Lupin as a future protein source in Sweden

– Food safety aspects, prospects and challenges

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- livsmedelssäkerhet, möjligheter och utmaningar

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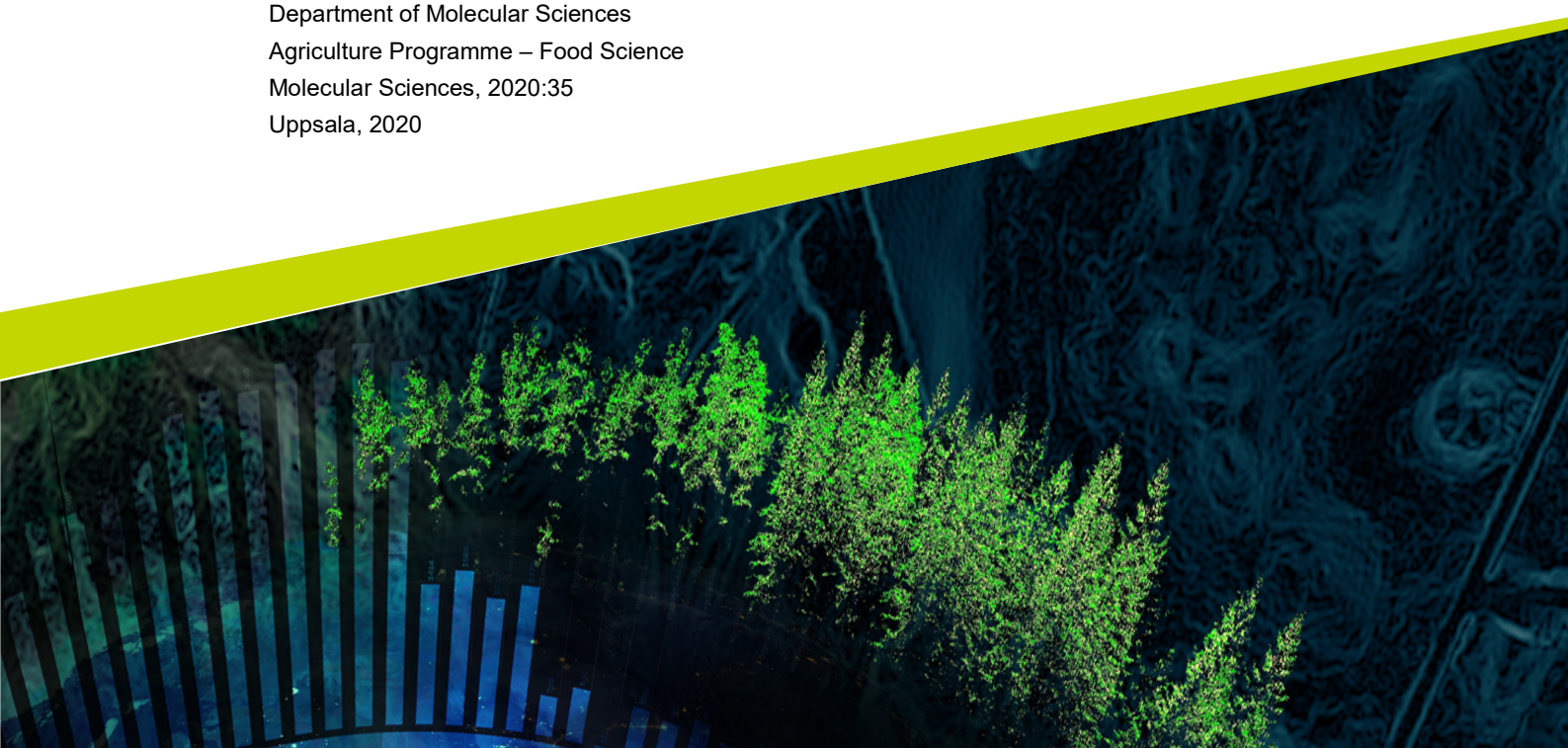

Lupin as a future protein source in Sweden – Food safety aspects, prospects and challenges

Lupin som en framtida proteinkälla i Sverige – livsmedelssäkerhet, möjligheter och utmaningar

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Abstract

The modern food system is a key driver of environmental and social burdens. A step towards becoming more sustainable has led to an increased demand for protein-rich leguminous crops. Lupin is a legume with a high nutritional value and well-adapted for cultivation in Sweden. The current view on lupins by producers and the market in Sweden was investigated. One of the challenges with lupin for human consumption is the lack of knowledge regarding its content of alkaloids. A purpose of this study was, therefore, to compare the alkaloid contents in cultivars of yellow lupin (Baryt, Bursztyn, and Mister), narrow-leafed (NL) lupin (Boregine, Mirabor, and Samba) and Andean lupin (Bolivian variety). For NL lupin, effects of year of harvest and of soaking time on alkaloid levels were also investigated. Another aim was also to determine if the alkaloids were present in the lupin seed cotyledon or the hulls. Alkaloids were extracted from lupin seed flour and the combined content of all forms of alkaloids was estimated based on the precipitation of an alkaloid-bismuth complex followed by solubilisation and spectrophotometric quantification. The total content of alkaloids varied from 0.29 to 1.37 %. The year of harvest strongly influenced the total alkaloid content in the two cultivars investigated. Soaking had an ambiguous effect on total alkaloid content. The lupin seed cotyledon contained the majority of the alkaloids. This study indicates that more research is needed to assure the safety of lupins for food purposes, which is essential to promote increased utilisation of lupin-based products.

Keywords: quinolizidine alkaloids, lupin, narrow-leafed lupin, yellow lupin, Andean lupin, future protein crop, commercialisation of lupin, utilisation of lupin

Sammanfattning

Det moderna livsmedelssystemet har en stor påverkan på både miljö och samhälle. För att bli mer hållbart har efterfrågan av proteinrika baljväxter ökat. Lupin är en baljväxt med ett högt nutritionellt värde och är en gröda som är väl anpassad för odling i Sverige. Därför undersöktes lupinens status på den svenska marknaden och hos producenter. En av utmaningarna med lupin för livsmedelskonsumtion är emellertid bristen på kunskap om dess innehåll av alkaloider. Syftet med denna studie var därför att jämföra det totala alkaloidinnehållet i lupinsorter från gul lupin (Baryt, Bursztyn, Mister), smalbladig lupin (Boregine, Mirabor, Samba) och Andisk lupin (Boliviansk kultivar). För den smalbladiga lupinen undersöktes även effekten av skördeår och blötläggningstidens påverkan på alkaloidhalt. Ytterligare ett syfte var att bestämma vart i lupinfröet som alkaloiderna ackumuleras. Alkaloider extraherades ur mjöl av malda lupinfrön. Det kombinerade innehållet av alla typer av alkaloider kunde uppskattas och baserades då på en fällning av alkaloid-vismutkomplex följt av en spektrofotometrisk kvantifiering. Den totala koncentrationen av alkaloider i lupinfröna varierade från 0,29 till 1,37 %. Skördeår hade en stark påverkan på det totala alkaloidinnehållet i de två studerade sorterna. Blötläggning hade en tvetydig effekt på alkaloidhalten. Det visade sig också att majoriteten av alkaloiderna återfanns i lupinfröets kärna. Slutsatsen av resultaten i denna studie visar dock att mer forskning behövs för att kunna garantera att lupiner är säkert för livsmedelskonsumtion. Detta är viktigt för att främja ett ökat intag av lupinbaserade produkter.

Nyckelord: quinolizidinalkaloider, lupin, smalbladig lupin, gullupin, Andisk lupin, framtidens proteingröda, kommersialisering av lupin

“There are far, far better things ahead than any we leave behind.”

— C.S. Lewis

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Abbreviations

ANFs	Antinutritional Factors
BfR	The Federal Institute of Risk Assessment
bw	Body weight
CS	Thiourea
Cv	Cultivar
dw	Dry weight
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
LPI	Lupin protein isolate
MUFA	Monounsaturated fatty acid
NL	Narrow-leafed
OGTR	Office of the Gene Technology Regulator
PUFA	Polyunsaturated fatty acid
QA	Quinolizidine alkaloids
RQ	Research question
SFA	Saturated fatty acid
TI	Trypsin inhibitor
TIA	Trypsin inhibitor activity
α-AI	α- amylase inhibitor

1. Introduction

The modern food system is a complex network and currently a key driver of environmental and social burdens. Therefore, it is facing a huge global challenge; to become more sustainable (Food and Agriculture Organisation of the United Nations (FAO), 2018; Garnett *et al.*, 2016; Hallström *et al.*, 2015).

Today, the modern food system accounts for 19-30 % of the greenhouse gas emissions (GHGE) globally (Hjorth *et al.*, 2020; Garnett *et al.*, 2016). Further, it is a major contributor to deforestation, land degradation and desertification, loss in biodiversity, nutrient losses (Garnett *et al.*, 2016; Hajer *et al.*, 2016), deteriorating soil quality, and land-use change (Alexander *et al.*, 2015). Between 1970 and 2014, terrestrial and aquatic biodiversity decreased by 60 % (WWF, 2018). The majority of losses in biodiversity occurs in the tropical regions where rain forest deforestation is a verity. The amount of wildlife species constitutes 4 % of the total global mammal biomass, based on weight, while humans represent 36 %. The major constituent here is the livestock production representing as much as 60 %. The extensive livestock production is an emergent issue, which leads to further exploitation of land and land-use change (FAO, 2016; Alexander *et al.*, 2015).

Socially, a growing global population and change in diet patterns lead to several concerns regarding sustainability (FAO, 2018; Alexander *et al.*, 2015). People need food, shelter, and food security. Increasing wealth is leading to an inclination towards more resource-intensive food, *i.e.* meat, milk, and egg products (UNEP, 2016). The globalisation of food supply increases the trade-offs and puts further pressure on the intensification of agricultural practices and land-use change.

To turn things around and reduce the global impact of existing food systems, transitions in eating habits and production structures are needed. Many alternatives have been suggested, such as the adoption of healthier diets (Alexander *et al.*, 2015; Hallström *et al.*, 2015), reduced consumption of resource-intensive food (FAO, 2018), increased awareness on healthy and sustainable food choices (de Boer & Aiking, 2019), decreased dependency on imported soy (Prins *et al.*, 2019) and increased production of leguminous crops in the European Union (EU) (Manners *et al.*, 2020). Also, it is essential to develop less resource-intensive agricultural practices and thus find strategies to manage water use, nutrients, and agrochemicals in a sustainable manner (Foley *et al.*, 2011). Hallström (2015) concludes that adopting a diet according to the Nordic dietary guidelines could lower land-use by 20 %. Moreover, Röös *et al.* (2018) demonstrate a scenario where Swedish meat consumption is reduced by 50 % and replaced with an increased proportion of

legumes. Hypothetically, this scenario would result in a 20 % lower climate impact and a 23 % reduction in land-use change. At the same time, it would only require a 1 % increase in Swedish legume production.

1.1. Problem analysis

There is an evident gap between Swedish farmers and the food industry (Linnskog Rudh, 2018). The farmer needs to be guaranteed that the sale of the harvest is possible, while the food industry needs assurance of a continuous and regular supply of volume with acceptable quality (Linnskog Rudh, 2018). Today, it is more advantageous to procure protein-rich leguminous crops from other parts of the world than from Sweden, as the price is lower and no further processing is required for the delivered product (Jonson, 2018). Lupin could be a suitable option as it has a high protein content, and thus a good competitor to the imported soybean (Renmark, 2019; Nilsson, 2017). In Scandinavia, the lupin has mainly served as animal feed but has the potential to become a plant-based protein source for human consumption (Manners *et al.*, 2020; Nilsson, 2017). The protein crop is well-adapted to the Swedish climate (Manners *et al.,* 2020), not genetically modified, and valuable to integrate into crop rotation as it improves soil quality through nitrogen fixation (Calabro *et al.,* 2015).

However, introducing the lupin in the human diet requires post-harvest processing, such as cleaning, dehulling, milling, and packing of lupin grains (Linnskog Rudh, 2018). These are non-existent in Sweden today (Jonson, 2018) (Sörbring, 2020; Linnskog Rudh, 2018; Röös *et al.*, 2018). Also, other barriers to the commercialisation of lupins exist, such as the concern regarding its allergenicity (McNaughton, 2019; Prins *et al.*, 2019; European Food Safety Authority (EFSA), 2014), the limited insight in which species and varieties of lupins that are most suitable for cultivation, nutritional value, and lack of knowledge regarding antinutritional factors (ANFs) (McNaughton, 2019). Food safety aspects related to legume consumption is a major challenge. Therefore, it is important to gain knowledge on how to properly prepare lupins for food consumption, and thus removing the ANFs below safety limits (The Federal Institute of Risk Assessment (BfR), 2017).

1.2. Aim and objectives of the project

This study aims to provide a foundation for increased utilisation of lupins in Sweden and to create incentives for the Swedish food industry to venture lupin-based foods. Additionally, different lupin varieties will be studied with the ambition to identify prospects and challenges with further food processing of the dried seed, *i.e.* dehulling and soaking. Moreover, potential food application areas for lupin seeds will be evaluated.

Research questions

RQ1: How is lupin perceived as a protein source for human consumption in Sweden?

RQ2: Is the total alkaloid content varying between different lupin species and cultivars?

RQ3: Is the alkaloid concentration in lupin seeds affected by the year of cultivation?

RQ4: Are the majority of the alkaloids mainly located to the hulls of the lupin seeds or in the cotyledon?

RQ5: Is soaking affecting the alkaloid content in lupin seeds?

2. Theory

This section will provide background information of the lupin species of agronomic interest: *Lupinus angustifolius, Lupinus luteus, Lupinus albus,* and *Lupinus mutabilis*. It will also include nutrition and composition, levels of antinutritional factors (ANFs) and alkaloid content. Lastly, the health aspects of human consumption of lupins will be reviewed.

2.1. The *Lupinus* species

Lupinus spp. is a diverse and widespread genus of the legume family of *Fabaceae*, including 200 species of flowering plants (Anderberg & Anderberg, 2017; Fogelfors, 2015). Of these 200 species, only four is fully domesticated; *Lupinus angustifolius, L. albus, L. luteus,* and *L. mutabilis,* Table 1 (Gresta *et al.*, 2017). In Sweden, the flower lupin (*L. polyphyllus*) and Nootka lupin[1] (*L. nootkatensis*) are considered as domiciled[2]. These are commonly known as toxic, invasive, and a threat to biodiversity as it easily spreads on the verge of roads, meadows, gardens, and fringe of the forests (Bäckström, 2018).

