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Debittering of *Lupinus albus L.* using subcritical water extraction

Dissertation submitted in partial fulfillment
of the requirements for the degree of

Master of Science in
Chemical and Biochemical Engineering

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

March, 2021

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To my family.

Acknowledgements

Firstly, I want to thank Departamento de Química, FCT-UNL, and *Simbeja*. None of this work would be possible without the help and orientation of my adviser, Professor Susana Barreiros, and my co-advisor, Professor Pedro Simões. Also, I want to thank the collaboration and help that Professora Ana Lourenço gave me in the organics department and my colleague Diana for all the support and lovely words in the worst times.

To Carolina, thank you for the support throughout my work and for making possible your work followthrough.

I want to sincerely thank everyone from the REQUIMTE/LAQV for all the patience, kindness, and knowledge they gave me. To Bruno, who guided me at the beginning of the laboratory, taught me so much, and always pushed me forward even when it got a little frustrating. To Marta, for continuously being there for me, always helping me when I most needed it, and for the kind words throughout these last months. And to everyone else, Professor Alexandre, Tânia, Ângelo, Rita Craveiro, Rodrigo, Sara, Liane, Rita Gameiro, and Mónica thank you.

I sincerely want to thank Luz, from Laboratório de Análises, for all the patience, great hallway talks, and help even when it was not her duty.

I want to thank my friends for putting up with me even when I was hard to handle, always believing in me, and never giving up on me. Pedro, you were my most significant support during these last few months, and I deeply thank you for all of it.

Last but not least, I want to give my parents and sister the biggest thank you. It cannot be put into words the support and love I have from you.

Abstract

White lupin, *Lupinus albus L.*, is a legume used for human and animal feed. It usually grows and is cultivated in the Mediterranean and the Middle East. It is rich in proteins, fibers, and carbohydrates and can replace soy consumption while decreasing soy imports in Europe. White lupin has a high content of alkaloids, which gives it a bitter taste while making it toxic to humans and animals. Lupin requires a pre-treatment that consists of cooking the lupin, followed by successive washes with water.

The present work studied an alternative green method of extracting lupin alkaloids, intending to reduce water consumption. Extraction with subcritical water was the method chosen, in a batch reactor, with pressurized water to remain in a liquid state. The four parameters studied were temperature (between 100 and 140 ºC), solvent-to-solid ratio (20:1 and 40:1), simple extraction, or two successive extractions, and particle size. The white lupin seeds were crushed (particle sizes between 0.5 and 1 mm) and chemically characterized, presenting 31.5% protein, 37% carbohydrates, and 9% lipids. In the extraction studies with the lupin powder, the temperature was the parameter with the greatest impact on the remaining alkaloids content, followed by the solvent-to-solid ratio.

The best result was obtained at 100 ºC with a solvent-to-solid ratio of 20:1, leading to the extraction of 71% lupanine from the lupin. Other components were co-extracted, namely carbohydrates (7 g/100 g of lupin) and protein (5 g/100 g of lupin). At these conditions, 23.7 g/100 g lupin of protein out of 31.5 g/100 g lupin remained in the lupin residue. These extraction conditions also allowed 27.8 g/100 g lupin of carbohydrates out of 37.0 g/100 g lupin to remain in the matrix. Successive extractions at 100 ºC and a 20:1 solvent-to-solid ratio with both lupin powder and whole lupin seeds showed that the second extraction barely enhanced the extraction yield of lupanine.

Keywords: White lupin, *Lupinus albus L.*, Debittering, Alkaloids, Lupanine, Subcritical Water.

Resumo

O tremoço-branco, *Lupinus albus L.*, é uma leguminosa usada para alimentação humana e animal. Geralmente, cresce e é cultivado no Mediterrâneo e no Médio Oriente. Rico em proteínas, fibras e hidratos de carbono, pode substituir o consumo de soja enquanto diminui as importações desta na Europa. O tremoço-branco tem alto teor de alcaloides, o que lhe confere um sabor amargo, ao mesmo tempo que o torna tóxico para o consumo humano e animal. O tremoço requer um pré-tratamento que consiste em cozer o tremoço, seguido de lavagens sucessivas com água.

O presente trabalho estudou um método alternativo verde de extração de alcaloides do tremoço, com o objetivo de reduzir o consumo de água. A extração com água subcrítica foi o método escolhido, em reator descontínuo, com a água pressurizada para a fazer permanecer no estado líquido. Os quatro parâmetros estudados foram temperatura (entre 100 e 140 ºC), razão sólido-solvente (1:20 e 1:40), extração simples ou duas extrações sucessivas e tamanho de partícula. As sementes de tremoço-branco foram trituradas (granulometria entre 0.5 e 1 mm) e caracterizadas quimicamente, apresentando 31.5% de proteína, 37% de hidratos de carbono e 9% de lípidos. Nos estudos de extração com a farinha de tremoço, a temperatura foi o parâmetro com maior impacto no teor de alcaloides remanescentes, seguido pela razão sólido-solvente.

O melhor resultado foi obtido a 100 ºC com razão sólido-solvente de 1:20, levando à extração de 71% da lupanina do tremoço. Outros componentes foram extraídos em simultâneo, nomeadamente hidratos de carbono (7 g/100 g de tremoço) e proteínas (5 g/100 g de tremoço). Nestas condições, 23.7 g/100 g de tremoço de proteína do total de 31.5 g/100 g de tremoço permaneceram no resíduo de tremoço. Essas condições de extração também permitiram que 27.8 g/100 g de hidratos de carbono do total de 37.0 g/100 g de tremoço permanecessem na matriz. Extrações sucessivas a 100 ºC e uma razão sólido-solvente de 1:20 com a farinha de tremoço e o tremoço inteiro mostraram que a segunda extração pouco melhorou o rendimento de extração do tremoço.

Palavras-chave: Tremoço-branco, *Lupinus albus L.*, Processo de Lavagem, Alcaloides, Lupanina, Água Subcrítica.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

Lupin is a legume that has been used as human and animal nutrition for many years. It is known in different cultures but mainly in the Mediterranean area and the Middle East. It has many health advantages, great nutritional value, and it also is excellent for soils.

White lupin has been used for consumption and medical purposes for many years. It is considered an excellent substitute for soybean in Europe, as it is a protein source homegrown crop and can decrease carbon footprint due to soy importation. Also, due to the world supply-demand by the increasing world population, soybean agriculture has been responsible for many deforestations and natural habitat displacement, mainly in South America.[1], [2]

Lupin seeds are considered toxic due to alkaloids' presence, and it is imperative to remove them before consumption. This process is known for its extensive water and time consumption. After debittering lupin seeds are regarded as an important food source due to their protein, fiber, carbohydrates, and oil contents.

The introduction chapter refers to concepts and previous works related to the theme of this thesis.

The first subchapter gives insight into the types of lupins, the particular importance of *Lupinus albus L.*, its lignocellulosic composition, nutritional values, food applications, and health benefits. It is mentioned the importance of the use of lupin as a soy substitute.

The second subchapter introduces alkaloids, with a major reference to quinolizidine alkaloids (QAs) and their toxicity. It also includes various ways to debitter lupin, making it safer for consumption while showing their advantages/disadvantages.

The last section discusses subcritical water extraction as an alternative green method and the possibility of the debittering of *L. albus L.* using subcritical water. This will be the subject of study in this work as a possible solution to the massive waste of water, making this method advantageous because of its low environmental impact.

1.1 White lupin (*Lupinus albus L.*)

Lupin, a common name for the genus *Lupinus* plants, is a non-starch legume, and a source of protein and dietary fiber.[3] *L. albus L.* belongs to the *Leguminosae* family, and due to its high nutritional value, namely high protein and oil content, it is comparable to soybean. It is used as human and animal feed, as well as green manure. As a crop, it can fight soil erosion and improve soil quality by accumulating nitrogen and phosphorous (P).[4]–[7]

The seeds of white lupin contain 33 to 47% protein, and the oil content varies from 6 to 13%, mostly including a high concentration of polyunsaturated fatty acids.[8] The seed casing, being 18% of seed weight, is an important source of polysaccharides. These are located in the cell walls, and their main components are sugar units of galactose and arabinose, as well as uronic acid. The storage sugars, mainly fibers, have a great water holding capacity and nutritional values. These fibers can be used in the food industry.[6], [8], [9]

Lupin seed contains substantial quantities of health-promoting agents such as phenolic compounds, tocopherols, phytosterols, and squalene.[3] Phenolic compounds, such as flavones, isoflavones, and phenolic acids, are bioactive compounds present in lupin seeds responsible for their antioxidant activity. Also, antibacterial and antimutagenic activities of these phenolic compounds have been confirmed. However, phenolic compounds of lupin seeds have not yet been studied comprehensively.[3]

The lupin production is lower than that of many other protein plant ingredients being 2 million tons produced per year.[4], [10] The lupin market has great potential in the Western European countries, both on-farm and industrial use.[8]

Since the beginning of the century, lupin seeds from *L. albus. L.* have been introduced in the European markets since they wildly grow there. The seeds, high in quinolizidine alkaloids content, have traditionally been consumed as a snack food in Southern Europe and the Middle East, after debittering, to remove the alkaloids.[3], [4]

Worldwide, there are ca. 450 species of lupin, all containing QAs. About 12 of these species occur in Asia, Africa, the American continent, and Europe, where only a few species are considered edible, such as *Lupinus albus L.* (white lupin) and *Lupinus angustifolius L.* (narrow-leaved lupin).[4], [6], [11]

Just four of these species are domesticated and relevant for agriculture. *L. albus L.* is one of these. It has been known since the Late Neolithic period, probably cultivated during the Bronze Age in Greece, Cyprus, and Egypt.[6] In addition to *Lupinus albus L.*, used in Europe, edible and domesticated lupin species include narrow-leaved lupin, *Lupinus angustifolius L.*, from Australia, the pearl lupin, *Lupinus mutabilis Sweet L.*, consumed in South America, and yellow lupin, *Lupinus luteus L.*

Until now, the majority of the food products have been based on *L. albus L.* An increase in *L. angustifolius* use is being seen because of the resistance of this species to anthracnose.[12] All of these are enlisted in the EU Novel Food Catalogue as of 2008.[4]

Lupin seeds from white lupin are used as feed for pigs, beef cattle, dairy cows, chicken,

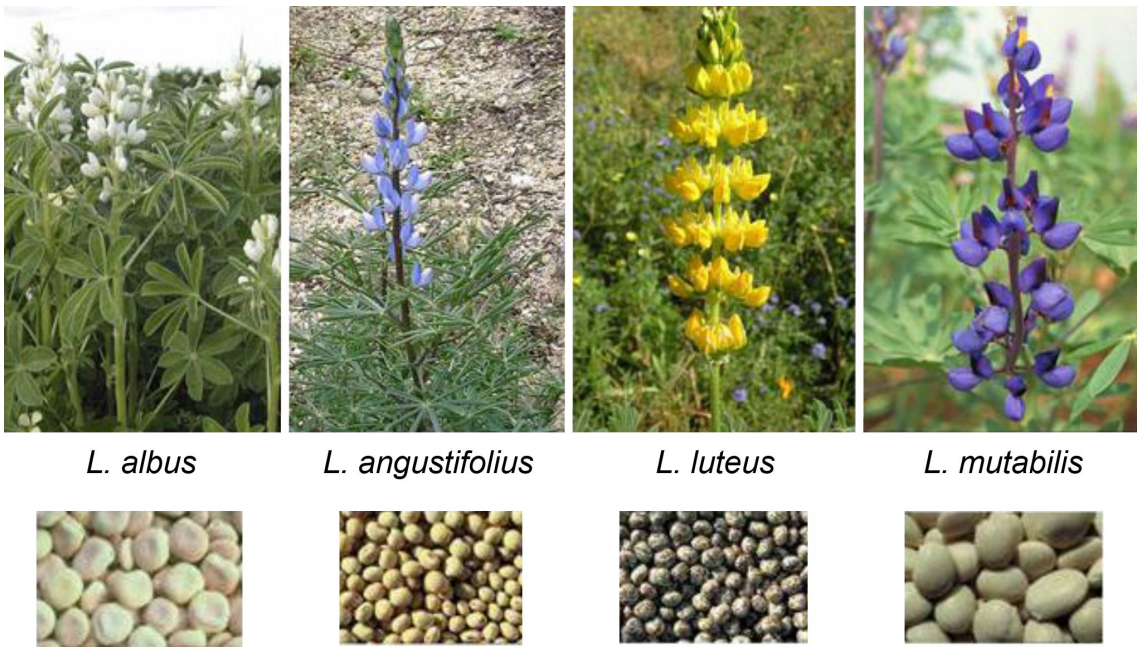


Figure 1.1: The four edible types of lupin, their plants, and seeds morphology.[13]

and sheep.[4] The four above mentioned edible species of lupin have also been used in aquaculture.[14] Plant-derived proteins have been used in aquafeeds for decades due to their nutritional values suitable for fish, being readily available on the global market, and their competitive prices compared to fish meal. The most used plant is soybean meal and its derivatives.[15] Many companies in Europe have shown interest in white lupin for its protein and fiber content. Besides the high protein content of lupin, there lies the significant nutritional value in fatty acid content, tocopherols, and low content of antinutritional factors, which are species that can inhibit the absorption of many essential nutrients.[12] Many food products have been produced and commercialized, such as pasta, bread, biscuits, muffins, crackers, etc., and also meat substitutes. An alternative to soybean food products is using lupin to substitute soybean sausages, ground beef, soy milk, and Asian fermented foods like *tempe* and *miso*.[4], [5], [11], [16] White lupin can substitute animal and plant proteins or even cereal flours.[5], [11]

In the last decades, genotypes of *Lupinus albus* and *Lupinus angustifolius* low on alkaloids have been selected and commercialized. Their genotypes present less than 1500 mg alkaloids/kg lupin (semi-bitter), semi-sweet varieties containing less than 500 mg alkaloids/kg lupin, or even sweet accounting for less than 200 mg alkaloids/kg lupin.[12] Bitter varieties of lupin have a total content of equal or more than 10,000 mg alkaloids/kg lupin.[4]

In the semi-bitter and sweet cultivars, lupanine is the most abundant alkaloid. However, it is complemented by generous amounts of albine, angustifoline, 13α-hydroxylupanine, and 13α-angeloyloxy-lupanine having, in some cases, trace amounts of α-isolupanine

and 13α-tigloyloxylupanine. In these seeds, the biosynthetic cut of the lupanine pathway prefers the production of minor alkaloids. In the bitter seeds, as in white lupin seeds, the percentage of lupanine is so high that it may reach almost 100%.[12]

Due to the presence of QAs, *Lupinus* acquire a bitter taste that must be removed before consumption.[5] Getting it ready for human consumption implies further treatment to remove the excess of QAs. This way, wild lupin seeds require pretreatment to ensure food safety while ensuring its antioxidant activity intake.[3]

1.1.1 Lignocellulosic matter in agro-food

Agro-food comprises lignocellulose, which is a compact, partially crystalline matrix. It consists of cellulose, hemicellulose, and lignin.[17] Lignocellulosic feedstocks are also a source of protein and lipids, which together with a high content in dietary fiber led to their integration into animal feed.[18]

Cellulose is the principal component of plant cell walls. It is a linear homopolymer of glucose units. It is mostly crystalline, for it shows excellent resistance to enzymatic hydrolysis. As for hemicellulose, it has a heterogeneous and easily hydrolyzable structure. This heteropolymer is made of sugar units of xylose, mannose, glucose, and galactose. Its composition differs from plant to plant, and it is what connects lignin and cellulose fibers. Lignin is an aromatic heteropolymer containing three building blocks. It has developed resistance to chemical and enzymatic degradation, compared to hemicellulose and cellulose.[17], [19]

Biomass can be fractionated into different useful streams to fully utilize its carbon content instead of being completely degraded as seen in composting or use in animal feed. There are many applications of the constituents of biomass [18]:

- Prebiotics originated from the hemicellulose oligomers,
- Upscale materials based on cellulose fibers and lignin,
- Edible oils,
- Meat protein alternatives, particularly in our study.

