The fate of quinolizidine alkaloids during the processing of lupins (*Lupinus* spp.) for human consumption

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Abstract

Lupin, a protein-rich grain legume, and products thereof, are becoming increasingly important in our diets. However, variable and high concentrations of quinolizidine alkaloids (QAs) may hamper this evolution. This study assessed the fate of QAs when processing *Lupinus albus* seeds and lupin-based foods, to give a first indication of the food industry's ability to sufficiently reduce the QA concentration. Typical unit processes, including toasting, dehulling, sterilization (sterilized jarred lupins), oven baking (cookies), frying (chips) and boiling in water (pasta), were simulated on lab-scale. A quantitative determination of five QAs and qualitative screening of other relevant QAs, in the derived fractions and lupin-based foods, was performed with a validated UHPLC-MS/MS and -HRMS method, respectively. Results revealed that the reduction in quinolizidine alkaloid content is highly dependent on the applied unit process, that QAs appear to be heat stabile, and that the depletion can be attributed to the leaching in cooking water.

Keywords

Lupin, Quinolizidine alkaloids, food production, unit processes, plant-based proteins

Chemical compounds studied in this article

(+)-lupanine (PubChem CID: 91471); (+)-13α-hydroxylupanine (PubChem CID: 250873); (-)-sparteine (PubChem CID: 644020); (-)-lupinine (PubChem CID: 91461); (-)-angustifoline (PubChem CID: 12306807)

1. Introduction

Given the current trajectory towards a more diversified protein supply and the shift in consumption towards plant-sourced proteins, the share of protein-rich small grain legumes and products thereof will become increasingly important in our diets (Aschemann-Witzel et al., 2020). Here, an interesting but often underappreciated legume is lupin, belonging to the diverse and widespread *Lupinus* genus, from the Genisteae tribe and Fabaceae family (Cowling et al., 1998). Out of more than 500 lupin species, four are considered of commercial and agricultural relevance: *Lupinus albus* (white lupin), *Lupinus angustifolius* (blue or narrow-leafed lupin), *Lupinus luteus* (yellow lupin) and *Lupinus mutabilis* (Andean lupin) (EFSA et al., 2019; Wink, 1993). Lupin has been used in food for many years now. In the Mediterranean area *lupini* beans are eaten as a pickled snack, and in the Andean highlands of South America lupin is a traditional food, locally known as "tarwi" or "chocho" (Petterson, 2004). Because of its interesting nutritional and chemical characteristics, it is used as a technological ingredient in a variety of processed foods, such as bakery and confectionary products, meat and dairy products (Villa et al., 2020). Literature indicates that lupin meal can be used in bread to enhance the water holding capacity, in that way increase the stability and the protein content of bread (Villarino et al., 2016). Lupin seeds or lupin protein isolates are being used in vegetarian meat alternatives (BfR, 2017; RIVM, 2015).

Lupin is a protein-rich crop with promising prospects, yet certain properties of the lupin plant urge caution. The sensitivity of consumers to lupin protein has led to its identification as a food allergen in Europe (Regulation EU 1169/2011). These allergic reactions were reported either as primary lupin allergy or due to cross-reactivity to other legumes such as soybean, pea, lentil and chickpea and peanut (Guillamón et al., 2010). Moreover, lupins are the main host for the fungus *Diaporthe toxica*, which produces phomopsins (PHOs), a specific group of 'emerging' and poorly studied mycotoxins (Battilani et al., 2011). PHOs are responsible for lupinosis, a

