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A green approach to the debittering of *Lupinus Albus L.*

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To my family.

Acknowledgements

The work presented in this thesis would not have been possible without several people.

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Abstract

Lupinus Albus L. has been widely used for human and animal consumption around the Mediterranean and Middle East, due to its interesting nutritional value, especially the high content of protein and carbohydrates.

However, white lupin seeds cannot be consumed directly due to the high content of alkaloids, compounds that are toxic and confer a bitter taste. The traditional way to avoid this is successive rinsing and boiling with water, which uses a lot of water that becomes wastewater.

The aim of this work is to propose an alternative method to extract the alkaloids from the seeds. Two methodologies were studied: extraction with water below its boiling temperature, and extraction with subcritical water, both under batch conditions. The main focus of this thesis was testing a set of extraction parameters: temperature, solvent-to-solid ratio, residence time, and successive extractions.

White lupin seeds were first characterized, revealing a high content of protein (37%), carbohydrates (42%), and lipids (13%).

The extraction study revealed a notable influence of temperature and solvent-to-solid ratio on the alkaloid yield.

The best result achieved with water below its normal point was at 80 °C, for 30 minutes and a 40:1 (water:lupin) ratio: 35.5% of the total amount of alkaloids present in the matrix were extracted.

The best extraction result achieved with subcritical water was at 100 °C, for 60 minutes and a 40:1 (water:lupin) ratio: 77.4% of the total amount of alkaloids present in the matrix were extracted. At those conditions, other components were co-extracted, namely about 8 g/100 g lupin of carbohydrates, 7 g/100 g lupin of protein, and 4 g/100 g lupin of lipids.

A second extraction assay, performed at the same experimental conditions as assay 1, using the lupin matrix obtained as solid residue in assay 1 but with fresh water, led to a negligible, further removal of alkaloids, both in experiments with water below its boiling temperature, and with subcritical water.

Keywords: White lupin, Debittering, Alkaloids, Subcritical water, Water extraction.

Resumo

O tremoço branco, também conhecido como *Lupinus Albus L.*, tem sido muito valorizado no consumo humano e animal pelo Mediterrâneo e Médio Oriente, devido ao seu alto teor em proteína e hidratos de carbono.

No entanto, as sementes de tremoço necessitam de um pré-tratamento antes de consumidas, uma vez que contêm compostos tóxicos e indigestos: alcaloides. O método tradicional para eliminar estes compostos passa por lavar e ferver sucessivamente em grandes quantidades de água, posteriormente desperdiçada.

O objetivo deste trabalho passa por propor um método alternativo para extrair os alcaloides das sementes de tremoço. Foram estudados dois métodos: extração com água abaixo do seu ponto de ebulição e extração com água subcrítica, ambas em reator descontínuo. O principal objetivo neste estudo passou por testar o efeito de vários parâmetros: temperatura, razão solvente/sólido, tempo de residência e sucessivas extrações.

Primeiramente foi realizada uma caracterização química ao tremoço, onde se relatou o seu alto teor em proteína (37%), hidratos de carbono (42%) bem como em lípidos (13%).

A temperatura e a razão solvente/sólido demonstraram ser os parâmetros que mais influenciam o rendimento de alcaloides nos métodos de extração estudados.

O melhor resultado obtido na extração com água abaixo do ponto de ebulição foi a 80 °C, durante 30 minutos e com uma razão 40:1 (água:tremoço): 35.5% do total de alcaloides presentes nas sementes foram extraídos.

O melhor resultado obtido com água subcrítica foi atingido a 100 °C, durante 60 minutos e com uma razão 40:1 (água:tremoço): 77.4% do total de alcaloides presentes nas sementes foram extraídos. Nestas condições, outros componentes foram também extraídos, como 8 g/100 g tremoço de hidratos de carbono, 7 g/100 g tremoço de proteína e 4 g/100 g tremoço de lípidos.

Um segundo ensaio, realizado às mesmas condições experimentais que o primeiro ensaio, usando o tremoço proveniente da primeira extração mas com água nova, demonstrou remover uma quantidade insignificante e residual de alcaloides, tanto na extração com água abaixo da sua temperatura de ebulição, quanto com água subcrítica.

Palavras-chave: Tremoço branco, Processo de lavagem, Alcaloides, Água subcrítica, Extração com água.

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1 | Introduction

Since ancient times, white lupin has been largely cultivated around the Mediterranean and Middle East, especially for human and animal consumption. The nutritional value, particularly the high protein content, promotes highly healthy diets. Despite this, the presence of alkaloids, toxic compounds that additionally confer a bitter taste, limits its direct use. A simple solution is using a pretreatment, commonly known as debittering treatment.

This chapter introduces fundamental concepts and reports on work related to the subject of this thesis.

The first section starts with an overview of *Lupinus Albus L.*, its characterization, nutritional value, and applications in the food industry. Then, a second section introduces the quinolizidine alkaloids concept, their toxicity, applications and analytical methods. The third section presents a brief overview of different debittering technologies that already exist in industry and their disadvantages. Lastly, the chapter ends with a description of two proposals for solving the problem.

1.1 White Lupin (*Lupinus Albus L.*)

Lupin is generating more interest in different industries due to its benefits in relation to it being an abundant source of protein, fiber and fat.

From the more than 200 species of the genus *Lupinus* known, white lupin, or *Lupinus Albus L.*, is the longest known crop [1], mainly cultivated in the Mediterranean Sea and South America [2]. In addition, blue lupin or *Lupinus angustifolius L.*, yellow lupin or *Lupinus luteus L.* and *Lupinus mutabulies*, also known as pearl lupin, are other familiar species of agriculture interest (Figure 1.1).

The use of lupin seeds as human and animal food increased in the 20th century because of its numerous health benefits.

White lupin seeds contain high amounts of proteins, from 33% to 38%, with essential amino acids like lysine, threonine, leucine and arginine [1], making *Lupinus Albus L.* seeds more suitable and valuable for vegan diets, and for celiac people [4]. Compared to soybean, peas and bean, lupin proteins have low anti-nutritive properties due to their minimum content of α-, β- and γ-conglutin, globulin proteins that may cause allergenic

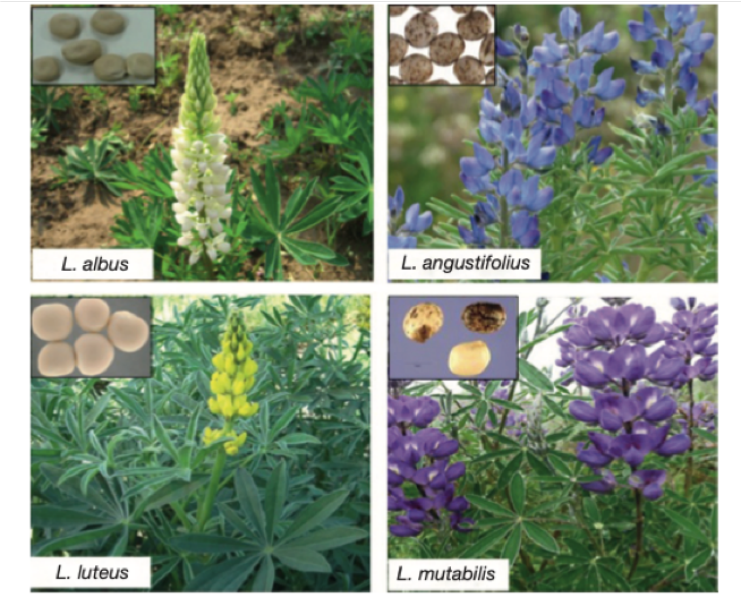

Figure 1.1: The most cultivated species of lupins and respective seeds [3].

effects. A proper diet includes this high protein content that can cause a significant decrease in LDL (bad colesterol) level, and reduce the blood pressure, preventing problems like diabetes and obesity [1].

White lupin is also a good source of carbohydrates, mono- and disaccharides, and non-starch polysaccharides being the major sugars in seeds. Mono- and disaccharides constitute about 6% of lupin seeds. Of these, sucrose was reported to be the most abundant (Table 1.1). These soluble sugars are important for seed germination due to their energy capacity [2].

The structural polysaccharides prevail, about 30% of the seed, mostly present in lupin hulls: cellulose and hemicellulose. These saccharides make white lupin an extraordinary source of fiber. Moreover, in contrast with other legumes, white lupin seeds do not contain many starch, resulting in a low glycemic index which prevents problems with insulin resistance [1].

Furthermore, the content in lipids (3–21%) plays an important role to maintain the energy at higher levels [5]. Mediterranean climate seeds, according to Annicchiarico *et al.*, normally have higher fat content [6]. In addition, lupin is a good source of unsaturated fatty acid moieties, e.g. ω-6 and ω-3. Nowadays, common diets have ratios of ω-6 to ω-3 up to 15 to 1. This excess of ω-6 acids is known to cause circulatory system diseases [1]. Andrzejewska *et al.* observed a 2:1 ratio in white lupin seeds, which reduces the risk of these health diseases and is advantageous for human health [7].

In addition to benefits to human and animal diet, literature studies have indicated that *Lupinus Albus L.* brings advantages for agriculture due to its ability to fix nitrogen via symbiotic nitrogen fixation (SNF). Nitrogen fixing crops can provide an alternative to the use of chemical fertilizers, which reduce the pollution problems and production costs

Mono and disaccharides	Mean value (g/kg)	Portion in total (%)
Sucrose	41.2 ± 1.5	70.7
Galactose	4.88 ± 0.32	8.4
Glucose	3.90 ± 0.27	6.7
Ribose	3.35 ± 0.04	5.8
Maltose	2.96 ± 0.23	5.1
Fructose	1.61 ± 0.04	2.8
Xylose	0.32 ± 0.05	0.6
Total	58.2	100

Table 1.1: Ratios of individual and total mono and disaccharide sugars of *Lupinus Albus L.* Values are reported on a dry matter basis [2].

[8, 9]. Additionally, soils with high nitrogen levels increase the yield and macroagregate stability [10]. Kristina Staples *et al.* reported that white lupin seeds can fix 150-200 kg/ha nitrogen [9]. Therefore, lupin contributes to reducing/eliminate the excessive use of nitrogen fertilizers and consequently generates sustainable agriculture.

Moreover, with a characteristic deep root system, white lupins tolerate nutrient-poor soils and access deeper water resources. These roots, when exposed to soils with nutrient deficiency, form clustered lateral rootlets (CRs), bringing several advantages. CRs provide access to a larger soil volume due to the increase of the surface area. They can absorb 10 times more nutrients, e.g., phosphorus, than root systems without cluster roots. This characteristic has been useful in understanding the physiology of nutrient acquisition, and consequently breeding other crops with this root system [11].

Annually, about 500,000 tons of lupin derived products are consumed in Europe such as pasta, biscuits, bread, milk substitutes and sauces. Additionally, some regions eat the seeds as a snack [12]. This consumption is due to lupin having been presented as an alternative to soybean and other legumes/grains. In fact, lupins are a great protein substitute and have low saturated fatty acid content. The high levels of oil with a high unsaturated fatty acids proportion is advantageous for human health [2] (Table 1.2).

		Lupin	Corn	Soybean	Wheat
Protein (%)		32.2	3.3	40.5	12.7
Fatty acids profile (%)	Saturated	13.5	7,5	15.5	21.1
	Monounsaturated	55.4	16.5	27.7	15.7
	Polyunsaturated	31.1	76.0	60.8	63.2

Table 1.2: Protein content and fatty acid profile composition of *L. albus* and other grains. Values are reported on a dry matter basis [2].

Although lupin seeds have great nutritional value, they also contain alkaloids in their composition, which renders the seeds improper for consumption. This anti nutritional compound has been related to the bitter taste present in some lupin species, especially in the *Lupinus Albus L.* and also with health problems (neurological effects leading to loss of motor coordination and muscular control) [13].

Seeds of *Lupinus Albus L.* are known as bitter based on their high levels of alkaloids, exceeding the limit of 0.200 mg/g [14]. Because of this, industries have been using different debittering pretreatments.

1.2 Quinolizidine Alkaloids

Quinolizidine alkaloids (QA) are frequently named as lupin alkaloids since they mostly occur in species from the genus *Lupinus*. These compounds play an important role, since they protect plants against pathogen microorganisms and herbivorous animals [13]. Also, Staples *et al.* observed that bitter lines, with higher alkaloid concentrations, had higher root nodulation for symbiotic nitrogen fixation (SNF) [9]. Despite that, as previously mentioned, QA are the principal reason why lupin seeds are toxic to humans and mammals.

Within the 170 alkaloids identified in different *Lupinus* species [13], lupanine is the major alkaloid present in *L. Albus* (see Table 1.3) and consequently correlated with the total alkaloid content, which varies from 0.02% to 13% [15].

Quinolizidine alkaloids	**Mean value (%)**
Lupanine	76.06
13-Hydroxylupanine	8.23
Multiflorine	5.52
Albine	4.48
Angustifoline	2.07
11,12-Seco-12,13-Didehydromultiflorine	1.74

Table 1.3: Total quinolizidine alkaloids content of *Lupinus Albus L.* [16]

1.2.1 Biosynthesis

Normally, alkaloids derive from the products of metabolism of amino acids, quinolizidine alkaloids being lysine-derived [13].

The biosynthetic pathways have been reported by a few researchers and it was established that the first step is the decarboxylation of L-lysine, by the enzyme lysine decarboxylase (LDC)[17]. From the decarboxylation, cadaverine is formed and, with a reaction catalyzed by the enzyme 17-oxosparteine synthetase, converted into 17-oxosparteine.

From this intermediate, lupanine is formed (Figure 1.2). This alkaloid serves as a precursor to other alkaloids, such as 13-hydroxylupanine.