However, the species of *L. angustifolius, L. albus* and *L. luteus* belong to the group of Old World species, originating from the European, Mediterranean and North African areas (Gresta *et al.*, 2017; Office of the Gene Technology Regulator (OGTR), 2013). There are 12 species included to this group of which all are annual crops with a larger seeds size. These species can further be divided into groups based on the seed-coat texture, where Malacospermae characterise a smooth seed coat, and Scabrispermae a rough seed coat. However, the species named gives seeds with a smooth seed coat, thus, belonging to the group of Malacospermae. It is the Old World lupin species that are recognised as most important from an agricultural perspective, due to their suitability as food and feed, and which has been improved through plant breeding.

The species of *Lupinus mutabilis* is a New World lupin. This group of lupins includes approximately 130 species (Gresta *et al.*, 2017). New World lupins are less specialised compared to the Old World species, but are mainly characterised as

[1] In Sweden, Nootka lupin is commonly called sand lupin.

[2] Meaning that these lupin species are a part of the natural flora in Sweden

herbaceous perennials, cross-pollinators and by their monopodial[3] type of branching (Gresta *et al.*, 2017; OGTR, 2013). The New World lupin is thought to originate from South, Central and North America, but the species within this group are poorly defined.

The cultivation of lupins in Europe includes a total area of 150 000 ha. Germany, Poland, the Russian Federation, Belarus and Ukraine are the only European countries that grow the crop on more than 10 000 ha (Gresta *et al.*, 2017).

Table 1. Overview of lupin species of agronomic interest and main cultivation areas

Latin name	Common name	Species group	Main cultivation area
L. angustifolius	Narrow-leafed lupin, blue lupin, sweet lupin	Old World	Poland, Germany, Australia
L. luteus	Yellow lupin	Old World	Poland
L. albus	White lupin	Old World	Italy, Spain, France, Australia
L. mutabilis	Andean lupin, chocho, tarwi	New World	Ecuador, Peru and Bolivia

2.1.1. *L. angustifolius*

The domestication of the Old World species of *Lupinus angustifolius* occurred in the late 19th century in the Baltic countries and Germany, with the purpose to serve as green manure (OGTR, 2013). Furthermore, sweet and edible varieties of the species were developed in Germany, Sweden and the Russian Federation in the late 20th century. Today, it is widely distributed in Mediterranean countries (Lim, 2012) and has domiciled in Australia, South Africa and North America and thus been included as a part of the natural flora (OGTR, 2013).

The species *Lupinus angustifolius* has many names, *i.e.* the Australian sweet lupin, blue lupin or narrow-leafed lupin (Lim, 2012). The domesticated cultivars of this species are often denoted as narrow-leafed lupin or Australian sweet lupin and give white or blue flowers and seeds suitable for feed and food (Alemu *et al.*, 2018; OGTR, 2013). Noteworthy is the name Australian sweet lupin, which reflects the extensive production in the specific continent, representing 95 % of the total lupin grain production (OGTR, 2013). However, to avoid misconception, the *Lupinus angustifolius* will henceforth be referred to as narrow-leafed (NL) lupin in this thesis.

This NL lupin has received the most attention, as it is well-adapted to temperate climate, is tolerant to abiotic stresses, *e.g.* drought, waterlogging (Renmark, 2019),

[3] Monopodial branching means that the crop grows upward with the terminal bud as a central leader. This gives the plant a pyramidal shape, as the lateral shoots remain subordinate to the top shoot (Encyclopaedia Britannica, 2020).

and maintains soil fertility (Lim, 2012). Initially, the edible seeds of NL lupin were used as animal feed but have slowly shifted to become more accepted as human food. Further, the seeds have a thick coat that constitutes 19-25 % of the seed weight (Petterson, 2004).

The cultivation of the NL lupin species in Europe is mainly concentrated in Germany and Poland (Gresta *et al.*, 2017). It is the most suitable lupin species for more extensive cultivation at northern latitudes (Manners *et al.*, 2020).

2.1.2. *L. luteus*

The domestication of the Old World species of *Lupinus luteus* or yellow lupin occurred at the same time and purpose as the NL lupin (OGTR, 2013). To date, it is commonly distributed in the pan-Mediterranean region, but also extensively cultivated in Australia.

The yellow lupin is a self-pollinator and gives yellow flowers. The seeds are mainly used as animal feed and to a lesser extent food (Petterson, 2004). The seed coat of the yellow lupin is thicker than of the other domesticated lupin species and comprises approximately 30 % of seed weight (Petterson, 2004).

The cultivation of the yellow lupin in Europe is centred to Poland (Gresta *et al.*, 2017). Among the four domesticated species, the yellow lupin is most sensitive to polluted soils (Baciak *et al.*, 2015). It is well adapted to sandy soils with low pH and fertility, and transient waterlogging, but less adjusted to alkaline soils (OGTR, 2013).

2.1.3. *L. albus*

This lupin species was the first to become domesticated (OGTR, 2013). In ancient Greece and Egypt, 2000 BC, the *Lupinus albus* was cultivated for multiple purposes, *i.e.* food, feed, cosmetics and medicine. In ancient Rome, 1000-800 BC, it was utilised as green manure. The development of sweet varieties occurred at the same time as the other two Old World species.

Today, the white lupin is widely distributed in the pan Mediterranean region and is mainly used for human consumption, particularly the larger and bitter seeds are used as snacks after debittering (Petterson, 2004). The seed coat is not as thick as for the yellow and NL lupin and comprises 15% of the total seed weight.

The white lupin prefers fertile and alkaline soils; thus, it is less adapted for sandy soils with low pH (OGTR, 2013). The crop is also sensitive to transient waterlogging and therefore requires well-drained cultivation areas.

The main producers of the white lupin are located in the southern parts of Europe and include Italy, France and Spain. The area of cultivation ranges from 3000 ha in Spain to 5000 ha in Italy (Gresta *et al.*, 2017). Additionally, Australia is a large producer (30 000 ha in 2009) (OGTR, 2013) and exporter of white lupin, exporting thousands of tonnes, particularly to the Middle East region (Petterson, 2004).

2.1.4. *L. mutabilis*

The species of *Lupinus mutabilis* originates from Andean countries, where it has been cultivated since ancient times. Some common names for *L. mutabilis* are chocho, tarwi (Breña et al., 2019; von Baer, 2019), pearl lupin, Tauri and Andean lupin (BfR, 2017). Hereinafter, the common name Andean lupin will be used throughout the thesis.

The crop is of less significance for human consumption globally, but is commonly produced for food, feed and green manure in Ecuador, Peru and Bolivia (Villacrés *et al.*, 2019; Święcicki *et al.*, 2015) and the Andean regions at altitudes of 2000-4000 m (OGTR, 2013). The Andean lupin has large seeds, which is an exception compared to other New World lupin species characterised by their small seed-size (Święcicki *et al.*, 2015). The seed-coat is rather thin and comprises 12 % of the total seed weight (Petterson, 2004). Important to acknowledge is that the seeds from Andean lupin require a debittering process by soaking before consumption (Córdova-Ramos *et al.*, 2019; Carvajal-Larenas *et al.*, 2015).

Moreover, the Andean lupin species is relatively resistant towards pests, diseases, drought and frosts. Nevertheless, the species struggles with some shortcomings, such as a prolonged crop maturation under long photoperiods and humid weather (Święcicki *et al.*, 2015). Additionally, the Andean species has low adaptation ability, which makes it incapable to compete with the NL lupin and yellow lupin cultivated in Europe and Australia (OGTR, 2013).

2.2. Composition and nutrition of the lupin seed

2.2.1. Macro- and micro nutrients

The lupin seed kernel has a dicotyledon structure, containing the majority of the energy stored in thickened cell-wall material, which comprises approximately 25 %, and in oil bodies (Petterson, 2004). Further, the seeds are covered with a thick seed coat (~25%) mainly consisting of cellulose, hemicellulose and a minor part of lignin and varies from 21-27 %, 4-11 %, and 0.35-34 %, respectively (Parmdeep *et al.*, 2015; Bähr *et al.*, 2014). The seed coat of lupins comprises a larger part of the total seed weight compared to other agronomic grain crops (Petterson, 2004). The total fibre content of the lupin seed ranges from 6.2 to 15.4 % in dry weight (dw), Table 2.

The starch content (SC) in lupin seeds reaches approximately 6 % (Table 2) and are higher than the SC in soybean (*Glycine max*) (≤0.91 %), but considerably lower in comparison to field peas (*Pisum sativum* L.) and chickpeas (*Cicer arietinum*) with an SC ranging from 50 to 70 % of its dw (Tayade *et al.*, 2019; Petterson, 2004). Furthermore, the protein content varies within and between lupin species but ranges from 15.8 to 52.6 % of dw, (Table 2) (Musco *et al.*, 2017; Parmdeep *et al.*, 2015; Lim, 2012). Likewise, the fat content in lupin seeds varies (Carvajal-Larenas *et al.*, 2016) and ranges from 4.9 to 24.6 % of the total seed (Table 2), with diverse levels

of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), Table 2 (Musco *et al.*, 2017). In NL and yellow lupin, the PUFA (~34-60 %) levels are more dominant than that of SFA and MUFA (Table 3). In white lupins the MUFA are dominant. Musco *et al.* (2017) declare that the PUFA composition in white lupin is comparable with canola oil with an n-3/n-6 ratio of 0.45, while yellow and NL lupin is more similar to olive oil (0.13) and soya bean oil (0.15). The fat in Andean lupin is dominant in the MUFA of C18:1 (46.4 %) and PUFA of C18:2 (33.1 %) but also contains some amounts of SFA, *i.e.* C16:0 (10.4 %), and C18:0 (4.7 %), and MFA; C16:1 (13.9%). (Carvajal-Larenas *et al.*, 2016). Although, the debittering process tends to increase the MUFA of C18:1 to 52.5 %

Considering the lupin kernel protein content, the NL lupin contains approximately 32-45 % (Święcicki *et al.*, 2015; Wäsche *et al.*, 2001) to 40 % of protein (Bähr *et al.*, 2014; Chew *et al.*, 2003), Table 2. Protein content of the white lupin cultivars, yellow lupin and Andean lupin is considered to reach the same levels as for soya bean, 40 % (Wäsche *et al.*, 2001), 20.7 to 41.1 % (Parmdeep *et al.*, 2015; Strakova *et al.*, 2006) and 34 to 50 % (Villacrés *et al.*, 2019; Święcicki *et al.*, 2015), respectively, Table 2. However, post-processing (*i.e.* dehulling) of the white lupin could increase the protein content in the lupin kernel to 44-50% (Chew *et al.*, 2003). Furthermore, the amino acid profile in seeds from all lupine species are deficient in the sulphur amino acids cysteine and methionine, thus it is inferior to that of eggs and milk casein.

The mineral content of lupins varies widely within and between species, Table 4 (Karnpanit *et al.*, 2017; Musco *et al.*, 2017; Carvajal-Larenas *et al.*, 2016; Lim, 2012; Strakova *et al.*, 2006). Here, calcium, magnesium and phosphorus are the most abundant minerals ranging from 120-430 mg/100 g, 160-330 mg/100 g, and 300-880 mg/100 g, respectively (Table 3). The Andean lupin contains high amounts of potassium (1130-1400 mg/ 100 g) compared to the NL lupin (Carvajal-Larenas *et al.*, 2016). Other minerals such as sodium, copper, iron, manganese, zinc, molybdenum, cobalt and selenium are only present in smaller amounts (Table 4).

Table 2. Macronutrients in whole seeds from four lupin species (g/ 100 g of DM except for moisture)

Species	DM	Carbohydrates	Proteins (N × 6.25)	Fat	Fibre	Starch	Ash
NL lupin	90.3[d]– 91.75[a*]	41[c]	15.8[e] – 40[f]	5.5[a*] – 8.0[e]	12.9[a*]-15.4[b]	5[e]-8[a*]	2.7[b] – 4.15[d]
Yellow lupin	89.6[a]-90.8[d]	38.8[c]	20.7[e]-41.1[a]	4.9[a]-9.1[e]	12.93[a]	3.1[e]-3.7[a]	4.23[a]-5.67[d]
White lupin*	91.3[d]-92.5[a*]	39.3[c]	24.1[e]-36.2[a*]	9.7[a, e*]	10.28[a*]	3.9[e]-7.45[a*]	3.78[a*]-5.25[d]
Andean lupin	90.1–93.8 [c]	26.1-43.2[c]	32–52.6[c]	13-24.6[c]	6.2-11[c]		2.4–5.2[c]

[a] values based on own calculations from (Strakova *et al.*, 2006), [b] (Lim, 2012), [c] (Carvajal-Larenas *et al.*, 2016), [d] (Musco *et al.*, 2017), [e](Parmdeep *et al.*, 2015), [f](Bähr *et al.*, 2014), (*) indicates mean values, empty box indicates that data is missing

Table 3. Fatty acid classes (g/ 100g) in whole lupin seeds and omega-3/ omega-6 ratio (Musco et al, 2017)

Species	SFA	MUFA	PUFA	n-3 PUFA	n-6 PUFA	n-3/n-6
NL lupin	21-26.7	29.5-38.7	34.7-49.5	5.9-7.7	28.7-43.2	0.146-0.267
Yellow lupin	15.1-16.9	24.6-29.5	54.3-60.3	6.7-9.5	47.6-50.9	0.142-0.186
White lupin	16.5-17.2	50.9-55.8	27.3-32.0	8.6-12.4	17.9-23.0	0.452-0.658

SFA (saturated fatty acids (FA)): C14:0, C15:0, C16:0, C18:0, C20:0, C22:0, C24:0; MUFA (monounsaturated FA): C16:1, C17:1, C18:1 n-9, C20:1 n-7, C22:1 n-9; PUFA (polyunsaturated FA): C18:2 n-6, C18:3 n-3, C20:2 n-6

Table 4. Nutrient composition of minerals in whole seeds of the four different lupin species (mg/ 100g of DM)

Species	Ca	Mg	P	K	Na	Cu	Fe	Mn	Zn	Mo	Co	Se
NL lupin	220[b] – 350[a]	160[b] – 184[a]	300[b] - 526[a]	800[b]	40[b]	0.47[b]-0.5[c]	2.74[e]-6.1[c]	1.9[b]-2.1[c]	3.4[e]-3.6[c]	0.16[b]	7.8[b]	8.9[b]
Yellow lupin	210[c] -380 [a]	225 [a]	610[c] -720 [a]			0.9[c]	9.3[c]	8.6[c]	5.6[c]			
White lupin*	200[c]-430[a*]	190[a*]	360[c]-507[a*]			0.5[c]	2.6[c]	83.5[c]	3.0[c]			
Andean lupin	120–180 [c]	240–330 [c]	600–880 [c]	1130-1400[c]		0.8-1.1[c]	5.0-7.3[c]	2.6-3.7[c]	3.4-3.6[c]			

[a] values based on own calculations from (Strakova *et al.*, 2006), [b] (Lim, 2012), [c] (Carvajal-Larenas *et al.*, 2016), [d] (Musco *et al.*, 2017), [e](Karnpanit *et al.*, 2017)

2.2.2. Alkaloids

Alkaloids are a group of phytochemicals[4] that belongs to the class of secondary metabolites (Hanson, 2003) and is considered an anti-nutritional factor (ANF) (Cortés-Avendaño *et al.*, 2020). They come in a diverse range of structures, although all alkaloids are nitrogen-containing bases which originates from an amino acid precursor, *i.e.* lysine, tyrosine, ornithine or tryptophan (Hanson, 2003). Nicotine, caffeine, and morphine are some alkaloids that have attracted considerable interest throughout the years of research. Looking at legume plants such as the genus *Lupinus*, another group of these secondary metabolites are of particular interest, namely the quinolizidine alkaloids (QA).

