. angustifolius* has been reported as 80% and 86.3% for the flour and pH 12 protein isolate respectively (Lqari, Vioque, Pedroche, & Millán, 2002). These results are similar to the ones depicted in **Figure 5.11**, where is clear the null effect of ultrasound. When comparing isolates from *L. mutabilis* with *L. angustifolius* both are up to 90 % with no strong differences among them.

With the data for digestibility and the amino acid profile, the PDCAAS was calculated and in the case of *L. angustifolius,* the PDCAAS reported so far was 0.53 (Carvajal-Larenas, 2015; Pastor-Cavada *et al.*, 2009), a lower value when compared with results from **Figure 5.11**. PDCAAS for control, 10 and 15 min ultrasound treatments were similar among them, but with a slight tendency to increase with the ultrasound time (15 min ultrasound treatment had the higher PDCAAS: 0.82). This is result was associated to a 0.23 g/ g crude protein increase on methionine concentration besides a small increase on *in-vitro* protein digestibility.

5. 3. 2. 3 Electrophoretic profile of *L. angustifolius* isolates obtained from US assisted alkaline extraction process.

The changes in protein fractions can be elucidated by SDS-PAGE. The results of the electrophoresis pattern of the protein isolates obtained from *L. angustifolius* are illustrated in **Figure 5.12.**

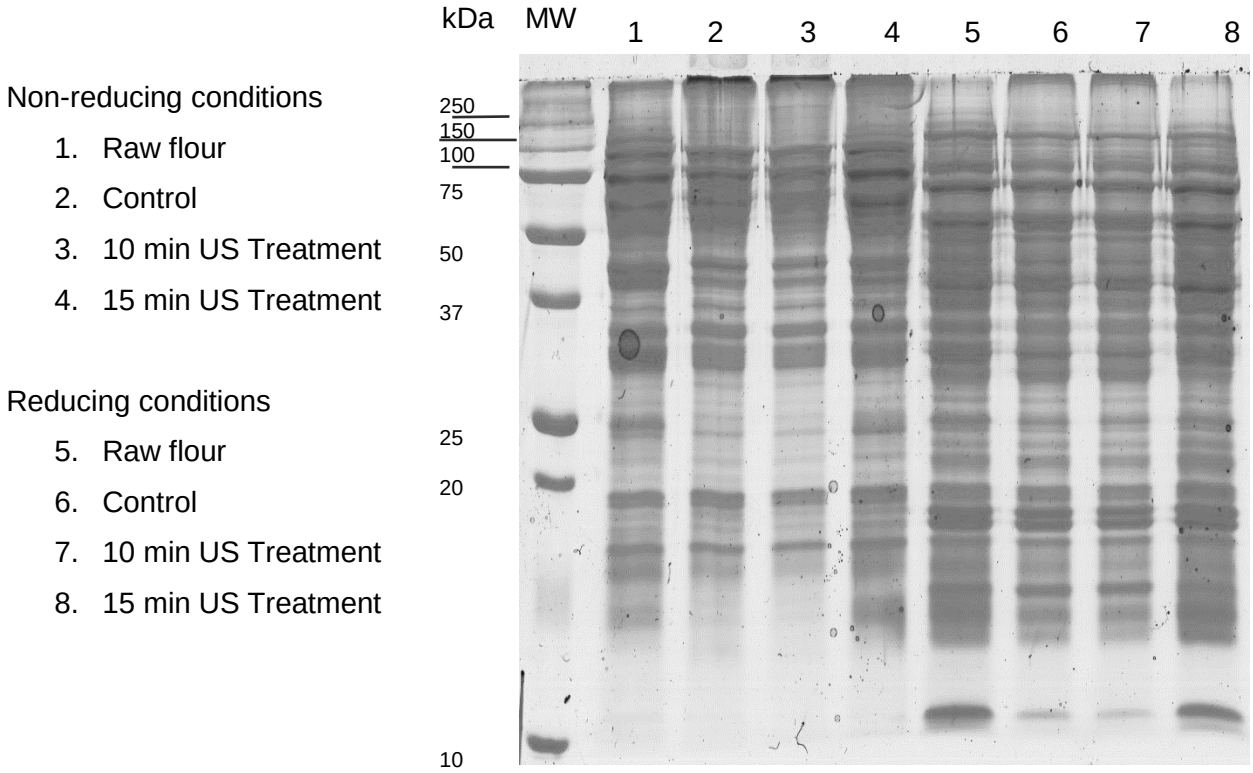


Figure 5.12 Reducing and non-reducing SDS-PAGE of *Lupinus angustifolius* protein extracts from the isolates obtained during US assisted alkaline extraction and isoelectric precipitation.

MW is the standard for molecular weight.

L. angustifolius protein isolates shown no significant differences between treatments and control except for the intensity in certain bands that could be due to different protein concentration. This result explains the similar behavior of the protein isolates from the different treatments. The presence of a band between 37-50 kDa can be attributed to γ-conglutin, a nutraceutical protein which several activities like hypoglycemic, anti-cholesterolemic and ACE-inhibitory activity has been attributed (Czubinski *et al*., 2015; Fontanari *et al*., 2012; Boschin *et al*., 2014).

5. 3. 2. 4 Amino Acid Profile of protein isolates from *L. angustifolius* obtained with US assisted alkaline extraction process.

In **Table 5.11,** the amino acid profile and amino acid score of *Lupinus angustifolius* protein isolates are presented. The most remarkable increase (10% difference compared with control) were for threonine, valine, alanine, methionine, lysine and tryptophan whereas serine, glutamic acid and cysteine were reduced with ultrasound treatment. Loss of polar amino acids and concentration of non-polar amino acids are expected when aqueous processing is executed, and protein agglomeration leads the under exposure of hydrophobic amino acids to the medium protecting them from physical or chemical damage. The main difference between the amino acid profile for flour and protein isolates was the enhanced nutritional properties, because of the concentration of five essential amino acid due to the ultrasound treatment.

Table 5.11 Amino acid profile of *L. angustifolius* protein isolates obtained during US assisted alkaline extraction and isoelectric precipitation.