The most used techniques to depolymerize/hydrolyze lignocellulosic structures are acid, alkaline, or enzymatic treatment. Still, all of them show setbacks in sugar degradation, corrosion problems, long processing times, and cost increase.[18]

1.1.2 Nutritional importance

According to Carmali [5], *Lupinus* usually contains 36-52% protein, 5-20% oil, and 30-40% fiber. QAs quantity varies from 1.9% to 2.7%, depending on the plant sample. The oil composition is similar to most edible oils, but *L. albus L.* presents oils derived from erucic acid.

Ingredient	Dry matter (DM)	Protein	Lipid	Ash	CHO (carbohydrate)	Gross energy (MJ/kg DM)
Anchovetta (fish) meal	926	668	75	186	4	21.8
Krill meal	917	687	129	117	0	21.5
Narrow-leaf lupin kernel meal[a]	910	402	70	33	497	20.7
Yellow lupin kernel meal[b]	937	496	67	41	410	21.0
White lupin kernel meal[c]	922	455	137	36	405	23.1
Lupin protein concentrate	937	716	81	25	115	23.4
Soy protein concentrate	920	871		4	14	66.0
Field pea meal	903	257	12	28	703	18.6
Expeller canola meal	938	380	136	59	425	22.0
SE canola meal	889	432	30	74	464	19.7
SE soybean meal	890	503	12	88	397	19.2
Full-fat soybean meal	909	416	196	53	336	23.4
Poultry offal meal	919	559	109	147	104	21.3
Blood meal	887	844	1	16	0	20.4
Meat and bone meal	950	560	88	324	27	19.3
Wheat	905	117	20	9	760	16.7
Wheat gluten	924	656	40	7	133	21.2

[a] *L. angustifolius*.
[b] *L. luteus*.
[c] *L. albus*.
SE, solvent extracted.
Source: Data derived from Glencross (2014), unpublished.

Figure 1.2: Chemical composition of different species of protein plants. All values g/kg DM (dry matter).[20]

According to Figure 1.2, the white lupin kernel meal is characterized by having ca. 8% humidity, 45% protein, 14% lipids, and carbohydrates account for around 40%. The amount of lipids in protein plants differs according to the source. Full-fat soybean meal has a more significant lipid content than the other protein plant meals. Most lupin kernels have lower content in lipids (<100 g/kg) than other plants, like rapeseed.

1.1.3 Soybean substitute

Interest in plant foods has increased due to their environmental sustainability and great health welfares. Other than soybeans, which still are the most used and cheapest legumes available, others have gained importance in preparing dairy-free, gluten-free, and meat-free products.[12]

One of the largest plant protein meals and feed ingredient resources in the world are soybean meals. In 2011, around 177 million tons per year of soybean were available.[20]

In the 2019-2020 period, world production accounted for about 337 million tons.[21]

With the increase in the exploitation of human and animal feed of soybean derivatives, the global market price has increased from 2000 to 2018. On the other hand, the global soybean production increase has made deforestation an issue and has displaced other food production systems in tropical and sub-tropic nations.[15]

Worldwide, soybean is the most produced and used plant protein source in aquaculture diet formulations for various species. Nevertheless, other plant protein sources like lupins, field peas, and rapeseed meals have shown potential in aquaculture feed ingredients.[20]

Even in colder temperate climates like northern Europe, lupin crops have grown in importance in regional agriculture. With this lupin meal potential, the dependence on imported soybean meal in aquaculture decreases. White lupin can substitute 25% of soy protein concentrate in carp diets. This substitution still allows for growth performance, body composition, feed utilization, and physiological status. This way, the use of lupin in aquafeeds meals can help prevent the global soybean demand in aquacultures and, therefore, decrease the environmental impact due to its production like deforestation. Consequently, the greenhouse gas emission drops, and the environmental impact such as deforestation diminishes as well.[15]

Even though lupin can be seen as an alternative to soybean in many ways – high protein content, digestible protein, soluble and non-soluble non-starch polysaccharides, which prevent digestion [15], and energy values – lupin contains toxic and bitter QAs and this is one of the reasons why soy production is still so much higher. But by removing such QAs by boiling and soaking in water, its use has been increasing.[12]

In Tunisia, and Europe in general, there is a significant dependence on imported soybean, and it is imperative to find an alternative in local sources. The *Lupinus* species can be a great alternative.[6], [22]

1.1.4 Cultivars

There are no significant differences between wild and cultivated lupin seeds, while there are some differences between the wild genotypes. The phenolic compound content of both wild and cultivated *L. albus L.* seed extracts was shown to be similar, as well as protein content.[3]

To accomplish some cellular processes like energy metabolism and signal transduction, phosphorous is an essential element. Even though it is abundant in the farm fields, it is often unavailable for crops as a result of its low solubility, making this a significant constraint in the production of the crop. Some plants have developed mechanisms of adaption by altering their roots architecture or biochemical processes. *L. albus L.* is one of the plant species that is P-deficient tolerant, and it is adaptive to a P stress environment. While there is P deficiency, the roots of white lupin are developed laterally with hairs, making them increase in surface area and dissolve the phosphorous better.[23]–[25]

When white lupin is intercropped with wheat, it has been seen improvements in wheat shoot growth and shoot phosphorous uptake without affecting its development and nutrition. This can be explained by the aptitude of the lupins to form cluster roots due to phosphorous starvation.[6] These cultivars seen their fertilizer utilization reduced due to the lupin's ability to undertake nitrogen from the air because of the symbiosis between nitrogen-fixing bacteria on the roots and lupins.[4]

The nutritional value of lupin depends greatly on the origin, species, regions, and processing steps.[14]

1.1.5 Health benefits

A wide range of effects is seen in one or more components of the living tissues of bioactive compounds. The latter is naturally sourced like plants, algae, foods, by-products, or even synthetic products. Bioactive compounds, such as phenolics, have shown a major positive impact on human health and wellbeing.[26], [27]

In vivo and *in vitro* studies revealed that adding lupin seeds powder or lupin-based processed foods to diets reduces the threat of diabetes, obesity, bowel dysfunction, and hypertension. Significant differences can be observed among wild and cultivated edible plant varieties. With this, phenolic compound quantities and antioxidant potency vary with species and cultivar of lupin.[3]

Lupin has been investigated because of its chemical composition and potential in preventing lipid disorders. It is one of the highest protein content plants and a good source of fibers, making it suitable for consumption.[28]

Fontanari [28] studied the whole lupin and protein isolate potential in reducing total cholesterol and plasma non-HDL cholesterol. The protein content of the lupin has a significant impact on this reduction. They also showed a hepatoprotective effect, inhibiting the accumulation of fats in the organism.

Experiments on animals, or clinical trials, have demonstrated that lupin has great health potential:

- Anti-atherogenic,
- Hypotensive,
- Hypoglycaemic activity,
- Hypocholesteroaemic.

1.2 Alkaloids

Murphy [29] defined alkaloids as: "Any of the complex nitrogen-containing heterocyclic organic compounds, mainly of plant origin, with potent pharmacological properties in

humans". Due to environmental and genetic diversities, the alkaloid composition of *Lupinus* varies.[5]

For some plants to defend themselves against predator attacks, they develop some biologically active substances.[30] These substances are a chemical defense mechanism for plants. They can be found in different plant species. They are diverse and vary in concentration from plant to plant.[20], [31] There are several bioactive substances with likely toxic effects on humans, mainly naming the quinolizidine alkaloids present in lupin seeds.[4]

Alkaloids are a kind of antinutritional factor. They can be bicyclic, tricyclic, or tetracyclic derivatives of the quinolizidine. The alkaloids are more frequently found in peas and beans, but high levels are found in some lupins. In terms of commercial values, the number of alkaloids present must never exceed 200 mg/kg.[20]

There are many groups of alkaloids, for instance, pyridine, quinolone, isoquinoline, indole, quinazoline, and many more. They can be divided into groups: true-alkaloids, proto-alkaloids, pseudo-alkaloids, peptides, cyclopeptide alkaloids, and more.

Even though most alkaloids are poisonous in large quantities or are pharmacologically active, they are often consumed daily in foods. When consuming cocoa beans, coffee, and tea leaves, there is ingestion of alkaloids like theophylline, caffeine, and theobromine.[31]

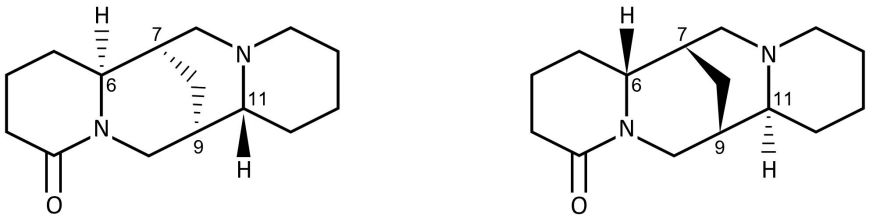
Alkaloids have been explored for their pharmacological properties. Around 10% of plant species produce alkaloids as a secondary metabolite wherein the primary function is to defend against pathogens and herbivores. And although other organisms can make alkaloids, these species are often isolated from plants and are useful as pure compounds.[29], [30]

Different approaches are made to make sweet *Lupinus*. There is a bacterial removal process in the literature to remove the alkaloids but it has not been implemented in the industry.[32] Another way is the cultivation of low-alkaloid content species, which makes the debittering process obsolete. However, this approach is more likely to happen in places where *Lupinus* is not endemic, such as Australia and Eastern Europe. As the sweetness of the *Lupinus* is a recessive genetic characteristic, this approach is not viable for areas where it is spontaneous.[5]

1.2.1 Quinolizidine alkaloids

Lupanine is a quinolizidine alkaloid with a symmetric structure, making it a good starting material for other alkaloids' semisynthesis. It is also presented as a high blood pressure reducer and hyperglycemia agent. This way, the recovery of lupanine from the leaching waters is an excellent area of interest.

L. albus L. leaching waters are full of QAs, mostly lupanine, an alkaloid containing a quinolizidine nucleus. Biosynthesis of this QA is still not fully implemented due to the number of steps that have to be done for the nucleus formation, and the overall yield is too low.[5]



(-)-(6*S*,7*R*,9*R*,11*R*)-lupanine (+)-(6*R*,7*S*,9*S*,11*S*)-lupanine

Figure 1.3: Enantiomeric forms of Lupanine.[5]

Alkaloid biosynthesis has gained a lot of interest since plants naturally produce great complex mixtures of alkaloids. The desirable types are in low concentrations making the production of the desirable alkaloids expensive. Alkaloids have been extracted and isolated from crude extracts from plants for many millennia as the folk medications' primary goal. However, pure and isolated alkaloids have been produced since the twentieth century as fine chemicals. Such alkaloids represent high complexity molecules, making them very hard to create by applying chemical synthesis, making crude plant extraction the most economical strategy.

With the improvement of plants' genetic manipulation, studies have been done focusing on the alkaloid engineering biosynthesis. This is made by generating transgenic plants or cell lines that can overproduce specific alkaloids or even inhibit undesirable ones' production.[29]

1.2.2 Quinolizidine alkaloids toxicity and debittering

The toxicity of lupin affects humans, especially children. The LD_{50} values for the QAs ingestion in rats go from 1,700 to 2,300 mg/kg of body weight, while in humans, according to toxicity observed, it is estimated that this number is two orders of magnitude lower. Toxicity studies in animals have shown decreased body weight and reduction of food intake.

The ingestion of lupin with alkaloids caused American calves deficiency during pregnancy if ingested by the mother and in Australia's lambs. Lupin used as food can cause allergies, mycotoxins, and high manganese content, which is not linked to alkaloids.[4]

Consuming white lupin with alkaloids can lead to respiratory problems and liver damage.[33]

It is imperative to remove the QAs before safely consuming the lupin. This is possible by cooking the seeds, following soaking that includes daily renewal and water disposal. It has been introduced varieties of sweet lupin in Europe since the '90s for consumption, but there are issues dealing with these kinds of low QAs lupin.[4] Most of the lupin alkaloids are water-soluble. Traditionally, they are soaked in running water, brined, or scalded to decrease alkaloids' content from 0.5 - 4% to 0.04%. When this legume is no longer

considered toxic, it is a great waste of potable water.[33] Usually, the QAs content of the wild bitter genotypes is above the consumption limit, being up to 2.7 mg/100 mg of dry matter.[3]

There are different methods to debitter lupin seeds, such as cooking/soaking with aqueous, acid, and alkaline thermal processes. Cooking and soaking in water have been the most used and effective process. For this purpose, lupin seeds were boiled in water with a 3:1 (water:seed) ratio for 75 minutes. After boiling the seeds, three possible treatments can proceed (Table 1.1) which shows the tremendous effort and amount of water that is put into the traditional debittering process.[11], [34]

Table 1.1: Traditional debittering processes of lupin.

Traditional debittering processes			
Procedure	**Temperature (°C)**	**Time (h)**	**Renewal (h)**
Standard aqueous	25 ºC	144	12
Thermal aqueous	55-60 ºC	144	12
0.5% sodium bicarbonate solution	25 ºC	144	12

Even though sweet lupin has been made to prevent the content of alkaloids in the plant, it is proven that they infer an important resistance to pathogens, which makes the plant more susceptible to diseases, and to make it successful cultivation it is imperative the use of pesticides. To not cause environmental problems, pesticides are frowned upon since they pollute the waters and soils.[3], [35]

1.2.3 Alkaloids extraction and quantification methods

In the laboratory, the method of extraction of alkaloids was first studied by Muzquiz [36] in 1993 and reapplied by Muzquiz [35] in 1994. The lupin seed was finely milled, homogenized with trichloroacetic acid. After centrifugation, sodium hydroxide was added. The alkaloids were extracted with dichloromethane, dried off, dissolved in methanol, and added codeine solution.