The QA in lupins are biosynthesized from the amino acid lysine in the leaf chloroplast and translocated throughout the plant by the phloem (Magalhães *et al.*, 2017; Wink *et al.*, 1995; Paech & Tracey, 1955). They are further stored in the seeds and epidermal cells of the plant (Wink *et al.*, 1995) and play an important role as a chemical defence towards biotic and abiotic stresses, *e.g.* herbivores, UV radiation, and pathogens (Magalhães *et al.*, 2017). Correspondingly, the bitter taste in lupin seeds is caused by the QAs. Thus, lupins are divided into groups of sweet and bitter varieties, where sweet varieties give seeds containing a lower amount of QA and bitter varieties give seeds with higher levels (Cortés-Avendaño *et al.*, 2020; Magalhães *et al.*, 2017; Święcicki *et al.*, 2015; Paech & Tracey, 1955).

The most abundant QAs in lupins are sparteine, lupinine and lupanine (BfR, 2017; Carvajal-Larenas *et al.*, 2016; Wink *et al.*, 1995). Other QAs like albine, 3-hydroxylupanine, 13-hydroxylupanine, angustifoline, α-isolupanine and multiflorane are present in lower amounts. The QAs are toxic if consumed in high doses and affect the nervous, circulatory, and digestive systems (BfR, 2017; Malmgren *et al.*, 2016). Severe lupin alkaloid intoxication can lead to anticholinergic syndromes, resulting in symptoms like anxiety, worry, agitation, delirium, dysarthria[5], myoclonus[6], dry skin and mucous membranes, fever, sinus tachycardia, hypertension, urine retention and reduced bowel activity (Malmgren *et al.*, 2016).

Thus, the safety limit set by health authorities, in some countries, is a total alkaloid content of 0.02 g/ 100 g of seeds in dry matter (DM) (Magalhães *et al.*, 2017). The acute lethal dose is estimated to be 10 mg/ kg body weight (bw) for children and infants, and 25 mg/kg bw for adults (BfR, 2017; Carvajal-Larenas *et al.*, 2016).

The total alkaloid level in lupin seeds varies hugely between species, but also among different cultivars within a species (BfR, 2017; Calabro *et al.*, 2015). To illustrate, the total alkaloid content (Table 5) in white lupin cultivars varies from 0.005 to 1.53 g/ 100 g seeds in dw (Calabro *et al.*, 2015), NL lupin 0.015 to 1.4,

[4] The term phytochemicals include compounds that are not reckoned as nutrients. Phytochemicals can have a positive health impact on mammals although, it could also have adverse effects.

[5] Speech impediment

[6] Involuntary and momentary twitches in muscles

yellow lupin 0.008 to 1.5, and Andean lupin 0.007 to 4.5 (Musco *et al.*, 2017; Carvajal-Larenas *et al.*, 2016; Lim, 2012; Wäsche *et al.*, 2001).

Further, older lupin varieties generally contained a higher content of alkaloids, which complicated the utilisation of the seeds for food and feed purposes (Fogelfors, 2015; Święcicki *et al.*, 2015). Also, extensive cultivation of the Andean lupin has been limited due to its high-alkaloid levels (Villacrés *et al.*, 2019; Święcicki *et al.*, 2015). Lately, plant breeding towards a lower alkaloid level has resulted in sweet varieties, safer for consumption (Magalhães *et al.*, 2017).

Table 5. Total alkaloid content (g/100g seed dw) and a selection of specific quinolizidine alkaloids (%) present in four different lupin species

	White lupin	NL lupin	Yellow lupin	Andean lupin
Total content (g/100 g seed dw)	0.005-1.53[c,d,e]	0.015-1.4[c,d]	0.009-1.5[c,d]	0.007-4.5[c]
Composition (%)				
Lupanine	70[c]-89.2[d*]	40.7[d*]-70[c]	5[d*]-60[c]	64.4 (46-84.5)[c]
D-lupanine	n.a[c]	n.a[c]	n.a[c]	13[c]
Sparteine	0.3[d*]	1.9[d*]	30[c]-94.6[d*]	12.6 (6.6-19.1)[c]
Albine	15[c]	n.a[c]	n.a[c]	n.a[c]
3-hydroxylupanine	n.a[c]	n.a[c]	n.a[c]	12[c]
13-hydroxylupanine	6.5[d*]-8[c]	12[c]-43.9[d*]	10.3[d*]	9.5 (1.6-14.9)[c]
Angustifoline/ oxoasparteine	3.3[d*]	10[c]-14.7[d*]	ND[d]	2.3 (0.6-5.4)[c]
Multiflorine	3[c]	n.a[c]	n.a[c]	1 (0.1-1.8)[c]
α-Isolupanine	1.2	ND	ND	0.3[c]

[c](Carvajal-Larenas et al., 2016), [d] numbers based on own calculations from values presented by (Musco *et al.*, 2017) ,[c](Calabro *et al.*, 2015), asterisk (*) indicate mean value, not available (n.a), not detected (ND)

2.2.3. Other antinutritional factors in lupins

Compounds included as ANFs are commonly the proteins α-amylase inhibitors (αAI), trypsin inhibitors (TI) and lectins, as well as glycosides; saponins, and α-galactosides, and other substances like phytate, phytic acid, tannins, and oxalate (Henriksson, 2017; Embaby, 2010). Although lupin seeds generally contain low amounts of ANFs, there are some variations within and between species (BfR, 2017; Henriksson, 2017). The most common ANFs in lupins, with alkaloids as an exception, are phytic acid, tannins, and saponins (Carvajal-Larenas *et al.*, 2016). Other articles also mention trypsin inhibitor activity (TIA), lectins, oligosaccharides, and α-galactosides as prevalent ANFs in lupin seeds (Musco *et al.*, 2017; Embaby, 2010).

Phytic acid is a natural substance in plants which strongly binds to dietary minerals such as iron, phosphorus and calcium, but it also has a high affinity to bind proteins and starch (Parmdeep *et al.*, 2015). At physiological pH, phytic acid is present in its ionic form, phytate, with six negatively charged phosphate groups. In lupin seeds

phytate is mainly located in the cotyledon, ranging from 0.5 to 2.7 %, Table 6 (Carvajal-Larenas *et al.*, 2016; Embaby, 2010; Petterson, 2004). These levels are 2- to 4-fold higher than the amount of phytic acid in chickpeas (Parmdeep *et al.*, 2015). Additionally, Embaby (2010) observed that processing of the lupin seeds, *e.g.* dehulling, increased the relative concentration of phytate.

Tannins are a common substance in plants (Petterson, 2004). Condensed tannins have an antinutritive effect affecting the gut enzymes and give an astringent taste to the legume grain. Tannins are mainly located in the lupin seed coat. Thus, dehulling is a simple practice for tannin removal. Conversely, Lampart-Szczapa *et al.* (2003) found that tannins were most abundant in lupin cotyledon, and dehulling only led to an increase in these substances. This is also consistent with the findings of Embaby (2010). Generally, tannin levels in lupin seeds vary widely between species and varieties and ranges from 10 to 134 mg/ 100 g. Table 6 (Parmdeep *et al.*, 2015; Embaby, 2010; Petterson, 2004).

Saponins are glycosides and common in all type of plant material (Petterson, 2004). Similar to the tannins, saponins affect the digestive system, especially the permeability of the mucosa in the small intestine. Saponins give a bitter taste to the seed and could, therefore, act as a food and feed deterrent. The presence of saponins in lupin seeds ranges from 5.5 mg/ 100 g seeds of dw in yellow lupin to 1700 mg in Andean lupin, Table 6.

The TIA is generally low in lupins (Parmdeep *et al.*, 2015; Petterson, 2004). TIA levels range from 13 (Petterson, 2004) to 174 mg/ 100 g of seed dw (Embaby, 2010), Table 6.

Lectins are heat-sensitive proteins and therefore significantly decreased by heat-processing of legume grains (Embaby, 2010). In lupin seeds, the initial levels of lectins are very low and only trace amounts can be detected, Table 6 (Petterson, 2004).

The oligosaccharides present in lupin seeds are mainly stachyose, raffinose and verbascose (Musco *et al.*, 2017; Carvajal-Larenas *et al.*, 2016; Gross *et al.*, 1988). The levels vary from 5.2 % in NL lupin to 15.4 % in some Andean lupin varieties (Petterson, 2004)

Lastly, the αAI activity ranges from 8.6% to 18.3% in some lupin cultivars (Parmdeep *et al.*, 2015). Notably, αAI activity in some cultivars of white and yellow lupin was not detected at all, showing a wide variation among species and cultivars.

Table 6. ANFs in whole lupin seeds

	White lupin	NL lupin	Yellow lupin	Andean lupin
Oligosaccharides (%)	7.5[a]	5.2[a]	12.3[a]	14.8-15.4[e]
αAI (%)	9.4-18.3[b]	9.1-12.6[b]	8.6-9.1[b]	
Saponins (mg/100g)	144-193[b]	5.7[a]-275[b]	5.5[a]-247[b]	1700[e]
Condensed tannins (mg/100g)	10[a]-134[b]	61-106[b]	20[a]-68[b]	60[d]
Lectins[c]	Trace[a]	Trace[a]	Trace[a]	
TIA (mg/100 g)	13[a]-174[f]	14 [a]	29 [a]	
Phytate (%)	0.79[a]	0.58[a]	0.96[a]	2.74[e]

[a](Petterson, 2004), [b](Parmdeep & Singh, 2017), [c]hemagglutination activity, [d](Carvajal-Larenas et al 2017), [e](Gross et al., 1988), [f](Embaby, 2010), empty box indicates that data is missing

2.2.4. Allergenic compounds

The increase in consumption of lupin seeds and lupin-based food has been followed by a growing number of allergic reactions to lupins (EFSA, 2014). It has further been noted that individuals with peanut- or soybean allergy also tend to react to lupins.

Most of the research done on this topic has analysed varieties of the NL lupin. This lupin species has several seed storage proteins, where the globulin proteins of α-conglutin, β-conglutin, δ-conglutin and γ-conglution are the most abundant (Schlegel *et al.*, 2019). Studies have shown that the β-conglutin is the major allergen and therefore the cause of allergenic response in lupin seeds (Lima-Cabello *et al.*, 2019; Schlegel *et al.*, 2019; EFSA, 2014; Lim, 2012). Consequently, this particular protein has been classified as a recognized allergen by the allergen nomenclature subcommittee (Schlegel *et al.*, 2019), and since 2006, lupins are included to the list of allergens by the EU (EU Directive 2006/142/EC). Thus, lupin as an ingredient has to be highlighted on food labels (Schlegel *et al.*, 2019).

2.2.5. Health aspects

Inclusion of white lupin dry extract in the diet has indicated antidiabetic and hypolipemic effects in individuals with diabetes type 2. Thus, lowering the concentrations of fats in the blood (Bouchoucha *et al.*, 2016). Also, whole white lupin seeds and its lupin protein isolates (LPI) have been observed to have cholesterol-lowering effects (Fontanari *et al.*, 2012). The high content of dietary fibres in lupin can also have a hypocholesterolemic effect (Pollard *et al.*, 2002). Lupin protein hydrolysates of NL lupin have indicated an anti-inflammatory effect in humans, reducing chronic inflammation (Cruz-Chamorro *et al.*, 2019; Millán-Linares *et al.*, 2014). Moreover, the relatively high content of oligosaccharides in lupin can have positive effects on the digestive system, promoting the development of beneficial gut microbiota, and thus stimulate the digestion in the large intestine (Sobotka *et al.*, 2016).

2.3. Processing and utilisation of lupins

2.3.1. Traditional cooking

Further, soaking of lupin seeds can be performed on either dehulled or lupin seeds with the hull. Soaking or debittering is essential for the removal of QA (BfR, 2017; Carvajal-Larenas *et al.*, 2016). Normally, the debittering process is implemented by soaking seeds in water for 24 hours, but the process can be extended to up to 5 days with a change of water three times a day (BfR, 2017). Soaking is followed by boiling, although boiling of lupin seeds are different compared to other legumes that tend to go soft after some time. This is not the case for lupins as the seeds remain its hard texture. Boiling reduces the amount of QA, as they are sensitive to thermal treatment (Jiménez-Martínez *et al.*, 2001), but could also increase the number of off-flavours (Roland *et al.*, 2017; Stephany *et al.*, 2016; Stephany *et al.*, 2015). After soaking and boiling, lupins are commonly used in products such as tempeh, falafel, miso, and soy (Petterson, 2004).

2.3.2. Industrial processing

The different fractions of relevance for lupin seed fractionation are proteins, oils and fibres. Each fraction has different purposes within the food industry (Muranyi *et al.*, 2016). The thick seed coat of lupin seeds makes it more difficult to dehull than other legumes although there is specialised equipment developed (Streckel & Schrader, 2020). The concept of dehulling lupin seeds comprises two peeling stones, where one is still and the other is moving, creating a force just enough to let the seed coat snap. Thereafter, the hulls and cotyledons are separated by an air duct where the air is flowing upwards. The light-weighted hulls rise with the air stream and the cotyledons remain on the bottom ready to be collected. Lupin hull fibre is used as a source of fibre in food. In Chile, hulls of the white lupin are toasted finely ground and sold as a dietary fibre supplement, VITAFIBER® (OGTR, 2013). In Australia, ground hulls of NL lupin have been included in bread at inclusion rates of around 4% for many years.