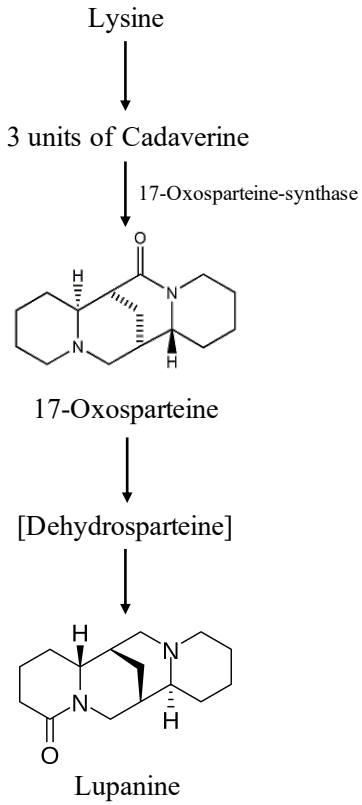

Figure 1.2: Biosynthesis pathways of lupanine. Adapted from [18].

The concentration of QA may vary depending on genotype and environment, which causes different alkaloid content between different lupin species. Some factors can influence this alkaloid concentration/synthesis: environmental conditions such as temperature, season, sunlight, soil properties (nitrogen levels and moisture) or even the presence of pathogen agents [13].

1.2.2 Toxicity

QA have the particularity of not being toxic to the legumes which produce them but toxic or very toxic to others.

According to the latest studies, tetracyclic alkaloids have the highest toxicity. One of these is lupanine, the major alkaloid from the seeds of white lupin. Results showed a moderate toxicity in vertebrates and acute in mammals, causing different effects like convulsions, tremors and death from heart attack or respiratory arrest [13][14]. In animals like sheep, QA toxicity can lead to depression, pulmonary paralysis and trembling. On human health, effects on the nervous system, gastrointestinal or cardiovascular systems are noted and can be observed if seeds are not debittered or ripe [13].

The acute oral LD_{50} (median lethal dose) values for lupanine (Figure 1.3), according to the latest literature results, was 159 mg/kg. The toxicity of other alkaloids was also determined, such as 13-hydroxylupanine and angustifoline, which are also present in white lupin seeds [19].

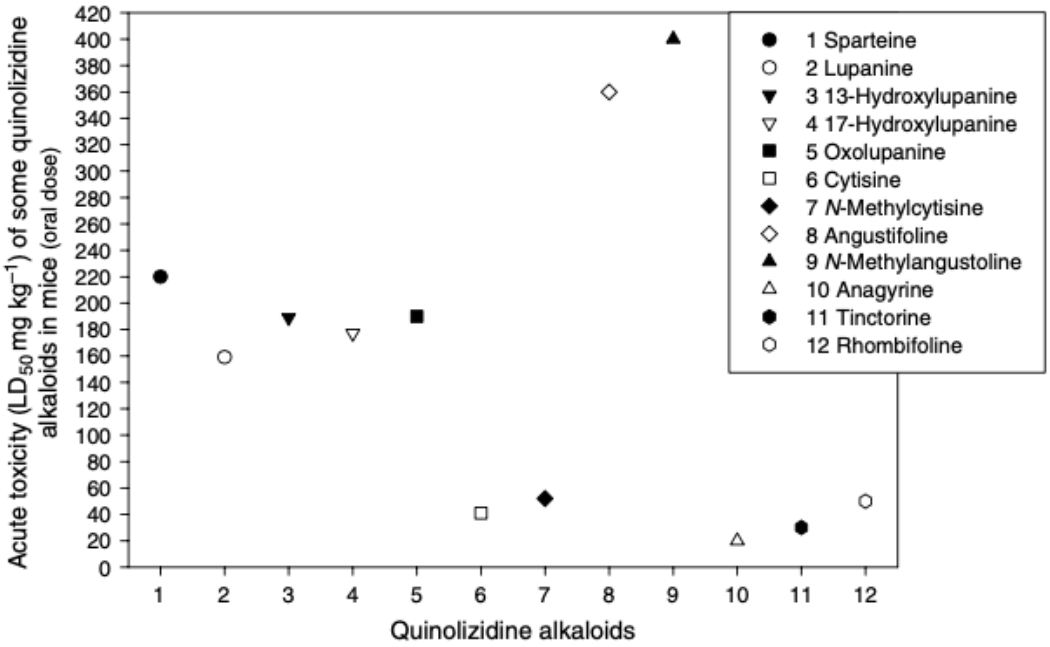

Figure 1.3: Quinolizidine alkaloids toxicity (LD_{50}) [19].

1.2.3 Analytical methods

Different techniques have been developed over the years to quantify QA. The extraction is normally performed with non-polar or low polar solvents as a consequence of the low polarity of quinolizidine alkaloids [13].

The determination has been made by several methods such as taste testing, seed color, calorimetry, thin layer chromatography (TLC), fluorescence, high performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry (MS).

Taste testing is a non-chemical and qualitative method where animals or humans are used as testers. It is because QAs have a bitter taste [20].

Seed color is also a qualitative method and it is based on the simple observation that dark seeds are bitter and white seeds are sweeter. This technique is not credible, especially in *Lupinus Albus L.*, because even very white seeds can have high alkaloid levels [20].

Calorimetry was used for the first time with quinolizidine alkaloids in 1940 and the idea is to compare unknown compounds with a calibration curve obtained with known standard compounds. It is based on the characteristic chemical reaction of these molecules. The limitations of these procedures lie in the reactions that are chosen [20].

TLC is an application known for its speed of analysis and can be qualitative or semi-quantitative. It is performed on a sheet of aluminum foil, coated with a silica gel layer (stationary phase). First, the sample is applied to the plate and, with a mobile phase, the mixture moves from one side to another. Afterwards, separation is achieved because analytes move at different rates. For qualification purposes, the distance covered by the sample is divided by the total distance (R_f) and to detect the substances, the spots are visualized by UV light onto the plate [20]. This method has limitations in the analyses of samples that have different alkaloids [13].

Fluorescence is a technique only used to qualify the alkaloids and is based on the fluorescence emission of some alkaloids at different wavelengths. Lupanine and its derivatives emit fluorescence after light absorption at 366 nm. The humidity of seeds is a critical factor and can limit the credibility of the results [20].

HPLC is useful to separate, identify and quantify the components of a sample mixture, when commercial standards are available [20]. By the fact that pure standards of QA are not easily available, HPLC is used by a few researches for alkaloids quantification [13]. The procedure relies on passing the sample through a column with a liquid or solid stationary phase (adsorbent). Each component interacts differently with the adsorbent, leading to different flow rates and to the separation.

GC is an alternative to HPLC, providing an easy and quick way to qualify but also quantify the alkaloids. Some temperature stability is required, to ensure that the substance is in the gas state. Another method is needed to identify the different alkaloids. GS coupled to a mass spectrometer (MS) has been a good alternative. MS allows the separation and identification of complex mixtures [20].

1.2.4 Applications

Alkaloids have been useful in different areas like agriculture and medicine.

Throughout history, some studies reported that plants with alkaloids have been important to improve health problems such as diabetes. Wiedemann *et al.* reported that lupanine can have antidiabetic properties due to its ability to reduce blood glucose levels, acting as a positive modulator of insulin release [21].

Alkaloids from many plants, especially lupin, have been considered as biological fertilizers due to their ability to establish complexes with the rhizosphere and with the soil. Later research confirmed that the use of Lupinex, a natural fertilizer which contains QA, increases the yield of legumes, cereals, oil plants and other crops [22].

Other research reported the possibility of using alkaloids as natural plant protector. For example, lupanine extract can be used to reduce the development of larvae [23]. This new possibility has been considered as a new alternative for synthetic products, such as pesticides [22].

1.3 Debittering Processes

As previously mentioned, one downside of white lupin seeds is their high content in toxic alkaloids, which causes a bitter taste. Lupin seeds need a pretreatment capable of extracting this antinutritional compound, to make the seeds proper and safe for consumption.

Different debittering methods have been used in industry: soaking/cooking with aqueous, acid and alkaline thermal procedures, germination, fermentation, and also breed species with low-alkaloid content [13].

Soaking and cooking has been the most effective process. This method has been tested at different conditions. According to one report, first the seeds were boiled in water (1:3, seed:water) for 75 min and then divided into three debittering procedures:

1. **Normal aqueous treatment:** Debittered with water at room temperature for 144 hours, water renewed at 12-h intervals;

2. **Thermal aqueous treatment:** Debittered with water at 55–65 °C for 144 hours, water renewed at 12-h intervals;

3. **Alkaline treatment:** Debittered with 0.5% sodium bicarbonate solution at room temperature for 144 hours, solution renewed at 12-h intervals.

Afterwards, the seeds are washed with water for 10 seconds and then soaked for 12 hours in salty water [24].

Authors reported that the total quinolizidine alkaloid content was lower in the seeds after alkaline treatment than after normal treatment, but both below the toxicity safety limit for humans and animals. On the other hand, some sensory properties (appearance, color, texture, etc.) and nutrients (mainly proteins and carbohydrates) loss occurs during these three treatments. Because of this, debittering with water at room temperature was found to be better, it is more straightforward to apply and the market loss in the food value of the seeds is minimized. Higher temperatures and organic solvents increase the secondary effects. Despite all this, large amounts of water are needed and wasted in these processes, which is a problem for the environment [13, 24–26].

Germination process can degrade some antinutritional factors and lead to products with better quality for animal and human diets. Chilomer *et al.* reported that total alkaloid content in blue and yellow lupin species reduced about 30% after 4-day germination in distilled water, at 24 °C in the dark. However this procedure takes time and does not garantee total removal of alkaloids, but can be a good complement [27].

Fermentation can also improve the nutritional value of lupin seeds and may result in a decrease of antinutritional compounds, which could be a faster and cheaper complement [28]. This process is one of the oldest for refining feed, where microorganisms are used, for example *Saccharomyces cerevisiae*, to transform the structure of protein and reduce the antinutritional factors, e.g. alkaloids. However, Zaworska *et al.* reported that lupin alkaloids are resistant to fermentation processes, it was observed that the alkaloids level

was higher in fermented seeds, compared with raw seeds. Moreover, a reduction of protein and non-structural sugars in the biomass was also observed [29].

Breeding species with low-alkaloid content has been investigated to solve the toxicity problem. However, since alkaloids are fundamental due to their strong genetic physiological function and background in plants, a decrease in yield and resistance has been observed [13]. Moreover, this has ecological consequences because bitterness is a dominant genetic characteristic [30].

An alternative method that has also been investigated is to detoxify lupin flours using bacteria able to degrade the alkaloids. The results suggest that this procedure may be an alternative debittering process [31].

Another methodology under investigation is the use of supercritical carbon dioxide extraction. Caetano *et al.* reported that using supercritical extraction with carbon dioxide 99.95% at 298K and 313K, i.e. above its critical point (parameter that will be mentioned in the section 1.5) only extracted lupanine and the quantities were small. In addition, this process always decreases the nutritional value due to the fact that lipids are simultaneously extracted with alkaloids [32].

1.4 Water extraction

The necessity to reduce both the waste of water and the use of organic solvents for extracting alkaloids has been led to alternative approaches. The process of extraction bioactive compounds is quite difficult in terms of selectivity. Besides alkaloids, several other soluble molecules are extracted, as previously mentioned in the section 1.3, causing significant decrease in nutritional value. Jimenez-Martinez *et al.* have reported that 120-270 g/kg of solids are lost during the aqueous, and alkaline thermal debittering procedures [26].

The efficiency of the extraction depends on multiple factors such as temperature, extraction time, solvent type, matrix particle size, solvent-to-solid ratio, and extraction conditions.

Researchers have shown that long extraction times might change the color and aspect of lupin seeds, due to the loss of pigments and water-soluble vitamins into the water [25]. Also, it was reported that significant reductions in crude protein and crude ash occur [33].

Though increasing temperature has a good effect on the extraction efficiency, it was reported that higher temperatures cause side effects on product quality such as degradation in color, and sensory properties, as well as thermally labile compounds [25, 34].

To obtain higher surface contact between solvent and matrix, smaller particle sizes should be used, facilitating the mass transfer of solutes [35]. Furthermore, Cacace *et al.* reported that increasing the solvent-to-solid ratio shows large effects on the yield, observing that there was a significant increase with the increase of the solvent-to-solute ratio [36].

As regards reactor configuration, batch solid-liquid extraction under continuous stirring is noted to be an easier and cheaper method for the extraction of natural compounds from plants. The continuous stirring allows the mixture to be constantly mixed, achieving turbulent conditions. Besides, the concentration gradient decreases, and mass transfer is privileged by the movement of vortexes [37].

A process under continuous conditions requires the use of more units such as peristaltic pumps, a solvent bath and an extraction column, making it more complex to operate [37].

This method has been applied in different studies such as in the extraction of antioxidant compounds from *Sterculia apetala* [37], phenolic compounds from milled berries [36], protopine from *Fumaria officinalis* [38], andrographolide from plants [39], and polyphenols from grape seeds [35].

1.5 Subcritical water extraction

The use of subcritical water extraction (SWE) is an interesting and environmentally benign alternative for extraction processes. It allows extracting a variety of solutes from different matrices such as plants [34].

Water is a nonflammable, green solvent. The water molecule is polar, and therefore liquid water at normal temperature and pressure is a good solvent for polar compounds, and salts. At atmospheric pressure, water boils at 100 °C. To avoid vaporization, pressure must be applied. *Subcritical water*, or *hot compressed water* (HCW), is the designation given to water above its normal boiling point and below its critical point (374 °C) [40], under sufficient pressure to remain in the liquid state (Figure 1.4). At the critical point, the density of the liquid and the gas become identical. Above it, the fluid is said to be supercritical, with a combination of liquid-like and gas-like properties [34, 41, 42].