liver disease common in grazing animals (Plumlee, 2004; Williamson et al., 1994). Characteristic to lupins are inherent plant toxins or 'lupin alkaloids', a group of secondary metabolites that comprises quinolizidine alkaloids (QAs), in addition to other alkaloids like piperidine alkaloids, *e.g.* ammodendrine, and simple indole alkaloids, *e.g.* gramine (ANZFA, 2001). Quinolizidine alkaloids are the most abundant and toxicologically relevant lupin alkaloids. They serve as nitrogen reserves and defense against pathogens and other predators, *e.g.* insects and herbivores (Boschin et al., 2008). Over 170 QA structures have been reported in different *Lupinus* species. They occur as bicyclic, tricyclic and tetracyclic structures in lupin (Wink, 1993). Naturally occurring wild lupins are toxic and may contain over 10 000 mg kg^{-1} QAs, while so-called 'sweet' lupins have a QA content of less than 500 mg kg^{-1} (Pilegaard & Gry, 2008). Maximum levels for QAs in food products of 200 mg kg^{-1} dry weight have been implemented by national food authorities (Pilegaard & Gry, 2008). The QA levels of bitter lupin seeds are traditionally reduced by soaking, cooking and washing lupin seeds for several days in large volumes of salted water. This process removes between 88 and 97% of QAs (Carvajal-Larenas et al., 2016; EFSA, 2019). In humans, QAs may inhibit acetylcholine receptors (AChRs) in both the central nervous- and peripheral autonomic system, which could cause respiratory failure and eventually death (EFSA et al., 2019; Tsiodras et al., 1999). The CONTAM panel (EFSA, 2019) and BfR (2017) have attributed QA intoxications and the few lethal cases to an insufficient debittering process by consumers. Most consumers cannot differentiate between sweet and bitter lupins. Studies have now also shown that there are so-called 'sweet' lupin varieties with high alkaloid concentrations, as these varieties tend to reacquire their bitterness over time, as a result of mutations, cross-breeding or recombinations (Uauy et al., 1995). While there is abundant literature available on the aqueous extraction of quinolizidine alkaloids as a debittering process (Carvajal-Larenas et al., 2016), the stability of QAs during typical unit processes in food processing like milling, dehulling, baking, soaking,

cooking, sterilization or frying, implemented at industry level, remains unclear. It is expected that physical and aqueous unit processes and heat treatments or the combination of one or two will affect the concentration of quinolizidine alkaloids in lupins. The objective of this study was to investigate how the concentration of some abundant and most toxic quinolizidine alkaloids evolve during processing in the derived fractions and lupin-based products, with the aim to give a first indication of the food industry's ability to sufficiently reduce the QA concentration in foods, in order to reduce the exposure of consumers to QAs in the shift to plant-based proteins.

2. Materials and Methods

2.1. Raw material lupin seeds

A single batch of 20 kg *Lupinus albus* seeds [white sweet lupins] harvested in Ansbach, Bavaria, Germany, in autumn 2021, was used as raw material throughout the complete study to avoid variability amongst batches of seeds. This particular batch was suggested by the plant breeder specifically because of its higher quinolizidine alkaloid content.

2.2. Identification and selection of unit processes and relevant foods

An analysis of the lupin agri-food chain and market study for foods containing lupins available on the European market (period autumn 2021 - spring 2022) was the starting point for the identification of relevant unit processes and lupin-containing foods. A label inventory was made of commercial available foods in supermarkets, small (organic) specialty shops and online shops of foods containing lupin. A long list of about 160 unique foods was made up and the final selection of unit processes and food products was done according to the importance of the unit process in the processing of lupin, the share of lupins in the food and feasibility to mimic the process on lab-scale. The selected unit processes include toasting, dehulling, sterilizing, boiling, frying and baking. Food products include sterilized jarred lupin seeds, cookies containing lupin meal, lupin pasta and lupin chips.

2.3. Lab-scale simulations of unit processes

Each production experiment was performed on two consecutive days. After each relevant unit process, representative samples (before and after application of the unit process) were collected from the produced fractions for analysis. An overview of the experimental design and sampling plan is given in Table 2. Samples were collected in LDPE bags kept at -20 °C until analysis. Flow diagrams and ingredient lists are given in supplementary files S1 to S8. An overview of the experimental plan and sampling plan is given in S9.

2.3.1. Milling: whole lupin flour

The objective of producing the whole lupin flour was to prepare a starting batch of raw material to produce the more complex matrices. Therefore, 3 kg of cleaned and sorted lupin seeds was weighed and grinded using a laboratory scale mill (FOSS: Hammertec – 50HZ, 2017, China) with 800 µm mesh and sieved through a 500 µm standard sieve.