In 2007, the extraction was replicated with some changes in the solvents used. First, the material was homogenized in hydrochloric acid. After centrifugation, the pH was adjusted with ammonium hydroxide. A solid extraction was carried out using an Isolute column. The alkaloids were then eluted with dichloromethane, and the solvent evaporated in a vacuum. The analysis of the quantification of the alkaloids was done with a GC-MS (capillary gas chromatography-mass spectrometry) apparatus.[37] These methods use non-environmental friendly organic solvents, take a long time to extract, and there can be found organic solvent residues in the desired products.[38]

There are analytical methods to quantify alkaloids, such as TLC (Thin Layer Chromatography), gas chromatography equipped with flame ionization detector (GC-FID) or a

mass spectrometer (GC-MS), HPLC (High-Performance Liquid Chromatography) or with NACE (Non-aqueous Capillary Electrophoresis) comprising UV or MS detection.[11]

FID is the most used gas chromatography detection method and can analyze a wide range of components from hydrocarbons to fatty acids, responding to pretty much all organic compounds. It is more sensitive, reliable, and reproducible than an MS detector. FID is also cheaper and easier to operate. While the MS detector has a lower LOD (limit of detection), GC-FID shows less deviation in reproducibility.[39]–[41]

1.3 Subcritical water

Subcritical fluid extraction technology is one of those methods of green extraction techniques.[26]

Subcritical water extraction (SBW) allows the recovery of health-promoting compounds present in many plants and other natural matrices. This technique and supercritical carbon dioxide extraction are considered economical and efficient ways to recover compounds without compromising the extracted products, or residue, quality, through control of experimental parameters. Both of these methods have a low environmental impact.[26], [42]–[44]

In the literature, water at elevated temperatures and pressures find different terminologies. In supercritical water, the definition well establishes that water is above its critical point (374 ºC e 22.1 MPa).[45] As for subcritical water, the concept is less defined. The most used term can be HCW or SBW, which shows water above 100 ºC, or 150 ºC, depending on the source, and different pressures to remain in the liquid state at these conditions.[3], [17], [46], [47]

Technically, subcritical water is not a defined physical state, as we can see in Figure 1.4. It is water below the critical point and above the triple point.[46]

In SBW extraction, there are main process parameters, among which temperature, solvent to solid ratio, particle size, extraction time, but also mixing, pressure, and flow rate in the case of semi-continuous processes.[26]

Temperature is a very important parameter because as temperature increases, SBW becomes more than an extraction solvent, being able to promote catalysis. This happens because the ionic product of water (K_w) increases, namely by three orders of magnitude as the temperature increases from 25 ºC to 300 ºC. Higher concentrations of H^+ and OH^- ions make the water become a reagent, as well as a solvent. Therefore, ionic reactions are triggered.[46], [48], [49]

On the other hand, the dielectric constant of water decreases with temperature because of the breaking of hydrogen bonds.[27], [50] As a consequence, nonpolar compounds, as in fats and lipids, have their solubility in SBW increase with the increase in temperature and the decrease of the dielectric constant.[46], [49], [51]

The properties of water change according to the different regions of temperature, as seen in Figure 1.5.[46]

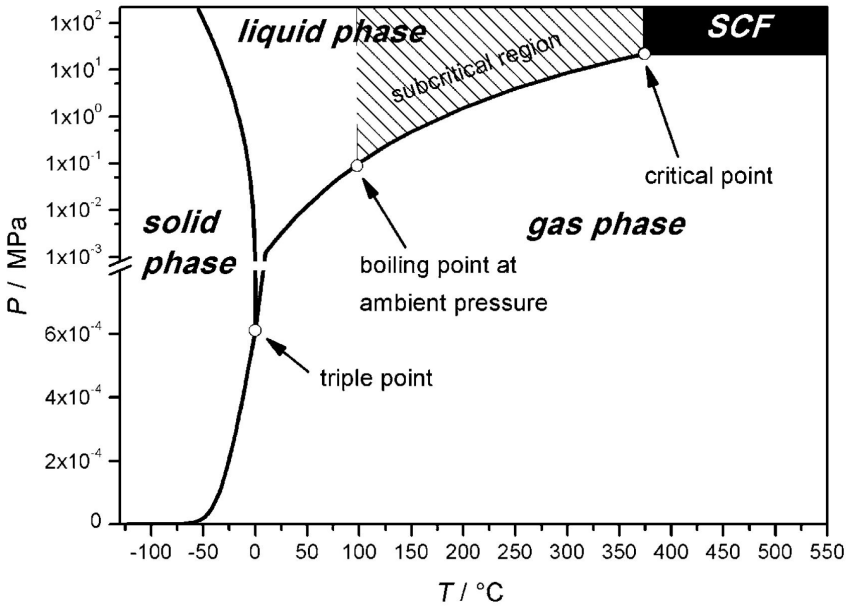


Figure 1.4: Phase diagram of water.[46]

	Ambient temperature	Subcritical water	Supercritical water
Temperature [°C]	0–100	100–374	>374
Vapor pressure [MPa]	0.003 (24 °C)[a]	0.1 (100 °C)–22.1 (374 °C)[a]	>22.1[a]
Aggregate state	liquid	liquid	no phase separation
Density [g cm^{-3}]	0.997 (25 °C)[a]	0.958 (101 °C, 0.11 MPa)[a] 0.692 (330 °C, 30 MPa)[b]	between gas-like and liquid-like densities,[c] for example, 0.252 (410 °C, 30 MPa)[b]
Viscosity [μPa s][d]	L: 884 G: 9.9 (25 °C)[a]	L: 277 G: 12.3 (101 °C)[a] L: 50.4 G: 30.7 (371 °C)[a]	low[e]
Heat capacity C_p [J g^{-1} K^{-1}][d]	L: 4.2 G: 2.0 (25 °C)[a]	L: 4.2 G: 2.1 (101 °C)[a] L: 69 G: 145 (371 °C)[a]	1300 (400 °C, 25 MPa)[f]
Dielectric constant	78.5 (25 °C, 0.1 MPa)[e]	27.1 (250 °C, 5 MPa)[g] 18.2 (330 °C, 30 MPa)[b]	5.9 (400 °C, 25 MPa)[g] 10.5 (400 °C, 50 MPa)[g]
Compressibility	no	slightly increased, but still a liquid (at 370 °C)[b]	yes
Ion product K_w [mol^2 L^{-2}]	10^{-14} (increasing to 10^{-12} at 100 °C)	increases from 10^{-12} (100 °C) to 10^{-11} (300 °C)[e,h,i]	strongly decreasing to below 10^{-20} (400 °C) and below 10^{-23} (550 °C); increases slightly with p[h]

[a] Ref. [61]; [b] ref. [25]; [c] ref. [62]; [d] L = liquid phase, G = gas phase; [e] ref. [64]; [f] ref. [66]; [g] ref. [53]; [h] ref. [63]; [i] Ref. [65].

Figure 1.5: Chemical and physical properties of water at different temperatures.[46]

Temperature affects reaction kinetics and equilibrium and is therefore a key variable. On the other hand, most of the SBW physical properties are not dependent on pressure. SBW has a lower viscosity and lower surface tension than water at ambient conditions, improving mass transfer to and from solid matrices.

An optimal solvent to solid ratio can maintain the concentration gradient between the bulk solvent and the sample matrix during extraction.[26]

The extraction time varies according to the extraction temperature, nature of the material, mode of extraction, and target compounds. Yields of extraction can first increase with time and then decrease because of the decomposition of previously extracted compounds. Collecting samples at fixed intervals could assist in understanding the composition of the extract with time.

The particle size of the sample is an essential factor for the smaller it is, the higher the contact surface area between solvent and solute, leading to a higher yield of extraction. The right particle size should increase the product yield and avoid particle agglomeration.

The mixing system is vital to reduce temperature gradients within the reactor. By increasing the mixing speed, the mass transfer improves because of the convective transfer rates of solutes between the solid matrix and the solvent. On the other hand, if the mixing speed is too high, some unwanted accumulation of particles on the reactor wall decreases the yield of extraction.[26]

Hydrolysis using SBW is a green alternative process that does not involve the use and later the need to recover additional chemicals. It also does not cause corrosion issues, is relatively fast and can be done using somewhat simple equipment.[17]

It was studied the influence of the temperature and water flow rate on the extraction/hydrolysis efficacy of SBW up to 210 ºC.[17] Previous studies show that hemicellulose dissolves between 120 to 190 ºC, while cellulose hydrolysis requires the use of temperatures above 230 ºC.[52] Another study evidenced the dissolution of hemicellulose at temperatures over 130 ºC. Submitting the material to this temperature for some time appeared to depolymerize the structure. The growth in temperature from 130 ºC to 190 ºC and the change in water properties mentioned before, specifically higher ionic products, favored the hemicellulose's dissolution with the increase in the number of carbohydrates recovered.[52] As for cellulose, after 2 minutes at 180 ºC, merely 5% was converted to glucose, a value that increased to 80% at 260 ºC.[46]

When processing disaccharides and polysaccharides with subcritical water, the decomposition step is considered the rate-limiting step of the reaction, and it increases with temperature, favoring the production of the oligomers and monomers as temperature increases.[46] The SBW mechanism includes solute transfers from active sites in the matrix to the extraction medium.

Relevant products obtained from glucose and fructose in SBW include acetic acid, formic acid, lactic acid, levulinic acid, and, most commonly, 5-hydroxymethylfurfural (5-HMF). There is a promising future for 5-HMF for very noticeable products of SBW

biomass treatments. The most critical point being in the automotive industry as a precursor for "green fuels".[46], [53]

Water is the most used solvent for subcritical extraction even though other fluids are used, such as butane, dimethyl ether, and propane. For instance, subcritical propane extracts antioxidant compounds and essential oils from mango leaves and sapucaia nuts. These techniques intend to promote the eco-friendly use of solvents in extractions while being safe and showing high standard extracts.[26]

1.4 The objective of this thesis and outline

The major challenge in consuming lupin seeds has been the massive amounts of water and time it takes to remove the alkaloids to make lupin seeds safe for consumption. This work aims to find an alternative green method to remove the alkaloids while keeping the major nutritional components of lupin mostly intact. In particular, it aims to reduce significantly the duration of the debittering process and the amount of water consumed, whereas maintaining the costs at a minimum and still ensuring food safety for animals and humans.

Previous work [54] has revealed a decrease in the amount of alkaloids in the extracts as temperature increased from 100 to 140 °C. To clarify the effect of temperature on the extraction of alkaloids, namely to verify if that trend was due to the degradation of extracted alkaloids at the highest temperature, a mass balance for alkaloids is required. This can be accomplished by measuring the amount of alkaloids remaining in the lupin residue after SBW extraction, not done previously. Such measurement requires eight consecutive extractions.

Also in previous work [54] the total amount of alkaloids was assumed to be the weight of the solid material recovered from the dichloromethane used as elution solvent of the Isolute column. In the present work, however, the solid material recovered by evaporating dichloromethane was submitted to GC analysis targeting lupanine.

The following tasks were thus pre-established to make this thesis possible:

1. Characterize the white lupin matrix so that a comparison with debittered lupin can be made and the overall impact of the SBW treatment can be established.

2. Use SBW to debitter the lupin using a batch reactor, varying the operation parameters: temperature, solvent-to-solid ratio, the effect of successive extractions, and the impact of particle size.

3. Assess the impact of extraction conditions on the composition of the lupin extracts obtained, on the remaining lupin residue, and in the latter case include alkaloids in the mass balance.

4. Reduce the alkaloid content of the resulting lupin seeds below toxicity levels of bitter white lupin, while preserving the nutritional value of lupin seeds.

The organization of the rest of the document is as follows:

- Chapter 2 includes the materials and methods used in this process.
- Chapter 3 shows the results and discussion of the data obtained throughout this work.
- Chapter 4 presents a general conclusion of the work.
- Chapter 5 proposes future work.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 *Lupinus albus L.*

Simbeja company provided the raw material used in this study. The seeds were cleaned to remove leaves and other impurities, and some of them were grounded in a household mill.

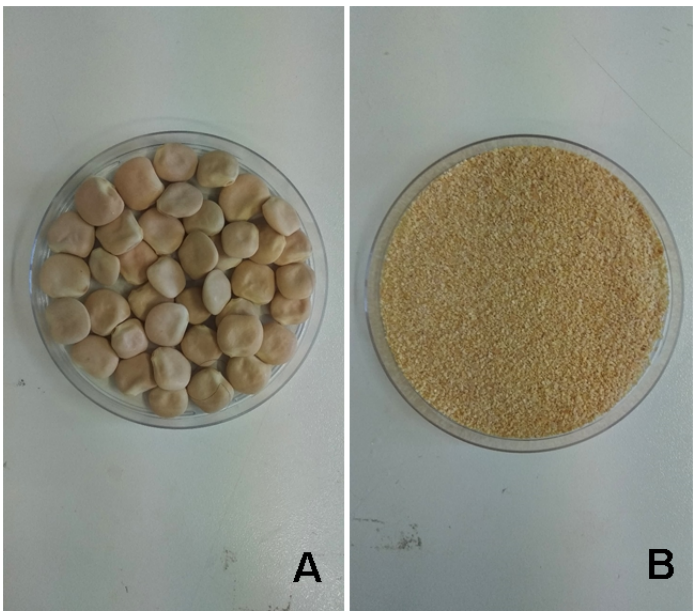


Figure 2.1: White lupin seeds (A), provided by *Simbeja*, and powder (B).

After that, the particles were sieved in an ASTME-11 laboratory test sieve. The particle size distribution after grinding is shown in Table 2.1. In this work, the particle size used

was between 0.5 and 1 mm (Figure 2.1-(B)) and stored in a freezer.

Table 2.1: Particle size distribution of lupin seeds after grinding.

Particle size (mm)	Mean value (%)
>1	35.3
0.5-1	31.4
0.355-0.5	10.9
<0.355	21.2

2.1.2 Reagents

Phenol (99%) and D-Glucose were from *Sigma-Aldrich*. Acetic acid glacial, ethanol, and n-hexane were from *Carlo Erba*, and dichloromethane was from *Labchem*. Ammonium Hydroxide and sulfuric acid were from *Honeywell*, and Isolute HM-N was from *Biotage*.

2.2 Chemical Characterization

2.2.1 Humidity content determination

The humidity content of *L. albus L.* powder was measured by placing 1 g of lupin in a Kern Dab 200-2 hygrometer at 105 ºC. The result obtained was in the mass percentage of humidity.

Figure 2.2: Humidity measurement in the hygrometer.