HCW has a higher ionic product than water at ambient conditions. As temperature increases, the ionic product can become three orders of magnitude higher. Due to the high H^+ and OH^- concentrations, water behaves not only as a solvent, but also as a catalyst [44].

HCW is also slightly less dense than normal water, much less viscous, which allows the overcoming of mass transfer limitations, and has a lower dielectric constant, similar to alcohols, due to the fast movement of the water molecules, which disorders the intermolecular hydrogen bonding [34, 45]

Several studies show that extraction methods with subcritical water are more efficient, less expensive, and a good alternative to organic solvents, which avoids negative environmental impacts. It is a viable solution to extract antioxidants from different matrices [46–49], hemicellulose [50, 51], and alkaloids from *Sophora flavescens* and goldenseal [34, 41].

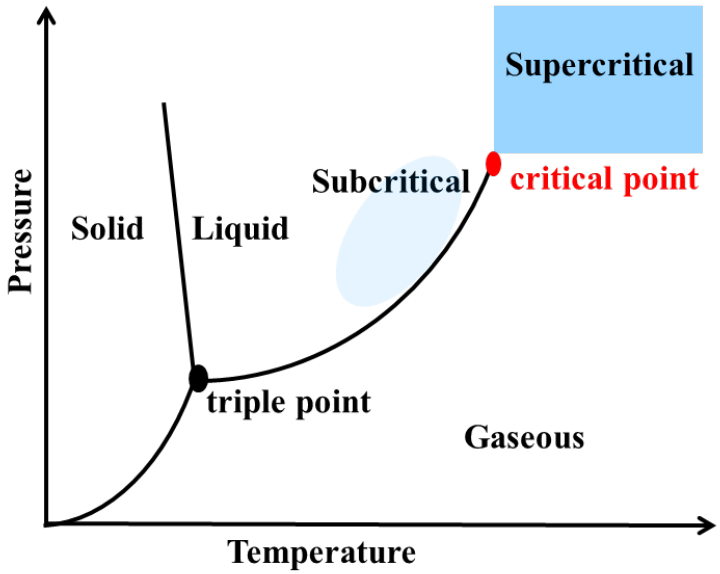

Figure 1.4: Water phase diagram indicating the subcritical and supercritical region, critical point, and triple point [43].

1.6 Motivation for this work, and thesis outline

Although widely adopted by the industry, the debittering pretreatment still faces some challenges. Lupin seeds debittering treatment usually includes cooking, and soaking in running water/aqueous alkaline solutions for several days, decreasing the alkaloid content to non-toxic levels. Large amounts of water/organic solvents are used and wasted, causing critical environmental impacts. Moreover, long operating times are needed, which consequently increase the process cost.

The aim of this thesis is to propose an alternative method for the debittering process, optimizing the operating parameters, especially the amount of water. Furthermore, costs must be as low as possible in the intended process, considering the limitations of the food and livestock feed industries.

To achieve the aim of this work, the following tasks were defined:

1. Determination of the chemical characterization of white lupin in order to know the content of some of its main components, for comparison with debittered lupin.

2. Debittering of white lupins using two different approaches, namely extraction with water below its boiling temperature, and subcritical water extraction, in both cases using batch reactors, varying several operation parameters such as temperature, residence time, and solvent-to-solute ratio.

3. Optimization of the extraction process, by bringing the alkaloid content under toxicity levels while minimizing the extraction of other valuable compounds.

The remainder of this document is organized into the following chapters:

- **Chapter 2** describes the materials and methods used to achieve the objective.
- **Chapter 3** reports and discusses the results obtained throughout this work.
- **Chapter 4** presents a short overview and conclusion on the obtained results.
- **Chapter 5** proposes future work.

2 | Materials and Methods

2.1 Materials

The material used during this project - white lupin seeds (*Lupinus Albus L.*) - was provided by the company Simbeja.

The seeds were previously cleaned (removal of leaves and other plant residues) and then fragmented in a coffee grinder, into a particle size between 0.5 and 1 mm (Figure 2.1). The resulting lupin seed powder was stored in a freezer.

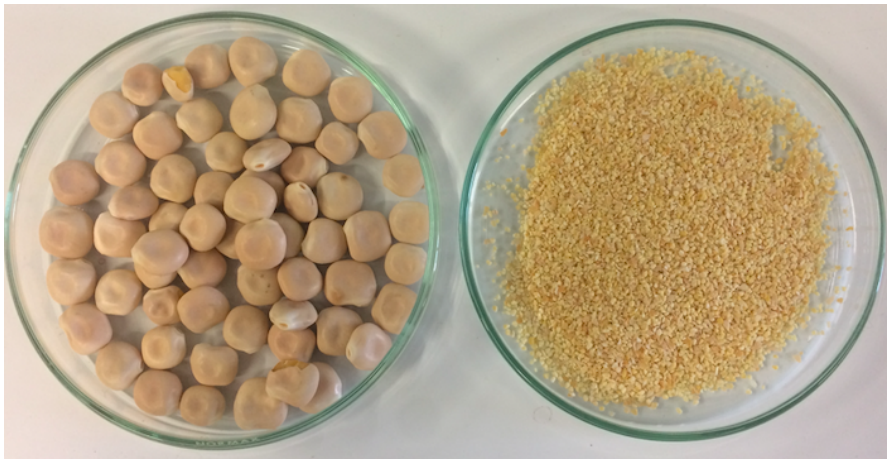

Figure 2.1: White lupin seeds and powder.

2.2 Chemical Characterization

2.2.1 Determination of protein content

In order to determine the crude protein content of lupin seeds, nitrogen content was determined by elemental analysis (at Laboratório de Análises, REQUIMTE-LAQV) and multiplied by a factor 6.25 [52].

To determine the soluble protein content, 5 g of ground white lupin seeds were mixed with distilled water (20:1, water:lupin) and incubated at 50 °C for 90 minutes, under constant stirring. Afterwards, the solution was filtered and the liquor obtained was lyophilized. Nitrogen content was determined in the remained solid, by elemental analysis.

The protein content of extracts was also determined by elemental analysis.

2.2.2 Determination of ash content

Ash content of lupin seeds was determined using 0.8 g of lupin seeds powder. The powder was put in a porcelain crucible, placed in a muffle at 550 °C for 6 hours. Afterwards, the crucible was placed in a desiccator to cool down and, through mass difference, the ash content was determined [53].

2.2.3 Determination of water content

Water content of lupin seeds was determined by using a hygrometer (KERN DAB 200-2) at 105 °C. For that purpose, 1 g of lupin seeds powder was weighed, and placed in the hygrometer, which calculated the water content values in mass percentage.

2.2.4 Determination of phenolic compounds content

To determine the total phenolic content of lupin seeds, first a hydro-alcoholic extraction was performed, and then phenolics were quantified in the resulting extract using the *Folin-Ciocalteu* method.

1. **Hydro-alcoholic extraction:** To 1 g sample of lupin seeds powder was added 40 mL of ethanol:water solution (60:40 v/v). The mixture was incubated at 50 °C for 90 minutes under constant magnetic stirring (150 rpm). Afterwards, it was filtered and the solution obtained was used to calculate the total phenolic content.

2. ***Folin-Ciocalteu* method:** The quantification was performed using the *Folin-Ciocalteu* method [54], with a calibration curve built with gallic acid monohydrate (Sigma 98%). For that purpose, solutions with different concentrations (25, 50, 100, 250, 500 and 750 mg/L) were prepared from a 5 g/L stock solution of gallic acid in milli-Q water.

   In order to avoid any interference, the solution obtained from the hydro-alcoholic extraction was centrifuged (Biofuge 13, Heraeus Sepatech) for 15 minutes at 12000 rpm, based on the procedure described by Sivaraman et al. [55].

   In test tubes were added 20 μL of the standard gallic acid solutions as well as the recovered supernatant from the centrifugation, 1.58 mL of distilled water and 100 μL of *Folin-Ciocalteu* reagent (MERK). The mixtures were stirred and incubated at room temperature, for five minutes. Afterwards, 300 μL of sodium carbonate solution (Sigma) was added and incubation in a dry bath (*Accu BlockTM Digital Dry Batch*) was carried out at 40 °C for 30 minutes.

   Lastly, the absorbance was measured at 750 nm (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA) and the concentration of the samples was calculated using the calibration curve, and expressed in g/L GAE (gallic acid equivalent).

2.2.5 Determination of lipid content

The lipid content was determined by the *Soxhlet* method (Figure 2.2), where 2 g of lupin seeds powder was defatted with 70 mL of *n*-hexane (Carlo Erba Reagents) for 3 hours. The resulting residue was dried overnight at 40 °C and, the solvent present in the oil solution was removed through evaporation. The remaining oil was weighed.

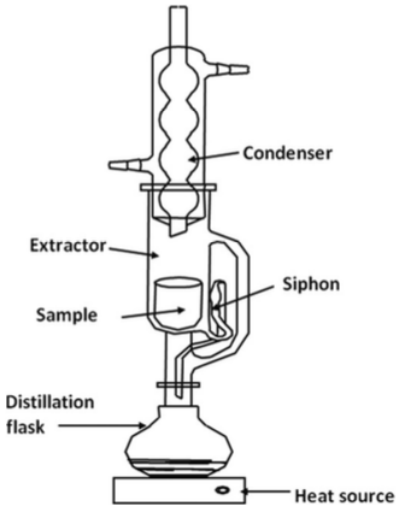

Figure 2.2: *Soxhlet* apparatus [56].

2.2.6 Determination of carbohydrates content

Carbohydrates are an important part of biomass samples and can be structural and non-structural. Because of this, two extractions were done: hydro-alcoholic extraction [57] (that extract the nonstructural sugars) and acid hydrolysis extraction [58] (where the structural sugars are removed). Both extractions were done with defatted lupin seeds.

The quantification of the sugars was performed by the colorimetric *phenol-sulfuric* method [59].

1. **Hydro-alcoholic extraction:** The residue obtained from the former step, 0.8 g, was first extracted with 40 mL of an ethanol:water solution (80:20 v/v) in an ultrasonic bath for 15 minutes at room temperature. Afterwards, the solid and liquid phases were separated by centrifugation for 10 minutes at 10000 rpm and the supernatant was collected. This procedure was repeated three times.

   The ethanol from the combined supernatants was evaporated under vacuum by a rotary evaporator at 50 °C. The soluble sugar-containing solution was diluted with 80 mL of water and used to quantify the carbohydrates.

2. **Acid hydrolysis extraction:** The resulting solid from the hydro-alcoholic extraction was dried overnight at 40 °C. From this residue, 0.3 g was weighed and to it were added 3 mL of 72% (w/w) H_2SO_4. This mixture was incubated in a 30 °C water

bath for 1 hour under constant stirring and diluted to 4% (w/w) by adding 84 mL of water.

Lastly, it was incubated in a silicone bath at 121 °C for 1 hour under constant stirring and afterwards it was filtered. The solution was then used to calculate the structural carbohydrates.

3. ***Phenol-sulfuric* method:** To quantify the nonstructural and structural carbohydrates by this method, a calibration curve was built with D+ glucose monohydrate (SIGMA Aldrich). For that purpose, glucose solutions with different concentration (0.005, 0.01, 0.025, 0.05, 0.075 and 0.1 g/L) were prepared from a 3 g/L stock solution in milli-Q water.

   To avoid any solids in the solution of the acid hydrolysis extraction, it was centrifuged (Biofuge 13, Heraeus Sepatech) at 12000 rpm for 15 minutes.

   Afterwards, to 500 μL of the recovered supernatant and of the standard D+ glucose monohydrate solutions was added 1.5 mL of 96% (w/w) H_2SO_4 and 300 μL of a 5% (w/v) aqueous solution of phenol. The mixtures were then stirred and incubated in a dry bath (*Accu BlockTM Digital Dry Batch*) at 90 °C for 5 minutes. After the incubation, the absorbance was measured and the concentration was calculated using the calibration curve, and expressed in g/L glucose equivalent.

2.2.7 Determination of lignin content

To calculate the lignin present in the lupin, the residue from the acid hydrolysis was washed with water, dried overnight at 105 °C and weighed [58].

2.2.8 Determination of quinolizidine alkaloids content

The first step to quantify the alkaloids was to add 30 g of lupin seeds powder to 100 mL of 1M acetic acid, and stir for 30 minutes. Then, in order to separate the solid and liquid phases, the mixture was centrifuged (Beckman Coulter, J-26 XPI) for 20 minutes at 12000 rpm. The supernatant was collected and its volume measured. This step was repeated 8 times.

The second step was to basify the aqueous solution (supernatant) with ammonium hydroxide (Honeywell) that was fed to a chromatographic solid phase extraction column (Isolute HM-N, Biotage), and eluted with dichloromethane (CH_2Cl_2, Honeywell). Finally, the CH_2Cl_2 was evaporated under vacuum by a rotary evaporator at 40 °C and the residue that remained (alkaloids) was dried overnight and weighed.

To determine the alkaloids content in the liquors from water and subcritical water extractions, only the second step was performed. 30 mL of liquor was basified with ammonium hydroxide (Honeywell), fed to the chromatographic solid phase extraction column (Isolute HM-N, Biotage), and eluted with dichloromethane (CH_2Cl_2, Honeywell).

Afterwards, the CH_2Cl_2 was evaporated at 40 °C under vacuum by a rotary evaporator, and the remaining residue was weighed.

2.2.9 Water extraction

Water extraction were performed with water below its boiling temperature, under continuous stirring. Lupin seeds powder was treated with water for different periods of time (30, 180 and 360 minutes), solvent-to-solid ratios (10:1, 20:1, 40:1) and temperatures (50 and 80 °C), in order to reach the highest possible concentration of the extracted compound (alkaloids) in the liquor.