2.3.2. Dehulling: lupin kernels and hulls

The cleaned whole lupin seeds were dehulled to separate the hulls (seed coat) from the kernel with a pilot-scale dehuller (JK Machinery, KMPP 300, Czech, Prague). Two different seed portions 500 g and 1000 g were weighed. The former was used for cleaning the dehuller, before the actual 1000 g portion. The seeds were gradually fed into the dehuller through the hopper and the dehulled samples were collected from the output. The yield collected after dehulling was weighed (925 g) and was made up of hulls, broken lupin kernels and lupin grits. The recovered dehulled seeds were further separated into different fractions using a pilot-scale seed cleaning equipment (KamasWestrup Dubois, Belgium) with 10 outputs graded by size. The cleaned seeds, clean hulls, broken hulls and lupin grits less than 2 mm were all collected from different outlets. The cleaned seeds and the clean hulls were weighed 667.5 g and 174 g

127 respectively. A 500 g portion of the clean lupin kernels was further milled into flour using a
128 laboratory scale miller (FOSS: Hammertec – 50HZ, 2017, China) with 800 µm mesh.

129 ### 2.3.3. Toasting: toasted whole- and dehulled lupin flour

130 The wet toasting experiment included two lupin toasting methods. The first was a process of
131 steam cooking and drying of the seeds, a method adopted by the lupin-processing food and feed
132 industry in Europe. The second was a high pressure treatment which involved autoclaving and
133 drying the seeds, as described by Heuzé et al. (2022) and Yu et al. (1999). Both whole lupins
134 and dehulled kernels were used in the toasting experiments, to obtain toasted whole lupin flour
135 and toasted dehulled lupin flour, respectively. As such, the impact of combining dehulling and
136 toasting on the quinolizidine alkaloid concentration of lupin derivatives was assessed. For the
137 steam cooking method, a 300 g portion of the cleaned lupin seeds was steamed in a household-
138 level steam cooker with two perforated layers and a lid (ASEB Convenient Series, VC145160,
139 China) at 100 °C for 40 minutes. While for the autoclavation, a 300 g portion of the cleaned
140 lupin seeds was processed for 4 min at 121°C in a laboratory scale autoclave (SANYO Labo
141 Autoclave MLS-242OU, Japan). The steamed and autoclaved seeds were allowed to cool, after
142 which they were dried in a hot air food dehydrator (KLARSTEIN, WEE. Nr: DE 46506833,
143 Germany) at 50 °C for 5.5 hours to reduce the water content to the initial water content of the
144 seeds. After cooling down, a 200 g portion of the dried seeds was milled into flour, in analogy
145 with the production of whole lupin flour (Section 2.3.1).

146 ### 2.3.4. Sterilization: sterilized jarred lupins

147 A modified version of the method described by Parmar et al. (2016) and the method provided
148 by a European-based company which produces canned lupins, formed the basis for the
149 production process of the sterilized jarred lupins. This involves three steps – hydration, cooking
150 and canning in a brine. A 1000 g portion of cleaned and sorted lupin beans was soaked in

151 distilled water in a 1:3 ratio (seed: water, w/v) at room temperature for 24 hours. The soaked
152 seeds were then drained, weighed and cooked at 100 °C for 30 min in distilled water in the ratio
153 of 1:3 (seed: water, w/v). The cooked seeds were drained and allowed to cool. The cooked lupin
154 seeds were canned in a brine solution of 2% NaCl in glass jars and sterilized in an autoclave at
155 121 °C for 20 min. The processed canned lupin was stored in a cool and dry room (21 °C) for
156 7 days and thereafter stored at -20°C prior to evaluation.

157 **2.3.5. Boiling (wet heating): lupin pasta**

158 The objective of this experiment was to determine the fate of QAs when cooking lupin pasta.
159 To produce the pasta, a fine durum semolina flour (Le Macinate, De Cecco) and the whole lupin
160 flour with particle size of 500 µm were used. Pasta was produced following the method
161 described by Jayasena & Nasar-Abbas (2012). Lupin flour and durum semolina were
162 thoroughly mixed in a ratio of 70:30 (durum semolina:lupin flour; w/w) in a bowl. This mixture
163 was poured in the pasta making machine (Lineapasta Equipment, Pasta Maker MPF 1,5N
164 Fimer, Italy), and distilled water at room temperature was added gradually while mixing. The
165 dough was mixed for approximately 10 min to form a non-sticky dough crumble in the pasta
166 machine before commencing the extrusion process. The mixed pasta dough was extruded in the
167 form of Tagliolini pasta through a 3.0 mm diameter die. The Tagliolini pasta strands were cut
168 into approximately 20 cm lengths, and were hanged on a wooden roller to dehydrate for 24
169 hours at 21 °C. Next, 100 g of the dried pasta was cooked in 1000 mL boiling distilled water
170 for 5 min. The cooked pasta was then drained through a cooking sieve and allowed to cool
171 before weighing and packaging. The weight of the pasta and the cooked water were measured
172 and recorded.