Flaking of lupin seeds can be performed in a flake roller mill consisting of rollers with high hydraulic pressure, which crushes the seeds to flakes (Streckel & Schrader, 2020). For lupin seeds, the flaking process results in yellow flakes, which further can be extracted from oil. Unlike for example canola and soybean, lupin is not considered as a true oilseed (Bhardwaj *et al.*, 2004), but the oil can be utilised within the baking industry or in pasta, sausages and diet goods (Prolupin, 2020a).

Furthermore, the white flakes remaining from the oil extraction can further be processed into lupin protein isolates (LPI). This process also gives fibre as a by-product (Prolupin, 2020a). The LPI comprises of more than 90 % of protein in DM (Prolupin, 2020b) and has received much attention as they are neutral in taste, exhibit good emulsifying and foaming properties (Lqari *et al.*, 2002). Therefore, LPI has a high value in further food processing, functioning as fat-replacer but also substituting egg and dairy in ice cream production and improving texture in pasta, bakery products and sausages (Muranyi *et al.*, 2016; Lqari *et al.*, 2002).

Fermentation of LPI or lupin milk (Snowden *et al.*, 2007) can provide lupin-based dairy alternatives (Hickisch & Schweiggert-Weisz, 2019; Hickisch *et al.*, 2016).

Additionally, whole lupin seeds can be finely ground in a wind sifter, or hammer cutter and a roller mill, or by dry milling followed by air classification, and used as a flour additive in bread (Villarino *et al.*, 2016; Petterson, 2004). It has been observed that the addition of lupin flour to bread improves the technological properties, sensory characteristics and the nutritional value (Piasecka-Jóźwiak *et al.*, 2018). Piasecka-Jóźwiak *et al.* (2018) observed positive effects on bread quality by adding 15 % of lupin flour in sourdough bread with a base of wheat flour. Adding more than 30 % of lupin flour in baking can result in low loaf volume, a “beany” flavour, and “grassy”, “hay-like” odour, thus reducing the sensory acceptance (Villarino *et al.*, 2016).

3. Method

This section describes the methodology implemented in this thesis. It is separated into three sections which include a literature review, a market analysis of the present situation in Sweden, and an experimental part, Figure 1. The literature review (2. Theory) focused on providing information regarding the lupin in general; including composition, nutritional value, anti-nutritional factors (ANFs), *e.g.* alkaloids, food processing methods and application areas within the food industry. The market analysis will include conversational material with Swedish actors within food processing and manufacturing and thus clarify current attitudes towards lupin utilisation. Lastly, the practical section will constitute an analysis of the total alkaloid content in lupin seeds from different species and varieties.

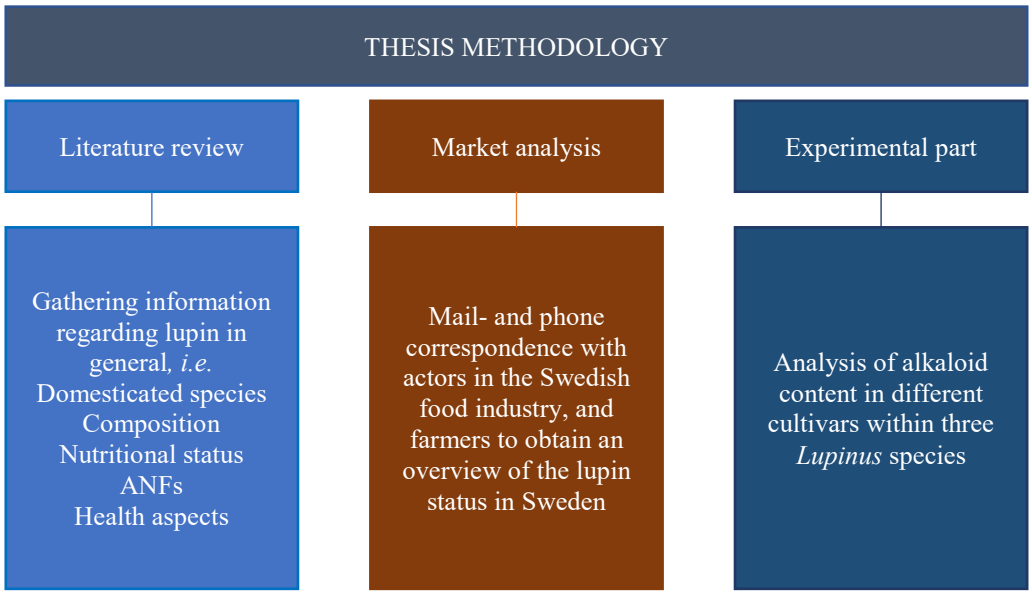

Figure 1. Schematic overview of method applied in this thesis

3.1. Literature review

Scientific articles, journal articles governmental documents, and books have been retrieved from databases, *i.e.* Primo, Google Scholar, Web of Science, Scopus and PubMed. Articles have also been retrieved from newspapers, *i.e.* Land, Livsmedel

i Fokus, and ATL. Information has been summarised for different purposes. The first section (2. Theory) of the thesis aims to provide general aspects on the topic and constitutes a foundation based on existing scientific studies.

3.2. Market analysis of lupins in Sweden

To get an overview of the utilisation of lupins in Sweden, a market analysis was implemented. Unstructured interviews were performed, and the respondents were asked how they perceive lupin as a protein crop, and as a food product. All respondents had some kind of relation to lupins and consisted of two farmers that cultivates lupins today, four actors within the food industry, and one researcher at the Swedish University of Agricultural Sciences (SLU). Also, statistics were obtained from Statistics Sweden.

3.3. Experimental procedure

Whole lupins seeds with hull were analysed for total alkaloid concentration. The different species and varieties are displayed in Table 7, together with the year of harvest and country of origin.

The yellow lupin seed varieties of Baryt, Mister and Burzstyn and the NL lupin cultivar (*cv.*) Samba was obtained from RISE and originated from Poland, the harvest year of 2017. One seed sample of the NL lupin cultivar Boregine was also supplied by RISE. This sample was harvested in Sweden, but the year is unknown.

Further, two seed samples of NL lupin *cv.* Mirabor harvested in 2018 and 2019, respectively, and two seed samples of Boregine harvested in 2017 and 2018, were collected from Nordisk Råvara. These four samples originated from a farmer located in north-western parts of Skåne, Kvidinge, Sweden. Additionally, another sample of *cv.* Boregine harvested in 2019 was collected directly from a farmer located in central parts of Skåne, Harlösa.

Seeds from a Bolivian variety of Andean lupin was supplied by SLU. The seeds were harvested in Sweden the year of 2015.

The cultivars Boregine, Mirabor, Mister, and Baryt are considered as sweet varieties of lupin. The Bolivian cultivar belongs to the group of bitter varieties. No information on which group the lupin cultivars Samba and Bursztyn considered to belong to was found.

Table 7. Lupin seeds obtained for this study, declaration of species, cultivar, year of harvest, code used in analysis, supplier and country of origin

Species and cultivar	Harvested	Code	Supplier	Seeds harvested
Yellow lupin				

Baryt	2017	LYBA	RISE	Poland
Bursztyn	2017	LYBUR	RISE	Poland
Mister	2017	LYMI	RISE	Poland
NL lupin				
Samba	2017	NLSAM	RISE	Poland
Boregine	Not specified	BORI	RISE	Sweden
Mirabor	2018	MIRA18	Nordisk Råvara	Sweden
Mirabor	2019	MIRA19	Nordisk Råvara	Sweden
Boregine	2017	BORE17	Nordisk Råvara	Sweden
Boregine	2018	BORE18	Nordisk Råvara	Sweden
Boregine	2019	BORE19	Ekenäs farm	Sweden
Andean lupin				
Variety from Bolivia	2015	MUTA	SLU	Sweden

3.3.1. Sample preparation

In total, 23 samples of lupin seeds from three different species (NL lupin, yellow lupin and Andean lupin) and seven cultivars (MUTA, Samba, Boregine, Mirabor, Baryt, Bursztyn, and Mister) were prepared for total alkaloid analysis. Of these, 10 seed samples (BORI1-BORI7, NLSAM1-NLSAM3) were soaked and oven-dried before milling as a food processing step, to investigate how much of the alkaloids that left the seeds during soaking, Table 8.

A sample of Boregine (BORI) was dehulled (by Streckel & Schrader, Hamburg, Germany) and collected as dicotyledons and hulls separated. The intention of dehulling the seeds was to study the distribution of total alkaloids in the lupin seeds (BORI-H and BORICOT), Table 8.

Moreover, five samples (BORICONT, NLSAMCONT, LYBACONT, LYMICONT, and LYBURCONT) with dry lupin seeds were milled without any additional soaking and drying procedure. The aim was to contribute with reference alkaloid concentration values to the soaked samples, Table 8.

The five samples of NL lupins (MIRA18, MIRA19, BORE17, BORE18, BORE19) were also milled, but without any processing, Table 8. The intention was to investigate whether cultivation conditions, *e.g.* weather fluctuations and water availability during plant growth, affected the levels of alkaloids in the seeds. While the purpose of including seeds from an Andean lupin (MUTA) was to analyse the total alkaloid content of a bitter lupin species.

Lastly, fat removal was performed for another flour sample, BORICONT, to test whether fat influenced on the determination of total alkaloid concentration, as it was described by Wallebroek (1940).

Table 8. Total number of flour samples analysed for total alkaloid concentration

Sample code		No. of samples	Soaked and dried	Soaking time
NL lupin				
BORI1-BORI7		7	Yes	1–7 days
BORICONT		1	No	N/A
BORI-H	Hulls only	1	No	N/A
BORICOT	Cotelydon only	1	No	N/A
BORAF	Defatted flour	1	No	N/A
NLSAM1-NLSAM3		3	Yes	1–3 days
NLSAMCONT		1	No	N/A
BORE17		1	No	N/A
BORE18		1	No	N/A
BORE19		1	No	N/A
MIRA18		1	No	N/A
MIRA19		1	No	N/A
Yellow lupin				
LYMICONT		1	No	N/A
LYBACONT		1	No	N/A
LYBURCONT		1	No	N/A
Andean lupin				
MUTA		1	No	N/A
Total		**24**		

3.3.2. Soaking and drying

The soaking process was implemented as an treatment according to the methods described by BfR (2017) and Carvajal-Larenas *et al.* (2013), with some modifications.

The seed samples (á 100 g) of each lupin variety (Table 9) were soaked in tap water in a 1:6 lupin to water ratio. One replicate was performed for each treatment. Soaking time ranged from one to seven days. The soaking water was changed three times during the daytime, with six-hour intervals.

After soaking was completed, the samples were rinsed, spread onto an oven sheet and dried in 50°C in a household oven for 12 hours.

Table 9. Species and cultivars of the soaked samples, including number of samples and number of days in soaking

Material	No. of samples	Sample code	Soaking time
NL lupin			
Boregine	7	BORI24-BORI168	1–7 days

Samba	3	NLSAM24-NLSAM72	1–3 days

3.3.3. Milling

The soaked and dried samples of BORI24 to BORI168, NLSAM24 to NLSAM72, and the samples of NLSAMCONT, BORICONT, LYBURCONT, LYBACONT and LYMICONT were initially milled in a sample mill (Cemotec 1090 sample mill, Foss A/S) at level 4 and again at level 2, to achieve a particle size of coarse grist. Thereafter, a fine flour was obtained with Ultra Centrifugal Mill (Retsch ZM200) to a final particle size of 0.5 mm. The samples were stored in a -18°C freezer until time of analysis.

The samples of MIRA18, MIRA19, BORE17, BORE18, and BORE19 were milled in a coffee grinder (Easy Grind, OBH Nordica) to a fine powder for approximately 30 seconds.

The seeds from the Andean Lupin (MUTA) were crushed in a pestle after applying liquid nitrogen.

3.3.4. Defatting procedure

The defatting procedure followed the method described by Newton (2014).

The lupin flour (10 g) was combined with hexane (≥99 %, Sigma-Aldrich) (30 ml), 1:3 (w/v). The beaker was covered with aluminium foil and the solution was stirred for 2 hours at 360 rpm. The mixture was transferred to a 15 ml polycarbonate centrifugal bottle and then centrifuged (Avanti centrifuge J-26 XPI, Beckman Coulter, USA) for 15 min at 4°C, 5000*g*. Thereafter, the hexane was decanted, and the remainder, *i.e.* the lupin flour, was evenly distributed on a glass tray and left to dry under a fume hood for 24 hours to allow the rest of the hexane to evaporate.

3.4. Alkaloid analysis

The total alkaloid content of 24 samples was analysed using Dragendorff's reagent (DR) and a spectrophotometric method, Table 8. The method is referred to as an analysis of total content since it includes all kinds of alkaloids combined and present in the samples. The applied method was based on a study by Sreevidya and Mehrotra (2003). The concept was to precipitate the alkaloids present in a lupin seed extract by the addition of DR containing bismuth. The alkaloid-bismuth complex was dissolved in sodium sulphide and nitric acid and measured in a spectrophotometer, after the addition of thiourea. The absorbance values were thereafter inserted in the linear equation from a calibration curve.

In this way, it was possible to estimate the total concentration of alkaloids as bismuth-equivalents, as the formation of the alkaloid-bismuth complex is 1:1. Thus, the amount of bismuth present in the sample reflects the level of alkaloids in the plant material. The detailed protocol is declared in Appendix 2.

3.4.1. Solutions and reagents

For the extraction of alkaloids from the lupin flour, diluted glacial acetic acid (2 %) was used. Further, throughout the alkaloid analysis, reagents as ethanol (70 %), 1 % solution of sodium sulphide nonahydrate (Na_2S) (Sigma-Aldrich; purity 99.99+ %), and concentrated HNO_3 and thiourea (CS) (3 %) was added to the samples.

The Dragendorff's reagent ($KBiI_4$) consisted of a mixture of bismuth(III) nitrate pentahydrate ($Bi(NO_3)_3 \cdot 5\,H_2O$) (Sigma-Aldrich; purity 98 %), glacial acetic acid, potassium iodide (KI), and distilled water.