800 mL of distilled water were used and placed in a 2 L cylindrical schott vessel containing different quantities of ground lupin seeds, to give the solvent-to-solid ratios indicated above. The schott vessel was placed in a thermostated water bath under stirring (magnetic stirrer, 200 rpm). In the end, in order to separate the lupin powder from the liquor, the samples were filtered under vacuum. The liquors were used to quantify the carbohydrates, protein, and alkaloid contents.

2.2.10 Subcritical water extraction

For the subcritical water extraction, the reactor apparatus used in this work is represented in Figure 2.3.

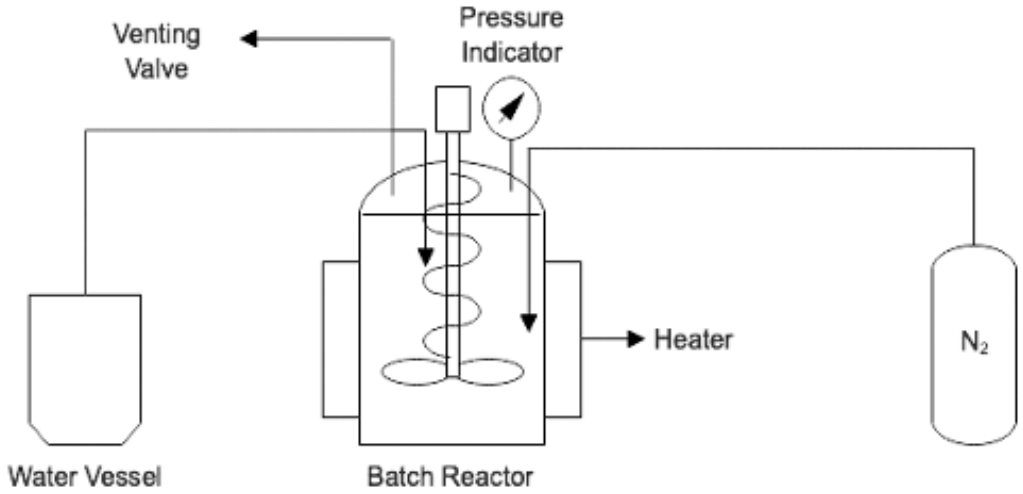

Figure 2.3: Subcritical water reactor apparatus.

It comprises a distilled water container, connected to a cooling coil in the reactor, which helps to regulate the temperature, and an agitator. The reactor is a stainless steel tube with a 1,2 L volume, and is covered by a heater, in order to reach the desired temperature. A nitrogen bottle is also connected to the reactor, with a valve, to pressurize.

Pressure in the reactor is measured with a pressure indicator. The venting valve is used to relieve the pressure at the end of the extraction.

Two different parameters were studied: temperature and solvent-to-solid ratio. In all the assays the residence time was 1 hour. Three temperatures were used: 100, 120 and 140 °C, with 20:1 and 40:1 solvent-to-solid ratio. The pressure used was only that necessary to maintain the liquid state, 10 bar at 100 and 120 °C, and 15 bar at 140 °C.

To perform an assay, first the lupin powder and distilled water were placed in the reactor. When the pressure reaches the desired value, the agitator and the heater were turned on. After the temperature is reached, the experiment started for 1 hour. At the end, the sample (lupin residue and liquor) was collected and filtered under vacuum, in order to separate the residue from the liquor. Each liquor sample was used to determine the carbohydrates, protein, and alkaloids content.

3 | Results and discussion

3.1 Chemical characterization

As previously mentioned, the first task of this thesis was to determine the chemical composition of white lupin seeds, provided by the company Simbeja. The main components determined were total carbohydrates, crude protein, oil, ash, phenolic compounds, as well as lignin. The composition is presented in Table 3.1. As expected, the dominant components identified were protein, carbohydrates, and lipids.

Components	g/100 g lupin
Carbohydrates	42.2 ± 0.3
Protein	36.5 ± 0.1
Lipids	13.2 ± 1.0
Ash	3.4 ± 0.1
Phenolic compounds	0.6 ± 0.1
Lignin	0.48 ± 0.01

Table 3.1: Chemical composition of white lupin seeds (data are reported on a dry mater basis; mean value ± standard deviation, n=3)

Protein content (36.5%) was similar compared with previous works, and was higher compared with other legumes like lentil and haricot bean [2, 60, 61]. Soluble protein content was 6.5 g/100g lupin, corresponding to ca. 18% of the total amount of protein.

The total carbohydrates content of white lupin seeds used in this work was 42%, also reported by other authors [1, 62]. The determination of total carbohydrates required the separation of soluble sugars, directly available in seeds, from insoluble sugars, which are the structural sugars that make up the structures of hemicellulose and cellulose. The levels of soluble and insoluble sugars determined in the seeds are shown in Table 3.2, with a major proportion of structural sugars (34%) compared with only 8% of free sugars.

Total fat content is usually from 8% to 12%. The white lupin seeds used in this study showed a high lipid content (13%), explained by the fact that in the Mediterranean climate white lupin is characterized by an 8% higher lipid content than in other regions [1]. Ash, phenolic compounds, and lignin were the minor components identified in the

seeds, which agrees with previous studies [2, 63, 64].

Total carbohydrates	g/100 g lupin
Soluble sugars	8.2 ± 0.1
Structural sugars	34.0 ± 0.5

Table 3.2: Free and structural sugars identified in white lupin seeds (data are reported on a dry mater basis; mean value ± standard deviation, n=3)

The main objective of this thesis was to extract the alkaloids present in white lupin seeds, to make them safe for consumption.

The determination of total alkaloid content was already done in a previous project (Lourenço *et al.*). As mentioned in the section 2.2.8, the procedure required 8 extractions to quantify the total alkaloids. In this work, only the first extraction was performed and the result, 1.28%, is in a good agreement with the result from the previous project, 1.25%. The total content of alkaloids present in white lupin seeds, as well as the total alkaloids extracted in each extraction is presented in Table 3.3, reproduced from the work of Lourenço *et al.*.

Extraction	Alkaloids (g/100 g lupin)
1	1.25
2	1.15
3	0.51
4	0.22
5	0.11
6	0.05
7	0.02
8	0.02
Total	3.34

Table 3.3: Total alkaloids content in white lupin seeds. Data are reported on a dry mater basis.

As expected, the result of total alkaloid content, 3.34 g/100 g lupin, demonstrated that these species of lupin, *Lupinus Albus L.*, is a bitter line. The high concentration of alkaloids exceeds the limit of 0.02 g/100 g lupin [14], and makes the seeds toxic to humans and animals.

The total alkaloid value 3.34 g/100 g lupin will be used for reference during this study.

3.2 Extraction study

The aim of this thesis concerns the extraction of alkaloids from the seeds of white lupin using two different approaches: water extraction with water below its boiling point, and subcritical water extraction. Both extractions were performed under batch conditions.

The yield of water soluble compounds was determined by measuring the amount of extract obtained. The latter was determined by lyophilizing the liquor that remained in the reactor after the assay.

The influence of several parameters on the yield of alkaloids was studied, namely the effects of temperature, solvent-to-solid ratio, residence time, and successive extractions. The yield (Y) of alkaloids is given by Equation 3.1:

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

Total carbohydrates and proteins were analysed in the extracts in order to study the impact of extraction on the nutritional value of lupin after the debittering process.

3.2.1 Water extraction

The yield of extraction of compounds from a food matrix depends on different parameters. In order to start with lower temperatures, which means lower operation costs, water below its boiling point and at atmospheric pressure was studied first.

Firstly, the influence of solvent-to-solid ratio on the extraction of alkaloids was studied at 50 °C, for extraction times of 3 and 6 hours. Table 3.4 shows that increasing solvent-to-solid ratio significantly increased the yield of alkaloids. In the 3 hours experiments, as that ratio varied from 10:1 to 40:1, the yield of alkaloids increased from below 1% to over 21%. The same trend was observed in the 6 h experiments. Table 3.4 also shows that the duration of the experiments in the time interval considered – 3 to 6 h – had a negligible effect on the yield of alkaloids.

The increase in the yield of alkaloids with the increase in solvent-to-solid ratio can be explained by the principles of mass transfer, where the concentration gradient is considered to be the main driving force. This concentration gradient is higher with higher solvent-to-solid ratios, which consequently increases the diffusion rate. Similar results were seen by Cacace *et al.* [36] and Lovasoa *et al.* [38].

To determine the impact of the extraction process on the lupin matrix, the yield of water soluble compounds was also assessed. In the 3 hours assays (Figure 3.1), increasing the solvent-to-solid ratio from 10:1 to 20:1 increased the amount of material extracted from the lupin matrix from 16% to about 23%. On the other hand, a change in that ratio from 20:1 to 40:1 had a negligible effect.

Extraction conditions			Yield of alkaloids
Temperature (°C)	Time (min)	Ratio	g/100 g lupin
50	180	10:1	0.02 (0.60%)
50	180	20:1	0.39 (11.6%)
50	180	40:1	0.73 (21.9%)
50	360	10:1	0.02 (0.60%)
50	360	20:1	0.37 (11.2%)

Table 3.4: Extraction with water below its boiling point: The influence of solvent-to-solid ratio on the yield of alkaloids. In parenthesis are given the percentage of the total amount of alkaloids extracted.

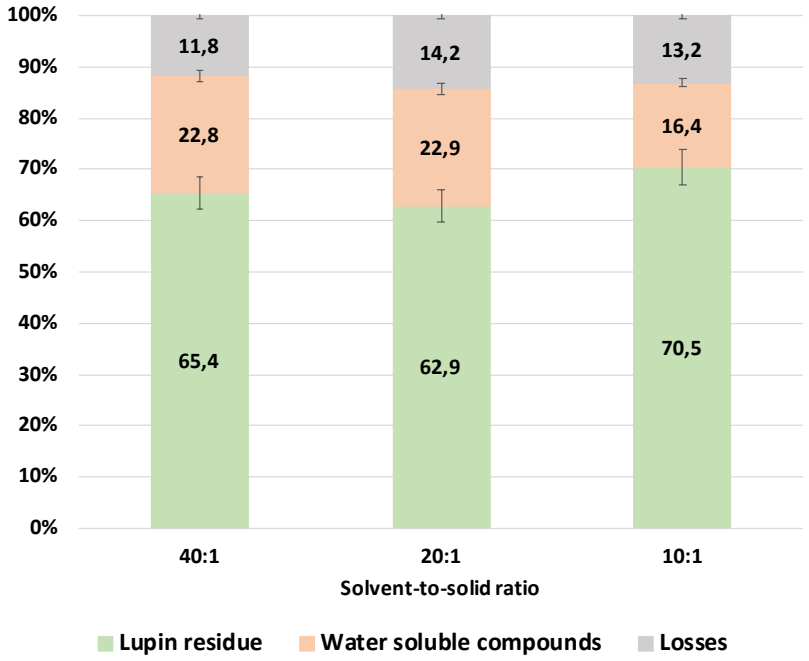

Figure 3.1: Extraction with water below its boiling point: The influence of solvent-to-solid ratio on the yield of water soluble compounds (temperature: 50 °C; time: 180 min). Error bars are given.

In the 6 hours experiments (Figure 3.2), very similar results were obtained. Increasing the solvent-to-solid ratio is advantageous for the alkaloids extraction, but on the other hand, it promotes the extraction of other compounds from the lupin matrix. This might be due to the fact that a larger volume of solvent can solubilize a higher amount of solutes, even at similar concentrations.

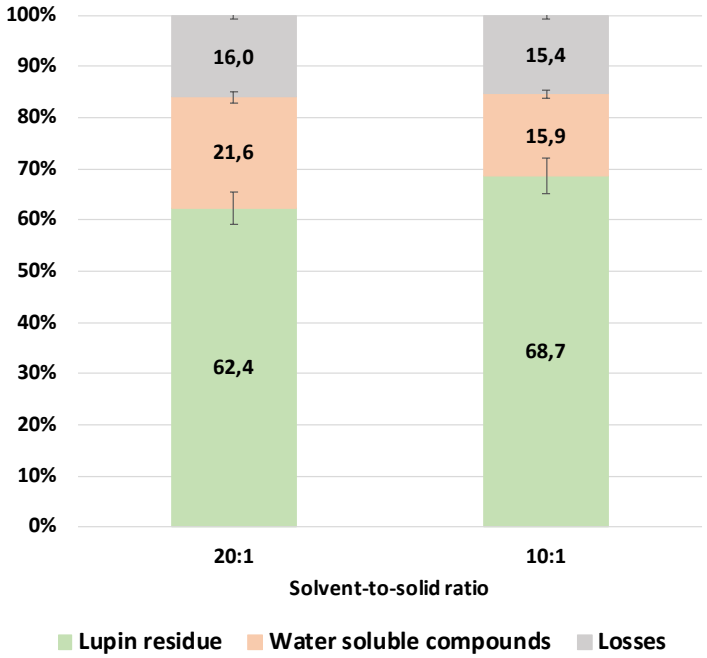

Figure 3.2: Extraction with water below its boiling point: The influence of solvent-to-solid ratio on the yield of water soluble compounds (temperature: 50 °C; time: 360 min). Error bars are given.

In all the assays loss of material was observed, as measured by the difference in the weight of lupin powder placed in the extractor, and the sum of lupin residue and water soluble compounds recovered at the end of the experiment. This can be explained by the fact that the lupin residue is first dried and then weighed, when compared to the original material placed in the extractor, which has a water content of about 10%. During the procedure there may also be loss of material when carrying out the collection of the residue from the extraction, and in the filtration procedure.