Amino acid	g/100 g Crude Protein		
	Control	10 min Treatment	15 min Treatment
Aspartic Acid	10.35	10.40	10.45
Threonine	3.12	3.59	3.62
Serine	4.69	4.05	3.98
Glutamic Acid	24.15	20.88	20.59
Proline	4.41	4.57	4.52
Glycine	4.08	4.42	4.41
Alanine	3.23	3.77	3.78
Cysteine	1.60	1.41	1.42
Valine	4.08	4.79	4.88
Methionine + Cys	2.16	2.17	2.21
Methionine	0.56	0.76	0.79
Isoleucine	4.69	4.91	4.98
Leucine	7.61	7.94	8.08
Tyrosine	3.63	3.93	3.92
Phenylalanine + Tyr	7.84	8.53	8.61
Phenylalanine	4.21	4.60	4.69
Lysine	4.61	5.31	5.31
Histidine	2.60	2.73	2.76

Arginine	11.29	10.47	10.37
Tryptophan	0.99	1.16	1.10
Amino acid Score[1]	**0.83**	**0.83**	**0.85**

[1] Amino acid score based on cysteine + methionine as limiting amino acid.

5. 3. 2. 5 Mass balance for Quinolizidine alkaloids in all fractions produced from US assisted protein extraction process for L. *angustifolius* (Fate of alkaloids during a *L. angustifolius* process for protein extraction).

In **Figure 5.13** the QA concentration in ppm obtained by GC-MS are presented. As can be seen, the ultrasound treatment had a negative effect, increasing the alkaloid content on the protein isolates. On the other hand, the QA concentration on bagasse and serum was lower in ultrasound treatments than the control. This phenomenon could be due to the enhanced water absorption of the protein isolates, because of the carbohydrate concentration of the final protein curd. The ultrasound might have liberated the alkaloids disrupting seed cells and more glycoproteins but being the latter capable of absorb alkaloid rich solvent, this reduces the QA concentration in the serum during protein precipitation. The alkaloid concentrations in the protein curd was 1.6, 4.2 and 6.1 mg of lupanine/100g dw, this represent a decrease of 94, 85 and 78 % for control, 10 and 15 min ultrasound treatments applied to *L. angustifolius* flour, respectively.

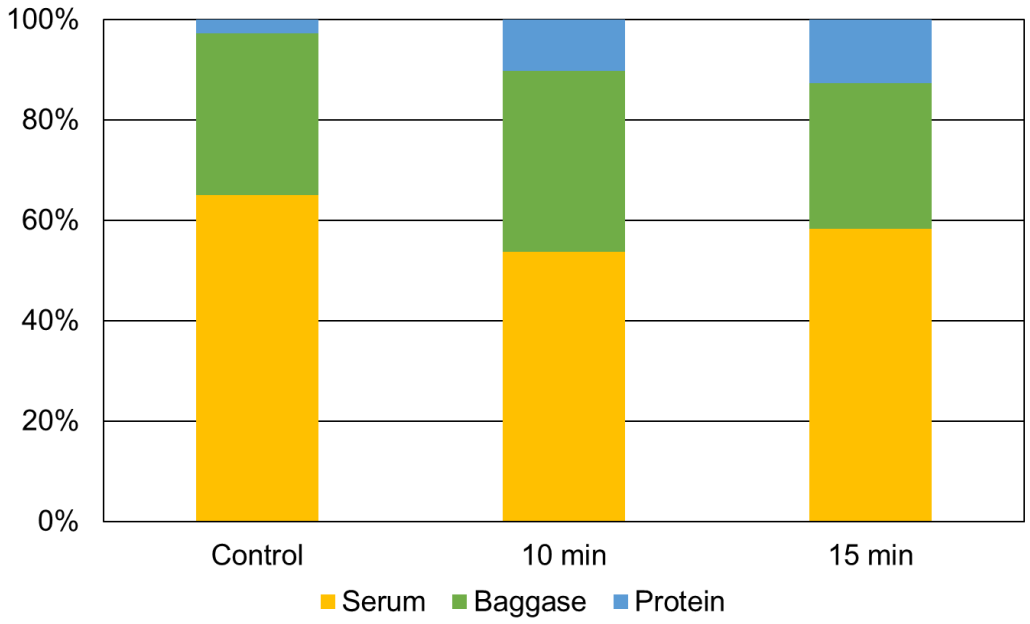


Figure 5.13 Quinolizidine alkaloids (QA) distribution in each fraction (serum, bagasse and protein curd) obtained during alkaline extraction assisted with US and isoelectric precipitation from *L. angustifolius*.

These results are crucial for toxicity determination. The safe dose of total QA is 35 mg/kg/day for adults. Regarding this, a 70 kg adult can safely consume 153, 58 and 40 kg of control, 10 and 15 min protein isolates daily, respectively. This amount of protein overcome any protein nutritional requirement (amino acids and total protein).

5. 3. 2. 6 Trypsin Inhibitor Activity (TIA) for *L. angustifolius* protein isolates produced with a US assisted alkaline extraction process.

Lupinus angustifolius protein isolates did not present any trypsin inhibitor activity. Even when 1% of Polyvinylpyrrolidone (PVP) was added to avoid the phenolic compounds interference, reproducible results were no obtained.

6 Conclusions and Recommendations

6.1 Conclusions

Three different species of *Lupinus* (*angustifolius, mutabilis* and *albus*) were characterized yielding differences in: 1) flour particle size for *L. angustifolius* (the highest median particle size: 178.4 µm vs 46 µm of the other two species) and 2) proximate analysis (fat, protein and carbohydrates). The highest protein concentration (46%) was observed in *L. mutabilis,* and the lowest (28%) in *L. angustifolius.* Protein solubility profile showed the presence of a high percentage of water-soluble fraction while amino acid profile confirmed the low cysteine and methionine concentration reported by the literature. Alkaloid concentration was the most useful data obtained from lupin flours (1.75% for *mutabilis* and 0.04% for *angustifolius* expressed as lupanine percentage). It helped to make the decision of which specie had the better potential for monitoring both the quinolizidine alkaloids fate and the safest protein isolate from these raw materials. Based on these results, protein extraction procedures assisted with ultrasound (control, 10 and 15 min ultrasound treatments) were executed in *L. mutabilis* and *L. angustifolius* flours. The effect of ultrasound over protein yield was beneficial for *L. mutabilis* due to an increase of 14% with 10 min treatment whereas for *L. angustifolius* there was no significant difference with the use of this technology. The functional property more affected by ultrasound in *L. mutabilis* was water absorption (200% more absorption than control) whereas water and nitrogen solubility decreased by half. A remarkable result was that *in-vitro* digestibility, not affected with treatment despite SDS-PAGE showed an increase of protein agglomerates due to ultrasound in the alkaline extraction procedure. This result was also confirmed with the secondary structure analysis, where a high proportion of aggregated strands and unordered structures appeared in 10 and 15 min treatments. The change in secondary structure was responsible for the augment of the denaturation temperature measured by DSC. In the case of *L. angustifolius*, water solubility, absorption and NSI were lower when compared with isolates from *L. mutabilis*. This different effect of ultrasound in lupin species indicates the difference in protein profile of both seeds. QA fate in the extraction process was highly affected by ultrasound. The results obtained for *L. mutabilis* protein isolates indicates a QA concentration reduction for both bagasse (81% less than control with 10 min ultrasound treatment) and protein isolate (50% less than control with 10 min ultrasound treatment). The opposite was observed for *L. angustifolius* because an increase in QA content for