Further, the standard bismuth nitrate solution was prepared as a stock solution for the calibration curve and consisted of ($Bi(NO_3)_3 \cdot 5\,H_2O$), concentrated nitric acid (HNO_3), and distilled water. To form a yellow-coloured bismuth complex, 3 % solution of thiourea (Sigma-Aldrich, Reagentplus TM, >=99.0 %) was added to the standard bismuth nitrate pentahydrate stock solution prior to the spectrophotometric measurement at the wavelength of 435 nm.

3.4.2. Extraction of alkaloids

The extraction of alkaloids from the different lupin flours was made in triplicates. Initially, 0.25 to 0.5 g of lupin flour was weighed into 15 ml falcon tubes and 2.5 ml of a 2 % solution of acetic acid was added. The tubes were put on a shaker (Rotaflex RM1, program F1) and shaken at 60 rpm for ten minutes and then centrifuged (Jouan C3i centrifuge, SA) at 4000 rpm for 5 minutes. Thereafter, the supernatant was collected by pipetting it into new falcon tubes. These steps were repeated two times. Lastly, the pH of the extracts was maintained between 2-2.5 with 1M HCl.

3.4.3. Procedure for Assay of Alkaloids and Plant extract

Initially, 1 ml from each extract was pipetted to Eppendorf tubes (2 ml) and 400 μl of DR was added. A cloudy orange precipitate was formed, according to the formula (I).

(I) $$KBiI_4 + plant\ extract \rightarrow (BiI_3)(Alkaloid \cdot HI)$$

Thereafter, the tubes were centrifuged in Heraeus Pico 21 Centrifuge (Thermo Scientific) at 13000 rpm for 5 minutes. Thereafter, another 100 µl was added to the tubes to control full precipitation and was followed by centrifugation. Thereafter, the supernatants were decanted completely and meticulously. The remaining pellets were washed with by adding 1 ml of 70% solution of ethanol and the tubes were run in a shaker (Rotaflex RM1, program F1) at 60 rpm for 5 minutes. The washing procedure was implemented to remove residual bismuth that did not form a complex with the alkaloids. The samples with ethanol were centrifuged, and the supernatant was discarded. The remaining pellet was treated with 400 µl of 1 % solution of sodium sulphide to release the bismuth from the alkaloidal complex, and a black-brown precipitate was formed. The samples were vortexed until the pellets were completely black.

Further, 400 µl of concentrated HNO_3 was added to the tubes to dissolve the pellets completely. Thereafter, the dilution up to 2 ml was made with distilled water and the tubes were centrifuged at 13000 rpm for 5 minutes. Lastly, 50 µl of the centrifuged solution was pipetted into new Eppendorf tubes and 150 µl of a mixture containing concentrated HNO_3, Na_2S and distilled water in a ratio of 1:1:4 (v/v) was added, creating a 4x dilution. To this, 1 ml of CS was added to form a yellow bismuth complex, according to the formula (II).

(II) $$\{Bi[CS(NH_2)_3]\}(NO_3)_3$$

Hereafter, the 1 ml was added to cuvettes and absorbance was measured at 435 nm with a spectrophotometer (Biochrom Ltd., WPA Biowave II UV/VIS, Nordic biolabs) against a blank containing HNO_3 and CS.

3.4.4. Procedure for calibration curve

Series dilution of the standard bismuth nitrate pentahydrate stock solution was performed by pipetting 1, 2, 3, 4, 5, 6, 7, 8, 9 ml of the solution into falcon tubes. The solutions were diluted to 10 ml of volume. Thereafter, 1 ml from each dilution was mixed in new falcon tubes with nitric acid, and thiourea to obtain a yellow-coloured mixture. Each mixture was measured in a spectrophotometer (Biochrom Ltd., WPA Biowave II UV/VIS, Nordic biolabs) at 435 nm to obtain a calibration curve.

3.4.5. Data processing

The absorbance values obtained from the spectrophotometric measurements were integrated into the linear equation received from the calibration curve. By this, it was possible to obtain an estimation of the total concentration of alkaloids, *i.e.* bismuth equivalents, from each lupin seed sample. As the plant extracts were made in triplicates, a mean concentration together with standard deviation and critical difference for various parameters was calculated for each sample by using Minitab Express software ($p < 0.05$, ANOVA). Additionally, multiple comparisons were studied using Minitab, *i.e.* ANOVA, and Tukey's *post hoc* test.

4. Results

This paragraph will present the main results of the thesis divided into two sections. Starting with the narratives from the market analysis (4.1) and closing with the result from the alkaloid analysis (4.2).

4.1. Market analysis of lupins in Sweden

The market analysis is based on unstructured interviews with several actors within the Swedish food industry. After summarising the material, it can be noted that many of the respondents experienced that lupin is a rather unknown crop in Sweden, and it is neither included as a horticultural crop nor an agricultural[7]. Therefore, no basic framework, supportive networks or infrastructure exists. Thus, the knowledge remains low regarding the usage of lupins for cultivation *(e.g.* potential pests and diseases, and how to control these factors) and in food use (*e.g.* how to handle post-harvest, non-existent processing facilities, allergenicity, utilisation of different fractions of the seeds and their application in food).

In recent years, Swedish farmers have started to cultivate lupins more exclusively for human food consumption[8,9]. The harvest can go directly to products such as minced legume mix (Baljväxtfärs, marketed in 2019)[10] or as tempeh. The minced legume mix consists of field peas (*Pisum sativum cv. arvense*), broad bean (*Vicia faba)*, and NL lupin seeds, and only require cleansing and milling of whole seeds before production. The lupin tempeh is produced in Sweden and requires dehulling prior to fermentation[11]. Further, Bengtsson[8] mentioned that immature green lupin seeds had a milder and sweeter taste compared to soaked and boiled mature lupin seeds that could exhibit a bitter flavour. Also, another Swedish company sells dried and whole NL lupin seeds directly to consumers[12].

There is a processing company[7] focusing on post-harvest processing of peas and lentils for human consumption. The company might be interested to expand their

[7] Olof Christersson, CEO at Agortus AB, personal communication 20th of February 2020

[8] Magnus Bengtsson, Farmer at Körslätts Farm in Kvidinge, personal communication 13th of May 2020

[9] Ronny Andersson, Farmer at Ekenäs Farm in Harlösa, personal communication, 15th of January 2020

[10] Anna Henning Moberg, Project developer at Axfoundation, personal communication, 14th of February 2020

[11] Eslam Salah, CEO at Lupinta, personal communication, 9th of June 2020

[12] Gunnar Backman, CEO at Nordisk Råvara, personal communication, 3rd of February 2020

business to include lupin seeds but finds the allergenicity of lupin seed as a main limitation.

Lupin is not a part of the official harvest statistics in Sweden as the total area of cultivation is below 500 ha[13]. However, data of the total cultivated area was possible to retrieve[12], Figure 2. The largest area was reached in 2004 when 350 ha of land was used for lupin cultivation. The majority of the harvest was used as animal feed[14]. Thereafter, the year of 2011 and 2012 peaked with approximately 300 ha. In 2019, ~175 ha of land was used for lupin crops. According to Statistics Sweden, all lupin seeds for cultivation in Sweden are imported. Boström[13] adds that there was a great interest in growing lupins, initially, but it steadily decreased as grain yields were unreliable and the sowing seeds were expensive to purchase. Also, financial supports from the EU are important from a cultivation perspective and often determines how much protein crops that are cultivated. This could be an affecting factor to the low cultivation area in 2009[13] in combination with a lower interest of lupins in general. Germany is one of the countries in the EU that has cultivated lupins extensively for human consumption. Also, Denmark cultivated lupins to a broad extent but experienced a radical decrease as a result of diseases[13].

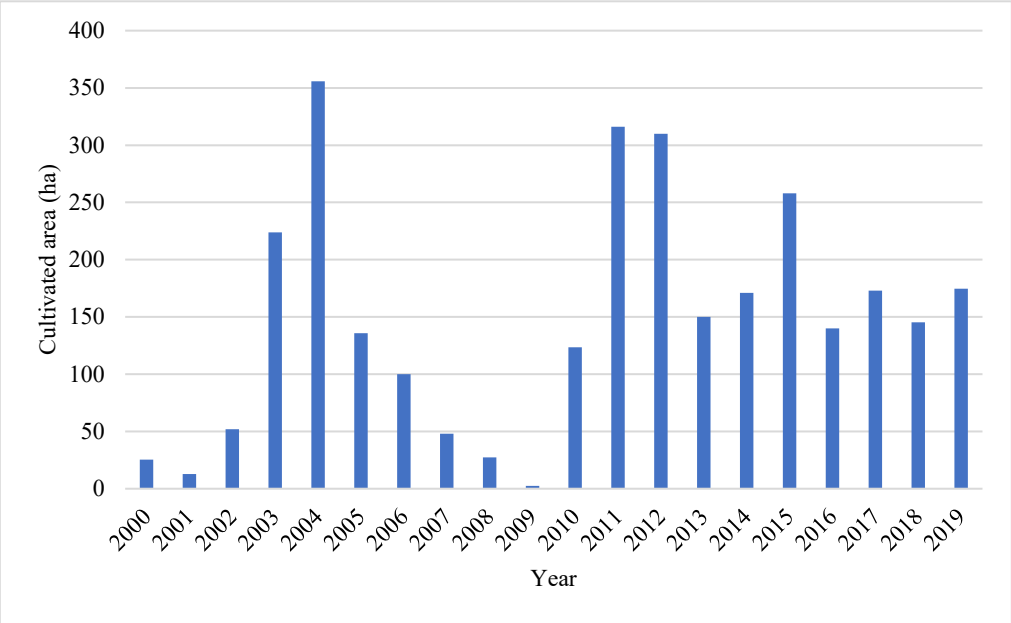

Figure 2. The total cultivation area (ha) of lupins in Sweden from 2000- 2019[12]

[13] Gerda Ländell, Department of agricultural- and energy statistics at Statistics Sweden, e-mail correspondence, 25th of February 2020

[14] Ulla-Lena Boström, research group leader, Department of Crop Production Ecology, personal communication, 20th of May 2020

4.2. Alkaloid analysis

4.2.1. Total alkaloid content in different cultivars

The mean alkaloid concentration in dw for the five different lupin cultivars obtained from RISE varied from 0.45 % to 1.37 %, Figure 3. A higher concentration was observed for the Andean lupin variety MUTA, the yellow lupin Baryt, and NL lupin Boregine reaching values of 1.37 ± 0.09 %, 1.03 ± 0.01 %, and 0.81 ± 0.07 % respectively. The yellow lupin cultivar of Bursztyn contained 0.66 ± 0.01 % of alkaloids, while the lower amount was seen for the NL lupin Samba (0.46 ± 0.03 %) and the yellow lupin variety Mister (0.46 ± 0.01 %).

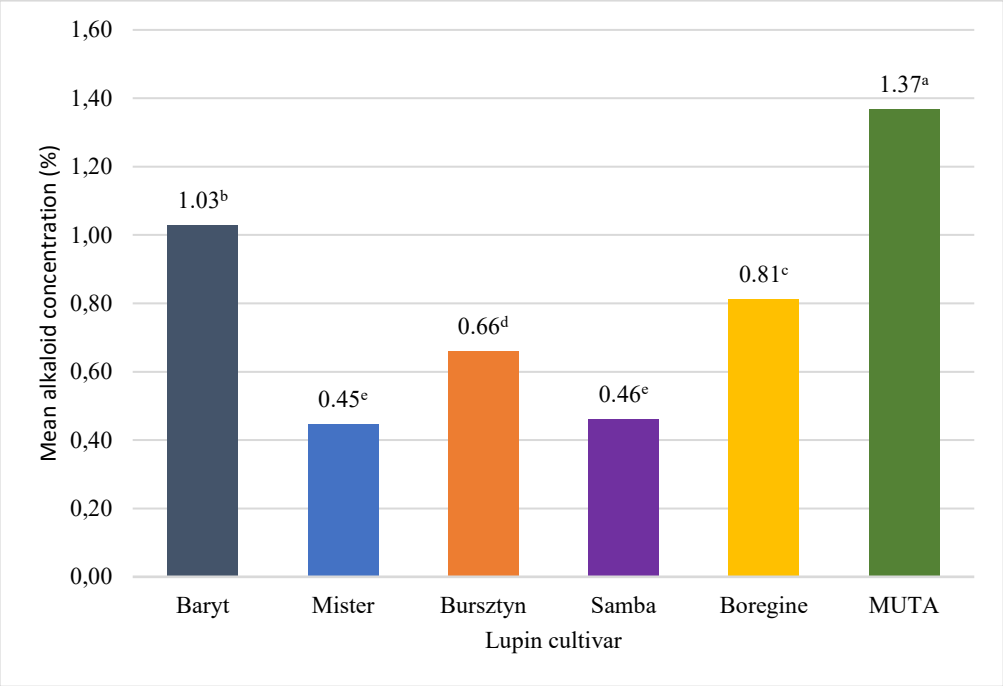

Figure 3. Alkaloid concentration (%) for three yellow lupin varieties (Baryt, Bursztyn and Mister), two cultivars of NL lupin (Samba and Boregine) and one Andean lupin cultivar (MUTA). Means that do not share a letter are significantly different ($p < 0.05$)

The ANOVA showed a significant difference ($p < 0.05$) in the total alkaloid concentration between all the different cultivars, except for the cultivars of Samba and Mister. The *post-hoc* comparison analysis showed that the lupin seeds from the cultivars of MUTA, Baryt, Bursztyn and Boregine were significantly different ($p < 0.05$) in alkaloid content to each other, and to the cultivars of Samba and Mister (Figure 3). Although, no significant difference was observed between the cultivars of Mister and Samba on a significance level of 95%.

4.2.2. The effect of year of harvest on alkaloid content

The NL lupin seeds *cv* Boregine and Mirabor from harvest years of 2017, 2018 and 2019 originating from two different farmers showed varying mean alkaloid concentrations, Figure 4.

Boregine lupin seeds harvested in 2017 reached a mean alkaloid level of 0.41 ± 0.03 %, Figure 4. Further, the seeds harvested 2018 resulted in a mean alkaloid concentration of 0.65 ± 0.06 % and 0.57 ± 0.03 %, for the cultivar of Boregine and Mirabor, respectively. Lower amounts of alkaloids were observed in lupin seeds from 2019, whereas Boregine contained 0.29 ± 0.02 % and Mirabor 0.3 ± 0.02 %.

The ANOVA indicated a significant difference ($p < 0.05$) between the three harvest years of *cv.* Boregine, Figure 4. This was also observed for the Mirabor cultivar.