Moreover, in order to study more specifically the impact of water extraction of alkaloids on the lupin nutritional value, carbohydrates and protein contents in the extracts were determined, since these are the main compounds present in the white lupin seeds. The methods used were the same as described in chapter 2.

The results obtained showed that increasing the solvent-to-solid ratio had a small effect on the relative amounts of carbohydrates. On the other hand, the protein extracted increased about 2 g from 10:1 to 20:1 or 40:1 (Table 3.5).

This can be explained by the fact that at the temperature of the assays, water can only extract soluble carbohydrates and soluble protein of the lupin matrix, of nonstructural nature. As seen in the section 3.1, lupin seeds have 8.2 g of soluble sugars per 100 of lupin and 6.5 g of soluble protein per 100 of lupin, values that are close to those given in Table 3.5. In the case of soluble protein, a consistent increase in the amount extracted can be observed as the solvent-to-solid ratio increases from 10:1 to 20:1 or 40:1, which might be due to the capacity of a higher amount of water to solubilize this protein.

Extraction conditions			Yields (g/100 g lupin)	
Temperature (°C)	**Time (min)**	**Ratio**	**Carbohydrates**	**Protein**
50	180	10:1	8.34 ± 0.21	5.15 ± 0.15
50	180	20:1	8.58 ± 0.19	7.23 ± 0.15
50	180	40:1	8.06 ± 0.20	7.51 ± 0.18
50	360	10:1	8.33 ± 0.22	5.37 ± 0.13
50	360	20:1	8.02 ± 0.17	7.15 ± 0.18

Table 3.5: Extraction with water below its boiling point: The influence of solvent-to-solid ratio on the yields of carbohydrates and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

In addition, a second extraction was performed at 50 °C for 180 minutes, and with a solvent-to-solid ratio of 10:1. This consists of using the residue from the first extraction, which was submitted to a second extraction assay. The yield of water soluble compounds was only 6.7 g/100 g lupin (Figure 3.3), of which 4.6 g were protein, 1.7 g were carbohydrates and only 0.003 g were alkaloids (Table 3.6). The result for protein is somewhat surprising, since the amount of soluble protein was determined to be 6.5%. However, this value was determined with a matrix that was not the one submitted to the second extraction assay. It is possible that the changes in the matrix due to the removal of certain compounds made some of the soluble protein more accessible. Alkaloids were detected in the extract, but at very low levels, indicating that at these conditions, alkaloids were mostly removed in the first extraction.

Extraction conditions				Yields (g/100 g lupin)		
Temp (°C)	**Ratio**	**Time (min)**	**Extraction**	**Alkaloids**	**Carbohydrates**	**Protein**
50	10:1	180	1	0.02	8.34 ± 0.21	5.15 ± 0.15
50	10:1	180	2	0.00	1.77 ± 0.11	4.62 ± 0.16
			Total	0.02	10.1 ± 0.16	9.76 ± 0.16

Table 3.6: Extraction with water below its boiling point: The influence of multiple extractions on the yields of alkaloids, carbohydrates, and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

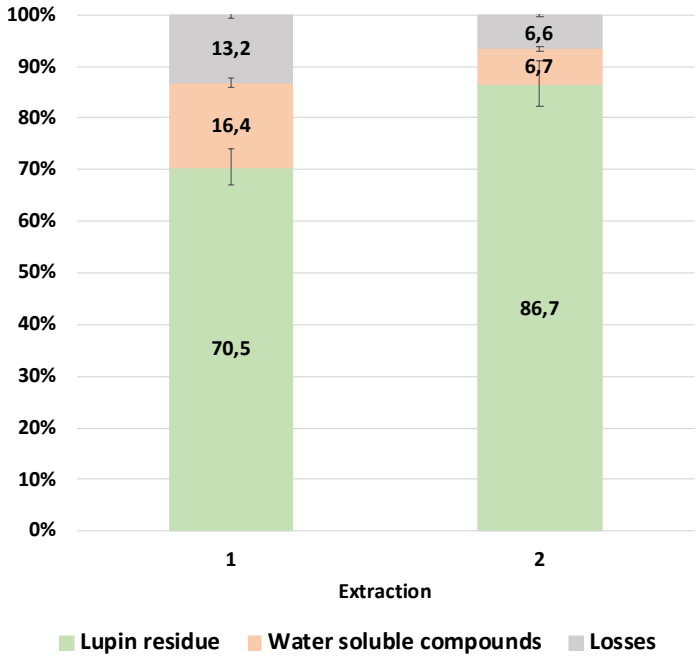

Figure 3.3: Extraction with water below its boiling point: The influence of multiple extractions on the yield of water soluble compounds (temperature: 50 °C; solvent-to-solid ratio: 10:1; time: 180 min). Error bars are given.

As reported, temperature is one of the most important variable in the extraction process.

The effect that temperature has on the alkaloids extraction from the lupin seeds was studied at 50 °C and 80 °C, in 30 minutes assays, with a solvent-to-solid ratio of 40:1, since it previously led to higher yields. Table 3.7 shows that increasing the temperature from 50 °C to 80 °C, notably increased the yield of alkaloids, from 14% to 36%. The increase in temperature facilitates, at the same time, the solubilization and diffusion of alkaloids from the matrix to the solvent, which results in better alkaloid yields.

Extraction conditions			Yield of alkaloids
Ratio	**Time (min)**	**Temperature (°C)**	**g/100 g lupin**
40:1	30	50	0.47 (14.0%)
40:1	30	80	1.19 (35.5%)

Table 3.7: Extraction with water below its boiling point: The influence of temperature on the yield of alkaloids. In parenthesis are given the percentage of the total amount of alkaloids extracted.

Furthermore, the water soluble compounds that were extracted also increased with temperature. At 50 °C, 16% of total solids were extracted, and at 80 °C it was observed an increase of 6%, i.e. from 16% to 23% (Figure 3.4). Within the water soluble compounds,

total carbohydrates and protein were analysed and the values were similar at both temperatures. Soluble sugars, about 8 g per 100 of lupin, and 7 g per 100 of lupin of the total proteins were extracted. As previously mentioned, only the non structural sugars and proteins are extracted at low temperatures. In addition, the lipid content in these extracts was also quantified and it was observed that 3 g/100 g lupin were extracted at 80 °C (Table 3.8).

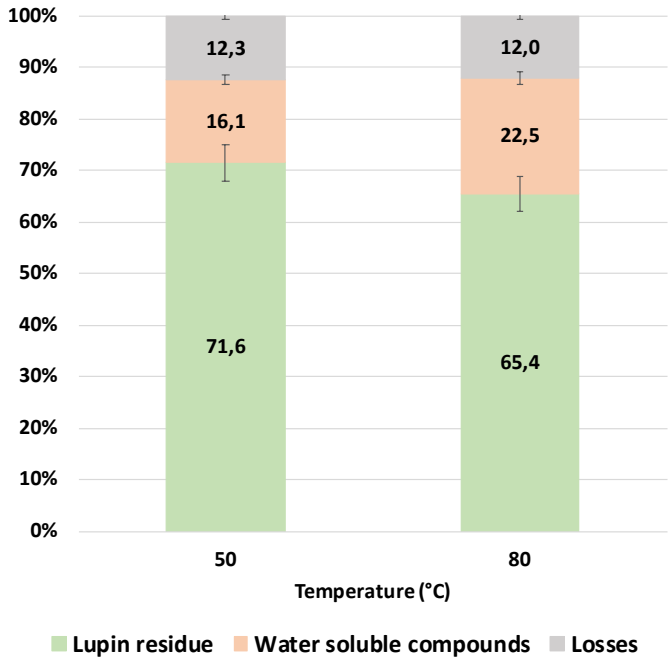

Figure 3.4: Extraction with water below its boiling point: The influence of temperature on the yield of water soluble compounds (solvent-to-solid ratio: 40:1; time: 30 min). Error bars are given.

Extraction conditions			Yields (g/100 g lupin)		
Ratio	**Time (min)**	**Temperature (°C)**	**Carbohydrates**	**Protein**	**Lipids**
40:1	30	50	8.79 ± 0.19	7.31 ± 0.14	0.02 ± 0.09
40:1	30	80	8.96 ± 0.20	7.64 ± 0.15	2.81 ± 0.10

Table 3.8: Extraction with water below its boiling point: The influence of temperature on the yields of carbohydrates, protein and lipids (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

In the interest of achieving better alkaloid yields, a second extraction was performed at 80 °C. As previously mentioned, this second extraction consists of using the residue collected from the first extraction, with fresh water.

It was observed that, from the first to the second extraction, the amount of water soluble compounds increased only by about 3 g/100 g lupin (Figure 3.5). The alkaloid yield did not show much better results with a second extraction. Only 0.08 g/100 g

lupin of alkaloids were additionally extracted (Table 3.9). This suggests that in the first extraction the solvent accessed those components that were accessible at the conditions of the experiment. By keeping those conditions constant in the second extraction, the solvent found a matrix that was nearly exhausted. The total amount of carbohydrates extracted barely changed with two extractions. However, 3 more grams of protein were removed per 100 g of lupin (Table 3.9).

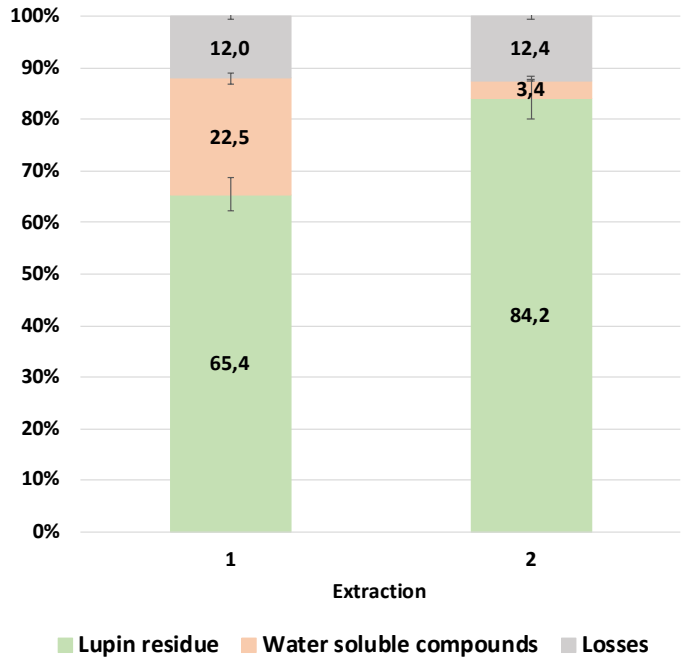

Figure 3.5: Extraction with water below its boiling point: The influence of multiple extractions on the yield of water soluble compounds (temperature: 80 °C; solvent-to-solid ratio: 40:1; time: 30 min). Error bars are given.

Extraction conditions				Yields (g/100 g lupin)		
Temp (°C)	**Ratio**	**Time (min)**	**Extraction**	**Alkaloids**	**Carbohydrates**	**Protein**
80	40:1	30	1	1.19	8.96 ± 0.20	7.64 ± 0.15
80	40:1	30	2	0.08	0.15 ± 0.11	3.01 ± 0.17
			Total	1.27	9.11 ± 0.16	10.6 ± 0.16

Table 3.9: Extraction with water below its boiling point: The influence of multiple extractions on the yields of alkaloids, carbohydrates, and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

Within the study that solvent-to-solid and temperature have on the extraction of alkaloids, the effect of residence time was also analysed.

It was reported that residence time also had influence on the alkaloid yield. From 30 to 180 minutes, at 50 °C and with a solvent-to-solid ratio of 40:1, the yield increased

from 14% to 22%. On the other hand, in the 180 and 360 minutes assays, the alkaloid yield remained constant, verified at two ratios, 20:1 and 10:1 (Table 3.10). An explanation could be the fact that over time the solid has imbibed more solvent, which dissolves better the compounds to extract, where the maximum is achieved in 180 min. After 180 minutes, the solvent appears to be saturated, and the thermodynamic equilibrium was reached.

Moreover, the results of the total carbohydrates and proteins showed similar values in all the experiments, because, as previously mentioned, temperature is the main variable that influences the extraction of these compounds. At 50 °C only non structural sugars and soluble proteins were extracted (Table 3.10).

Extraction conditions			**Yields (g/100 g lupin)**		
Temp (°C)	**Ratio**	**Time (min)**	**Alkaloids**	**Carbohydrates**	**Protein**
50	40:1	30	0.47 (14.0%)	8.79 ± 0.19	7.31 ± 0.14
50	40:1	180	0.73 (22.0%)	8.06 ± 0.20	7.51 ± 0.18
50	20:1	180	0.39 (11.6%)	8.58 ± 0.19	7.23 ± 0.15
50	20:1	360	0.37 (11.2%)	8.02 ± 0.17	7.15 ± 0.18
50	10:1	180	0.02 (0.60%)	8.34 ± 0.21	5.15 ± 0.15
50	10:1	360	0.02 (0.60%)	8.33 ± 0.22	5.37 ± 0.13

Table 3.10: Extraction with water below its boiling point: The influence of residence time on the yields of alkaloids, carbohydrates and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3). In parenthesis are given the percentage of the total amount of alkaloids extracted

Throughout the water extraction study, it was observed that about 16-22 g/100 g lupin of water soluble compounds are always extracted from the matrix to the solvent. Jimenez Martinez *et al.* also reported that 12-27 g/100 g lupin of solids are removed during the thermal debittering process, which is in a good agreement with our results [26].

Within the water soluble compounds, soluble sugars, protein and also lipids are the main nutrients that are extracted besides alkaloids. These results were also observed by Erbas: when the seeds were debittered with water at 65 °C, carbohydrates, protein and lipids contents decreased in the seeds [2].