2.2.2 Protein content determination

The protein content of lupin seeds powder was determined by an elementary analysis that yielded the nitrogen content of the material. This analysis was performed at Laboratório de Análises, REQUIMTE-LAQV. A 6.25 conversion factor of nitrogen-to-protein was used. Both the lupin residue that remained in the high-pressure reactor and the extract obtained from lyophilizing the liquor in the reactor were submitted to an identical analysis to determine protein content.[55]

2.2.3 Ash content determination

To determine the lupin seeds' ash content, 0.8 g of powder was placed in a previously weighed porcelain crucible. The crucible was then placed in a muffle at 550 ºC for 5 hours. Upon cooling down the crucible, it was weighed, and the ash content was determined by mass difference.[56]

2.2.4 Lipid content determination

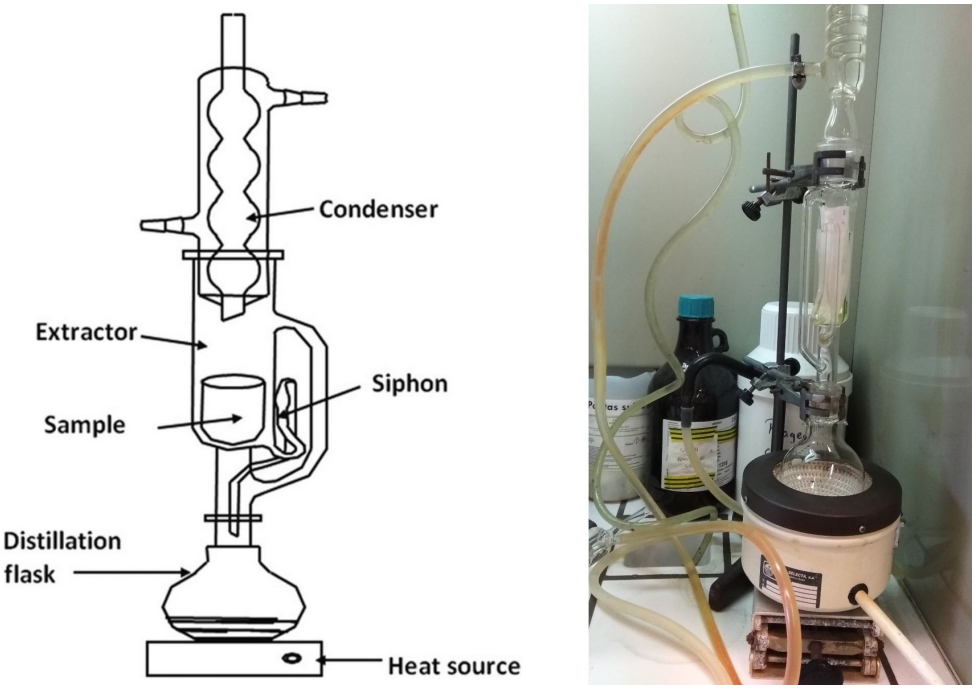


Figure 2.3: Soxhlet extraction apparatus.[57]

The defat of the lupin powder was performed in a Soxhlet extraction. This procedure was carried out for 3 hours, using 2 g of lupin powder and 70 mL of n-hexane. The solvent in the solution was evaporated with nitrogen, and the oil was weighted. The solid residue was dried overnight at 40 ºC to remove the solvent and was weighed.

This experiment was replicated twice.[17], [57]

2.2.5 Carbohydrates content determination

The determination of carbohydrates is done with previously defatted lupin seeds. This process takes 2 steps to remove the non-structural and structural sugars. The first step consists of a hydro-alcoholic extraction, and the second of an acid hydrolysis extraction, respectively.

To quantify the total sugars after the extractions, the colorimetric phenol-sulfuric method was performed.

First, to isolate the soluble (non-structural) sugars, 0.8 g of defatted lupin powder were extracted, in duplicate, with 40 mL of an (80:20, v/v) ethanol:water solution in an ultrasonic bath, at room temperature, for 15 minutes, before a 10,000 rpm centrifugation (BeckmanCoulter, J-26 XPI), for 10 minutes at 4 ºC. This process was done 3 times, the 3 supernatants were combined after filtration and evaporated in a rotary evaporator at 50 ºC, under vacuum, to remove the ethanol. The remaining solution was diluted to 100 mL and used for carbohydrate quantification.[58]

The acid hydrolysis was performed using the solid residue, defatted lupin powder, after extraction of the soluble carbohydrates that was dried overnight at 40 ºC. To hydrolyze these structural carbohydrates, 0.3 g of this solid was added 3 mL of 72% (w/w) H_2SO_4. This solution was incubated in a water bath for 30 ºC, with agitation, for 1 hour. After that, the mixture was diluted to 4% (w/w) by adding 84 mL of water and incubated in a silicone bath, for 1 hour, under stirring, for 121 ºC. The mixture was vacuum filtered, and the supernatant was analyzed to quantify carbohydrates.[17], [58], [59]

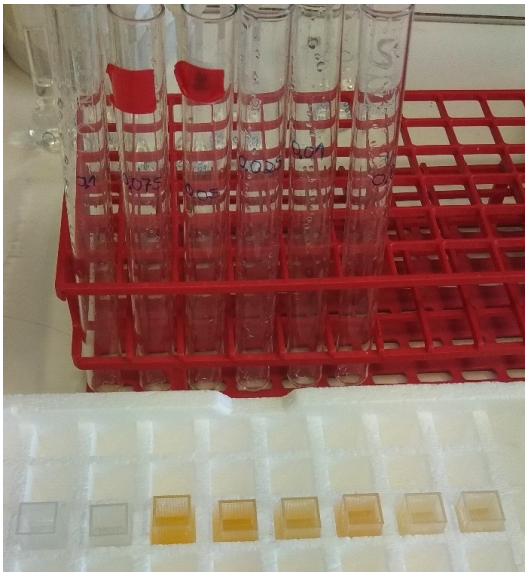


Figure 2.4: Preparing to build the D-Glucose calibration curve for the colorimetric method.

The carbohydrate analysis was carried out by quantifying the sugar-rich liquids' reducing sugar content of a standard solution or a solution sample. A calibration curve was

built with D-glucose monohydrate using a 1 g/L stock solution to prepare the solutions with concentrations of 0.005, 0.01, 0.025, 0.05, 0.075, and 0.1 g/L in deionized water. The blank was deionized water as well.

Before preparing the solution to quantify the sugars, a dilution of 50:1 was applied to the samples. To 500 μL of the sample were added 1.5 mL of 96% (w/w) H_2SO_4 and a 300 μL of a 5% (w/v) aqueous solution of phenol. These mixtures were well stirred and incubated for 5 minutes at 90 ºC in an Accu $Block^{TM}$ Digital dry bath from Labret International, Inc. The absorbance was measured at 490 nm using a Thermo Scientific Genesys 50 UV-visible spectrophotometer. The results obtained are expressed in g/L glucose equivalent.[17], [60]

2.2.6 Lignin content determination

Lignin content was calculated by mass difference after drying the acid hydrolysis residue overnight at 105 ºC.[58], [59]

2.2.7 Alkaloids content determination

To quantify the alkaloids in the original lupin seeds and in the residue that remained in the high-pressure reactor after extraction with SBW, 3 g of solid material were stirred for 30 minutes in 30 mL of 1M acetic acid. This mixture was centrifuged for 20 minutes at 12,000 rpm to separate the liquid and solid phases. The supernatant's volume was measured to know the adequate quantity of isolute and dichloromethane to use.

After that, the pH was adjusted with ammonium hydroxide, and a solid extraction was carried out using a solid-phase extraction Isolute column (Isolute HM-N, *Biotage*).

The alkaloids were then eluted with dichloromethane, and the solvent evaporated under vacuum in a rotary evaporator at 40 ºC. This process was done 8 times. In the case of the extracts obtained by lyophilizing the liquors produced in the reactor, the process was simplified because it was only extracted once.

The alkaloid extraction residue was dried with nitrogen and sent to Laboratório de Análises, REQUIMTE-LAQV, to quantify the major alkaloid present in the mixture, lupanine, using an Agilent Technologies, model 6890 GC-FID (Gas Chromatography using Flame-Ionization Detection) equipped with a GC Autosampler, HTA sampling for Science, model HT3100A. The analysis was performed with caffeine as an internal standard and purified lupanine for the calibration curve.[5], [61]

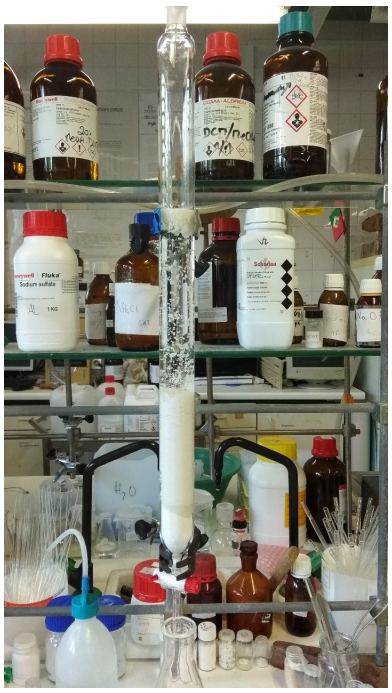


Figure 2.5: Alkaloids extraction apparatus.

2.2.8 Subcritical water extraction

The apparatus for the subcritical water extraction was a batch pressure reactor from Parr, model 4547, and it is represented in Figure 2.6 and Figure 2.7.

The reactor is a stainless steel tube with a 1.2 L volume. It includes a stirrer with 2 flat blade turbines and a heater for achieving the desired temperatures. There is also a distilled water container connected to a cooling coil inside. To raise the pressure inside the reactor, nitrogen from a bottle is used, and it is connected to the reactor with a valve.

Temperature, solvent-to-solid ratio, consecutive extractions, and different raw material particle sizes were studied. All the assays had a residence time of 1 hour, and all first assays were done using 500 mL of water. The temperatures studied were 100, 120, and 140 ºC, with a 20:1 solvent-to-solid ratio. A ratio of 40:1 was applied at 100 ºC as well. Lupin was extracted twice, as powder and as received (whole), at 100 ºC and 20:1 ratio. The pressure of 50 bar maintained the liquid state in all assays.

At the beginning of the assay, lupin and distilled water were added to the reactor. The first thing to do after closing the reactor is to pressurize it. Then, the stirrer and heater were turned on, and the assay started after the desired temperature was reached. After 1 hour, the temperature was decreased by setting the thermocouple to 25 ºC. The reactor has to cool down, and only then can it be depressurized and opened to collect the lupin residue and liquor from the extraction.

Samples from the lupin residue were centrifuged 3 times to accurately separate the solid from the liquid, while only the first supernatant was used for liquor analysis. Both liquor and residue were frozen using liquid nitrogen and lyophilized under vacuum for 3

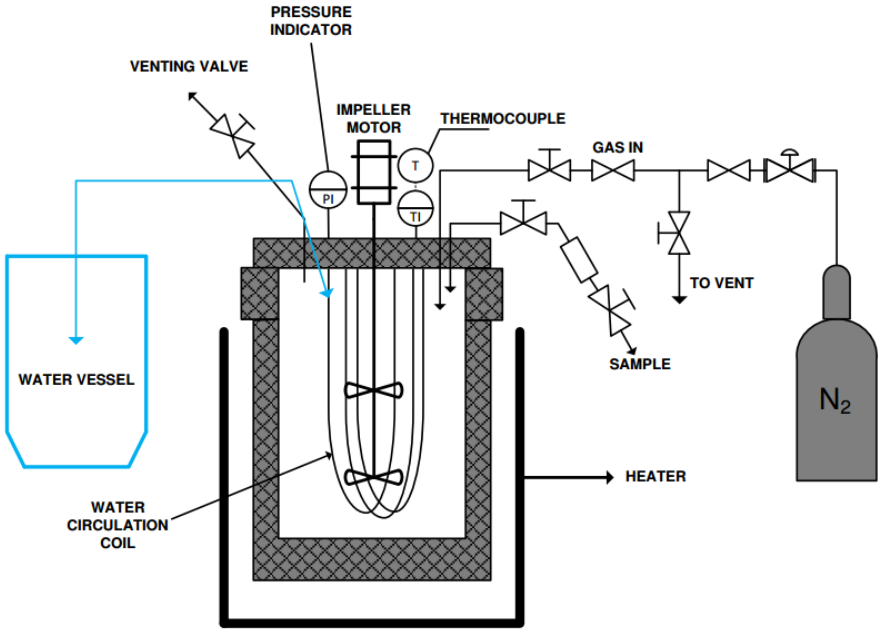


Figure 2.6: Batch reactor apparatus drawn using *Microsoft Visio* software based on Essien [26].

Figure 2.7: Laboratory-scale batch reactor apparatus.

days in a CHRIST ALPHA 1-4, Braun Biotec International lyophilizer. The dried residue and the lyophilized liquor were used to determine lipids, protein, carbohydrates, and alkaloids content.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Chemical Characterization

The first task was to determine the chemical composition of white lupin seeds to assess the content of its main components before debittering and compare after the batch assays with subcritical water. The main species of interest are total carbohydrates, protein, lipids, ash, and lignin. Values for these components given in the literature vary within an interval. This interval derives from the fact that many factors play a role in biomass composition, such as species or cultivar.[3]

The total amount of carbohydrates of this work was 37%, of which 29% make up the structural sugars, while only 8% make up the free sugars content, which is lower than Carmalia *et al.* measured [5] but higher than other authors [33]. As mentioned before, this was a two-step procedure, where the soluble sugars were separated from insoluble sugars that make up the hemicellulose and cellulose structures.[33], [62]

As seen in Table 3.1, the most significant components by mass are carbohydrates, protein, and lipids.

Table 3.1: White lupin powder chemical characterization (data are reported on a dry matter basis; mean value ± standard deviation, n=3)

Components	**g/100 g lupin**
Carbohydrates	37.0 ± 1.6
Protein	31.5 ± 1.3
Lipids	8.6 ± 0.2
Ash	2.8 ± 0.1
Lignin	0.4 ± 0.1

The protein content is 31.5%, which is a little lower than literature but still higher

than other legumes like rapeseed and field pea.[20], [62]

The lipid content is usually between 6 to 13%.[8] The results showed this lupin has around 9% of fat content, which brings us to middle of the interval reported. Ash and lignin were quantified as well and are considered minor components of white lupin.

The humidity content of this lupin was around 10%.

As mentioned before, this work's main objective was to extract the alkaloids from the white lupin, making it safe for consumption while preventing all the major components from degrading. This procedure was referred to in Subsection 2.2.7, and to quantify all alkaloids present by that laboratory procedure, an eight-time consecutive extraction of the same solid was done. Table 3.2 shows the total amount of lupanine present in the debittered lupin as well as the lupanine mass extracted each time.

Table 3.2: Total lupanine content in white lupin seeds. Data are reported on a dry matter basis.

Extraction	**Lupanine (g/100 g lupin)**
1	0.66
2	0.55
3	0.12
4	0.02
5	$1.0e^{-4}$
6	$1.3e^{-3}$
7	$4.0e^{-4}$
8	$6.0e^{-4}$
Total	1.35

The total alkaloid content of the white lupin provided by *Simbeja* was 1.35 g/100 g lupin, which shows that this species is a bitter type because it is over the 1.0 g alkaloids/kg lupin mark.[3], [4], [36] Since this value exceeds the value indicated by the regulatory bodies of 0.02 g/100 g of lupin [12], these seeds are not safe for consumption and, hence, must be treated.

In this work, 1.35 g/100 g lupin will be used as a reference for the total alkaloid content.

3.2 Extraction study

White lupin seeds were submitted to SBW extraction in a batch reactor to remove alkaloids, one of the objectives of this thesis.

A SBW experiment yields a liquor and a residue. One way to find out if the extraction was successful was first to measure the amount of water-soluble compounds in the reactor, after lyophilization of the liquor and production of an extract. The extract was then analyzed for lupanine.