173 **2.3.6. Baking (dry heating): lupin cookies**

The cookies were produced according to AACC micro method (No. 10-52). A flour proportion of 30 % lupin flour with particle size of 500 µm and 70 % wheat flour was used. Fat (Solo, Belgium), sugar (Tiense Suikerraffinaderij, Belgium), non-iodized salt (Everyday, Belgium), and sodium bicarbonate (Cérébos, France) were mixed at low speed in a mixing machine (HOBART Planetary Mixer N-50, Germany) for 3 min. The sucrose solution and the distilled water were added and mixed further for 1 minute at low speed and 1 minute at moderate speed. The flour mixture was added and further mixed for 2 min at low speed and scraped every 30 seconds. The dough was removed from the kneading bowl and divided into 3 portions on a parchment paper. The dough pieces were flattened with the palm of the hand. With the help of slats of 6.2 mm thickness, the dough was rolled out at that thickness by one forward and one backward movement with a dough rolling pin. The cookies are cut to size with a round cookie cutter (with a diameter of 63.5 mm), remaining dough was packaged and stored in the freezer until analysis. After weighing the ready-to-bake dough pieces (average weight of 19.18 ± 0.81 g), all sixteen cookies were placed on one aluminum tray, lined with a non-stick silicon baking mat, placed in the middle of the oven (MIWE Aero Oven, Germany), and immediately baked for 9 min at 205°C. After 30 minutes of cooling, the cookies were packaged and stored in the freezer until analysis.

2.3.7. Frying (oil heating): lupin chips

Lupin chips were produced based on the method described by Adrianus (2016). 300 g of whole lupin flour (< 500 µm), 195 g egg white (Everyday, Belgium) and 2 g non-iodized salt (Everyday, Belgium) were mixed in a dough mixer and gently mixed well therein for 10 minutes. The dough was rolled out with a baking roller onto a baking slab and turned over at least twice. A pasta machine (Sailnovo, Italy) was used to roll out the dough to a thickness of about 0.4 mm. The dough was cut out in triangular shapes (6.5 x 6.5 x 6.5 cm) with an average weight of 2.61 g. The shaped dough was then fried in 2500 mL of vegetable oil (Solo, Belgium)

in a deep-frying bath (FRIFRI 5848 DUOFIL, Belgium) at a temperature of 170 °C for 1.5 minutes. Ten pieces of dough were fried each time in the bath and allowed to drain and cool. The experiment was done on two consecutive days with fresh vegetable oil.

2.4. Analysis of raw materials and produced lupin fractions

2.4.1. Crude protein content

The crude protein content of the whole lupin flour and dehulled lupin flour was determined using the Kjeldahl method according to AACC method 46-10. Analyses were performed in duplicate for each production replicate.

2.4.2. Dry matter content

Dry matter content was determined for all produced fractions, except liquids, with the ICC Standard Method No. 110/1.

2.5. Analysis of quinolizidine alkaloids in lupin seeds, high-fat matrices and other derived matrices

2.5.1. Chemicals and instrumentation

Solvents (acetonitrile, methanol and water, LC/MS grade) were purchased from Biosolve (Valkenswaard, Netherlands). Formic acid (98-100%) (FA), triphenyl phosphate (TPP) and ammonium formate (99.0%) were purchased from Merck (Darmstadt, Germany). Analytical standards (sparteine, lupanine, lupinine, 13-hydroxylupanine and angustifoline) (purity > 97%) were obtained from Sanbio B.V. (Uden, The Netherlands) and Merck (Darmstadt, Germany). Preliminary tests, validation and quantitative analyses were performed with an Acquity UPLC Binary Solvent system coupled to a XEVO TQ MS detector. Data acquisition and data treatment were done with MassLynx version 4.1 software and TargetLynx software, respectively (all from Waters, Millford, MA, USA).