3.2.2 Subcritical water extraction

Given that the alkaloid yield from water extraction was not enough to make the lupin seeds safe for consumption, subcritical water extraction was performed. The application of subcritical water as an extraction enables the use of higher temperatures, which can improve the mass transfer and solubility effects.

In this experiment, the influence of temperature on the yield of alkaloids, as well as other components, was studied at 100, 120 and 140 °C, with a solvent-to-solid ratio of

20:1, and a residence time of 60 min. Pressure was only the necessary pressure to keep water liquid (Table 3.11).

Extraction conditions			
Temperature (°C)	**Time (min)**	**Ratio**	**Pressure (bar)**
100	60	20:1	10
120	60	20:1	10
140	60	20:1	15

Table 3.11: Subcritical water extraction: Assay conditions.

Data obtained when subcritical was used indicated a downward tendency with the increase in temperature from 100 °C to 140 °C (Table 3.12). The alkaloid yield reached its maximum at 100 °C, with a value of 47.9%.

The decrease of alkaloids yield could be explained by the fact that these compounds are sensitive to heat, and higher temperatures may degrade the alkaloids. The degradation was significantly noted above 100 °C, demonstrating a possible degradation at higher temperatures.

Extraction conditions			Yield of alkaloids
Ratio	**Time (min)**	**Temperature (°C)**	**g/100 g lupin**
20:1	60	100	1.60 (47.9%)
20:1	60	120	1.45 (43.5%)
20:1	60	140	1.27 (37.9%)

Table 3.12: Subcritical water extraction: The influence of temperature on the yield of alkaloids. In parenthesis are given the percentage of the total amount of alkaloids extracted.

Wang *et al.* also reported these results, the total alkaloid yield from *Sophora flavescens* increased firstly with temperature from 70 to 100 °C, and then decreased from 100 to 190 °C, reaching its maximum at 100 °C [41].

Moreover, increasing the temperature from 100 °C to 140 °C increased the total amount of water soluble compounds from 22.5 % to 42.6 % (Figure 3.6) as expected, due to the fact that solubility of many compounds increases with temperature. With the increase in temperature, water also becomes a better catalyst for biomass hydrolysis. Because of this, some valuable compounds are extracted, and the nutritional value of white lupin seeds is negatively affected.

As previously mentioned, in all the assays there exist losses, explained by the fact that the lupin residue is first dried and then weighted, losing its water content, of about 10%.

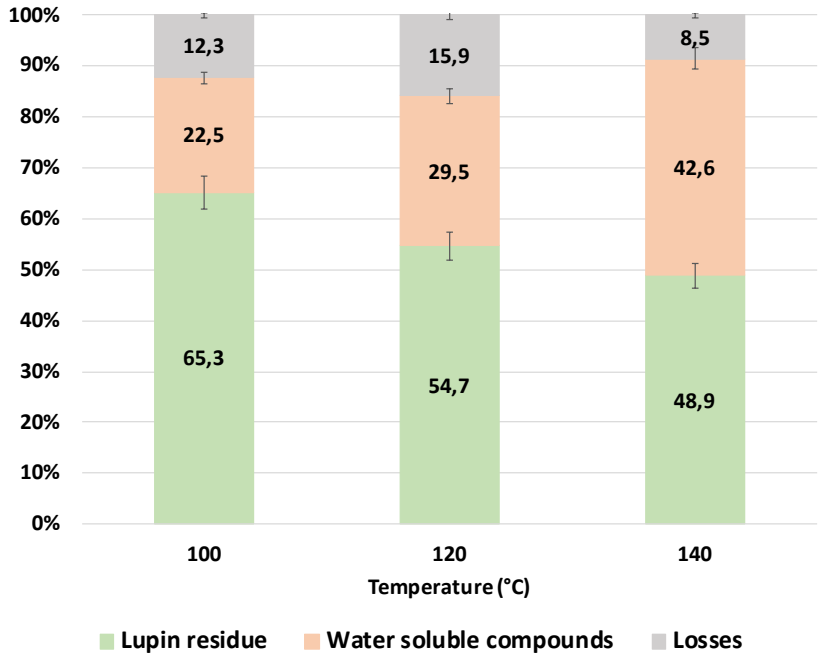

Figure 3.6: Subcritical water extraction: The influence of temperature on the yield of water soluble compounds (solvent-to-solid ratio: 20:1; time: 60 min). Error bars are given.

Moreover, in the collection of the residue from the reactor and in the filtration procedure there may also be losses.

White lupin seeds are a rich source of protein and carbohydrates (Table 3.1) so, in order to study the impact that subcritical water application has on the lupin nutritional value, total carbohydrates and protein contents were analysed in these extracts, using the same methods described in the chapter 2.

The values of total carbohydrates content (Table 3.13) in the extract increased as temperature increased from 100 to 140 °C. At 100 °C it was observed that only the soluble sugars were extracted, but from 120 to 140 °C some structural sugars are also extracted, explained by the fact that water becomes more reactive at higher temperatures. Increasing the temperature, raises the ionic product of water, and consequently water catalyses the hydrolysis of biomass.

The same was observed with protein content (Table 3.13), the yield increased as temperatures increases.

Extraction conditions			Yields (g/100 g lupin)	
Ratio	**Time (min)**	**Temperature (°C)**	**Carbohydrates**	**Protein**
20:1	60	100	8.17 ± 0.21	7.01 ± 0.11
20:1	60	120	10.1 ± 0.20	8.34 ± 0.18
20:1	60	140	13.1 ± 0.21	11.9 ± 0.15

Table 3.13: Subcritical water extraction: The influence of temperature on the yields of carbohydrates and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

Afterwards, the influence of solvent-to-solid ratio on the yield of alkaloids was studied. The temperature and time chosen in the experiment were 100 °C and 60 min, since the maximum alkaloid yield was obtained at these conditions. The solvent-to-solid ratios studied were 20:1 and 40:1.

With the increase in solvent-to-solid ratio, the yield of alkaloids in the extract significantly increased. From 20:1 to 40:1, the yield of alkaloids was raised by about 30 percentage points (Table 3.14). This could be explained with the principles of mass transfer. With higher solvent-to-solid ratios, concentration gradient is better, which results in an increase of diffusion rate [36].

Extraction conditions			Yield of alkaloids
Temperature (°C)	**Time (min)**	**Ratio**	**g/100 g lupin**
100	60	20:1	1.60 (47.9%)
100	60	40:1	2.59 (77.4%)

Table 3.14: Subcritical water extraction: The influence of solvent-to-solid ratio on the yield of alkaloids. In parenthesis are given the percentage of the total amount of alkaloids extracted.

On the other hand, the increase in solvent-to-solid ratio barely changed the amount of water soluble compounds extracted (Figure 3.7). At 100 °C, water does not have the ability to attack the hemicellulose structure of lupin, and therefore carbohydrates, proteins, and also lipids are extracted in similar amounts, irrespective of solvent-to-solid ratio. The results obtained at 100 °C are similar to those obtained at 80 °C (Figure 3.5).

The yields of carbohydrates and proteins are reported on table 3.15 . As expected, at 100 °C only the soluble sugars and proteins were extracted. Additionally, lipids were also analysed in the extract at 100 °C, for a solvent-to-solid ratio of 40:1. It was observed that 4 g/100 g lupin of lipids were removed from the matrix to the solvent, at these conditions. This value is higher than the value obtained at 80 °C, and shown in Table 3.8, which can be explained by the effect of an increase in temperature.

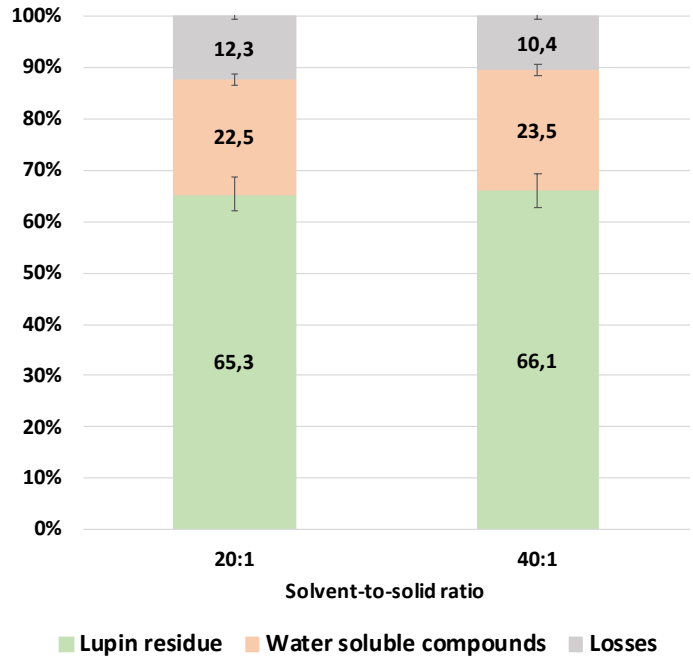

Figure 3.7: Subcritical water extraction: The influence of solvent-to-solid ratio on the yield of water soluble compounds (temperature: 100 °C; time: 60 min). Error bars are given.

Extraction conditions			Yields (g/100 g lupin)		
Temperature (°C)	Time (min)	Ratio	Carbohydrates	Protein	Lipids
100	60	20:1	8.17 ± 0.21	7.01 ± 0.11	-
100	60	40:1	7.54 ± 0.17	7.35 ± 0.09	4.01 ± 0.08

Table 3.15: Subcritical water extraction: The influence of solvent-to-solid ratio on the yields of carbohydrates, protein and lipids (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

A second extraction was performed at 100 °C, for 60 minutes, with a solvent-to-solid ratio of 40:1. The residue collected from the first extraction was used with fresh water.

It was observed that a second extraction barely improved the total amount of alkaloids removed from the lupin, only an additional 0.05 g/100 g lupin being extracted (Table 3.16).

Moreover, in the second extraction the amount of water soluble compounds increased by only 7 g per 100 of lupin (Figure 3.8). The total amount of carbohydrates extracted barely changed, but 3 g of protein per 100 of lupin were additionally removed with a second extraction (Table 3.16). These results are similar to those shown in Figure 3.5, for a temperature of 80 °C.

Extraction conditions				Yields (g/100 g lupin)		
Temp (°C)	**Ratio**	**Time (min)**	**Extraction**	**Alkaloids**	**Carbohydrates**	**Protein**
100	40:1	60	1	2.59	7.54 ± 0.17	7.35 ± 0.09
100	40:1	60	2	0.05	0.33 ± 0.13	3.04 ± 0.15
			Total	2.64	7.87 ± 0.15	10.4 ± 0.12

Table 3.16: Subcritical water extraction: The influence of multiple extractions on the yields of alkaloids, carbohydrates, and protein (data are reported on a dry mater basis; mean value ± standard deviation, n=3).

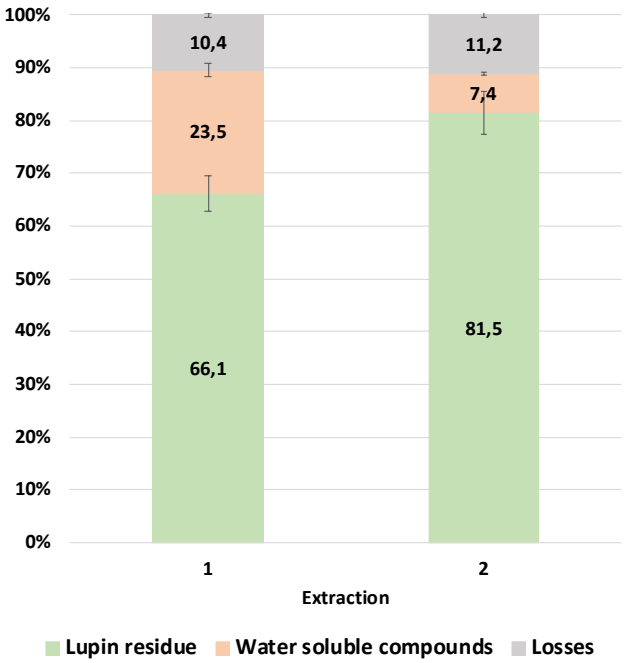

Figure 3.8: Subcritical water extraction: The influence of multiple extractions on the yield of water soluble compounds (temperature: 100 °C; time: 60 min; solvent-to-solid ratio: 40:1). Error bars are given.

4 | Conclusion

The purpose of this thesis was to develop an alternative and environmentally friendly method for the extraction of alkaloids, which are toxic compounds present in the white lupin seeds that confer a bitter taste. The alternative methods used were extraction with water below its boiling point, and extraction with subcritical water.

The influence of a series of parameters (temperature, solvent-to-solid ratio, residence time, and multiple extractions) on the yield of alkaloids was analysed. Special attention was also given to the effect that the extraction methods had on the chemical composition of lupin seeds that constituted the residue of those extraction processes.

Firstly, a chemical characterization of white lupin seeds was performed. It was found that white lupin seeds have a high content of protein, about 37%, which makes lupin more valuable comparing with other legumes. A high content of carbohydrates was also revealed, 42%, where 8% are free sugars and 34% are structural sugars. Furthermore, the lipid content in the seeds was 13%, quite higher than normal but characteristic in seeds from the Mediterranean.

The extraction yield of alkaloids was determined at five temperatures (50, 80, 100, 120 and 140 °C), three solvent-to-solid ratios (10:1, 20:1, 40:1), and at different residence times. The best alkaloid yield was obtained at 100 °C, 40:1 ratio, residence time of 60 min, and with a process consisting of two consecutive extractions: 2.64 g of alkaloids per 100 of lupin were extracted. However, the result obtained is not enough for bringing the alkaloid content under the desired toxicity levels. The assays at 50 °C led to very low yield in alkaloids, which suggest that low temperatures are not efficient. Furthermore, the results suggest that white lupin alkaloids are thermally sensitive and temperatures at or above 120 °C may lead to degradation, which must be taken in consideration in process optimization.