both bagasse and protein curd fractions was associated with the ultrasound treatment. Ultrasound for *L. mutabilis* resulted to be a useful tool due to the increase in protein yield and the new functional and thermal properties associated with the change in protein structure registered as a different thermal, electrophoretic and secondary structure profile. The association between ultrasound time and functional changes described herein can be helpful to create new protein profiles with more or less solubility, water absorption and thermal resistance. Moreover, the environmental and economic implications that comes with the use of this technology for lupin extraction process are highly relevant in the pursuit of new protein sources. Less water usage, processing time, waste production and energy are the main advantages of ultrasound processing.

6.2 Recommendations

With the knowledge generated with this work, the following further activities can be recommended:

- To evaluate the economic feasibility and scalability potential of the ultrasound assisted lupin protein isolate production.
- To develop better methods for flour defatting in order to take advantage of the lupin high fat content without using and wasting organic solvents.
- To evaluate the effect of the lupin oil refinement over the alkaloid concentration, fatty acid profile and oxidation stability.
- To investigate of the functional and nutritional properties of the bagasse to create high-value food ingredients.
- To improve purification of quinolizidine alkaloids and research in further applications such as drug or pesticide.
- To design and test cheaper drying process for protein isolates like spray drying to reduce more the cost of the product.
- To determinate QA reduction from protein curd by adding washing steps to the process.

7 Bibliography

1. AACC International. Approved Methods of Analysis, 11th Ed. Method 30-20.01. Crude Fat in Grain and Stock Feeds. Approved April 13, 1961. AACC International, St. Paul, MN, U.S.A. http://dx.doi.org/10.1094/AACCIntMethod-30-20.01

2. AACC International. Approved Methods of Analysis, 11th Ed. Method 55-10.01. Test Weight per Bushel. Approved April 13, 1961. AACC International, St. Paul, MN, U.S.A. http://dx.doi.org/10.1094/AACCIntMethod-55-10.01

3. AOAC (1992). American Organization of Analytical Chemists International. Official Methods 962.09, 978.02, 925.10, 923.03, 982.30. Determination of crude fiber, total nitrogen, moisture, ashes, amino acid profile. Washington, DC.

4. AOCS (2006). Official and Tentative Methods of the American Oil Chemist's Society. American Oil Chemist's Society (AOCS). Method Ba 11–65. Nitrogen Solubility Index. Urbana, IL, USA.

5. Aguilera, J. M., & García, H. D. (1989). Protein extraction from lupin seeds: a mathematical model. *International Journal of Food Science & Technology*, *24*(1), 17–27. https://doi.org/10.1111/j.1365-2621.1989.tb00615.x

6. Alberta Agriculture and Food. (2007). Using 1,000 Kernel Weight for Calculating Seeding Rates and Harvest Losses.

7. Aniszewski, T. (2015). *Alkaloids: chemistry, biology, ecology, and applications.* Elsevier.

8. Arntfield, S. D., & Murray, E. D. (1981). The Influence of Processing Parameters on Food Protein Functionality I. Differential Scanning Calorimetry as an Indicator of Protein Denaturation. *Canadian Institute of Food Science and Technology Journal*, *14*(4), 289–294. https://doi.org/10.1016/S0315-5463(81)72929-8

9. Ashokkumar, M., Lee, J., Kentish, S., & Grieser, F. (2007). Bubbles in an acoustic field: an overview. *Ultrasonics Sonochemistry*, 14(4), 470-475.

10. Ashokkumar, M., Lee, J., Zisu, B., Bhaskarcharya, R., Palmer, M., & Kentish, S. (2009). Hot topic: Sonication increases the heat stability of whey proteins. *Journal of Dairy Science*, 92(11), 5353-5356.

11. Australia New Zealand Food Authority (2001). Lupin Alkaloids in Food A Toxicological Review and Risk Assessment *Technical Report Series No. 3*; Available online: https://www.foodstandards.gov.au/publications/documents/TR3.pdf

12. Bader, S., Oviedo, J. P., Pickardt, C., & Eisner, P. (2011). Influence of different organic solvents on the functional and sensory properties of lupin (*Lupinus angustifolius L*.) proteins. *LWT - Food Science and Technology*, 44(6), 1396–1404. https://doi.org/10.1016/j.lwt.2011.01.007

13. Berghout, J. A. M., Marmolejo-Garcia, C., Berton-Carabin, C. C., Nikiforidis, C. V., Boom, R. M., & van der Goot, A. J. (2015). Aqueous fractionation yields chemically stable lupin protein isolates. *Food Research International*, 72, 82–90. https://doi.org/10.1016/j.foodres.2015.03.039

14. Biliaderis, C. G. (1983). Differential scanning calorimetry in food research-A review. *Food Chemistry*, *10*(4), 239–265. https://doi.org/10.1016/0308-8146(83)90081-X

15. Boschin, G., Scigliuolo, G. M., Resta, D., & Arnoldi, A. (2014). ACE-inhibitory activity of enzymatic protein hydrolysates from lupin and other legumes. *Food chemistry*, 145, 34-40.