The yield of extraction of lupanine is given by Equation 3.1.[45]

$$Y = \frac{\text{mass of lupanine in the extract}}{\text{mass of lupanine in the lupin seeds placed in the extraction vessel}} \times 100 \quad (3.1)$$

The protein and total carbohydrates content of the extract were also determined to assess the effect of the extraction on the nutritional value of the lupin during the debittering process.

To close mass balances, the contents of lupanine, protein, and carbohydrates in the lupanine residue left in the reactor were also measured, as well as its content in lipids. The latter component was not expected to be present in high amounts in the extract and thus was only monitored in the solid lupin residue, for comparison with the corresponding amount in the original lupin material.

All experiments were performed with lupin seeds powder, obtained by grinding the seeds in a household mill, as indicated in Chapter 2. The exception was the last experiments performed, where whole lupin seeds were used.

3.2.1 Subcritical Water Extraction

The extraction yield of compounds from a lignocellulosic matrix is dependent on several parameters [63], [64]:

- Nature of the solvent,
- Composition of the sample,
- Temperature,
- Solid-to-solvent ratio,
- Stirring system,
- Successive extractions,
- Particle size.

As previously mentioned in Section 1.3, temperature is one of the most critical variables in extraction studies. The first round of assays was performed by varying temperature from 100 to 140 ºC while keeping a constant 20:1 solvent-to-solid ratio (Table 3.3). All assays were done with a residence time of 60 minutes, to compare with previous work.[54]

The pressure used was always 50 bar for all assays as well, and it was not changed throughout the study since its variation shows a low impact on the extraction, as mentioned in Section 1.3.

The impact of extraction conditions on the lupin matrix was studied by measuring the amount of water-soluble compounds in the liquors produced by generating lupin

Table 3.3: Assay conditions for lupin powder.

Extraction conditions			
Temperature (°C)	**Time (min)**	**Ratio**	**Pressure (bar)**
100	60	20:1	50
120	60	20:1	50
140	60	20:1	50

extracts after lyophilization (Figure 3.1). In all the assays there was material that could not be accounted for at the end of the experiments. This was confirmed by calculating the difference between the amount of lupin placed in the reactor and the amount of lupin residue left in the reactor plus the mass of water-soluble compounds.

There is about 12-21 g/100 g lupin of solids lost during extraction. This value is considered normal as it falls within the ranges mentioned in the literature (12-27 g/100 g lupin).[65]

These losses are partly explained by the fact that there is ca. 10% of water in the lupin put into the reactor, and this amount is not accounted for during the mass balance. Correction for this would lower material losses by about 2-10%. Also, there can be some material losses when collecting the residue from the reactor, as well as in the 3-step washing/centrifugation process followed by filtration.

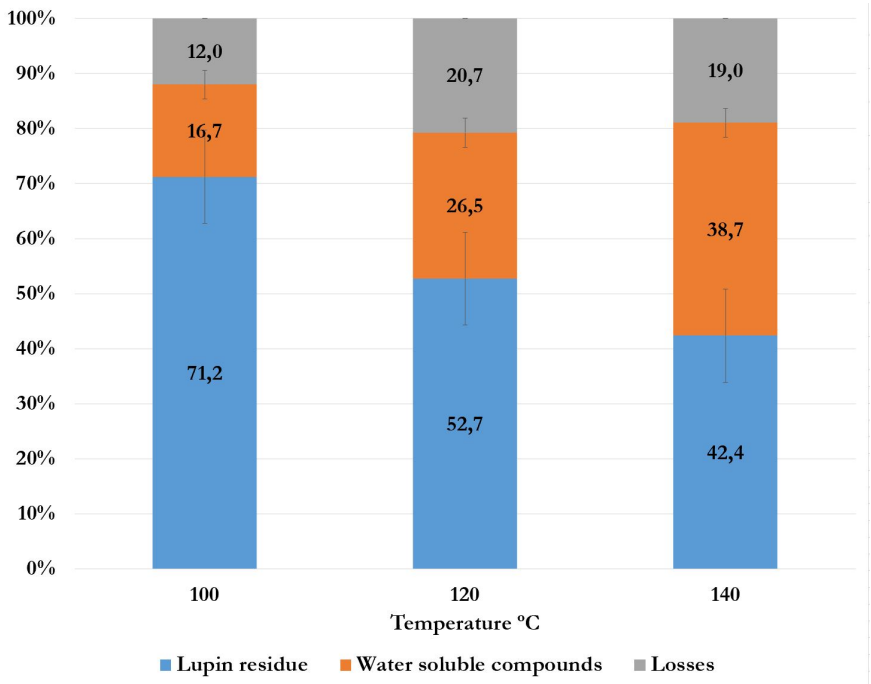


Figure 3.1: The influence of temperature on the yield of water-soluble compounds in the lupin powder extract (solvent-to-solid ratio: 20:1, time: 60 minutes). Error bars are given.

The amount of water-soluble compounds increase with temperature. This can be

partly explained by the increases in solubility of the compounds being extracted. On the other hand, with the increase in temperature, water becomes more reactive due to the increase in its ionic product, thereby becoming a catalyst for biomass hydrolysis.

Analysis of the lupin extracts produced to allow a more detailed assessment of the extraction assays, as seen in Table 3.4.

Table 3.4: The influence of temperature on the extraction yield of lupanine in the lupin powder extract. The values in parenthesis are the percentage of lupanine extracted, taking 1.35 g lupanine/100 g lupin as 100%.

Extraction conditions			**Yield of lupanine**
Ratio	**Time (min)**	**Temperature (°C)**	**g/100 g lupin**
20:1	60	100	0.95 (71%)
20:1	60	120	1.02 (76%)
20:1	60	140	0.02 (2%)

The results show that a temperature of 120 ºC leads to a slightly higher yield of extraction of alkaloids than 100 ºC: 76% extraction vs. 71% extraction, respectively, which still falls over the 200 mg of alkaloids per kg of lupin permitted by law. At 140 ºC, however, there is an abrupt decrease in the amount of alkaloids detected in the extract. Alkaloids are highly temperature-sensitive, so some degradation might occur. These results differ somewhat from those of previous work [54]. The latter work did not reveal such an abrupt decrease in the total amount of alkaloids as temperature varied from 100 to 140 ºC. However, as explained earlier, the method of analysis of alkaloids was not the same. In the present case, lupanine was singled out as target compound, and its content was quantified by GC.

One of this work's objectives was to maintain the nutritional value of the white lupin as intact as possible while still managing to remove alkaloids below the toxicity level. Therefore, as described in Chapter 2, carbohydrates and protein were determined in the extract, it being known that the water extraction also removes these components.

As shown in Table 3.5, only non-structural sugars (total of 8 g/100 g lupin, as obtained in the characterization of lupin seeds) and little protein are extracted at 100 ºC. With the increase in temperature, the amounts of carbohydrates, and protein extracted increased. At higher temperatures, water not only accommodates higher amounts of dissolved species, but it also becomes capable of degrading the structure of hemicellulose, thereby releasing additional sugar units. Entrapped protein should also become more accessible.

Overall, the results in Table 3.5 are similar to those of previous work [54]. From the standpoint of the integrity of the lupin matrix, the best temperature of extraction is 100 ºC, given that the yield of extraction of alkaloids is 71%, a value that increases only slightly as temperature increases to 120 ºC. At 100 ºC, operations costs are also lower.

The influence of solvent-to-solid ratio from 20:1 to 40:1 was studied at 100 ºC. Figure

Table 3.5: The influence of temperature on the yields of carbohydrates and protein in the lupin powder extract (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions			Yields (g/100 g lupin)	
Ratio	**Time (min)**	**Temperature (°C)**	**Carbohydrates**	**Protein**
20:1	60	100	6.8 ± 0.4 (18%)	4.8 ± 0.1 (15%)
20:1	60	120	12.7 ± 0.4 (34%)	7.3 ± 0.1 (23%)
20:1	60	140	15.9 ± 0.7 (43%)	12.4 ± 0.1 (39%)

3.2 shows that a higher amount of water-soluble compounds was obtained, as could be explained by the ability of a higher amount of solvent to accommodate compounds extracted from the lupin matrix.

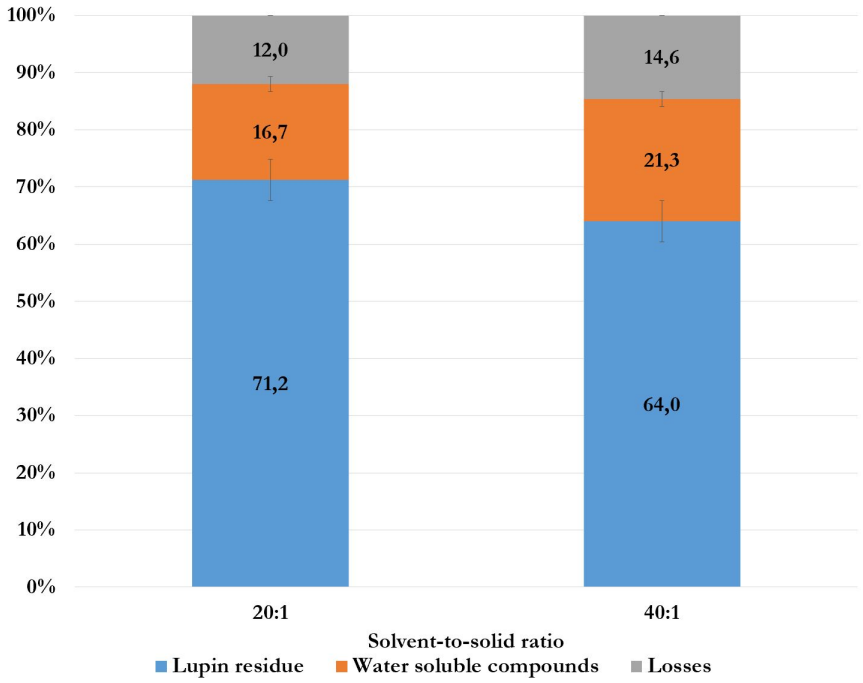


Figure 3.2: The influence of solvent-to-solid ratio on the yield of water-soluble compounds in the lupin powder extract (temperature: 100 °C, time: 60 minutes). Error bars are given.

Table 3.6 shows that the yield of extraction of lupanine did not increase significantly from a solvent-to-solid ratio of 20:1 to 40:1, thereby not showing an effect of improved mass transfer as the concentration gradient gets higher.[66] The trend observed in the extraction of alkaloids as temperature increased is not similar to that observed in previous work [54]. The latter work reported an approximately 25% increase in the extraction of alkaloids at 100 °C when the solvent-to-solid ration increased from 20:1 to 40:1. This could be due to the fact that the previous work assumed the final mass after alkaloid extraction being all alkaloids. The Isolute column may have let through other nonpolar compounds present in the extract.

Table 3.6: The influence of solid-to-solvent ratio on the yield of lupanine in the powder extract. The parenthesis gives the percentage of lupanine extracted. The values in parenthesis are the percentage of lupanine extracted, taking 1.35 g lupanine/100 g lupin as 100%.

Extraction conditions			Yield of lupanine
Ratio	**Time (min)**	**Temperature (°C)**	**g/100 g lupin**
20:1	60	100	0.95 (71%)
40:1	60	100	1.0 (74%)

As seen in Table 3.7, the amount of extracted protein is higher than that obtained with a 20:1 solvent-to-solid ratio, but not that much higher, which is a positive result, given that protein is a target nutritional component. Carbohydrates, on the other hand, seem to be extracted in amounts similar to those obtained at 120 ºC with a 20:1 solvent-to-solid ratio. At 100 ºC, the lupin matrix should be very resistant to hydrolysis, irrespective of the solvent-to-solid ratio. At this temperature, water does not have the ability to destroy the hemicellulose structure. Therefore, it was not to be expected that the amount of sugars extracted at 100 ºC with a 40:1 solvent-to-solid ratio would be much different than 8 g/100 g lupin, the value determined for soluble sugars in the original lupin matrix.

Table 3.7: The influence of temperature on the yields of carbohydrates and protein in the powder extract (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions			Yields (g/100 g lupin)	
Ratio	**Time (min)**	**Temperature (°C)**	**Carbohydrates**	**Protein**
20:1	60	100	6.8 ± 0.4 (18%)	4.8 ± 0.1 (15%)
40:1	60	100	10.9 ± 0.4 (30%)	6.6 ± 0.1 (21%)

Two consecutive extractions were performed at 100 ºC, at a 20:1 solvent-to-solid ratio, for 60 minutes, for both lupin powder and whole seeds. This consisted of using the residue from the first extraction, after lyophilization, by submitting it to a second extraction at otherwise identical experimental conditions as the first assay.

As shown in Figure 3.3, it seemed that the matrix had been nearly exhausted of extractable compounds in the first extraction, given the sharp decrease in the amount of water-soluble compounds recovered in the second extraction.

A more detailed analysis of the extracts obtained corroborates the above conclusion that the second extraction was not very effective, in that the increase in compounds extracted was very low.

The amount of lupanine extracted in the first assay is slightly lower than that given in Table 3.4 and Table 3.6 and should reflect the error associated with the measurement of that quantity.

With the objective to study the effect of particle size on the yield of extraction, the next

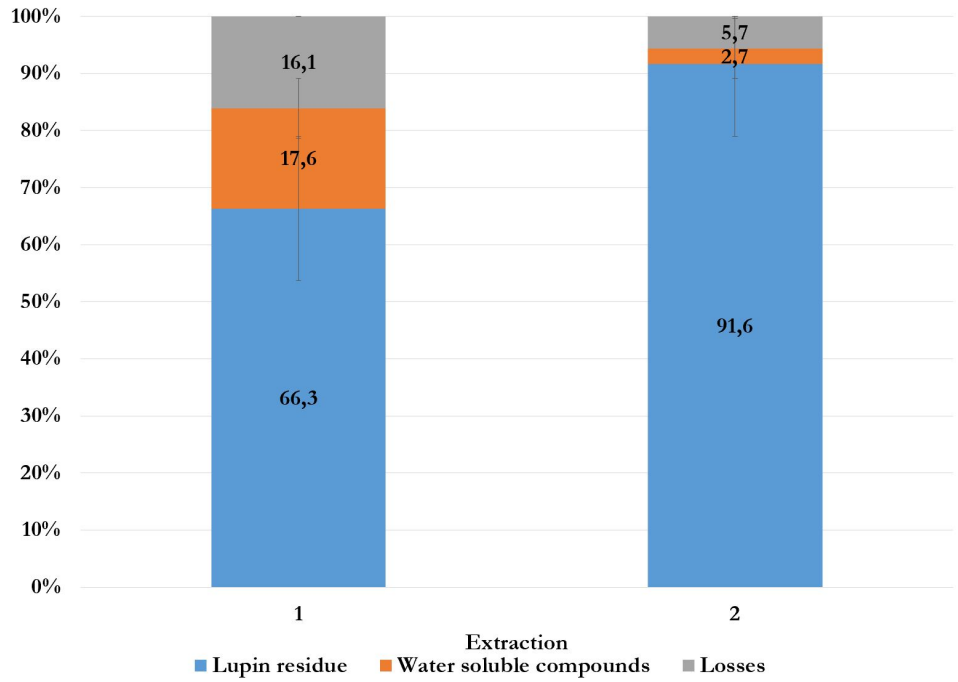


Figure 3.3: The influence of multiple consecutive extractions of lupin powder on the yield of water-soluble compounds in the lupin extract (temperature: 100 ºC, solvent-to-solid ratio: 20:1, time: 60 minutes). Error bars are given.

experiment was performed with whole lupin seeds. Again, two consecutive extractions were carried out at 100 ºC with a 20:1 solvent-to-solid ratio. The results obtained were similar for lupin powder and whole lupin seeds, as seen in Table 3.8 and Table 3.9.