The study of the influence of the above variables on the alkaloid yield showed that temperature and solvent-to-solid ratio had the largest effect, increasing these parameters increasing the yield of alkaloids. This could be a consequence of an improvement in the water solubility, mass transfer effects and surface equilibria. The diffusion of water through the matrix increases and allows greater extraction yields.

Increasing the residence time did not show significant effect but it was observed that the maximum alkaloid yield may occur in 3 hours extraction time. Moreover, a consecutive extraction with fresh water, performed at 50, 80 and 100 °C, barely improved the

extraction of alkaloids. A reason for these results could be the fact that the remaining alkaloids are trapped in deeper matrix areas, which hinders their extraction. The presence of higher pressures may facilitate the process.

Besides alkaloids, it was also analysed the water soluble compounds extracted throughout the process. Data reported that always about 16-22% of solids are removed from the white lupin seeds during the debittering. The lowest result was obtained at 50 °C, with a solvent-to-solid ratio of 10:1. As a result, one consequence of the debittering pretreatment is the decrease in the nutritional value. The nutrients extracted in higher amounts were found to be carbohydrates, more specifically the soluble sugars (8% of total lupin seeds), and proteins (about 7% of total lupin seeds). Lipids were also extracted at 80 °C (3 g/100 g lupin) and 100 °C (4 g/100 g lupin), increasing temperature having a positive effect.

5 | Future work

This thesis proposed two new approaches for the debittering pretreatment, testing a set of parameters. The results obtained indicate that the use of subcritical water is a better option for debittering lupinus, although none of the approaches used succeeded in reducing the alkaloid content to the target toxicity levels.

Future work can optimize subcritical water extraction and exploit, in particular, the effect of pressure that might facilitate diffusion within the matrix.

To clarify the results of the assays with subcritical water, namely the fact that the amount of alkaloids detected in the extracts decreased as temperature increased from 100 to 140 °C, a mass balance should be performed for alkaloids. This involves quantifying alkaloids not only in the extracts obtained, but also in the lupin matrix that remains in the reactor at the end of the experiments. As indicated earlier, analysis of alkaloids in the lupin matrix requires a laborious process of eight consecutive extractions. If the alkaloids content of the remaining matrix decreases as temperature increases, then the progressively lower content of alkaloids in the extracts should be due to thermal degradation of those compounds.

If it is possible to meet the target alkaloid content, then the impact of the subcritical water treatment on the lupin matrix must be carefully considered, and strategies to recover components of interest must be studied.

Bibliography

[1] P. Janusz. "White lupin (Lupinus albus L.)–nutritional and health values in human nutrition–a review." In: *Czech Journal of Food Sciences* 35.2 (2017), pp. 95–105.

[2] M. Erbaş, M. Certel, and M. Uslu. "Some chemical properties of white lupin seeds (Lupinus albus L.)" In: *Food Chemistry* 89.3 (2005), pp. 341 –345. ISSN: 0308-8146. DOI: https://doi.org/10.1016/j.foodchem.2004.02.040. URL: http://www.sciencedirect.com/science/article/pii/S0308814604002055.

[3] S. Islam and W. Ma. "Lupine." In: *Encyclopedia of Food and Health*. Ed. by B. Caballero, P. M. Finglas, and F. Toldrá. Oxford: Academic Press, 2016, pp. 579 –585. ISBN: 978-0-12-384953-3. DOI: https://doi.org/10.1016/B978-0-12-384947-2.00432-3. URL: http://www.sciencedirect.com/science/article/pii/B9780123849472004323.

[4] A. Arnoldi and S. Greco. "Nutritional and nutraceutical characteristics of lupin protein." In: *Nutrafoods* 10.4 (2011), pp. 23–29.

[5] A. Green and R. Oram. "Variability for protein and oil quality in Lupinus albus." In: *Animal Feed Science and Technology* 9.4 (1983), pp. 271 –282. ISSN: 0377-8401. DOI: https://doi.org/10.1016/0377-8401(83)90020-2. URL: http://www.sciencedirect.com/science/article/pii/0377840183900202.

[6] P. Annicchiarico, P. Manunza, A. Arnoldi, and G. Boschin. "Quality of Lupinus albus L. (White Lupin) Seed: Extent of Genotypic and Environmental Effects." In: *Journal of agricultural and food chemistry* 62.28 (2014), pp. 6539–6545.

[7] J. Andrzejewska, S. Ignaczak, and P. Barzyk. "Oil content and fatty acid profile in seeds of Polish breeding lines and cultivars of legumes." In: *Acta Scientiarum Polonorum. Agricultura* 15.2 (2016).

[8] C. Carranca, M. Torres, and J. Baeta. "White lupine as a beneficial crop in Southern Europe: I. Potential for N mineralization in lupine amended soil and yield and N2 fixation by white lupine." In: *European Journal of Agronomy* 31.4 (2009), pp. 183 –189. ISSN: 1161-0301. DOI: https://doi.org/10.1016/j.eja.2009.05.009. URL: http://www.sciencedirect.com/science/article/pii/S1161030109000483.

[9] K. Staples, A. Hamama, R. Knight-Mason, and H. Bhardwaj. "Alkaloids in White Lupin and Their Effects on Symbiotic N Fixation." In: *Journal of Agricultural Science* 9 (May 2017), p. 13. DOI: 10.5539/jas.v9n6p13.

[10] S. Clark. "Plant Guide for white lupine (*Lupinus albus L.*" In: *USDA- Natural Resources Conservation Service* (2014).

[11] J. Müller, V. Gödde, K. Niehaus, and C. Zörb. "Metabolic Adaptations of White Lupin Roots and Shoots under Phosphorus Deficiency." In: *Frontiers in Plant Science* 6 (2015), p. 1014. ISSN: 1664-462X. DOI: 10.3389/fpls.2015.01014. URL: https://www.frontiersin.org/article/10.3389/fpls.2015.01014.

[12] Toxinology.no. *Lupin allergens*. 2010. URL: http://toxinology.nilu.no/Researchareas/Foodallergens/Factsheets/Lupinallergens.aspx (visited on 02/08/2020).

[13] G. Boschin and D. Resta. "Alkaloids Derived from Lysine: Quinolizidine (a Focus on Lupin Alkaloids)." In: *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*. Ed. by K. G. Ramawat and J.-M. Mérillon. Berlin, Heidelberg: Springer Berlin Heidelberg, 2013, pp. 381–403. ISBN: 978-3-642-22144-6. DOI: 10.1007/978-3-642-22144-6_11. URL: https://doi.org/10.1007/978-3-642-22144-6_11.

[14] G. Boschin, P. Annicchiarico, D. Resta, A. D'Agostina, and A. Arnoldi. "Quinolizidine Alkaloids in Seeds of Lupin Genotypes of Different Origins." In: *Journal of Agricultural and Food Chemistry* 56.10 (2008). PMID: 18433102, pp. 3657–3663. DOI: 10.1021/jf7037218. eprint: https://doi.org/10.1021/jf7037218. URL: https://doi.org/10.1021/jf7037218.

[15] M. Kroc, W. Rybiński, P. Wilczura, K. Kamel, Z. Kaczmarek, P. Barzyk, and W. Świecicki. "Quantitative and qualitative analysis of alkaloids composition in the seeds of a white lupin (Lupinus albus L.) collection." In: *Genetic Resources and Crop Evolution* (Nov. 2016). DOI: 10.1007/s10722-016-0473-1.

[16] M. Kroc, W. Rybiński, P. Wilczura, K. Kamel, Z. Kaczmarek, P. Barzyk, and W. Święcicki. "Quantitative and qualitative analysis of alkaloids composition in the seeds of a white lupin (Lupinus albus L.) collection." In: *Genetic Resources and Crop Evolution* 64.8 (2017), pp. 1853–1860.

[17] S. Bunsupa, M. Yamazaki, and K. Saito. "Quinolizidine alkaloid biosynthesis: recent advances and future prospects." In: *Frontiers in Plant Science* 3 (2012). DOI: 10.3389/fpls.2012.00239. URL: https://doi.org/10.3389%2Ffpls.2012.00239.

[18] M Wink, T Hartmann, and L Witte. "Enzymatic synthesis of quinolizidine alkaloids in lupin chloroplasts." In: *Zeitschrift für Naturforschung C* 35.1-2 (1980), pp. 93–97.

[19] C. von Linné. "CHAPTER 3 - Biological Significance of Alkaloids." In: *Alkaloids - Secrets of Life*. Ed. by T. Aniszewski. Amsterdam: Elsevier, 2007, pp. 141 –180. ISBN: 978-0-444-52736-3. DOI: https://doi.org/10.1016/B978-044452736-3/50005-2. URL: http://www.sciencedirect.com/science/article/pii/B9780444527363500052.

[20] Seneca. "CHAPTER 2 - Alkaloid Chemistry." In: *Alkaloids - Secrets of Life*. Ed. by T. Aniszewski. Amsterdam: Elsevier, 2007, pp. 61 –139. ISBN: 978-0-444-52736-3. DOI: https://doi.org/10.1016/B978-044452736-3/50004-0. URL: http://www.sciencedirect.com/science/article/pii/B9780444527363500040.

[21] M. Wiedemann, C. M. Gurrola-Díaz, B. Vargas-Guerrero, M. Wink, P. M. García-López, and M. Düfer. "Lupanine improves glucose homeostasis by influencing KATP channels and insulin gene expression." In: *Molecules* 20.10 (2015), pp. 19085–19100.

[22] Plautus. "CHAPTER 4 - Applications." In: *Alkaloids - Secrets of Life*. Ed. by T. Aniszewski. Amsterdam: Elsevier, 2007, pp. 181 –204. ISBN: 978-0-444-52736-3. DOI: https://doi.org/10.1016/B978-044452736-3/50006-4. URL: http://www.sciencedirect.com/science/article/pii/B9780444527363500064.

[23] S. Bardocz, G. Hajós, and A. Pusztai. *Effects of antinutrients on the nutritional value of legume diets*. Office for Official Publications of the European Communities, 1999.

[24] M. ERBAS. "THE EFFECTS OF DIFFERENT DEBITTERING METHODS ON THE PRODUCTION OF LUPIN BEAN SNACK FROM BITTER LUPINUS ALBUS L. SEEDS." In: *Journal of Food Quality* 33.6 (2010), pp. 742–757. DOI: 10.1111/j.1745-4557.2010.00347.x. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1745-4557.2010.00347.x. URL: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1745-4557.2010.00347.x.

[25] N. Ertaş and N. Bilgiçli. "Effect of different debittering processes on mineral and phytic acid content of lupin (Lupinus albus L.) seeds." In: *Journal of food science and technology* 51.11 (2014), pp. 3348–3354.

[26] 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." In: *Journal of the Science of Food and Agriculture* 81.4 (2001), pp. 421–428. DOI: 10.1002/1097-0010(200103)81:4<421::AID-JSFA829>3.0.CO;2-U. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/1097-0010%28200103%2981%3A4%3C421%3A%3AAID-JSFA829%3E3.0.CO%3B2-U. URL: https://onlinelibrary.wiley.com/doi/abs/10.1002/1097-0010%28200103%2981%3A4%3C421%3A%3AAID-JSFA829%3E3.0.CO%3B2-U.

[27] K Chilomer, M Kasprowicz-Potocka, P Gulewicz, and A Frankiewicz. "The influence of lupin seed germination on the chemical composition and standardized ileal digestibility of protein and amino acids in pigs." In: *Journal of animal physiology and animal nutrition* 97.4 (2013), pp. 639–646.

[28] C. Jiménez-Martínez, H. Hernández-Sánchez, and G. Dávila-Ortiz. "Diminution of quinolizidine alkaloids, oligosaccharides and phenolic compounds from two species of Lupinus and soybean seeds by the effect of Rhizopus oligosporus." In: *Journal of the Science of Food and Agriculture* 87.7 (2007), pp. 1315–1322. DOI: 10.1002/jsfa.2851. eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1002/jsfa.2851. URL: https://onlinelibrary.wiley.com/doi/abs/10.1002/jsfa.2851.

[29] A. Zaworska, A. Frankiewicz, and M. Kasprowicz-Potocka. "The influence of narrow-leafed lupin seed fermentation on their chemical composition and ileal digestibility and microbiota in growing pigs." In: *Archives of animal nutrition* 71.4 (2017), pp. 285–296.

[30] H. Reinhard, H. Rupp, F. Sager, M. Streule, and O. Zoller. "Quinolizidine alkaloids and phomopsins in lupin seeds and lupin containing food." In: *Journal of chromatography. A* 1112 (May 2006), pp. 353–60. DOI: 10.1016/j.chroma.2005.11.079.

[31] F. C. Santana and J. Empis. "Bacterial removal of quinolizidine alkaloids from Lupinus albus flours." In: *European Food Research and Technology* 212.2 (2001), pp. 217–224.

[32] F. J. Caetano, M. L. B. da Costa, and M. G. Bernardo-Gil. "Extraction of alkaloids from Lupinus albus sp. using compressed carbon dioxide." In: *High Pressure Chemical Engineering*. Ed. by P. R. von Rohr and C. Trepp. Vol. 12. Process Technology Proceedings. Elsevier, 1996, pp. 475 –480. DOI: https://doi.org/10.1016/S0921-8610(96)80082-3. URL: http://www.sciencedirect.com/science/article/pii/S0921861096800823.

[33] A. Mubarak. "Nutritional composition and antinutritional factors of mung bean seeds (Phaseolus aureus) as affected by some home traditional processes." In: *Food chemistry* 89.4 (2005), pp. 489–495.