2.5.2. UHPLC-MS/MS method for quantification of QAs in lupins and high-fat matrices

For the quantitative determination of five QAs (sparteine, lupanine, lupinine, 13-hydroxylupanine and angustifoline) in lupin seeds and high-fat matrices (cookies and chips), a UHPLC-MS/MS method was developed and validated. The LC- and extraction methods by Horna (2014) and Hwang et al. (2020) were tested and evaluated on the basis of analytes' extraction efficacies (recoveries) and selectivity.

Regarding the LC-method, QAs were separated with the UPLC HSS T3 analytical column (2.1 x 100 mm, 1.8 µm particle diameter) and pre-column (2.1 mm x 5 mm) (Waters). The A and B mobile phases were 0.1% FA in H_2O and 0.1% FA in MeOH, respectively (flow rate: 0.4 mL min^{-1}). The gradient elution program was: 0-0.5 min, 95% A; 0.5-4.0 min, 0% A; 4.0-4.5 min, 0% A; and 4.5-5.0 min, 95% A, in accordance with Hwang et al. (2020). The injection volume was 5 µL. Some example chromatograms are given in supplementary material S10.

For the optimization of the mass spectrometry method, a 1 ppm dilution of the different analytes in methanol was used to tune the compounds and set relevant MS parameters. Fragments (m/z) of precursor ions with highest sensitivity were selected in Multiple Reaction Monitoring (MRM) mode, and source parameters were adjusted to achieve optimal sensitivity (S11). Ionization happened in positive electrospray ionization (ESI+) mode.

For the extraction of QAs, to a 1.0 g test portion of the homogenized sample 4 mL of the extraction solvent (70% methanol/30% water) was added. The mixture was shaken for 15 min in a shaker (Hersteller: Collomix GMBH, 892888, Germany) and centrifuged at 1942 g for 15 min in a centrifuge (Sigma Centrifuge 6- 16, Germany). The supernatant was filtered with a 0.45 µm syringe filter, which was further 1:2.5 diluted in ultrapure water. To an amber vial 980 µL of the diluted sample extract and 20 µL triphenylphosphate (TPP), as an injection-internal

246 standard, was added, for injection into the UHPLC-MS/MS system. To assure that analytes are
247 within the concentration range of the calibration curve, sample extracts were further diluted
248 with diluted blank matrix extract.

249 Matrix-matched calibration curves were prepared in blank soy flour extract and the extract of
250 cookies prepared with soy flour (AACC micro method No. 10-52), which were spiked at 8
251 different concentration levels (5, 10, 20, 50, 75, 100, 200 and 400 $\mu g\ kg^{-1}$). Calibration functions
252 (quadratic, 1/x weighing) were computed by the MassLynx 4.1 software.

253 The LC-MS/MS method was validated for two matrices (soy flour and soy cookie), to meet the
254 criteria of SANTE/11494R1/2021 as guidance. Linearity was assessed from the calibration
255 curves, made as described above. The repeatability relative standard deviation (RSD_r) was used
256 to assess the repeatability or intraday precision. This was done by fortifying five replicate blank
257 samples at the LOQ concentrations, which were 50 $\mu g\ kg^{-1}$ for sparteine and angustifoline and
258 100 $\mu g\ kg^{-1}$ for lupanine, lupinine and 13-hydroxylupanine, on one day. The intermediate or
259 interday precision was evaluated with the reproducibility relative standard deviation (RSD_R).
260 This was derived from recovery experiments executed in triplicate on three days at a low and
261 high QA concentration, i.e. 500 $\mu g\ kg^{-1}$ and 10 $mg\ kg^{-1}$, respectively.

262 QA concentrations in liquid samples were quantified using standard addition.

263 **2.5.3. High Resolution Mass Spectrometry**

264 UHPLC-HRMS was performed on a Q Exactive mass spectrometer coupled to an Accela binary
265 UHPLC system, existing of a binary UHPLC pump, open autosampler and column oven
266 (Thermo Fisher Scientific, Waltham, MA, USA). The system was running Tracefinder 2
267 software and all data analysis was performed using Compound Discoverer 3 (all from Thermo
268 Fisher Scientific). The UHPLC method from the targeted method was used and the MS was run
269 in data-dependent MS/MS mode ($ddMS^2$), acquiring a full scan at 70 000 resolution, followed