Table 3.8: The influence of successive extractions using lupin powder on the yields of lupanine, carbohydrates, and protein in the lupin extract (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions			**Yields (g/100 g lupin)**		
Temp (°C)	**Ratio**	**Extraction**	**Lupanine**	**Carbohydrates**	**Protein**
100	20:1	1	0.87 ± 0.1 (64%)	6.9 ± 0.7 (19%)	5.0 ± 1.3 (16%)
100	20:1	2	0.08 ± 0.1 (6%)	1.4 ± 0.2 (4%)	0.7 ± 0.1 (2%)
		Total	0.95 ± 0.2 (70%)	8.3 ± 0.4 (22%)	5.8 ± 0.2 (18%)

Overall the same trend is observed in Table 3.8 and Table 3.9. A higher amount of extracted protein in the second assay may be the result of increased accessibility to the matrix after the first treatment. The yield of extraction of lupanine was lower than for lupin powder, which may be explained by comparatively more difficult access to the inner portions of lupin seeds.

Carbohydrates accounted for 7.5 g sugars per 100 g lupin, which means all nonstructural sugars were extracted. In terms of protein, 6.3 g of protein per 100 g of lupin were removed, and it is an expected value since it is comparable to other assays at 100 ºC and

20:1 ratio. Lupanine extraction was low since 0.9 g/100 g lupin were extracted from the 1.35 g of lupanine present at the beginning of the assays, making this a 62% successful experiment only.

Table 3.9: The influence of multiple extractions using whole lupin seeds on the yields of lupanine, carbohydrates, and protein in the lupin extract (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions			**Yields (g/100 g lupin)**		
Temp (°C)	**Ratio**	**Extraction**	**Lupanine**	**Carbohydrates**	**Protein**
100	20:1	1	0.63 (47%)	6.1 ± 0.6 (17%)	4.6 ± 0.1 (15%)
100	20:1	2	0.14 (10%)	1.4 ± 0.2 (4%)	1.8 ± 0.2 (6%)
		Total	0.77 (57%)	7.5 ± 0.8 (20%)	6.4 ± 0.3 (20%)

The conclusions are evident in Figure 3.3 that shows a sharp decrease in the amount of water-soluble compounds from the first to the second extraction.

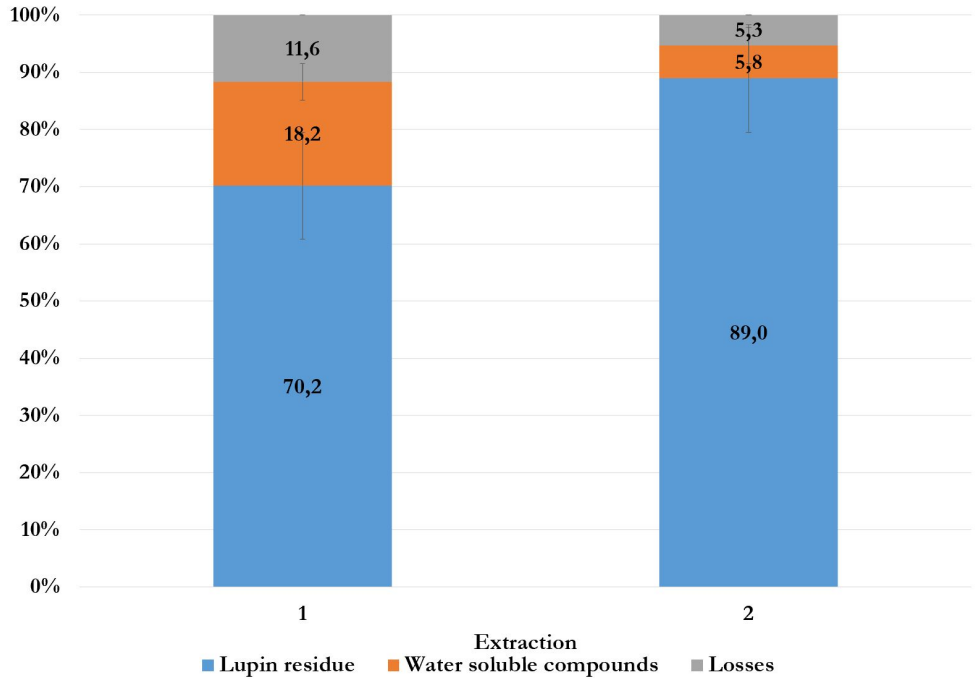


Figure 3.4: The influence of multiple consecutive extractions of whole lupin seeds on the yield of water-soluble compounds in the lupin extract (temperature: 100 °C, solvent-to-solid ratio: 20:1, time: 60 minutes). Error bars are given.

In this experiment, the agitator configuration and its shape were not adequate. Predictably, at the end of the assay, the seeds were not physically equal to those put in the reactor. The results obtained for the two particle sizes could have been more different if the whole seeds had remained intact.

In this study, the quantification of alkaloids was also done in the lupin residue, as a way to confirm if alkaloids were indeed being extracted in higher amounts at 140 ºC, but not being detected in the analysis of the extract due to their degradation in the reactor. Protein, carbohydrates, and lipids were also accounted for.

As can be seen in Table 3.10, the alkaloid content in the residue was higher at 100 ºC, while only 0.05 g/100 g of lupin is accounted for in the lupin residue of the 140 ºC assay. This implies that, at 140 ºC, there are major losses of lupanine and this can be due to heat degradation in the reactor, which makes it not available for analysis in the extract.

Table 3.10: The influence of temperature on the yields of lupanine, carbohydrates, protein, and lipids on the lupin powder residue (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions		**Yields (g/100 g lupin)**			
Temp (°C)	**Ratio**	**Lupanine**	**Carbohydrates**	**Protein**	**Lipids**
100	20:1	0.39 (29%)	21.9 ± 0.4 (59%)	23.7 ± 1.1 (75%)	6.6 ± 0.1 (77%)
120	20:1	0.13 (10%)	17.2 ± 0.2 (47%)	16.3 ± 0.1 (57%)	6.9 ± 0.2 (80%)
140	20:1	0.05 (4%)	14.7 ± 0.6 (40%)	14.1 ± 0.1 (45%)	7.8 ± 0.1 (91%)

Concerning the major nutritional values, the 100 ºC residue shows the best values for protein and carbohydrates compared to other temperatures and also other solvent-to-solid ratios (Table 3.11). Lipid content appears to increase in the residue with temperature. This should reflect the fact that lipids are essentially not being extracted at the conditions of the assay, its content varying within the experimental error.

Table 3.11: The influence of solvent-to-solid ratio on the yields of lupanine, carbohydrates, protein, and lipids on the lupin powder residue (data are reported on a dry matter basis; mean value ± standard deviation, n=3).

Extraction conditions		**Yields (g/100 g lupin)**			
Temp (°C)	**Ratio**	**Lupanine**	**Carbohydrates**	**Protein**	**Lipids**
100	20:1	0.39 (29%)	21.9 ± 0.4 (59%)	23.7 ± 1.1 (75%)	6.6 ± 0.1 (77%)
100	40:1	0.16 (12%)	8.1 ± 0.9 (22%)	22.2 ± 0.2 (70%)	6.9 ± 0.2 (80%)

Figure 3.5 shows a mass balance for lupanine and highlights the high amount of lupanine that is unaccounted for in the 140 ºC assay. The best lupanine yields in the extract occur at 100 ºC with a 20:1 and 40:1 ratios. Although the yields of extraction of lupanine appear similar in each case, the amount of lupanine in the 40:1 residue suggests that the higher this ratio, the higher the extraction of lupanine from the lupin matrix.

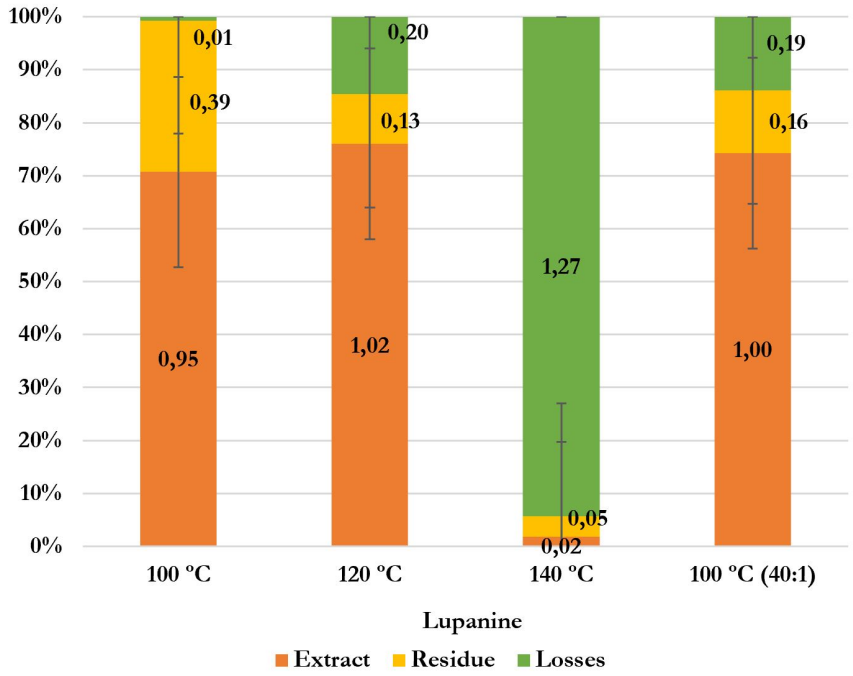


Figure 3.5: The influence of temperature and ratio on the yield of lupanine in the lupin powder (solvent-to-solid ratio: 20:1 and 40:1). Error bars are given. 100% = 1.35 g lupanine/100 g lupin.

As for the carbohydrates mass balance (Figure 3.6), it is known that soluble sugars are always extracted during the assays and after hitting the 8 g of sugar per 100 g of lupin mark, structural sugars are being removed from the lupin matrix. This is clear at 140 °C. By increasing the solvent-to-solid ratio to 40:1, the sugars in the residue decrease. Again, this was not anticipated since water at 100 °C does not have the ability to degrade the lignocellulosic matrix, and only soluble sugars should have been available for extraction.

In the protein mass balance, protein being probably the most important component for this study to accomplish soy substitution, it can be seen (Figure 3.7) that the best yield of extraction when analyzing the residue was obtained at 100 °C, with a 20:1 ratio, with the least amount of losses. The result for protein in the 40:1 ratio assay is similar to that obtained in the assay at 100 °C for a 20:1 ratio, and the fraction of protein in the residue should likewise be similar to that obtained in the 100 °C, 20:1 assay. In previous work [54] soluble protein was determined by extraction with water at 90 °C, at a 20:1 solvent-to-solid ratio, yielding a value of 6.5 g/100 g lupin, similar to the amount of protein measured in the extract.

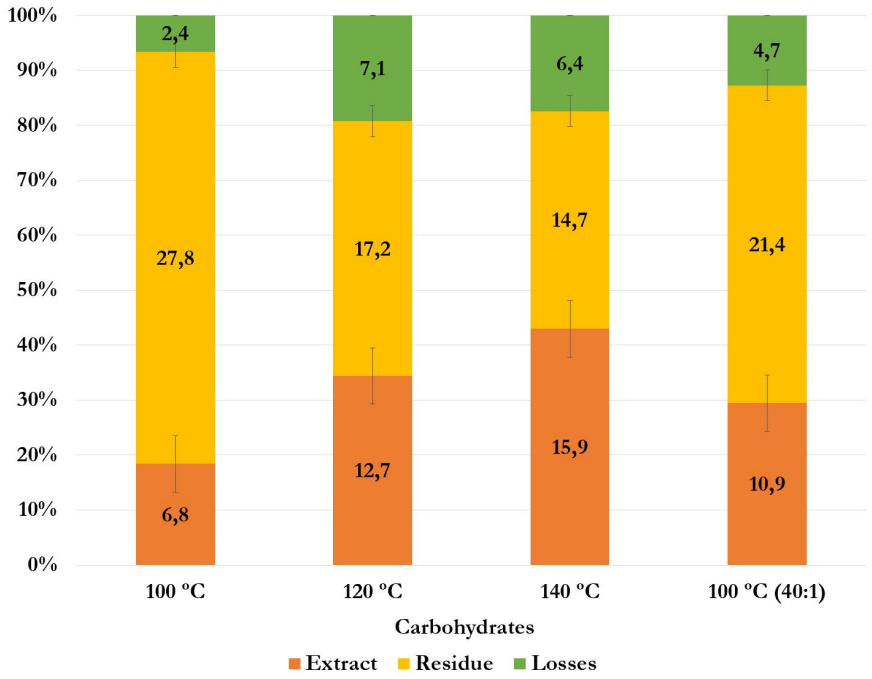


Figure 3.6: The influence of temperature and ratio on the yield of carbohydrates in the lupin powder (solvent-to-solid ratio: 20:1 and 40:1). Error bars are given. 100% = 37.0 g carbohydrates/100 g lupin.

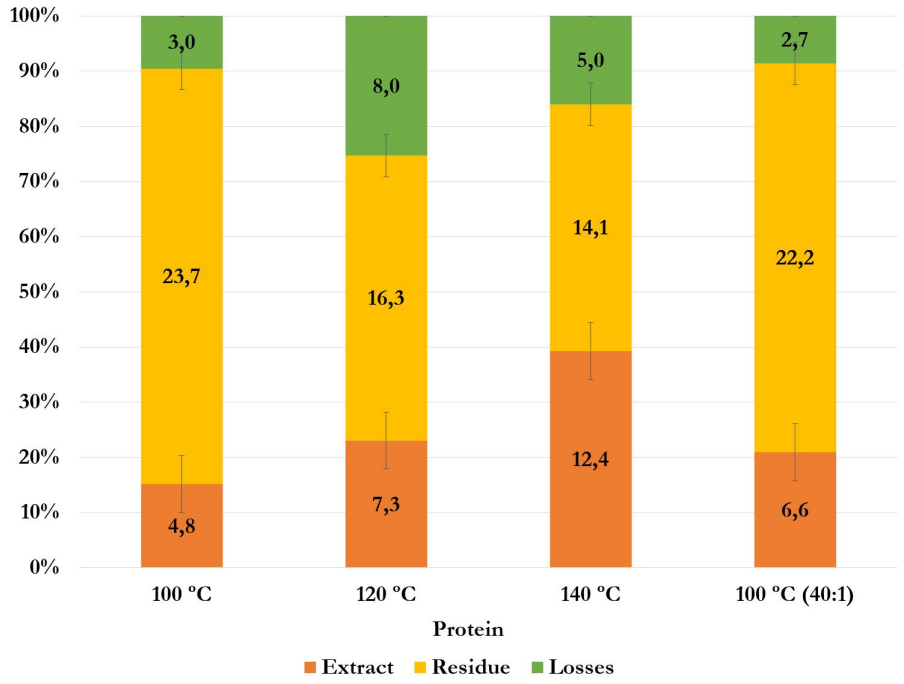


Figure 3.7: The influence of temperature and ratio on the yield of protein in the lupin powder (solvent-to-solid ratio: 20:1 and 40:1). Error bars are given. 100% = 31.5 g protein/100 g lupin.