The *post hoc* pairwise comparison for the seeds of Boregine from 2017, 2018 and 2019 also shows that all seed samples were significantly different ($p < 0.05$) in alkaloid content to each other, Figure 4. The results from the seeds of cultivar Mirabor showed a similar outcome as Boregine. However, there was no significant difference between the two cultivars harvested in 2018, Figure 4.

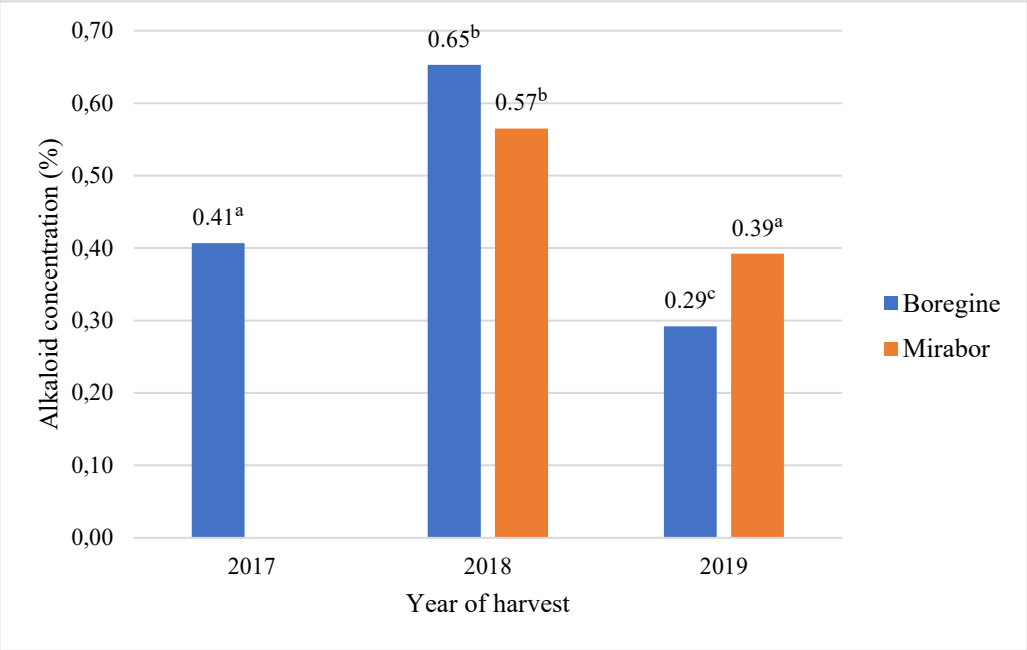

Figure 4. Total alkaloid concentration in NL lupin seeds from cultivars of Boregine harvested in 2017, 2018, and 2019 and Mirabor harvested in 2018 and 2019. Means that do not share a letter are significantly different ($p < 0.05$) in comparisons between years of the same cultivar.

The two-way ANOVA indicated no significant variation between the two cultivars of Boregine and Mirabor harvested 2018 and 2019 on a 95 % significance level, upper-right corner in Figure 5. However, the alkaloid concentration varied considerably between the two harvest years ($p < 0.05$). Further, the ANOVA indicated that cultivar × year interactions were significant ($p < 0.05$) for the total alkaloid concentration, showing non-parallel lines in the lower-left corner, Figure 5.

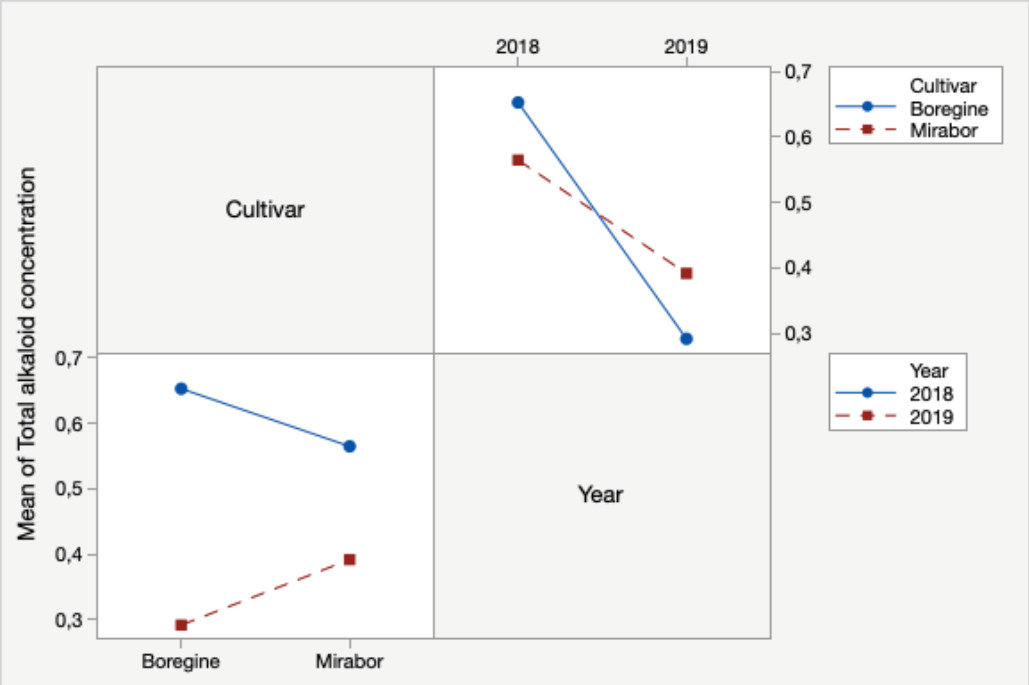

Figure 5. Interaction plot for total alkaloid concentration (%) between the cultivars of Boregine and Mirabor the harvest years of 2018 and 2019

4.2.3. Total alkaloid content in lupin seed cotyledon, and hull

The mean alkaloid content in the different seed components from cultivar Boregine showed values of 0.04 ± 0.02 % and 0.50 ± 0.04 % in seed hulls and seed cotyledon, respectively, Figure 6. The ANOVA test indicated a significantly lower concentration of alkaloids in lupin seed hulls compared to the seed cotyledon ($p < 0.05$). Further, the *post hoc* comparison test strengthens the hypothesis of a significant difference in mean alkaloid concentrations between the different seed material, Figure 6.

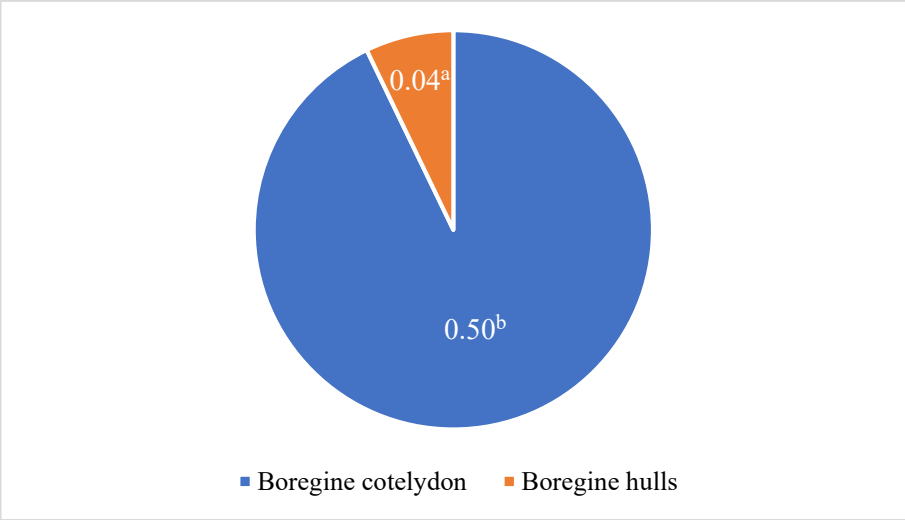

Figure 6. The total alkaloid concentration (%) for Boregine seed cotyledon and hulls, and its distribution throughout the whole lupin seed. Means that do not share a letter are significantly different

4.2.4. The effect of soaking on alkaloid content

The alkaloid concentration varied over time for the soaked seed samples of NL lupin *cv.* Boregine and *cv.* Samba (Figure 7). The seeds of Boregine that were soaked within a time interval of 1 to 7 days gave a mean alkaloid concentration (%) ranging from 0.41 to 0.74, Table 10. The mean alkaloid concentration in NL lupin *cv.* Samba soaked for 1 to 3 days varied from 0.55 to 0.70 %, Table 11.

There was a significant difference ($p < 0.05$) between the mean values for alkaloid concentration in the soaked Boregine samples, Table 10. Although, it is important to highlight that the sample soaked for 4 days only was grounded to coarsely grist, which may have influenced the extraction of alkaloids from the lupin material, see Discussion. A significantly ($p < 0.05$) lower alkaloid concentration was observed for the sample that was soaked for 24 hours, with a concentration 40 % lower than the control sample. Additionally, the *post hoc* test showed that the 24 hours-sample had significantly ($p < 0.05$) lower levels of total alkaloids in comparison to all the other soaked samples, Table 10. Further, differences ($p < 0.05$) between the control sample and the samples soaked for 2, 3, 3, and 7 days were non-significant, Table 10. Additionally, the sample soaked for 6 days deviated significantly ($p < 0.05$) in alkaloid concentration from the control samples and the samples soaked for 2 and 3 days but did not differ from the samples soaked for 5 and 7 days, Table 10.

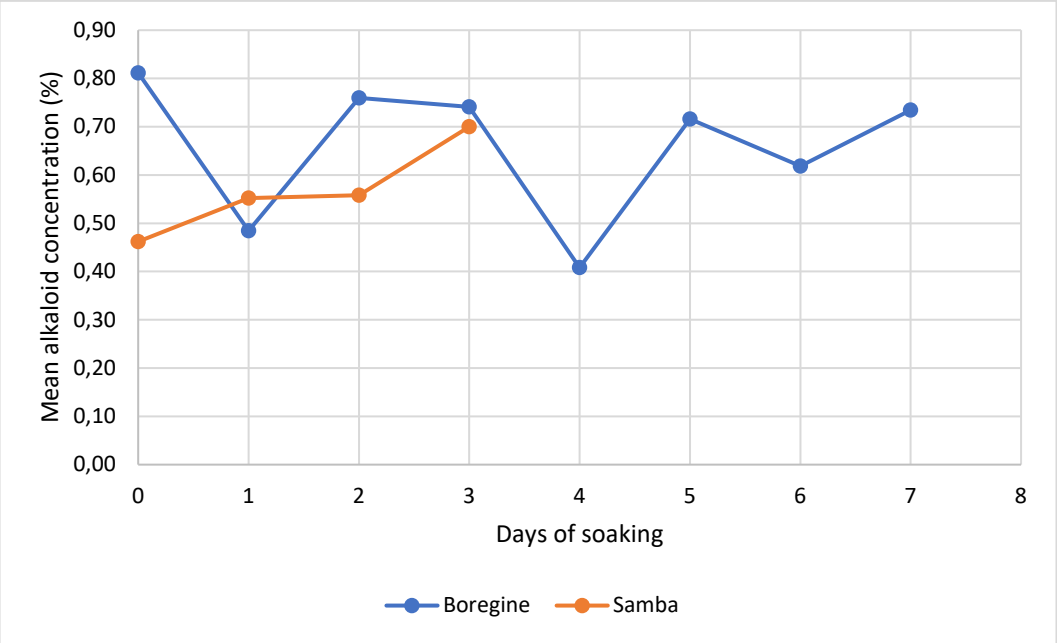

Figure 7. Average of the total alkaloid concentration (%) in the NL lupin cv. Boregine and Samba. Soaking time ranged from 1 to 7 days for Boregine and 1 to 3 days for Samba. The control samples consisted of whole seeds without soaking or drying (n=3)

Table 10. Average alkaloid concentration (%) for NL lupin cv. Boregine control sample and samples soaked for 1 to 7 days (n=3)

Days of soaking	Mean alkaloid concentration (%)	Change (%)
Control	0.81 ± 0.07 a	
1	0.48 ± 0.04 c	-40.3
2	0.76 ± 0.03 a	-6.4
3	0.74 ± 0.01 a	-8.7
4	0.41 ± 0.05 c	-49.7
5	0.72 ± 0.04 ab	-11.8
6	0.62 ± 0.02 b	-23.8
7	0.74 ± 0.04 ab	-9.5

± indicate standard deviation, means that do not share a letter are significantly different on a 95 % significance level

The ANOVA for the soaked samples of *cv.* Samba indicated no significant difference between the total alkaloid concentrations on a confidence interval of 95 %, Table 11. The soaking resulted in a non-significant increase in alkaloid content compared to the control sample, Table 11.

Table 11. Average alkaloid concentration (%) for NL lupin cv. Samba control sample and samples soaked for 1 to 7 days (n=3)

Days of soaking	Mean alkaloid content (%)	Change (%)
Control	0.46 ± 0.03 a	
1	0.55 ± 0.06 a	19.5
2	0.56 ±0.13 a	20.8
3	0.70 ± 0.16 a	51.5

± indicate standard deviation, means that do not share a letter are significantly different ($p < 0.05$)

4.2.5. Defatted lupin flour from *cv. Boregine*

The defatting procedure of Boregine lupin flour resulted in a total alkaloid concentration of 0.69 ± 0.05 % for sample BORAF. Meanwhile, the control sample with fat (BORF) showed a concentration of 0.81 ± 0.07 %, Figure 8. The mean alkaloid concentration in BORF sample was 14 % higher compared to BORAF sample. Although, no significant difference was observed between the two-sample means after running ANOVA ($p < 0.05$).