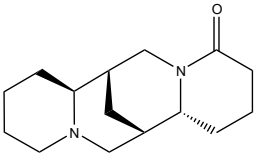
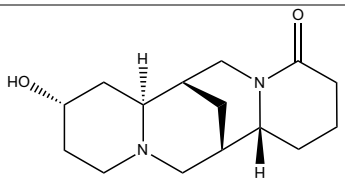
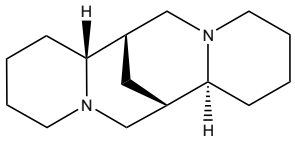
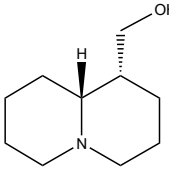
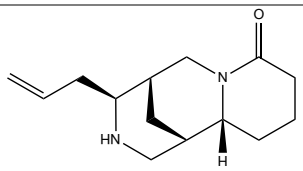
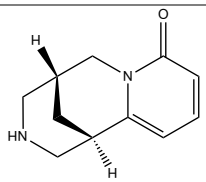
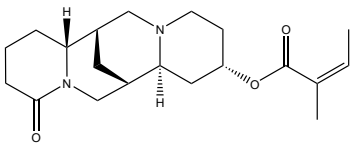
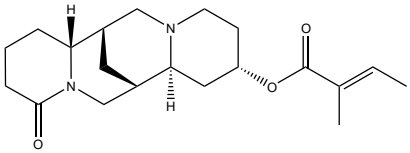
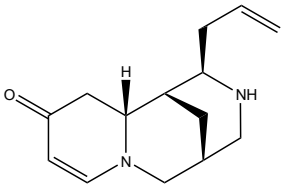
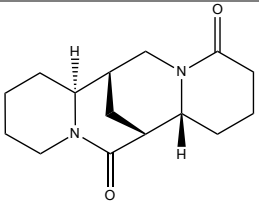
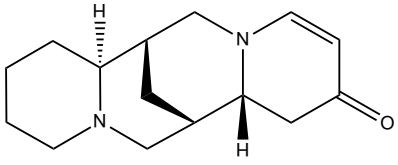
by five MS/MS scans of the most abundant MS peaks at 17 500 resolution, using a dynamic exclusion of 6 seconds. A list of possible quinolizidine alkaloids in white lupins was compiled from literature and these were searched for (supplementary file S12) . A compound was deemed identified when the MS spectrum showed the correct monoisotopic mass with an error below 2 ppm and at least two isotopes were detected at the expected intensity and when the MS/MS fragments showed more than four fragments expected from the in silico fragmentation prediction as calculated by Compound Discoverer. This does mean some isobaric quinolizidine alkaloids (for example multiflorine and 5,6-dehydrolupanine) cannot be distinguished, as the structures are very similar (the place of the double bond and carbonyl-function are shifted on the same ring) and the formula is the same ($C_{15}H_{22}N_2O$) and the same is true for some isomers, such as cis- and trans-13-tigloyloxylupanine.

2.6. Fate of Quinolizidine Alkaloids

Equation 1 was used to calculate the fate of quinolizidine alkaloids. For toasted dehulled lupin flour and the sterilized jarred lupins, the fate of QAs was calculated for both the sub-unit processes as well as the overall production process.

$$Fate = \frac{(final\ QA\ concentration - initial\ QA\ concentration)}{initial\ QA\ concentration} \times 100 \qquad \text{Equation 1}$$

Table 1 Physico-chemical properties of quinolizidine alkaloids included in the LC-MS/MS method and overview of QAs included in the LC-HRMS method.

Compound	Molecular formula	Structure	MW (g/mol)	Log P (est)	pKa
Quinolizidine alkaloids included in the LC-MS/MS method					
(+)-Lupanine	$C_{15}H_{24}N_2O$		248.370[a]	1.6[a]	9.4[b]
(+)-13α-hydroxylupanine	$C_{15}H_{24}N_2O_2$		264.369[a]	0.6[a]	8.8[b]
(-)-Sparteine	$C_{15}H_{26}N_2$		234.387[a]	2.5[a]	12[b]
(-)-Lupinine	$C_{10}H_{19}NO$		169.268[a]	1.2[a]	9.4[b]
(-)-Angustifoline	$C_{14}H_{22}N_2O$		234.343[a]	1.4[a]	10.3[b]
Quinolizidine alkaloids included in the HRMS method					
					
(-)-Cytisine		**13-Angeloyloxylupanine**		**13-Tigloyloxylupanine**	
					
(-)-Albine		**17-oxolupanine**		**Multiflorine**	

[a] EFSA (2019)