Chapter 4

Conclusions

This thesis aimed to find a green alternative for the debittering process of white lupin. This lupin cannot be consumed before alkaloids, which confer a bitter taste and are toxic, are removed. The method studied was extraction with subcritical water under batch conditions.

First, white lupin was chemically characterized to find a high value of protein of 31.5%, which is more than found in many other legumes. In terms of carbohydrates, it was found that around 29% are structural sugars, and 8% are non-structural, soluble sugars. The lipids content was about 9%.

Afterwards, subcritical water extractions under batch conditions were performed to study the best parameters for extracting alkaloids, namely temperature, solvent-to-solid ratio, and successive extractions, while maintaining the very high nutritional value of the lupin.

The subcritical water assays were performed at 100, 120, and 140 ºC involving two solvent-to-solid ratios with a constant residence time of 1 hour and 50 bar of pressure. The best lupanine extraction yield was achieved at 100 ºC and a 20:1 solvent-to-solid ratio: 0.95 g of lupanine per 100 g of lupin were extracted, accounting for approximately 71% of the lupanine present in lupin, while maintaining in the lupin matrix the major nutritional components (protein, carbohydrates, and lipids). As temperature increases above 120 ºC, alkaloid degradation occurs due to their sensitivity to high temperatures.

Consecutive extractions at 100 ºC and a 20:1 solvent-to-solid ratio with both lupin powder and whole lupin seeds showed that the second extraction barely enhanced the extraction yield of lupanine. This can happen because the remaining alkaloids are trapped in the lupin matrix, and water at these conditions cannot remove them. Even so, this demonstrated that the particle size did not play a major role in the yield of extraction since the yields of lupanine, protein, carbohydrates, and lipids were similar.

Water-soluble compounds were also analyzed throughout this process. It was expected a 12-27% loss of solids during the extractions, and indeed there were 12-21% losses in this work. The highest amount of water-soluble components was obtained at 140 ºC and a 20:1 solvent-to-solid ratio, namely 39 g/100 g of lupin. As a consequence of debittering at that temperature, the nutritional value of lupin decreases as well, higher amounts of protein and carbohydrates being removed from the lupin matrix.

Quantification of alkaloids was also done in the lupin residue not only to confirm the extraction yields but also to close mass balances. By submitting the lupin residue to 8 successive extractions, it was possible to confirm that alkaloids are indeed extracted from the lupin matrix in higher amounts at 140 ºC than at lower temperatures. If they are not detected in equivalent amounts in the lupin extracts, it must be because they are degraded in the reactor at 140 ºC. This finding clarifies previous work [54].

Protein, carbohydrates, and lipids were also analyzed in the residue after each assay, again to confirm the results obtained from analyzing the lupin extracts and to close mass balances, taking previous work [54] one step further. The best extraction conditions from the standpoint of keeping protein in the lupin matrix were indeed 100 ºC and a 20:1 solvent-to-solid ratio, where 23.7 g/100 g of protein out of 31.5 g/100 g lupin remained in the lupin residue. These extraction conditions also allowed 27.8 g/100 g lupin of carbohydrates out of 37.0 g/100 g lupin to remain in the matrix. Additionally, it was found that lipids essentially remain in the lupin residue. Even though these are positive outcomes, the amount of alkaloids in the lupin residue after extraction at the conditions indicated is still above the toxicity level allowed for consumption (0.02 g/100 g lupin).

GC-FID analysis made possible the quantification of lupanine, the major alkaloid present in lupin, in amounts reported to represent 80 to 100% of all alkaloids. Previous work [54] did not use a method of analysis focused only on lupanine, but rather on the amount of a solid containing alkaloids. This can explain higher amounts of alkaloids extracted when compared to those obtained in the present work. The work described in this thesis did not succeed in bringing the alkaloids of lupin under toxicity level when using subcritical water extraction. It confirmed that the quantification of alkaloids to close mass balances is a highly time-consuming process. It also showed the need to conduct several assays with the same experimental parameters to better quantify errors associated with the assays.

Chapter 5

Future work

- Lupanine was the alkaloid monitored in this study. It represents from 80 to almost 100% of the total content of alkaloids in white lupin. Future work should be done to account for the remaining alkaloids present in white lupin, such as albine, angustifoline, 13α-hydroxylupanine, and 13α-angeloyloxy-lupanine.[12]
- Future work should consider varying the residence time. At 100 ºC, 60 minutes might be a relatively short time for the extraction, as seen in the lupanine yield. Previous work [54] used also 60 minutes of residence time in assays with subcritical water. However, when performing extraction with water below its boiling point, there was an improvement in extraction yields from 30 to 180 minutes. The suggestion is thus to increase residence time in assays with subcritical water, to 90 minutes, or possibly higher.
- As mentioned earlier, it is also suggested to perform several extractions with the same experimental parameters in order to quantify errors better, namely those associated with the use of the batch reactor apparatus.

Bibliography

[1] P. D. Richards, R. J. Myers, S. M. Swinton, and R. T. Walker, «Exchange rates, soybean supply response, and deforestation in South America», *Global Environmental Change*, vol. 22, no. 2, pp. 454–462, 2012. DOI: 10.1016/j.gloenvcha.2012.01.004.

[2] D. Byerlee, J. Stevenson, and N. Villoria, «Does intensification slow crop land expansion or encourage deforestation?», *Global Food Security*, vol. 3, no. 2, pp. 1–7, 2014. DOI: 10.1016/j.gfs.2014.04.001.

[3] M. Karamać, H. H. Orak, R. Amarowicz, A. Orak, and W. Piekoszewski, «Phenolic contents and antioxidant capacities of wild and cultivated white lupin (*Lupinus albus L.*) seeds», *Food Chemistry*, vol. 258, pp. 1–7, 2018. DOI: 10.1016/j.foodchem.2018.03.041.

[4] Kirsten Pilegaard and Jørn Gry, *Alkaloids in edible lupin seeds*, 9. 2008, vol. 53, pp. 1–71, ISBN: 9788578110796.

[5] S. Carmalia, V. D. Alves, I. M. Coelhoso, L. M. Ferreira, and A. M. Lourenço, «Recovery of lupanine from *Lupinus albus L.* leaching waters», *Separation and Purification Technology*, vol. 74, no. 1, pp. 38–43, 2010, ISSN: 13835866. DOI: 10.1016/j.seppur.2010.05.005.

[6] S. Tounsi-Hammami, C. Le Roux, S. Dhane-Fitouri, P. De Lajudie, R. Duponnois, and F. Ben Jeddi, «Genetic diversity of rhizobia associated with root nodules of white lupin (*Lupinus albus L.*) in Tunisian calcareous soils», *Systematic and Applied Microbiology*, vol. 42, no. 4, pp. 448–456, 2019. DOI: 10.1016/j.syapm.2019.04.002.

[7] A. Scarafoni, A. Consonni, V. Galbusera, A. Negri, G. Tedeschi, P. Rasmussen, C. Magni, and M. Duranti, «Identification and characterization of a Bowman-Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds», *Phytochemistry*, vol. 69, no. 9, pp. 1820–1825, 2008. DOI: 10.1016/j.phytochem.2008.03.023.

[8] C. Huyghe, «White lupin (*Lupinus albus L.*)», *Field Crops Research*, vol. 53, no. 1-3, pp. 147–160, 1997. DOI: 10.1016/S0378-4290(97)00028-2.

[9] H. T. Phan, S. R. Ellwood, K. Adhikari, M. N. Nelson, and R. P. Oliver, «The first genetic and comparative map of white lupin (*Lupinus albus L.*): Identification of QTLs for anthracnose resistance and flowering time, and a locus for alkaloid content», *DNA Research*, vol. 14, no. 2, pp. 59–70, 2007. DOI: 10.1093/dnares/dsm009.

[10] E. M. Abraham, I. Ganopoulos, P. Madesis, A. Mavromatis, P. Mylona, I. Nianiou-Obeidat, Z. Parissi, A. Polidoros, E. Tani, and D. Vlachostergios, «The use of lupin as a source of protein in animal feeding: Genomic tools and breeding approaches», *International Journal of Molecular Sciences*, vol. 20, no. 4, pp. 1–27, 2019. DOI: 10.3390/ijms20040851.

[11] G. Boschin, A. D'Agostina, P. Annicchiarico, and A. Arnoldi, «Effect of genotype and environment on fatty acid composition of *Lupinus albus L.* seed», *Food Chemistry*, vol. 108, no. 2, pp. 600–606, 2008. DOI: 10.1016/j.foodchem.2007.11.016.

[12] J. D. Coïsson, M. Arlorio, M. Locatelli, C. Garino, D. Resta, E. Sirtori, A. Arnoldi, and G. Boschin, «The artificial intelligence-based chemometrical characterisation of genotype/chemotype of *Lupinus albus* and *Lupinus angustifolius* permits their identification and potentially their traceability», *Food Chemistry*, vol. 129, no. 4, pp. 1806–1812, 2011, ISSN: 03088146. DOI: 10.1016/j.foodchem.2011.05.107.

[13] T. Group, *Lupin allergens*, 2010. [Online]. Available: http://toxinology.no/research-areas/food-allergens/lupin-allergens/ (visited on 09/12/2020).

[14] M. D. Drew, T. L. Borgeson, and D. L. Thiessen, «A review of processing of feed ingredients to enhance diet digestibility in finfish», *Animal Feed Science and Technology*, vol. 138, no. 2, pp. 118–136, 2007. DOI: 10.1016/j.anifeedsci.2007.06.019.

[15] A. Anwar, A. H. Wan, S. Omar, E. El-Haroun, and S. J. Davies, «The potential of a solid-state fermentation supplement to augment white lupin *(Lupinus albus)* meal incorporation in diets for farmed common carp (Cyprinus carpio)», *Aquaculture Reports*, vol. 17, pp. 1–10, 2020. DOI: 10.1016/j.aqrep.2020.100348.

[16] Shawnna Clark USDA, *Plant Guide*. 2014, pp. 1–6, ISBN: 1800222122.

[17] B. Pedras, M. Salema-Oom, I. Sá-Nogueira, P. Simões, A. Paiva, and S. Barreiros, «Valorization of white wine grape pomace through application of subcritical water: Analysis of extraction, hydrolysis, and biological activity of the extracts obtained», *Journal of Supercritical Fluids*, vol. 128, pp. 138–144, 2017. DOI: 10.1016/j.supflu.2017.05.020.

[18] B. M. Pedras, G. Regalin, I. Sá-Nogueira, P. Simões, A. Paiva, and S. Barreiros, «Fractionation of red wine grape pomace by subcritical water extraction/hydrolysis», *Journal of Supercritical Fluids*, vol. 160, 2020. DOI: 10.1016/j.supflu.2020.104793.

[19] T. Rogalinski, T. Ingram, and G. Brunner, «Hydrolysis of lignocellulosic biomass in water under elevated temperatures and pressures», *Journal of Supercritical Fluids*, vol. 47, no. 1, pp. 54–63, 2008. DOI: 10.1016/j.supflu.2008.05.003.

[20] B. Glencross, «Understanding the nutritional and biological constraints of ingredients to optimize their application in aquaculture feeds», in *Aquafeed Formulation*, 2016, pp. 33–73, ISBN: 9780128009956. DOI: 10.1016/B978-0-12-800873-7.00003-8.

[21] *World Soybean Production | SOPA*, 2020. [Online]. Available: http://www.sopa.org/statistics/world-soybean-production/ (visited on 08/31/2020).

[22] M. M. Lucas, F. L. Stoddard, P. Annicchiarico, J. Frías, C. Martínez-Villaluenga, D. Sussmann, M. Duranti, A. Seger, P. M. Zander, and J. J. Pueyo, «The future of lupin as a protein crop in Europe», *Frontiers in Plant Science*, vol. 6, no. September, pp. 1–6, 2015. DOI: 10.3389/fpls.2015.00705.

[23] Y. Y. Zhu, H. Q. Zeng, C. X. Dong, X. M. Yin, Q. R. Shen, and Z. M. Yang, «MicroRNA expression profiles associated with phosphorus deficiency in white lupin (*Lupinus albus L.*)», *Plant Science*, vol. 178, no. 1, pp. 23–29, 2010. DOI: 10.1016/j.plantsci.2009.09.011.

[24] T. Mimmo, S. Hann, L. Jaitz, S. Cesco, C. E. Gessa, and M. Puschenreiter, «Time and substrate dependent exudation of carboxylates by *Lupinus albus L.* and *Brassica napus L.*», *Plant Physiology and Biochemistry*, vol. 49, no. 11, pp. 1272–1278, 2011. DOI: 10.1016/j.plaphy.2011.08.012.

[25] Z. Wang, A. B. Rahman, G. Wang, U. Ludewig, J. Shen, and G. Neumann, «Hormonal interactions during cluster-root development in phosphate-deficient white lupin (*Lupinus albus L.*)», *Journal of Plant Physiology*, vol. 177, pp. 74–82, 2015. DOI: 10.1016/j.jplph.2014.10.022.

[26] S. O. Essien, B. Young, and S. Baroutian, «Recent advances in subcritical water and supercritical carbon dioxide extraction of bioactive compounds from plant materials», *Trends in Food Science and Technology*, vol. 97, pp. 156–169, 2020. DOI: 10.1016/j.tifs.2020.01.014.

[27] P. P. Singh and M. D. Saldaña, «Subcritical water extraction of phenolic compounds from potato peel», *Food Research International*, vol. 44, no. 8, pp. 2452–2458, 2011. DOI: 10.1016/j.foodres.2011.02.006.

[28] G. G. Fontanari, J. P. Batistuti, R. J. D. Cruz, P. H. N. Saldiva, and J. A. G. Arêas, «Cholesterol-lowering effect of whole lupin (*Lupinus albus*) seed and its protein isolate», *Food Chemistry*, vol. 132, no. 3, pp. 1521–1526, 2012. DOI: 10.1016/j.foodchem.2011.11.145.

[29] D. J. Murphy, «Alkaloids», *Encyclopedia of Applied Plant Sciences*, vol. 2, pp. 118–124, 2017. DOI: 10.1016/B978-0-12-394807-6.00221-5.

[30] J. Jirschitzka, F. Dolke, and J. C. D'Auria, *Increasing the pace of new discoveries in tropane alkaloid biosynthesis*. 2013, vol. 68, pp. 39–72, ISBN: 9780124080614. DOI: 10.1016/B978-0-12-408061-4.00002-X.

[31] Z. Memariani, N. Gorji, R. Moeini, and M. H. Farzaei, *Traditional uses*. 2019, pp. 23–66, ISBN: 9780128153543. DOI: 10.1016/B978-0-12-815354-3.00004-6.