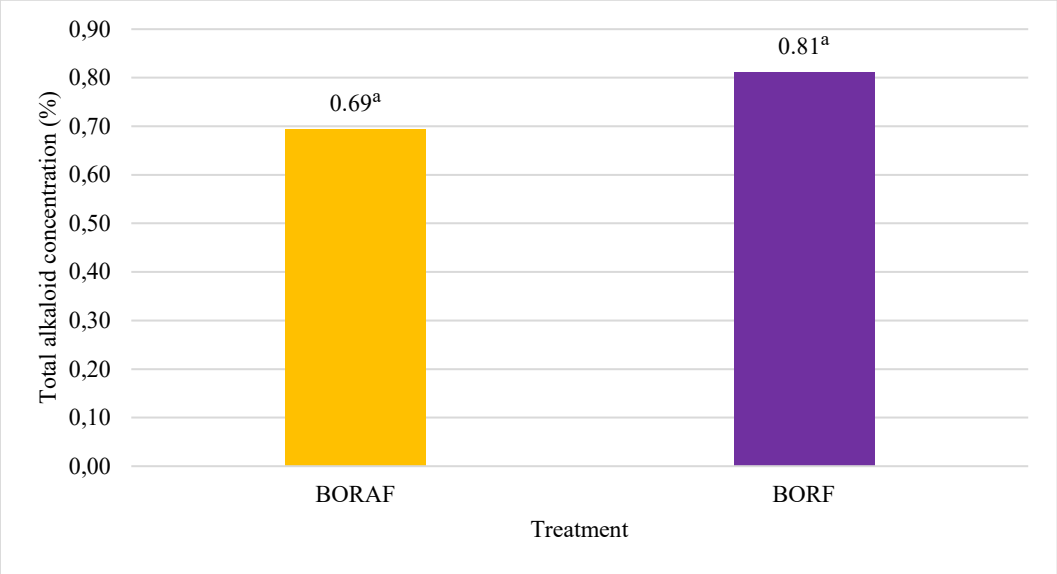

Figure 8. The total alkaloid concentration (%) for defatted lupin flour (BORAF) and control sample (BORF) with fat. Means that do not share a letter are significantly different

5. Discussion

The following section will discuss the findings from the market analysis which included how different actors perceived lupins for human consumption and possible solutions to some of the challenges. From the alkaloid analysis, elucidating aspects will be presented regarding the differences in alkaloid content between lupin species and cultivars, and how the alkaloid concentration was affected by year of cultivation. Furthermore, the alkaloid distribution in the lupin seed and the effect of soaking on alkaloid levels will be deliberated. Lastly, the applied method, in this study, will be contemplated, and the section will conclude with suggestions for further research.

5.1. Market analysis of lupins in Sweden

There are only a few Swedish lupin-based products on the market today and a small number of actors within the food industry focusing on lupin product development. The lack of processing facilities in Sweden limits further lupin utilisation and product development. Nonetheless, actors are perceived as motivated to expand the utilisation of lupins and to ultimately invest in lupin processing equipment. Similarly, many respondents experience that there is a demand for lupin seeds for human consumption. However, the lack of knowledge regarding post-harvest processing of lupin constitutes a limitation for the optimal introduction of the lupin-based products to consumers. A multi-corporate collaboration might be a part of a solution to this challenge (AGFO Talks, 2020). In a multi-corporate collaboration, stakeholders would put aside the competition and work together to develop a strategy on how to properly introduce Swedish grown protein crops on the market. Also, decision-makers could largely influence on increased cultivation of lupins in Sweden by providing agricultural subsidies to the primary producers. Construction of a governmental body of knowledge could be an alternative to increase expertise in the area (Emgardsson, 2020).

5.2. Alkaloid content

Starting with the seeds without any treatment; the highest mean alkaloid concentration was observed for the Andean lupin (1.37 %), which was expected as this lupin species could produce seeds with alkaloid levels up to 4.5 % (Carvajal-Larenas 2017). However, the concentration obtained for the yellow lupin cultivars Baryt (1.03 %) and Mister (0.45 %) was not expected to be as high, as earlier

findings indicated that these varieties should have considerably lower alkaloid concentration (in DM), only reaching 0.01 % (Księżak *et al.,* 2018) and 0.01-0.26 % (Księżak *et al.,* 2018; Musco *et al.*, 2017), respectively. The explanation for the high alkaloid content of the cultivar Baryt in this study could be that the seeds originated from the harvest year of 2017. Storage can increase the relative concentration of alkaloids (Dobiesz *et al.*, 2017). Likewise, the alkaloid content in Boregine (0.81 %) was higher in this study compared to literature. Here, Jansen *et al.* (2012) found that mean alkaloid content was 0.034 %. The Boregine seeds used in this part of the study were grown in Sweden, but the year of harvest is unknown. However, environmental factors could have affected the alkaloid in this particular sample, but as some information is missing, it is impossible to draw any conclusion.

Moreover, the comparison of the two different NL lupin varieties, Mirabor and Boregine, harvested 2018 and 2019, showed that the alkaloid content is more strongly correlated to the impact of harvest year than by cultivar. This is in line with the results observed in another study (Calabro *et al.*, 2015). However, Calabro *et al.* (2015) also observed that the alkaloid levels were strongly influenced by cultivar and the year×cultivar interaction. The significantly higher alkaloid level observed in the seeds harvested the year 2018, in this study, could have been a cause of a great many factors. The extreme drought in Sweden the year of 2018 could have been an affecting factor, as other studies found that drought stress increased the content of QAs in lupin seeds (Frick *et al.*, 2017; van de Noort, 2017; Carvajal-Larenas *et al.*, 2016). Another study concluded that high alkaloid content in the NL lupin seeds significantly correlated with high temperatures during pod ripening (Jansen *et al.* 2009). Also, soil pH (Jansen *et al.* 2012), low humidity (Cortés-Avendaño *et al.* 2020), deficiency of minerals (Frick *et al.* 2017) play an important role in alkaloid accumulation in lupin seeds. Important to highlight is that other lupin species and cultivars might have a different response to abiotic stresses (Staples *et al.*, 2017; Carvajal-Larenas *et al.*, 2016). Here, Magalhães *et al.* (2017) found that a yellow lupin cultivar experienced a lower alkaloid content when cultivated in the warmer climate of the Mediterranean regions. However, the aim of this study was not to investigate the effect of cultivation conditions on alkaloid content but to elucidate that the year of cultivation significantly affected the alkaloid concentration in the lupin seeds. Nonetheless, it is of relevance to emphasise that many factors affect the alkaloid accumulation in the lupin seed during cultivation. It is of great importance to identify the factors that have the largest impact on the alkaloid content to be able to grow lupin for human consumption.

Regarding the distribution of alkaloids in the lupin seed, it was observed that the seed coat contained considerably lower amounts of alkaloids compared to the cotyledon. This is consistent with other studies (Sumire-Qquenta *et al.*, 2019; Sedláková *et al.*, 2016; Parmdeep *et al.*, 2015; OGTR, 2013; Petterson, 2004), indicating that the seed hulls mainly contain cellulose, hemicellulose and lignin. Thus, the bitter taste that is believed to be caused by alkaloids cannot be diminished by industrial dehulling of the seeds. On the contrary, the dehulling process could instead increase the relative concentrations of the alkaloids and other bitter-tasting ANFs, *i.e.* saponins and tannins (Embaby, 2010). However, dehulling can also have

the positive effect of increasing the bioavailability of calcium, iron and zinc (Karnpanit *et al.*, 2017).

Implementing a debittering process has been proven to reduce the alkaloid content considerably in lupin seeds (Villacrés *et al.*, 2020; Córdova-Ramos *et al.*, 2019; Sumire-Qquenta *et al.*, 2019; Carvajal-Larenas *et al.*, 2016). Villacrés *et al.* (2020) were able to remove 80 % of total alkaloids in Andean lupin by hydrating and cooking the seeds in saline (0.5 % NaCl) water at temperatures of 80°C and 91°C, respectively. However, in this thesis, the hydrated lupin seeds were not subjected to any hydrothermal process. The reasoning behind this was that studies implied that high-temperature thermal treatments might increase the number of off-flavours (Roland *et al.*, 2017; Stephany *et al.*, 2015), thus lowering the sensory acceptance (Stephany *et al.*, 2015). The results from the soaking of lupin seeds had ambiguous effects on the alkaloid concentration. The seed flour from NL cultivar Boregine soaked for 1 day showed a 40 % decrease in alkaloid concentration compared to the control sample. Also, the sample soaked for 4 days showed almost 50 % decrease in QA content. Important to highlight is that this sample only was coarsely grounded, which may have made the alkaloid extraction less efficient than for the other soaked samples. Hence, conclusions cannot be drawn from this sample. The remaining samples (2, 3, 5, 6, and 7 days) only showed a non-significant declination in alkaloid content. Nevertheless, the soaked seeds of NL lupin cultivar Samba showed contradictive results. Here, soaking indicated a slight increase in alkaloid content, although, not significant. Possible cause of this is difficult to determine. Embaby (2010) reports a significant increase of 6.3 % in tannins in lupin seeds from sweet varieties after soaking for 24 hours. This is considered to be related to hydrolysis of high molecular weight insoluble polymers to small molecular weight soluble polymers (Embaby, 2010). It can, therefore, be hypothesised that this theory might apply to the lupin alkaloids, and thus, be the cause of the slight increase observed in the soaked seeds of Samba. Another explanation could be that there is an increase of the relative concentration of alkaloids in the lupin seeds, as water-soluble compounds, *i.e.* vitamins, minerals, and monosaccharides are leached to soaking water (Villacrés *et al.,* 2020). Also, the extraction efficiency of the alkaloids could have been affected by the soaking of lupin seeds. Thus, this could have been a factor causing the slight elevation of alkaloid content in seeds of Samba. However, if this was the case, the same trend should have been observed in the soaked seeds of Boregine as well, but it was not. To sum up, soaking affected the total alkaloid concentration in this study, although, ambiguously and in one case non-significantly. However, this conclusion is based on the statistical variation from the alkaloid analysis, performed in triplicates. Important to highlight is that the initial soaking of the lupin seeds was only carried out in one replicate for each sample. Therefore, it is not possible to determine the repeatability of the applied method. All things considered, it was not sufficient to remove the majority of alkaloids from the lupin seeds by hydration and oven-drying in this study. Thus, application of hydrothermal treatment could be essential from a food safety perspective (Villacrés *et al.,* 2020; Jiménez-Martínez *et al.*, 2001).

The reliability of the method chosen to quantify alkaloids in this study could be questioned. The analysis was performed without Soxhlet-extraction of the fat and with acetic acid. Nonetheless, no significant difference in alkaloid concentration was observed between the defatted lupin flour sample and the flour sample containing fat. This suggests that the fat in the lupin seed samples did not significantly influence the alkaloid content in the extract. Thus, this refutes the hypothesis of Wallebroek (1940) implying that fatty oil content had a disturbing effect on the alkaloids. Furthermore, the extraction by acetic acid might have resulted in precipitation of other interfering substances. On the contrary, acidic solutions are commonly used for alkaloid extractions (Christiansen *et al*., 1997, Frick *et al*., 2017). One could also speculate whether the structure of the alkaloids in the lupin samples might have influenced the precipitation with Dragendorff's reagent. However, to the best of authors' knowledge, there are no reports considering this matter. Moreover, the Dragendorff's method is an established method for analysing alkaloid content in comparative studies (Staples *et al.*, 2017; Sreevidya & Mehrotra, 2003) and expresses only the total alkaloid content (Harrison & Williams, 1982). To control the reliability of the results, the efficiency of the alkaloid extraction, qualitative and quantitative determination of the composition of QA in this study, other methods could have been implemented, such as GC-MS (Cortés-Avendaño *et al.*, 2020) or HRGC-MS (Musco *et al.*, 2017). However, there is no certified method for alkaloid detection and quantification today (Frick *et al.,* 2017). This indicates that an improved and comprehensive methodology is needed to be able to monitor the alkaloid content in lupin seeds for food safety purposes.

For future research, it would be interesting to test the debittering method described by Villacrés *et al.* (2020) and further investigate how soaking effects the alkaloids content in lupin seeds. Also, germination and fermentation have been observed to reduce the alkaloid content and other ANFs (*i.e.* phytate) considerably, while also increasing the sensory properties (Kaczmarska *et al.*, 2017; Carvajal-Larenas *et al.*, 2016; Kaczmarek *et al.*, 2016). Lastly, investigating the sensory properties of immature NL lupin seeds could be another suggestion for further research. As Bengtsson[15] mentioned that the flavour is sweeter and milder than of mature, dried NL lupin seeds.

[15] Magnus Bengtsson, farmer at Körslätts Farm, Kvidinge, Skåne, personal communication 13th of May 2020

Conclusion

In the present study, a market analysis was implemented to investigate how lupin is perceived as a protein crop for human consumption in Sweden. Also, an alkaloid analysis was carried out to compare the total alkaloid content between lupin species and cultivars. The effect of year of harvest and soaking on alkaloid concentration was studied as well as the distribution of alkaloids in the lupin seeds.

The market analysis indicated that further utilisation of lupin for human consumption is mainly limited by the absence of processing facilities, limited knowledge-base, and basic frameworks. Nonetheless, actors are perceived as motivated to expand their lupin cultivation and production of lupin products.

Significant variations in alkaloid content were observed between lupin species and cultivars. The year of cultivation strongly affected the alkaloid concentration in NL lupin seeds. Drought stress could be one explanation, but it is likely that many other factors also affect the alkaloid biosynthesis during cultivation.

It was confirmed that the alkaloids are mainly located in the lupin cotyledon. Thus, the bitter-tasting alkaloids of lupin cannot be removed by the dehulling process. An alternative for alkaloid removal is soaking of the lupin seeds. However, soaking had an ambiguous effect on the alkaloid content in this study. Therefore, soaking of lupin seeds in saline water followed by a hydrothermal treatment could reduce the alkaloids to a greater extent. This could diminish the bitter taste and increase the palatability of lupins in food products.

To the best of the authors' knowledge, this was the first study that included an alkaloid analysis on Swedish-grown lupin seeds. The concluding remarks are that more research is needed to identify the abiotic stresses that have the greatest impact on alkaloid accumulation in lupin seeds, gain knowledge on how to properly reduce the alkaloid content in lupin seeds by processing, and developing a suitable method for alkaloid detection and quantification. All these aspects are essential to provide a lupin product safe for human consumption.

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Appendix 1 Samples for alkaloid analysis

Table A1. Total number of samples; species, cultivar, number of samples per cultivar, soaking time. NL, and yellow lupin

Species	Cultivar	No. of samples	Soaking	Soaking time
NL	Boregine	7	Yes	1–7 days
NL	Boregine	1	No	N/A
NL	Boregine (husk + hull)	1	No	N/A
NL	Boregine (Cotelydon)	1	No	N/A
NL	Samba	3	Yes	1–3 days
NL	Samba	1	No	N/A
NL	Boregine 2017	1	No	N/A
NL	Boregine 2018	1	No	N/A
NL	Boregine 2019	1	No	N/A
NL	Mirabor 2018	1	No	N/A
NL	Mirabor 2018	1	No	N/A
YL	Mister	3	Yes	1–3 days
YL	Baryt	3	Yes	1–3 days
YL	Bursztyn	3	Yes	1–3 days
YL	Mister	1	No	N/A
YL	Baryt	1	No	N/A
YL	Bursztyn	1	No	N/A
Total		**31**		

Table A2. The species and cultivars analysed with the purpose to investigate the relation between alkaloid concentration in seeds and soaking processes.