[b] Hama et al. (2022)

3. Results and Discussion

3.1. Method validation

Coefficients of determination (R^2) of above 0.996 were obtained for all analytes in both matrices, indicating good linearity. In all cases, $\%RSD_r$ and $\%RSD_R$ values were below 20%. In contrary to what was reported by Khedr et al. (2023), the extraction method (70/30 methanol/water) resulted in little adverse matrix effects and satisfactory matrix recoveries, with average recoveries within the range of 70% and 120%. An overview of average recoveries at three concentration levels is given in S13. The LOQ corresponds to the lowest analyte concentration that complies with the validation acceptance criteria and were set so that a minimum signal-noise ratio of 10 was obtained (SANTE/11494R1/2021). This corresponds to 50 µg kg^{-1} for sparteine and angustifoline and 100 µg kg^{-1} for lupanine, lupinine and 13-hydroxylupanine. A theoretical LOD was derived from this as the minimum analyte concentration with a S/N ratio of 3. For lupin seeds, the LOD ranged from 2 µg kg^{-1} for sparteine and angustifoline to 19 µg kg^{-1} for lupanine, for high-fat matrices this ranged from 1 µg kg^{-1} for angustifoline to 12 µg $kg^{-1}$for lupanine.

Interference of alkaloid isomers has been noted in literature, e.g. sparteine and angustifoline, at the measured transitions has been put forward (Khedr et al., 2023). However, good selectivity was obtained with stable retention times (2.2 min for sparteine and 2.3 min for angustifoline) resulting in base line separation. No interference was observed throughout the validation and analyses in our research.

3.2. Quinolizidine alkaloid concentration in the raw material *L. albus* seeds

A detailed overview of measured mean concentrations and standard deviations of quinolizidine alkaloids in the whole lupin seed flour and all produced fractions is given in S14. The results are corrected for the water content (g kg^{-1} dw). The whole lupin seed flour had a total QA

316 concentration, *i.e.* sum of five QAs in this study, of 1.43 ± 0.08 g kg^{-1} dw, with lupanine clearly
317 the most abundant alkaloid (Figure 1), accounting for 90%. The lowest reported QA was
318 lupinine (< LOD) and remained below the detection limit throughout the study. This result is
319 supported by the study of Boschin et al. (2008) and EFSA (2019) who reported lupanine as the
320 most abundant and detected QA found in white lupin seeds. The seeds, used as raw material,
321 were described as 'sweet' lupins by the breeder, but are clearly exceeding the generally
322 accepted 500 mg kg^{-1} limit. It has been established that alkaloid levels and patterns can vary
323 widely inside and between *Lupinus* species, e.g. due to abiotic environmental impacts, like the
324 geographical location, growth year and soil characteristics, or genetic modifications, such as
325 mutations or cross-breeding (Boschin et al., 2008; Wink, 2019).

326 Five additional quinolizidine alkaloids ((-)-cytisine, 13-angeloyl-oxylupanine or 13-tigloyloxy-
327 lupanine, 17-oxolupanine, albine and multiflorine) were identified in the raw material by LC-
328 HRMS. According to BfR (2017) and Blaschek et al. (2016), the most abundant QAs in *L.*
329 *albus* seeds are lupanine (55-75% of total alkaloids), albine (6-15%), multiflorine (3-14%), 13-
330 hydroxylupanine (4-12%) and 13-angeloyloxylupanine (1-3%). The HRMS analysis confirmed
331 the presence of albine, multiflorine and 13-angeloyloxylupanine, indicating that the current LC-
332 MS/MS method, is underestimating the total QA concentration.

333

3.3. The fate of QAs when dehulling lupin seeds

Table 2. Fate (%) of the five different QAs and total QA content (sum of five QAs) and the dry matter content, obtained for each produced fraction and food product in relation to the whole lupin seed flour (starting material) and/or dehulled lupin flour. Mean values for two production days (n = 2). ND: Not Detected