16. Camacho, L., Sierra, C., Marcus, D., Guzmán, E., Campos, R., Von Bäer, D., and Trugo, L. (1991). Nutritional quality of lupine (*Lupinus albus* cv. Multolupa) as affected by lactic acid fermentation. *International Journal of Food Microbiology*. 14: 277-286.

17. Carbonaro, M., Maselli, P., & Nucara, A. (2015). Structural aspects of legume proteins and nutraceutical properties. *Food Research International*, *76*(P1), 19–30. https://doi.org/10.1016/j.foodres.2014.11.007

18. Carvajal-Larenas, F. E., Linnemann, A. R., Nout, M. J. R., Koziol, M., & van Boekel, M. A. J. S. (2015). *Lupinus mutabilis*: Composition, Uses, Toxicology, and Debittering. Critical *Reviews in Food Science and Nutrition*, 56(9), 1454–1487. https://doi.org/10.1080/10408398.2013.772089

19. Castañeda-González, L., Angarita-Pabón, S., Bernal, F., & Coy-Barrera, E. (2014). Comparison of conventional and ultrasound-assisted extraction of alkaloids from Lupinus mirabilis leaves. *Planta Medica*. 80(10).

20. Cavalieri, F., Ashokkumar, M., Grieser, F., & Caruso, F. (2008). Ultrasonic synthesis of stable, functional lysozyme microbubbles*. Langmuir*, 24(18), 10078-10083.

21. Cerletti, P., Fumagalli, A., & Venturin, D. (1978). Protein composition of seeds of *Lupinus albus*. *Journal of Food Science*, 43(5), 1409-1411.

22. Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics Sonochemistry*, *18*(5), 951–957. https://doi.org/10.1016/j.ultsonch.2010.12.016

23. Chango, A., Villaume, C., Bau, H., Schwertz, A., Nicolás, J., & Mejean, L. (1998). Effects of casein, sweet white lupin and sweet yellow lupin diet on cholesterol metabolism in rates. *Journal of Science and Agriculture*, 76, 303–309.

24. Cheftel, J. C., & Cheftel, H. (1989). *Introducción a la bioquímica y tecnología de los alimentos.* Zaragoza, España: Acribia.

25. Contribution of Proteins in Total Dietary Consumption. Available online: http://chartsbin.com/view/1157

26. Czubinski, J., Barciszewski, J., Gilski, M., Szpotkowski, K., Debski, J., Lampart-Szczapa, E., & Jaskolski, M. (2015). Structure of γ-conglutin: Insight into the quaternary structure of 7S basic globulins from legumes. *Acta Crystallographica Section D: Biological Crystallography*, *71*, 224–238. https://doi.org/10.1107/S1399004714025073

27. D'Agostina, A., Antonioni, C., Resta, D., Arnoldi, A., Bez, J., Knauf, U., & Wäsche, A. (2006). Optimization of a pilot-scale process for producing lupin protein isolates with valuable technological properties and minimum thermal damage. *Journal of Agricultural and Food Chemistry*, *54*(1), 92–98. https://doi.org/10.1021/jf0518094

28. Dagnia, S. G., Petterson, D. S., Bell, R. R., and Flanagan, F. V. (1992). Germination alters the chemical-composition and protein quality of lupin seeds. *Journal of the Science of Food and Agriculture*. 60: 419-423.

29. Dabhade, A. R., Mokashe, N. U., & Patil, U. K. (2016). Purification, characterization, and antimicrobial activity of nontoxic trypsin inhibitor from *Albizia amara* Boiv. *Process Biochemistry*, 51(5), 659-674.

30. Deshpande, S. S., & Damodaran, S. (1989). Structure-Digestibility Relationship of Legume 7S Proteins. *Journal of Food Science*, *54*(1), 108–113. https://doi.org/10.1111/j.1365-2621.1989.tb08579.x

31. Duranti, M., Consonni, A., Magni, C., Sessa, F., & Scarafoni, A. (2008). The major proteins of lupin seed: Characterization and molecular properties for use as functional and nutraceutical ingredients. *Trends in Food Science and Technology*, *19*(12), 624–633. https://doi.org/10.1016/j.tifs.2008.07.002

32. Duranti, M., Sessa, F., Scarafoni, A., Bellini, T., & Dallocchio, F. (2000). Thermal stabilities of lupin seed conglutin γ protomers and tetramers. *Journal of Agricultural and Food Chemistry*, *48*(4), 1118–1123. https://doi.org/10.1021/jf9907384

33. Eaton-Mordas, C. A., & Moore, K. G. (1978). Seed Glycoproteins of *Lupinus angustifolius*. *Phytochemistry*, *17*, 619–621.

34. El-Adawy, T. A., Rahma, E. H., El-Bedawey, A. A., & Gafar, A. F. (2001). Nutritional potential and functional properties of sweet and bitter lupin seed protein isolates. *Food Chemistry*, *74*(4), 455–462. https://doi.org/10.1016/S0308-8146(01)00163-7

35. Erbas, M., Certel, M., & Uslu, M. K. (2005). Food Chemistry Some chemical properties of white lupin seeds (*Lupinus albus* L.), *89*, 341–345. https://doi.org/10.1016/j.foodchem.2004.02.040.

36. FAO Statistics Division 2010, *Food Balance Sheets*, Food and Agriculture Organization of the United Nations, Rome, Italy, viewed 17th March 2011, <http://faostat.fao.org/>.

37. Fontanari, G. G., Batistuti, J. P., Cruz, R. J. Da, Saldiva, P. H. N., & Arêas, J. A. G. (2012). Cholesterol-lowering effect of whole lupin (*Lupinus albus*) seed and its protein isolate. *Food Chemistry*, *132*(3), 1521–1526. https://doi.org/10.1016/j.foodchem.2011.11.145

38. Food and Agriculture Organization of the United Nations (1994). Definition and classification of commodities: 4. Pulses and derived products. www.fao.org/es/faodef/fdef04e.htm

39. Food and Agriculture Organization of the United Nations. (2016). 2016 International Year of Pulses. Retrieved March 29, 2018, from http://www.fao.org/pulses-2016/about/en/

40. Gdala, J., & Buraczewska, L. (1996). Chemical composition and carbohydrate content of seeds from several lupin species. *Journal of Animal and Feed Sciences*, 5, 403–416.