[34] J. Mokgadi, C. Turner, and N. Torto. "Pressurized hot water extraction of alkaloids in Goldenseal." In: *American Journal of Analytical Chemistry* 2013 (2013).

[35] A. Bucić-Kojić, M. Planinić, S. Tomas, M. Bilić, and D. Velić. "Study of solid–liquid extraction kinetics of total polyphenols from grape seeds." In: *Journal of Food Engineering* 81.1 (2007), pp. 236–242.

[36] J. Cacace and G Mazza. "Mass transfer process during extraction of phenolic compounds from milled berries." In: *Journal of Food Engineering* 59.4 (2003), pp. 379–389.

[37] F. Mosca, G. I. Hidalgo, J. Villasante, and M. Almajano. "Continuous or Batch Solid-Liquid Extraction of Antioxidant Compounds from Seeds of Sterculia apetala Plant and Kinetic Release Study." In: *Molecules* 23 (July 2018), p. 1759. DOI: 10.3390/molecules23071759.

[38] 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." In: *Separation and Purification Technology* 54.2 (2007), pp. 253–261.

[39] R. Wongkittipong, L Prat, S. Damronglerd, and C. Gourdon. "Solid–liquid extraction of andrographolide from plants—experimental study, kinetic reaction and model." In: *Separation and Purification Technology* 40.2 (2004), pp. 147–154.

[40] R. M. Smith. "Superheated water: the ultimate green solvent for separation science." In: *Analytical and Bioanalytical Chemistry* 385.3 (2006), pp. 419–421. ISSN: 1618-2650. DOI: 10.1007/s00216-006-0437-y. URL: https://doi.org/10.1007/s00216-006-0437-y.

[41] 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." In: *Journal of pharmaceutical and biomedical analysis* 58 (2012), pp. 146–151.

[42] D. Wen, H Jiang, and K. Zhang. "Supercritical fluids technology for clean biofuel production." In: *Progress in Natural science* 19.3 (2009), pp. 273–284.

[43] A. T. Quitain, C. Y. Herng, S. Yusup, M. Sasaki, and Y. Uemura. "Conversion of biomass to bio-oil in sub-and supercritical water." In: *Biofuels-Status Perspect* (2015), pp. 459–476.

[44] A Kruse and E Dinjus. "Hot compressed water as reaction medium and reactant: properties and synthesis reactions." In: *The Journal of supercritical fluids* 39.3 (2007), pp. 362–380.

[45] G Brunner. "Near critical and supercritical water. Part I. Hydrolytic and hydrothermal processes." In: *The Journal of Supercritical Fluids* 47.3 (2009), pp. 373–381.

[46] B. Aliakbarian, A. Fathi, P. Perego, and F. Dehghani. "Extraction of antioxidants from winery wastes using subcritical water." In: *The Journal of Supercritical Fluids* 65 (2012), pp. 18–24.

[47] E. Ibanez, A. Kubátová, F. J. Señoráns, S. Cavero, G. Reglero, and S. B. Hawthorne. "Subcritical water extraction of antioxidant compounds from rosemary plants." In: *Journal of agricultural and food chemistry* 51.2 (2003), pp. 375–382.

[48] L. He, X. Zhang, H. Xu, C. Xu, F. Yuan, Ž. Knez, Z. Novak, and Y. Gao. "Subcritical water extraction of phenolic compounds from pomegranate (Punica granatum L.) seed residues and investigation into their antioxidant activities with HPLC–ABTS+ assay." In: *Food and Bioproducts Processing* 90.2 (2012), pp. 215–223.

[49] P. P. Singh and M. D. Saldaña. "Subcritical water extraction of phenolic compounds from potato peel." In: *Food Research International* 44.8 (2011), pp. 2452–2458.

[50] M. Tanaka, A. Takamizu, M. Hoshino, M. Sasaki, and M. Goto. "Extraction of dietary fiber from Citrus junos peel with subcritical water." In: *Food and bioproducts processing* 90.2 (2012), pp. 180–186.

[51] H. Pińkowska, P. Wolak, and E. Oliveros. "Production of xylose and glucose from rapeseed straw in subcritical water–Use of Doehlert design for optimizing the reaction conditions." In: *Biomass and bioenergy* 58 (2013), pp. 188–197.

[52] B. Hames, A. Sluiter, C. Scarlata, and N. R. E. L. (U.S.) *Determination of Protein Content in Biomass: Laboratory Analytical Procedure (LAP) : Issue Date, 07/17/2005*. NREL/TP. National Renewable Energy Laboratory, 2008. URL: https://books.google.pt/books?id=NI8TkAEACAAJ.

[53] A. Sluiter and N. R. E. L. (U.S.) *Determination of Ash in Biomass: Laboratory Analytical Procedure (LAP) : Issue Date, 7/17/2005*. NREL/TP. National Renewable Energy Laboratory, 2008. URL: https://books.google.pt/books?id=RDX3vQAACAAJ.

[54] A. L. Waterhouse. "Determination of Total Phenolics." In: *Current Protocols in Food Analytical Chemistry* 6.1 (2002), pp. I1.1.1–I1.1.8. DOI: 10.1002/0471142913.fai0101s06. eprint: https://currentprotocols.onlinelibrary.wiley.com/doi/pdf/10.1002/0471142913.fai0101s06. URL: https://currentprotocols.onlinelibrary.wiley.com/doi/abs/10.1002/0471142913.fai0101s06.

[55] T. Sivaraman, T. Kumar, G. Jayaraman, and C. Yu. "The Mechanism of 2,2,2-Trichloroacetic Acid-Induced Protein Precipitation." In: *Journal of protein chemistry* 16 (June 1997), pp. 291–7. DOI: 10.1023/A:1026357009886.

[56] M. Castro, J. Ruiz-Jimenez, and L. Ayuso. "Environmental Applications | Soxhlet Extraction." In: Dec. 2013. ISBN: 9780124095472. DOI: 10.1016/B978-0-12-409547-2.04734-X.

[57] Q. Deng, M. H. Penner, and Y. Zhao. "Chemical composition of dietary fiber and polyphenols of five different varieties of wine grape pomace skins." In: *Food Research International* 44.9 (2011), pp. 2712–2720.

[58] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, and D. Templeton. "Determination of structural carbohydrates and lignin in biomass." In: *Biomass Anal Technol Team Lab Anal Proced* 2011 (Jan. 2004), pp. 1–14.

[59] 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." In: *Analytical biochemistry* 339.1 (2005), pp. 69–72.

[60] A. Sujak, A. Kotlarz, and W. Strobel. "Compositional and nutritional evaluation of several lupin seeds." In: *Food chemistry* 98.4 (2006), pp. 711–719.

[61] V Večerek, P. Suchỳ, E. Straková, M Macháček, et al. "Nutritive composition of seeds of the lupin varieties registered in the Czech Republic." In: *Lupins for health and wealth. Proceedings of the 12th International Lupin Conference, Fremantle, Western Australia, 14-18 September 2008*. International Lupin Association. 2008, pp. 123–126.

[62] A. Mohamed and P Rayas-Duarte. "Composition of Lupinus albus." In: *Cereal Chemistry* 72.6 (1995), pp. 643–647.

[63] 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." In: *Food chemistry* 258 (2018), pp. 1–7.

[64] D Gorecka, E Lampart-Szczapa, W Janitz, and B Sokolowska. "Composition of fractional and functional properties of dietary fiber of lupines (L. luteus and L. albus)." In: *Food/Nahrung* 44.4 (2000), pp. 229–232.

A | Calibration curves

A.1 Gallic acid standard curve

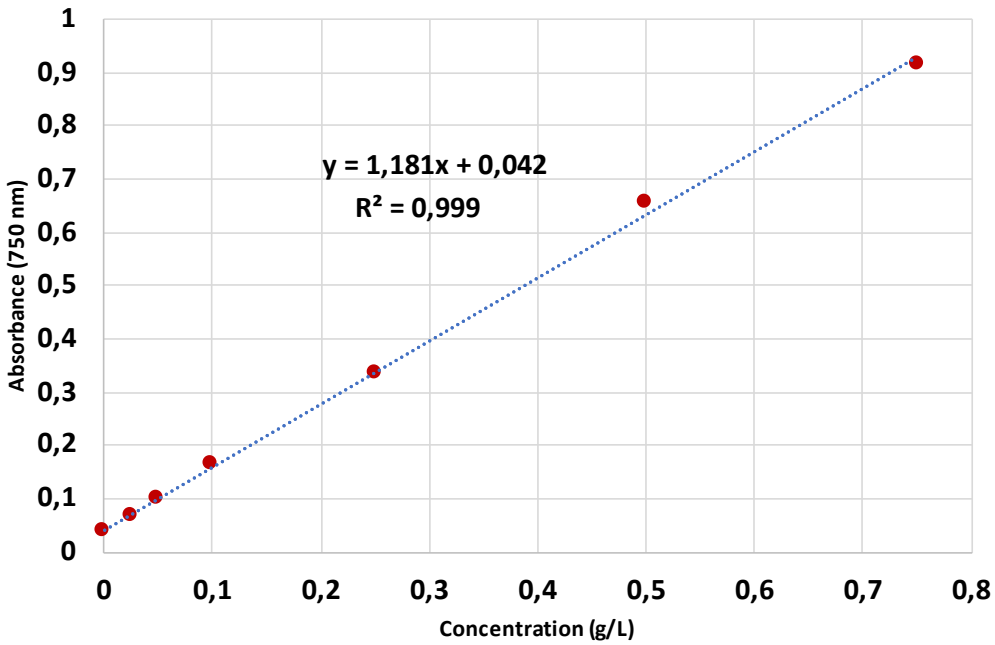

Figure A.1: Gallic acid standard curve.

A.2 Glucose calibration curve

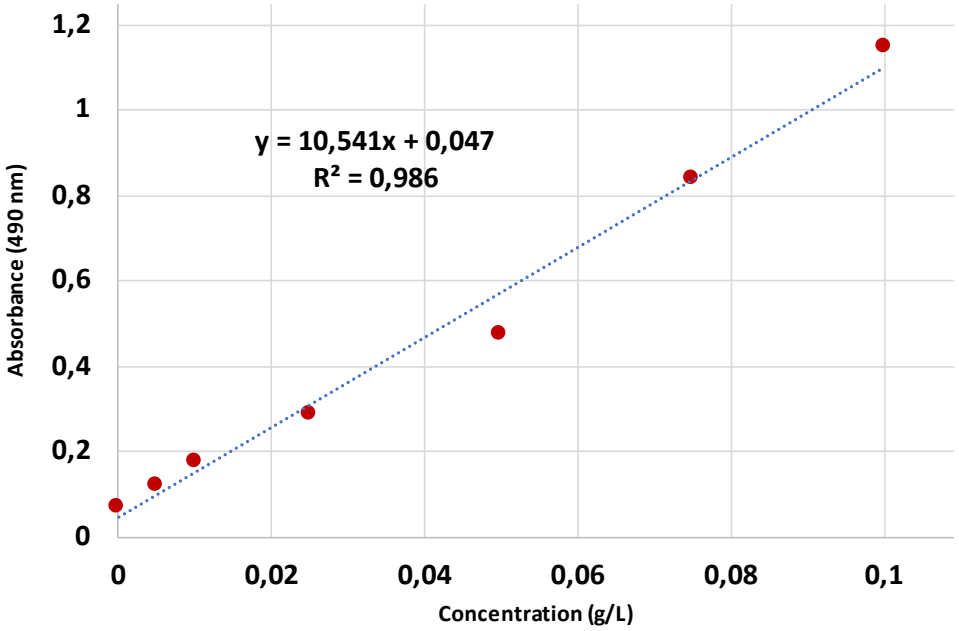

Figure A.2: Glucose calibration curve.

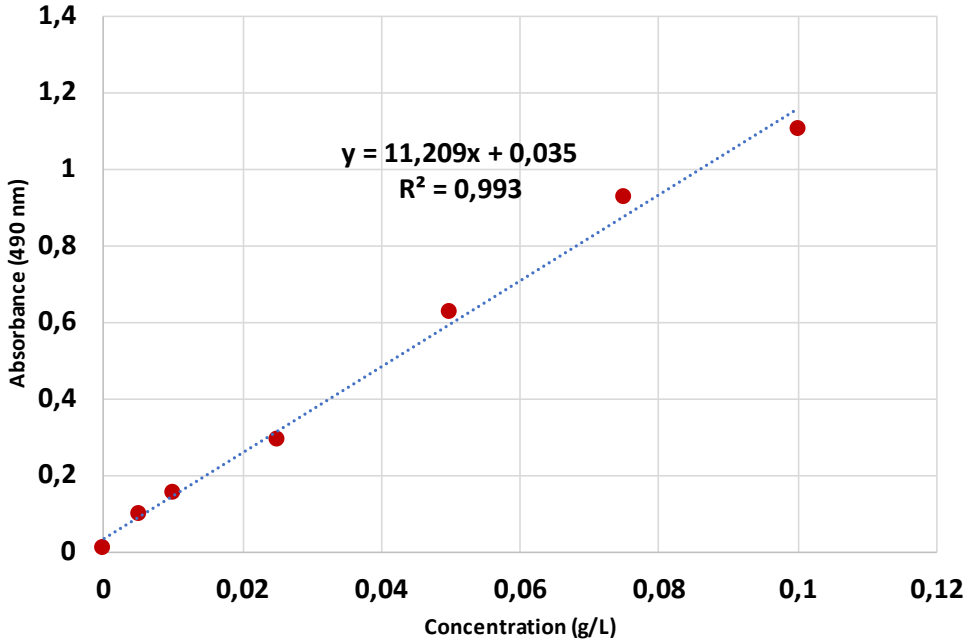

Figure A.3: Glucose calibration curve.