Objective: Is alkaloid concentration affected by soaking?

Species	Cultivar	No. of samples	Soaking	Soaking time
NL	Boregine	7	Yes	1–7 days
NL	Samba	3	Yes	1–3 days
YL	Mister	3	Yes	1–3 days
YL	Baryt	3	Yes	1–3 days
YL	Bursztyn	3	Yes	1–3 days
Total no. of samples		**19**		

Table A3. The species and cultivars prepared as control samples to the samples in table A3

Objective: Control samples			
Species	**Cultivar**	**No. of samples**	**Soaking**
NL	Boregine	1	No
NL	Samba	1	No
YL[b]	Mister	1	No
YL	Baryt	1	No
YL	Bursztyn	1	No
Total no. of samples		**5**	

Table A4. The species and cultivars analysed with the purpose to localize the alkaloids

Objective: Is alkaloid content located in husk + hull or in cotyledon?

Species	**Cultivar**	**No. of samples**	**Soaking**
NL	Boregine (husk + hull)	1	No
NL	Boregine (Cotelydon)	1	No
Total no. of samples		**2**	

Table A5. The species and cultivars prepared to analyse potential environmental impacts during cultivation, e.g. drought, frost

Objective: Is alkaloid content affected by year of harvest, *e.g.* drought

Species	**Cultivar**	**No. of samples**	**Soaking**
NL	Boregine 2017	1	No
NL	Boregine 2018	1	No
NL	Boregine 2019	1	No
NL	Mirabor 2018	1	No
NL	Mirabor 2018	1	No
Total no. of samples		**5**	

Appendix 2 Lab protocol

Apparatus
Spectrophotometer
Analytical balance
Digital pH-meter
Centrifuge

Material

Defatting of lupin flour
Filter paper
Funnel
Beakers; 50 ml
Magnetic stirrer

Alkaloid analysis
4 st 100 ml volumetric flasks
Falcon tubes (15 ml)
Eppendorf tubes, 2 ml
Pipettes

Chemical and reagents

Defatting of lupin flour
Hexane

Alkaloid analysis
Dragendorrf's reagent
Bismuth(III) nitrate pentahydrate ($Bi(NO_3)_3 \cdot 5\ H_2O$), standard solution; purity 99,8%
Thiourea, Reagentplus TM, >= 99.0%
Sodium sulfide (N_2S)
Concentrated nitric acid, (HNO_3)
8g Potassium iodide (KI)
Glacial acetic acid, purity: 99,9 %
Etanol, 95%
Dilute hydrochloric acid (HCL)

Solutions

a) Dragendorff's reagent (DR)
   1) prepare solution of 0,8g bismuth nitrate pentahydrate in 40 ml distilled water and 10 ml glacial acetic acid
2) 8 g of KI is dissolved in 20 ml distilled water
3) Mix the two solutions

b) Standard bismuth nitrate solution
Dissolve 10 mg bismuth nitrate pentahydrate ($Bi(NO_3)_3 \cdot 5\ H_2O$) in 5 ml concentrated nitric acid (HNO_3) and diluting to 100 ml with distilled water.

c) Thiourea, 3%
The thiourea solution was prepared by dissolving 3 g of thiourea in 100 ml distilled water

d) Sodium sulfide, 1%
The sodium sulfide nonahydrate solution was prepared by suspending 1 g of N_2S in 100 ml distilled water.

e) Acetic acid, 2%
The glacial acetic acid (99,9 %) was diluted to 2 % by taking 2 ml and add to 98 ml distilled water.

Procedure

Procedure for calibration curve
1. Pipette 1, 2, 3, 4, 5, 6, 7, 8, and 9 ml of standard bismuth nitrate solution into separate falcon tubes (15 ml)
2. Dilute to 10 ml with distilled water
3. Take 1 ml of this solution and add 5 ml thiourea, 3%.
4. Measure the absorbance value of the yellow solution at 435 nm against a colourless blank

Defatting of lupin flour
The fat removal was performed according to the method described by Newton (2014).

1) 10 gram of lupin flour is combined with 30 ml of hexane (1:3, w/v)
2) Stir the mixture for 2 hours at 360 rpm
3) Centrifuge for 15 min at 4°C, 5000*g* (Avanti® Centrifuge J-26 XPI, Beckman Coulter, USA)
4) Decant the hexane and leave the lupin flour to dry under a hood for 24 hours

Extraction of alkaloids from lupin flour
0,25 g finely powdered lupin seed flour was extracted with 2,5 ml aqueous acetic acid (2%) in a falcon tube (15 ml) and shaken with a Rotamix RM1, program F1, 60 rpm, at room temperature for 10 min. The sample was centrifuged (Jouan SA, C3i) at 4000 rpm for 5 min. The procedure was repeated 3 times. The extract was mixed and diluted to 10 ml with aqueous acetic acid (2%). The pH was maintained at 2-2.5 with 1M HCl (approx. 4 drops). Thereafter, 2 ml was transferred to an eppendorf and centrifuged at 13000 rpm (Heraeus Pico 21 Centrifuge, Thermo Scientific) for 5 min.

Procedure for Assay of Plant Extracts
1. Take 1 ml of the extract/ alkaloid solution and put in a new eppendorf
2. Add 400 μl of Dragendorff's reagent (DR). Precipitate will form
3. Centrifuge the sample at 13000 rpm for 5 min
4. Check the centrifugate for complete precipitation by adding 100 μl DR
5. Centrifuge again at 13000 rpm for 5 minutes
6. Decant the supernatant completely and meticulously. Pipette if necessary
7. Add 1 ml EtOH (70%) to the eppendorf tubes (washing-step)
8. Run the samples in Rotaflex, program F1, 60 rpm for 5 minutes
9. Centrifuge the samples in 13000 rpm for 5 minutes and discard the supernatant
10. Treat the remaining residue with 400 μl of sodium sulfide solution. A brownish black precipitate will form
11. Run the samples in Rotaflex, program F1, 60 rpm for 5 minutes
12. Check for complete precipitation by adding 2 drops of N_2S-solution
13. Dissolve the residue in 400 μl conc. HNO_3, with warming if necessary
14. Dilute this solution to 2 ml in an eppendorf tube with distilled water
15. Take 200 μl from this solution and add 1 ml thiourea solution
16. Measure the absorbance at 435 nm against a blank containing HNO_3 and thiourea

The amount of bismuth present in the solution was calculated by multiplying the absorbance values with the factor, taking suitable dilution factor into consideration. The factor is obtained from the standard curve, which is a constant for different concentrations.

$$Factor = \frac{concentration}{absorbance}$$

Lupin – a future protein crop in Sweden?

Our modern food systems have a large impact on our environment, contributing to 19-30 % of total greenhouse gas emissions. To shift towards a more sustainable food system, we should adopt healthier diets which contain more plant-based protein, such as legumes, and less meat and dairy. A decreased dependency on soy is wanted and will require increased production of other legumes. However, this is not problem-free as legumes contain antinutritional factors (ANFs), which is a limiting factor for increased consumption.

This is a popular scientific summary based on a master thesis in food science (30 hp), written by Sanna Pasanen at the Swedish University of Agricultural Science. The thesis with the title "Lupin as a future protein source in Sweden: food safety aspects, prospects and challenges" aimed to provide knowledge for increased utilisation of lupins in Sweden and to investigate how the crop is perceived as a potential protein source. The study also included an analysis of alkaloid content in lupins and its hulls and how different factors affect these levels.

Lupinus species

Lupinus is a wide and diverse genus with approximately 200 species of flowering plants. There are only four *Lupinus* species that are domesticated and used for food and feed purpose. These are the narrow-leafed lupin (*L. angustifolius*), the yellow lupin (*Lupinus luteus*), *the* white lupin (*Lupinus albus*) and the Andean lupin (*Lupinus mutabilis*). It is important to not confuse these lupins with the toxic wild lupin growing in the roadsides.

The global cultivation of lupin can be seen in Figure 1. Australia is the top producer of lupins. They are also grown in Russia, parts of Europe, and South America.

Nutritional composition

The lupin has a thick seed coat that mainly consists of dietary fibre. Lupin starch content, Table 1, is higher compared to the soybean (Glycine max) but considerably lower than the pea (Pisum sativum). The protein and fat content of lupin, Table 1, varies widely between species and cultivars. Lupin also contains micronutrients like various minerals.

Figure 1. The global cultivation of lupin

Table 1. Nutritional value of lupin seeds

	Starch	Protein	Fat
% of seed	6	15-52	4-25

ANFs in lupin

ANFs are undesirable substances in legumes that reduce the uptake of nutrients in the body. The most common ANF in lupins are the alkaloids, which comprise 0.005 – 4.5 % of the seed. Alkaloids help protect the plant against environmental threats like herbivores or UV light.

The bitter taste of lupin seeds is thought to be caused by the alkaloids. Therefore, lupins are divided into sweet and bitter varieties, where the sweet varieties contain lower levels of alkaloids and bitter varieties contain higher levels.
Alkaloids can be toxic if consumed in high doses and could affect the digestive system.

Processing of lupin

Lupins are traditionally prepared by soaking for 24 hours and boiling for one hour. Thereafter, the lupin can be used in foods like falafel, miso and soy.
Industrial processing can include milling of whole lupin seeds to flour, which can be used as an additive in bread-baking. Another type of industrial processing is fractionation. Here, each part of the lupin seed is isolated. The hulls can function as a dietary fibre supplement. The oil can be used in baking, pasta products or sausages. It is also possible to isolate the proteins from the lupin kernel, yielding a lupin protein isolate (LPI). This isolate has a high value in food processing and can substitute egg and dairy in ice cream production and improve texture in pasta and bakery products. Fermentation of LPI is useful in the development of plant-based dairy alternatives.

Prospects and challenges

There are both prospects and challenges with lupin production. Some positive aspects include the high-nutritional value of lupin and its suitability to substitute the imported soy. However, the biggest challenges are the absence of processing facilities for cleaning and dehulling lupin seeds in Sweden, but also the bitter taste of lupin which is difficult to mask.

Alkaloid content in lupins

In this study, most of the lupin alkaloids were found in the kernel, Figure 2. There was a wide variation in alkaloid levels between species and cultivars, Figure 3. Soaking of seeds showed ambiguous results on alkaloid removal. The seeds of one cultivar (Boregine) soaked for 24 hours removed 40 % of the alkaloids, while the soaked seeds of another cultivar (Samba) showed no decrease in alkaloids at all.

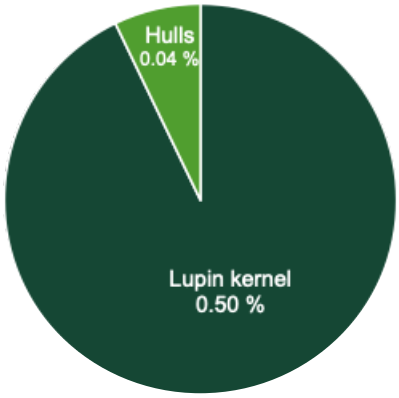

Figure 2. The majority of lupin alkaloids were found in the lupin kernel

The year of harvest of the lupin seed had a large impact on alkaloid content, Figure 4. The seeds harvested in 2018 showed considerably higher alkaloid content compared to the other harvest years. The drought of 2018 could be an explanation of the high levels, but the effect is not entirely clear.

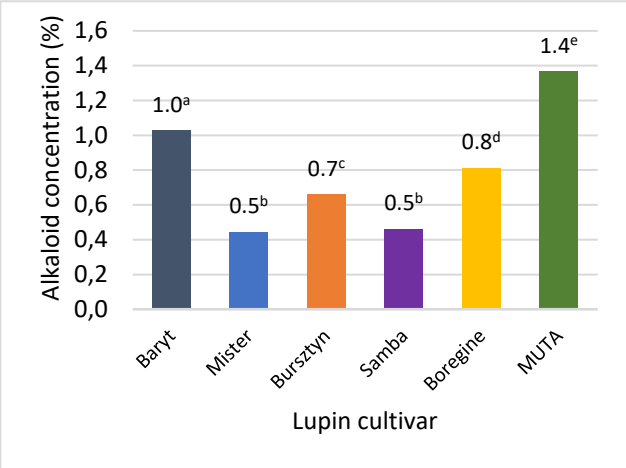

Figure 3. Alkaloid content in three different species of lupins including narrow-leafed lupin (Boregine and Samba), yellow lupin (Baryt, Bursztyn and Mister) and Andean lupin (Bolivian variety)

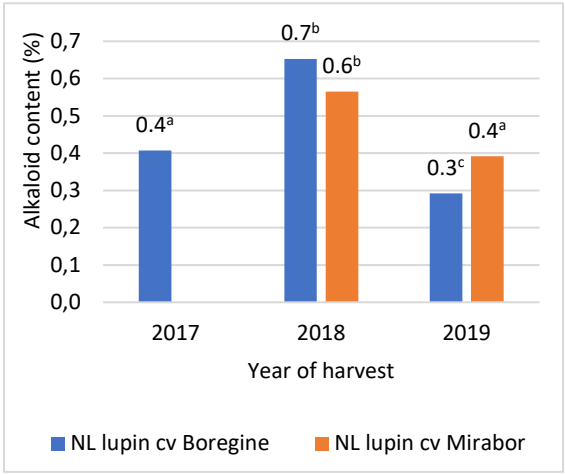

Figure 4. Alkaloid content in lupin seeds harvested different years.

Conclusion

Lupin possesses a good nutritional value, with a high content of protein, dietary fibre and oil. It is thus a good substitute for soy and a suitable legume for human consumption. The greatest obstacle for increased lupin production is the absence of processing facilities. To address this issue, we need to create incentives for actors to invest. Regarding the alkaloid content in lupin seed, more knowledge is needed about lupin alkaloids and how they are stored in the plant during growth. We also need to find a way to efficiently and thoroughly remove the alkaloids from the lupin seeds. All things concluded, lupin has great potential for cultivation and human consumption in Sweden. It is just a matter of time before it plays a leading role as a protein source on the plate of the Swedish consumer.

Figure 5. Lupin hulls (left picture) and lupin kernels (right picture) from narrow-leafed lupin cultivar Boregine