41. Ghosh, S., Suri, D., & Uauy, R. (2013). Assessment of protein adequacy in developing countries: Quality matters. *Food and Nutrition Bulletin*, 34(2), 244–246. https://doi.org/10.1017/S0007114512002577

42. Gueguen, J. (1983). Legume seed protein extraction, processing, and end product characteristics. *Plant Foods for Human Nutrition*, *32*(3–4), 267–303. https://doi.org/10.1007/BF01091191

43. Hamerstrand, G. E., Black, L. T., & Glover, J. D. (1981). Main content area Trypsin inhibitors in soy products: modification of the standard analytical procedure. *Cereal Chemistry,* 58(1), 42-45.

44. Hettiarachchy, N. S., & Ziegler, G. R. (1994). *Protein functionality in food systems*. CRC Press.

45. Hsu, H.W., Vavak, D.L., Satterlee, L.D., & Miller, G.A. (1977). A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, 42(5), 1269-1273.

46. Industry & Investment NSW. (2011). *Growth & Development* (1st ed.). New South Wales, Australia: PROCROP.

47. International Food Policy Research Institute. *Nutrition Country Profile: México*; 2015. Available online: http://ebrary.ifpri.org/utils/getfile/collection/p15738coll2/id/129974/filename/130185

48. Jackson, M., & Mantsch, H. H. (1995). The use and misuse of FTIR spectroscopy in the determination of protein structure. *Critical Reviews in Biochemistry and Molecular Biology*, *30*(2), 95–120. https://doi.org/10.3109/10409239509085140

49. Karki, B., Lamsal, B. P., Jung, S., van Leeuwen, J. (Hans), Pometto, A. L., Grewell, D., & Khanal, S. K. (2010). Enhancing protein and sugar release from defatted soy flakes using ultrasound technology. Journal of Food Engineering, 96(2), 270–278. https://doi.org/10.1016/j.jfoodeng.2009.07.023

50. King, J., Aguirre, C., & De Pablo, S. (1985). Functional Properties of Lupin Protein Isolates (*Lupinus albus* cv *Multolupa*). *Journal of Food Science*, *50*(1), 82–87. https://doi.org/10.1111/j.1365-2621.1985.tb13282.x

51. Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, *56*(3), 242–258. https://doi.org/10.1007/BF02671468

52. Knorr, D., Zenker, M., Heinz, V., & Lee, D. U. (2004). Applications and potential of ultrasonics in food processing. *Trends in Food Science & Technology*, 15(5), 261-266.

53. Koleva, I., van Beek, T. A., Soffers, A. E. M. F., Dusemund, B., & Rietjens, I. M. C. M. (2012). Alkaloids in the human food chain - natural occurrence and possible adverse effects. *Molecular Nutrition and Food Research*, 56(1), 30–52. https://doi.org/10.1002/mnfr.201100165

54. Lampart-Szczapa, E., Konieczny, P., Nogala-Kałucka, M., Walczak, S., Kossowska, I., & Malinowska, M. (2006). Some functional properties of lupin proteins modified by lactic fermentation and extrusion. *Food Chemistry*, *96*(2), 290–296. https://doi.org/10.1016/j.foodchem.2005.02.031

55. Lee, G. D. (1953). Determining Reference Test Weight per Bushel Value of Grains. *U.S. Department of Agriculture*, *4*(6), 1–4.

56. Lee, M. J., Pate, J. S., Harris, D. J., & Atkins, C. A. (2006). Synthesis, transport and accumulation of quinolizidine alkaloids in *Lupinus albus* L. and *L. angustifolius* L. *Journal of experimental botany*, 58(5), 935-946.

57. Li, H., Pordesimo, L., & Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. *Food Research International*, *37*(7), 731–738. https://doi.org/10.1016/j.foodres.2004.02.016

58. Lqari, H., Vioque, J., Pedroche, J., & Millán, F. (2002). *Lupinus angustifolius* protein isolates: Chemical composition, functional properties and protein characterization. *Food Chemistry*, 76(3), 349–356. https://doi.org/10.1016/S0308-8146(01)00285-0

59. LUPINS Receival Standards Season 2017 / 2018.

60. Magni, C., Sessa, F., Accardo, E., Vanoni, M., Morazzoni, P., Scarafoni, A., & Duranti, M. (2004). Conglutin γ, a lupin seed protein, binds insulin in vitro and reduces plasma glucose

levels of hyperglycemic rats. *Journal of Nutritional Biochemistry*, *15*(11), 646–650. https://doi.org/10.1016/j.jnutbio.2004.06.009

61. Malgarini, G., & Hudson, B. J. F. (1980). Valutazione della caratteristichi chimiche e di alcune propieta funzionall di prodotti derivati dal lupino (*Lupinus albus*). *Riv. Ital. Sost. Grasse*, 57, 378.

62. Monteiro, M. R. P., Costa, A. B. P., Campos, S. F., Silva, M. R., da Silva, C. O., Martino, H. S. D., & Silvestre, M. P. C. (2014). Evaluation of the chemical composition, protein quality and digestibility of lupin (*Lupinus albus* and *Lupinus angustifolius*). *O Mundo da Saúde, São Paulo*, 38, 251-259.

63. Morad, M. M., EL-MAGOLI, S. B., & Aflfl, S. A. (1980). Macaroni supplemented with lupin and defatted soybean flours. *Journal of Food Science*, 45(2), 404-405.

64. Morsy, N., Rayan, A., Khaled, Y. (2015). Physico Chemical Properties, Antioxidant Activity, Phytochemicals and Sensory Evaluation of Rice-Based Extrudates Containing Dried *Corchorus olitorius* l. Leaves. *Journal of Food Processing & Technology*, *6*(1), 1–8. https://doi.org/10.4172/2157-7110.1000408

65. Muranyi, I. S., Volke, D., Hoffmann, R., Eisner, P., Herfellner, T., Brunnbauer, M., … Schweiggert-Weisz, U. (2016). Protein distribution in lupin protein isolates from *Lupinus angustifolius* L. prepared by various isolation techniques. *Food Chemistry*, *207*, 6–15. https://doi.org/10.1016/j.foodchem.2016.03.073

66. Muzquiz, M. (2000). *Alkaloids*. Gas Chromatography. 1938-1949

67. National Geographic. What the World Eats. Available Online: http://www.nationalgeographic.com/what-the-world-eats/

68. Ochoa-Rivas, A., Nava-Valdez, Y., Serna-Saldívar, S. O., & Chuck-Hernández, C. (2017). Microwave and ultrasound to enhance protein extraction from peanut flour under alkaline conditions: effects in yield and functional properties of protein isolates. *Food and Bioprocess Technology*, *10*(3), 543-555.