[32] F. C. Santana and J. Empis, «Bacterial removal of quinolizidine alkaloids from *Lupinus albus* flours», *European Food Research and Technology*, vol. 212, no. 2, pp. 217–224, 2001. DOI: 10.1007/s002170000221.

[33] M. Erbaş, M. Certel, and M. K. Uslu, «Some chemical properties of white lupin seeds (*Lupinus albus L.*)», *Food Chemistry*, vol. 89, no. 3, pp. 341–345, 2005. DOI: 10.1016/j.foodchem.2004.02.040.

[34] M. Erbas, «The effects of different debittering methods on the production of lupin bean snack from bitter *Lupinus albus L.* seeds», *Journal of Food Quality*, vol. 33, no. 6, pp. 742–757, 2010. DOI: 10.1111/j.1745-4557.2010.00347.x.

[35] M. Muzquiz, L. Carmen Cuadrado, G. Ayet, C. de la Cuadra, C. Burbano, and A. Osagiel, «Variation of Alkaloid Components of Lupin Seeds in 49 Genotypes of *Lupinus albus L.* from Different Countries and Locations», Tech. Rep., 1994, pp. 1447–1450. DOI: 10.1021/jf00043a011.

[36] M. Muzquiz, C. L. Ridout, K. R. Price, and G. R. Fenwick, «The saponin content and composition of sweet and bitter lupin seed», *Journal of the Science of Food and Agriculture*, vol. 63, no. 1, pp. 47–52, 1993. DOI: 10.1002/jsfa.2740630108.

[37] N. Erdemoglu, S. Ozkan, and F. Tosun, «Alkaloid profile and antimicrobial activity of *Lupinus angustifolius L.* alkaloid extract», *Phytochemistry Reviews*, vol. 6, no. 1, pp. 197–201, 2007. DOI: 10.1007/s11101-006-9055-8.

[38] H. Wang, Y. Lu, J. Chen, J. Li, and S. Liu, «Subcritical water extraction of alkaloids in *Sophora flavescens* Ait. and determination by capillary electrophoresis with field-amplified sample stacking», *Journal of Pharmaceutical and Biomedical Analysis*, vol. 58, no. 1, pp. 146–151, 2012. DOI: 10.1016/j.jpba.2011.09.014.

[39] T. Pacchiarotta, E. Nevedomskaya, A. Carrasco-Pancorbo, A. M. Deelder, and O. A. Mayboroda, «Evaluation of GC-APCI/MS and GC-FID As a Complementary Platform», *Journal of Biomolecular Techniques*, vol. 21, no. 4, pp. 205–2013, 2010.

[40] J. Vergilio, T. Claus, O. O. Santos Jr, L. U. Rovigatti Chiavelli, and S. Ara, «Analytical Aspects of the Flame Ionization Detection in Comparison with Mass Spectrometry with Emphasis on Fatty Acids and Their Esters», *Advances in Gas Chromatography*, pp. 39–56, 2014. DOI: 10.5772/57333.

[41] B. B. Misra, E. Bassey, and M. Olivier, «Comparison of a GC-Orbitrap-MS with Parallel GC-FID Capabilities for Metabolomics of Human Serum», *bioRxiv*, 2019. DOI: 10.1101/740795.

[42] S. H. Nile, A. Nile, E. Gansukh, V. Baskar, and G. Kai, «Subcritical water extraction of withanosides and withanolides from ashwagandha (*Withania somnifera L.*) and their biological activities», *Food and Chemical Toxicology*, vol. 132, pp. 1–9, 2019. DOI: 10.1016/j.fct.2019.110659.

[43] J. K. Yan, L. X. Wu, W. D. Cai, G. S. Xiao, Y. Duan, and H. Zhang, «Subcritical water extraction-based methods affect the physicochemical and functional properties of soluble dietary fibers from wheat bran», *Food Chemistry*, vol. 298, pp. 1–9, 2019. DOI: 10.1016/j.foodchem.2019.124987.

[44] H. U. Yoo, M. J. Ko, and M. S. Chung, «Hydrolysis of beta-glucan in oat flour during subcritical-water extraction», *Food Chemistry*, vol. 308, pp. 1–7, 2020. DOI: 10.1016/j.foodchem.2019.125670.

[45] X. Liu, Y. Wang, J. Zhang, L. Yan, S. Liu, A. A. Taha, J. Wang, and C. Ma, «Subcritical water extraction of phenolic antioxidants with improved α-amylase and α-glucosidase inhibitory activities from exocarps of Castanea mollissima Blume», *Journal of Supercritical Fluids*, vol. 158, pp. 1–9, 2020. DOI: 10.1016/j.supflu.2019.104747.

[46] M. Möller, P. Nilges, F. Harnisch, and U. Schröder, *Subcritical water as reaction environment: Fundamentals of hydrothermal biomass transformation*, 2011. DOI: 10.1002/cssc.201000341.

[47] S. W. Kim, M. J. Ko, and M. S. Chung, «Extraction of the flavonol quercetin from onion waste by combined treatment with intense pulsed light and subcritical water extraction», *Journal of Cleaner Production*, vol. 231, pp. 1192–1199, 2019. DOI: 10.1016/j.jclepro.2019.05.280.

[48] S. Haghighat Khajavi, Y. Kimura, T. Oomori, R. Matsuno, and S. Adachi, «Kinetics on sucrose decomposition in subcritical water», *LWT - Food Science and Technology*, vol. 38, no. 3, pp. 297–302, 2005. DOI: 10.1016/j.lwt.2004.06.005.

[49] A. Kruse and E. Dinjus, «Hot compressed water as reaction medium and reactant. 2. Degradation reactions», *Journal of Supercritical Fluids*, vol. 41, no. 3, pp. 361–379, 2007. DOI: 10.1016/j.supflu.2006.12.006.

[50] J. R. Vergara-Salinas, M. Vergara, C. Altamirano, Á. Gonzalez, and J. R. Pérez-Correa, «Characterization of pressurized hot water extracts of grape pomace: Chemical and biological antioxidant activity», *Food Chemistry*, vol. 171, pp. 62–69, 2015. DOI: 10.1016/j.foodchem.2014.08.094.

[51] M. Tanaka, A. Takamizu, M. Hoshino, M. Sasaki, and M. Goto, «Extraction of dietary fiber from Citrus junos peel with subcritical water», *Food and Bioproducts Processing*, vol. 90, no. 2, pp. 180–186, 2012. DOI: 10.1016/j.fbp.2011.03.005.

[52] B. M. Pedras, M. Nascimento, I. Sá-Nogueira, P. Simões, A. Paiva, and S. Barreiros, «Semi-continuous extraction/hydrolysis of spent coffee grounds with subcritical water», *Journal of Industrial and Engineering Chemistry*, vol. 72, pp. 453–456, 2019. DOI: 10.1016/j.jiec.2019.01.001.

[53] D. A. Iryani, S. Kumagai, M. Nonaka, Y. Nagashima, K. Sasaki, and T. Hirajima, «The hot compressed water treatment of solid waste material from the sugar industry for valuable chemical production», *International Journal of Green Energy*, vol. 11, no. 6, pp. 577–588, 2014. DOI: 10.1080/15435075.2013.777909.

[54] Carolina Isabel Coimbra Marques, «A green approach to the debittering of *Lupinus Albus L.*», Nova School University, 2020, pp. 19–37.

[55] B. Hames, C. Scarlata and A. Sluiter, «Determination of Protein Content in Biomass Laboratory Analytical Procedure (LAP)», Tech. Rep., 2008.

[56] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton, «Determination of Ash in Biomass Laboratory Analytical Procedure (LAP)», Tech. Rep., 2008.

[57] M. Luque de Castro, J. Ruiz Jiménez, and L. García Ayuso, «Environmental Applications | Soxhlet Extraction», *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*, no. June, pp. 1–8, 2013. DOI: 10.1016/b978-0-12-409547-2.04734-x.

[58] A. Sluiter, B. Hames, R. O. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. of Energy, «Determination of Structural Carbohydrates and Lignin in Biomass», Tech. Rep., 2008, pp. 1–14.

[59] F. M. Yedro, J. García-Serna, D. A. Cantero, F. Sobrón, and M. J. Cocero, «Hydrothermal hydrolysis of grape seeds to produce bio-oil», *RSC Advances*, vol. 4, no. 57, pp. 30 332–30 339, 2014. DOI: 10.1039/c4ra00429a.

[60] T. Masuko, A. Minami, N. Iwasaki, T. Majima, S. I. Nishimura, and Y. C. Lee, «Carbohydrate analysis by a phenol-sulfuric acid method in microplate format», *Analytical Biochemistry*, vol. 339, no. 1, pp. 69–72, 2005. DOI: 10.1016/j.ab.2004.12.001.

[61] M. Wink, C. Meißner, and L. Witte, «Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*», *Phytochemistry*, vol. 38, no. 1, pp. 139–153, 1995. DOI: 10.1016/0031-9422(95)91890-D.

[62] J. Prusinski, «White lupin (*Lupinus albus L.*) - Nutritional and health values in human nutrition - A review», *Czech Journal of Food Sciences*, vol. 35, no. 2, pp. 95–105, 2017. DOI: 10.17221/114/2016-CJFS.

[63] L. Rakotondramasy-Rabesiaka, J. L. Havet, C. Porte, and H. Fauduet, «Solid-liquid extraction of protopine from *Fumaria officinalis L.*-Analysis determination, kinetic reaction and model building», *Separation and Purification Technology*, vol. 54, no. 2, pp. 253–261, 2007. DOI: 10.1016/j.seppur.2006.09.015.

[64] F. Mosca, G. I. Hidalgo, J. Villasante, and M. P. Almajano, «Continuous or batch solid-liquid extraction of antioxidant compounds from seeds of *Sterculia apetala* plant and kinetic release study», *Molecules*, vol. 23, no. 7, pp. 1–12, 2018. DOI: 10.3390/molecules23071759.

[65] C. Jiménez-Martínez, H. Hernández-Sánchez, G. Alvárez-Manilla, N. Robledo-Quintos, J. Martínez-Herrera, and G. Dávila-Ortiz, «Effect of aqueous and alkaline thermal treatments on chemical composition and oligosaccharide, alkaloid and tannin contents of *Lupinus campestris* seeds», *Journal of the Science of Food and Agriculture*, vol. 81, no. 4, pp. 421–428, 2001. DOI: 10.1002/1097-0010(200103)81:4<421::AID-JSFA829>3.0.CO;2-U.

[66] J. E. Cacace and G. Mazza, «Mass transfer process during extraction of phenolic compounds from milled berries», *Journal of Food Engineering*, vol. 59, no. 4, pp. 379–389, 2003. DOI: 10.1016/S0260-8774(02)00497-1.

APPENDIX A

CALIBRATION CURVES

A.1 Colorimetric method

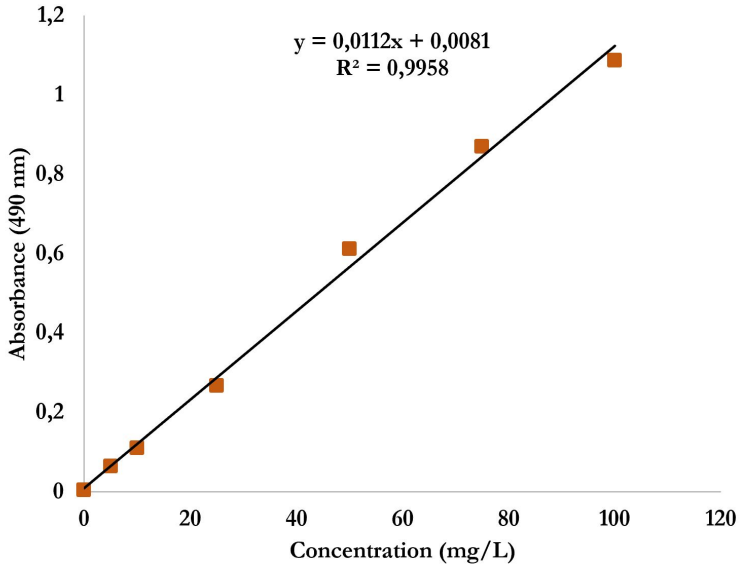


Figure A.1: Glucose calibration curve.

A.2 GC-FID method

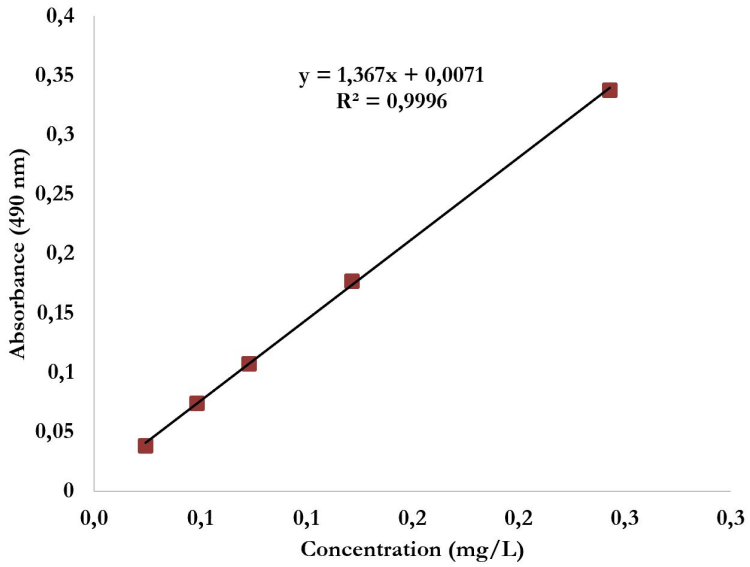


Figure A.2: Lupanine calibration curve (low range).

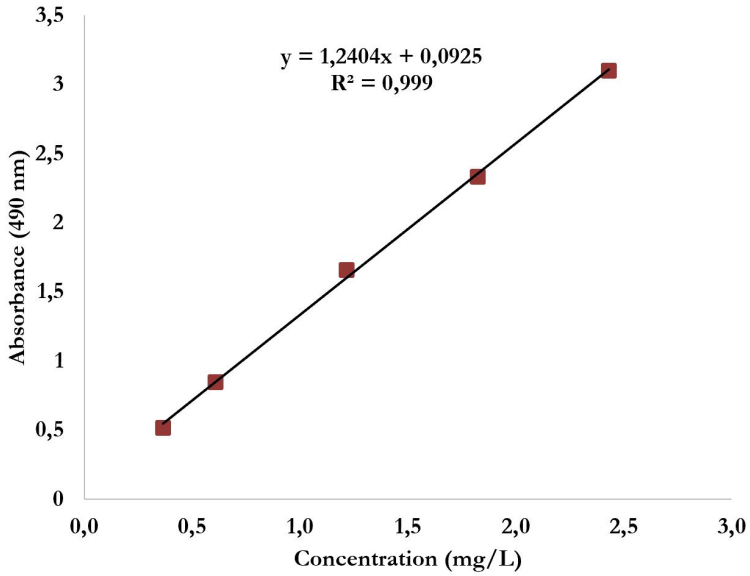


Figure A.3: Lupanine calibration curve (high range).