69. Ortiz, J. G. F., & Mukherjee, K. D. (1982). Extraction of alkaloids and oil from bitter lupin seed. *Journal of the American Oil Chemists Society*, *59*(5), 241-244.

70. Osborne, T. B., & Campbell, G. F. (1897). The proteids of lupin seeds. *Journal of the American Chemical Society*, *19*(6), 454-482.

71. Pastor-Cavada, E., Juan, R., Pastor, J. E., Alaiz, M., & Vioque, J. (2009). Analytical nutritional characteristics of seed proteins in six wild Lupinus species from Southern Spain. *Food Chemistry*, *117*(3), 466–469. https://doi.org/10.1016/j.foodchem.2009.04.039

72. Paulsson, M., & Dejmek, P. (1990). Thermal denaturation of whey proteins in mixtures with caseins studied by differential scanning calorimetry. *Journal of Dairy Science,* 73(3), 590-600.

73. Pozuelo, J. M., Lucas, M. M., De Lorenzo, C., Fernández-Pascual, M., Maldonado, S., & De Felipe, M. R. (2001). Immunolocalization of alkaloids and X-ray microanalysis of elements in lupin seeds. Protoplasma, 218(1-2), 104-111.

74. Protein sources by calories, value and portion size. Available Online: https://docs.google.com/spreadsheets/d/1PnUZ3NnjUxXRGoLcdMsV6fztsmon4AMSzYcnOCwlG2M

75. Pulse Australia. (2017). Australian Pulse Standards 2017 / 2018, (1).

76. Rodriguez, N. R., & Garlick, P. J. (2008). Introduction to Protein Summit 2.0: continued exploration of the impact of high-quality protein on optimal health. *American Journal of Clinical Nutrition*, 87, 1551-1553

77. Rozan, P., Lamghari, R., Linder, M., Villaume, C., Fanni, J., Parmentier, M., & Méjean, L. (1997). *In Vivo* and *in Vitro* Digestibility of Soybean, Lupine, and Rapeseed Meal Proteins after Various Technological Processes †. *Journal of Agricultural and Food Chemistry*, *45*(5), 1762–1769. https://doi.org/10.1021/jf960723v

78. Ruiz, L. P., Jr, & Hove, E. L. (1976). Conditions affecting production of protein isolate from lupin seed kernel. *Journal of the Science and Food Agriculture*, 27, 667–674.

79. Ruiz Jr, L. P., White, S. F., & Hove, E. L. (1977). The alkaloid content of sweet lupin seed used in feeding trials on pigs and rats. *Animal Feed Science and Technology*, 2(1), 59-66.

80. Salinas-Valdés, A., De la Rosa Millán, J., Serna-Saldívar, S. O., & Chuck-Hernández, C. (2015). Yield and Textural Characteristics of Panela Cheeses Produced with Dairy-

Vegetable Protein (Soybean or Peanut) Blends Supplemented with Transglutaminase. *Journal of food science,* 80(12).

81. Sambrook, J., & Russell, D. W. (2006). SDS-polyacrylamide gel electrophoresis of proteins. *CSH Protoc*, 1, 4.

82. Santos, C., Ferreira, R., & Teixeira, A. (1997). Seed Proteins of *Lupinus mutabilis*. *J. Agric. Food Chem.*, *45*(1994), 3821–3825. https://doi.org/10.1021/jf970075v

83. Sathe, S. K., Deshpande, S., & Salunkhe, D. K. (1982). Functional Properties of Lupin Seed (*Lupinus mutabilis*) Proteins and Protein Concentrates. *Journal of Food Science*, *47*, 491–497. https://doi.org/10.1111/j.1365-2621.1982.tb10110.x

84. Schwartzberg, H. G., & Chao, R. Y. (1982). Solute diffusivities in leaching processes. *Food Technology*, 36(2), 73-86.

85. Schwert, G. W., & Takenaka, Y. (1955). A spectrophotometric determination of trypsin and chymotrypsin. *Biochimica et biophysica acta*, 16, 570-575.

86. Serna-Saldivar, S. O. (2012). *Cereal grains: laboratory reference and procedures manual*. CRC Press.

87. Schoeneberger, H., Gross, R., Cremer, H. D., & Elmadfa, I. (1982). Composition and Protein Quality of *Lupinus Mutabilis*. *Journal of Nutrition*, *112*(November), 70–76.

88. Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., Duranti, M., Magni, C… & Arnoldi, A. (2004). Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *The Journal of nutrition*, 134(1), 18-23.

89. Sirtori, E., Resta, D., Brambilla, F., Zacherl, C., & Arnoldi, A. (2010). The effects of various processing conditions on a protein isolate from *Lupinus angustifolius*. *Food Chemistry*, *120*(2), 496–504. https://doi.org/10.1016/j.foodchem.2009.10.043

90. Smýkal, P., Vernoud, V., Blair, M. W., Soukup, A., & Thompson, R. D. (2014). The role of the testa during development and in establishment of dormancy of the legume seed. *Frontiers in Plant Science*, 5, 351.

91. Sosulski, F., & Youngs, C. G. (1979). Yield and functional properties of air-classified protein and starch fractions from eight legume flours. *Journal of the American Oil Chemists' Society*, *56*(3), 292–295. https://doi.org/10.1007/BF02671477

92. Sousa, I. M. N., Morgan, P. J., Mitchell, J. R., Harding, S. E., & Hill, S. E. (1996). Hydrodynamic characterization of lupin proteins solubility, intrinsic viscosity and molar mass. *Journal of Agricultural and Food Chemistry*, 44, 3018–3021.

93. Subcommittee on the Tenth Edition of the RDAs. (1989). *Recommended Dietary Allowances*. *Recommended Dietary Allowances: 10th Edition*. Washington, D.C.: National Academies Press. https://doi.org/10.17226/1349

94. Sujak, A., Kotlarz, A., & Strobel, W. (2006). Compositional and nutritional evaluation of several lupin seeds. *Food Chemistry*, *98*(4), 711–719. https://doi.org/10.1016/j.foodchem.2005.06.036

95. Taylor, M. J., & Richardson, T. (1980). Antioxidant activity of skim milk: effect of sonication. *Journal of Dairy Science*, 63(11), 1938-1942.

96. Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry - A review. *Innovative Food Science and Emerging Technologies*, 9(2), 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

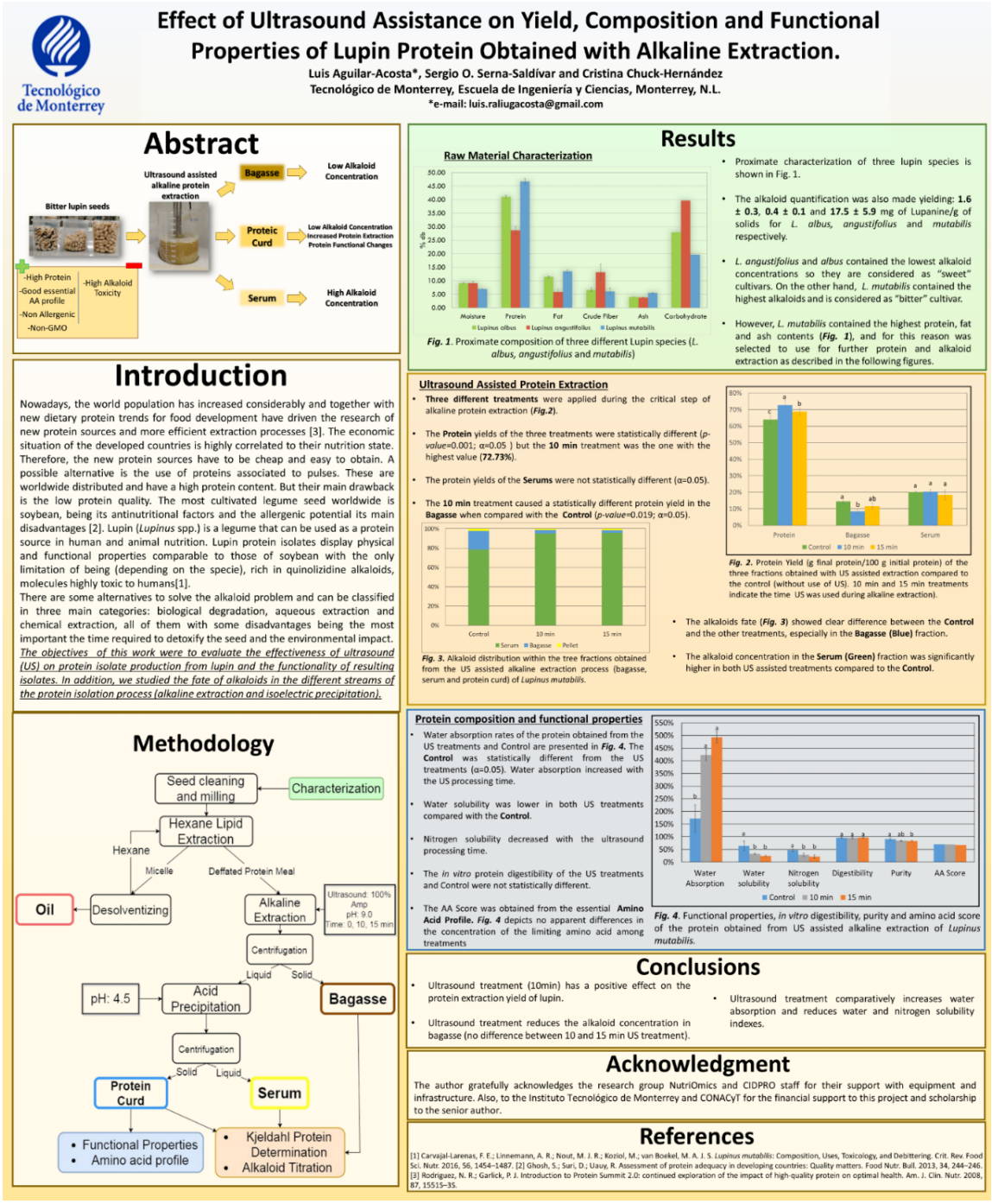
97. Wäsche, A., Müller, K., & Knauf, U. (2001). New processing of lupin protein isolates and functional properties. *Nahrung - Food*, *45*(6), 393–395. https://doi.org/10.1002/1521-3803

98. WHO/FAO/UNU Expert Consultation. (2007). Protein and amino acid requirements in human nutrition. *World Health Organization Technical Report Series*, (935), 1–265. https://doi.org/ISBN 92 4 120935 6

99. Wink, M. (1985). Metabolism of quinolizidine alkaloids in plants and cell suspension cultures: Induction and degradation. In *Primary and Secondary Metabolism of Plant Cell Cultures* (pp. 107-116). Springer, Berlin, Heidelberg.

8 Congress Presentations

8.1 AACCI Poster Presentation



Abstract

Ultrasound assisted alkaline protein extraction

Bitter lupin seeds

Bagasse → Low Alkaloid Concentration

Proteic Curd → Low Alkaloid Concentration, Increased Protein Extraction, Protein Functional Changes

Serum → High Alkaloid Concentration

-High Protein
-Good essential AA profile
-Non Allergenic
-Non-GMO

-High Alkaloid Toxicity

Introduction

Nowadays, the world population has increased considerably and together with new dietary protein trends for food development have driven the research of new protein sources and more efficient extraction processes [3]. The economic situation of the developed countries is highly correlated to their nutrition state. Therefore, the new protein sources have to be cheap and easy to obtain. A possible alternative is the use of proteins associated to pulses. These are worldwide distributed and have a high protein content. But their main drawback is the low protein quality. The most cultivated legume seed worldwide is soybean, being its antinutritional factors and the allergenic potential its main disadvantages [2]. Lupin (*Lupinus* spp.) is a legume that can be used as a protein source in human and animal nutrition. Lupin protein isolates display physical and functional properties comparable to those of soybean with the only limitation of being (depending on the specie), rich in quinolizidine alkaloids, molecules highly toxic to humans[1].

There are some alternatives to solve the alkaloid problem and can be classified in three main categories: biological degradation, aqueous extraction and chemical extraction, all of them with some disadvantages being the most important the time required to detoxify the seed and the environmental impact. *The objectives of this work were to evaluate the effectiveness of ultrasound (US) on protein isolate production from lupin and the functionality of resulting isolates. In addition, we studied the fate of alkaloids in the different streams of the protein isolation process (alkaline extraction and isoelectric precipitation).*

Methodology

Seed cleaning and milling ← Characterization

Hexane Lipid Extraction

Hexane

Micelle → Oil → Desolventizing

Deffated Protein Meal → Alkaline Extraction (Ultrasound: 100% Amp, pH: 9.0, Time: 0, 10, 15 min)

Centrifugation

Liquid → pH: 4.5 → Acid Precipitation

Solid → Bagasse

Centrifugation

Solid → Protein Curd

Liquid → Serum

- Functional Properties
- Amino acid profile

- Kjeldahl Protein Determination
- Alkaloid Titration

Results

Raw Material Characterization

Fig. 1. Proximate composition of three different Lupin species (*L. albus, angustifolius* and *mutabilis*)

- Proximate characterization of three lupin species is shown in Fig. 1.
- The alkaloid quantification was also made yielding: **1.6 ± 0.3**, **0.4 ± 0.1** and **17.5 ± 5.9** mg of Lupanine/g of solids for *L. albus, angustifolius* and *mutabilis* respectively.
- *L. angustifolius* and *albus* contained the lowest alkaloid concentrations so they are considered as "sweet" cultivars. On the other hand, *L. mutabilis* contained the highest alkaloids and is considered as "bitter" cultivar.
- However, *L. mutabilis* contained the highest protein, fat and ash contents (***Fig. 1***), and for this reason was selected to use for further protein and alkaloid extraction as described in the following figures.

Ultrasound Assisted Protein Extraction

- **Three different treatments** were applied during the critical step of alkaline protein extraction (***Fig.2***).
- The **Protein** yields of the three treatments were statistically different (*p-value*=0.001; α=0.05) but the **10 min** treatment was the one with the highest value (**72.73%**).
- The protein yields of the **Serums** were not statistically different (α=0.05).
- The **10 min** treatment caused a statistically different protein yield in the **Bagasse** when compared with the **Control** (*p-value*=0.019; α=0.05).

Fig. 2. Protein Yield (g final protein/100 g initial protein) of the three fractions obtained with US assisted extraction compared to the control (without use of US). 10 min and 15 min treatments indicate the time US was used during alkaline extraction).

Fig. 3. Alkaloid distribution within the tree fractions obtained from the US assisted alkaline extraction process (bagasse, serum and protein curd) of *Lupinus mutabilis*.

- The alkaloids fate (***Fig. 3***) showed clear difference between the **Control** and the other treatments, especially in the **Bagasse (Blue)** fraction.
- The alkaloid concentration in the **Serum (Green)** fraction was significantly higher in both US assisted treatments compared to the **Control**.

Protein composition and functional properties

- Water absorption rates of the protein obtained from the US treatments and Control are presented in ***Fig. 4.*** The **Control** was statistically different from the US treatments (α=0.05). Water absorption increased with the US processing time.
- Water solubility was lower in both US treatments compared with the **Control**.
- Nitrogen solubility decreased with the ultrasound processing time.
- The *in vitro* protein digestibility of the US treatments and Control were not statistically different.
- The AA Score was obtained from the essential **Amino Acid Profile.** ***Fig. 4*** depicts no apparent differences in the concentration of the limiting amino acid among treatments

Fig. 4. Functional properties, *in vitro* digestibility, purity and amino acid score of the protein obtained from US assisted alkaline extraction of *Lupinus mutabilis*.

Conclusions

- Ultrasound treatment (10min) has a positive effect on the protein extraction yield of lupin.
- Ultrasound treatment reduces the alkaloid concentration in bagasse (no difference between 10 and 15 min US treatment).
- Ultrasound treatment comparatively increases water absorption and reduces water and nitrogen solubility indexes.

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References

[1] Carvajal-Larenas, F. E.; Linnemann, A. R.; Nout, M. J. R.; Koziol, M.; van Boekel, M. A. J. S. *Lupinus mutabilis*: Composition, Uses, Toxicology, and Debittering. Crit. Rev. Food Sci. Nutr. 2016, 56, 1454–1487. [2] Ghosh, S.; Suri, D.; Uauy, R. Assessment of protein adequacy in developing countries: Quality matters. Food Nutr. Bull. 2013, 34, 244–246. [3] Rodriguez, N. R.; Garlick, P. J. Introduction to Protein Summit 2.0: continued exploration of the impact of high-quality protein on optimal health. Am. J. Clin. Nutr. 2008, 87, 1551S–3S.

8.2 AMIDIQ Oral Presentation

In the framework of the XXXIX Encuentro Nacional de la AMIDIQ 2018 carried out in San José del Cabo, Baja California Sur in May 1 to 4 of 2018, an oral presentation work titled “Evaluación del potencial alimenticio de la fracción protéica de tres especies de lupino” was accepted and presented.

AMIDIQ
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La Academia Mexicana de Investigación y Docencia en Ingeniería Química (AMIDIQ)

Otorga el presente

RECONOCIMIENTO

A:

Luis Alberto Aguilar Acosta, Sergio Román Othon Serna Saldívar, Cristina Elizabeth Chuck Hernández

Por la presentación del trabajo:

EVALUACIÓN DEL POTENCIAL ALIMENTICIO DE LA FRACCIÓN PROTEICA DE TRES ESPECIES DE LUPINO

ID: 476

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Campus Guanajuato | División de Ciencias Naturales y Exactas Departamento de Ingeniería Química

9 Curriculum Vitae

Luis Alberto Aguilar Acosta was born in Río Bravo, Tamaulipas, México in November 25, 1992. In June 2016 he graduated from the Universidad Autónoma de Nuevo León with the title of Químico Farmacéutico Biólogo. In August of the same year he started his M. Sc of Biotechnology studies in the Instituto Tecnológico y de Estudios Superiores de Monterrey. He focused his research on the project of this thesis under the advice and supervision of Cristina Chuck Hernández Ph. D.

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