Produced fraction/ food	Unit process	Dry matter content	Sparteine	Lupanine	Lupinine	13-OH-lupanine	Angusti-foline	Total QAs
Lupin hulls	Dehulling	0.882 ± 0.003	- 90%	- 87%	ND	- 95%	- 96%	- 88%
Dehulled lupin flour	Dehulling: dehulled lupin flour	0.924 ± 0.004	+ 21%	+ 31%	ND	+ 14%	+ 17%	+ 30%
Toasted Whole Lupin Flour - Autoclaved	Toasting	0.926 ± 0.005	- 23%	- 11%	ND	+ 15%	- 46%	- 11%
Toasted Whole Lupin Flour - Steam Cooked	Toasting	0.929 ± 0.002	- 29%	- 14%	ND	+ 21%	- 35%	- 13%
Toasted Dehulled Lupin Flour - Autoclaved	Toasting	0.931 ± 0.004	- 25%	- 24%	ND	+ 12%	- 43%	- 23%
	Toasting and dehulling	0.931 ± 0.004	-25%	- 32%	ND	- 1%	- 51%	- 32%
Toasted Dehulled Lupin Flour – Steam Cooked	Toasting	0.927 ± 0.004	- 17%	- 23%	ND	+ 2%	- 35%	- 22%
	Toasting and dehulling	0.927 ± 0.004	- 16%	- 15%	ND	+ 14%	- 24%	- 14%
Soaked lupin seeds	Soaking	0.352 ± 0.000	+ 3%	- 12%	ND	+ 103%	+ 21%	- 5%
Cooked lupin seeds (n = 6)	Cooking	0.332 ± 0.014	- 36%	- 27%	ND	- 26%	- 40%	- 28%
Sterilized jarred lupins (n = 6)	Auto-clavation	0.268 ± 0.003	- 31%	- 44%	ND	- 62%	- 64%	- 46%
	Whole production process	0.268 ± 0.003	- 55%	- 64%	ND	- 43%	- 74%	- 63%
Baked cookie	Baking	0.949 ± 0.003	+ 10%	- 15%	ND	- 3%	- 39%	- 15%

Lupin chips	Frying	0.972 ± 0.001	- 1%	- 19%	ND	- 25%	- 39%	- 19%
Cooked pasta	Boiling	0.329 ± 0.009	- 40%	- 53%	ND	- 50%	- 37%	- 52%

Whole lupin seeds and dehulled kernels had a crude protein concentration of 33.5 ± 0.2 g/100 g dw and 38.7 ± 2.0 g/100 g dw, respectively. Even though the focus of this study is not on the nutritional composition, it was interesting to determine the differences in the protein content in the whole lupin flour and dehulled lupin flour, as a control parameter of the dehulling procedure's efficiency. A 15.4% increase in the protein content was obtained by dehulling the lupin seeds. Smulikowska et al. (1995) reported an increase in crude protein of about 20% when dehulling white lupin seeds, indicating that the efficiency of the dehulling procedure may be lower, i.e. more remaining hulls in the kernel fraction. As seen from Figure 1, a remarkable difference in the total QA content in the hulls (0.17 ± 0.03 g kg^{-1} dw) and the kernel (1.58 ± 0.31 g kg^{-1} dw) was measured, increasing and decreasing the QA content with 30% and 88% in the dehulled flour and seed coats, respectively. Hence, it can be deduced that QAs are more concentrated in the cotyledon than the seed coats. The large variability in the mean QA concentration in the dehulled lupin kernels may be due to the varying efficiency of the dehulling procedure over multiple production days. With the HRMS method only 13-angeloyl-oxylupanine or 13-tigloyloxy-lupanine, albine and multiflorine were detected in the lupin hulls, confirming the LC-MS/MS findings. These results provide evidence on the distribution of quinolizidine alkaloids in lupin seeds, with the hulls having a notably lower QA content. As such, the hulls of seeds with a high quinolizidine alkaloid content, could potentially still find an application in the food chain, e.g. as a fiber source in breakfast cereals (Sipsas et al., 2008). Clearly, while a physical unit process such as dehulling may produce a protein-enriched fraction, the QA content will be enriched simultaneously. A similar result was reported by

Wang et al. (2012), with a broad difference in the distribution and contents of QA between the seed coat (6.4 ± 0.2 mg g^{-1}) and cotyledons (37.9 ± 1.0 mg g^{-1}) of *Sophora alopecuroides* seeds.

3.4. The fate of QAs when toasting lupin seeds

The QA analysis results of the toasting experiments for the whole lupin flour and dehulled lupin flour are given in Figure 1 and Table 2. These results indicate that wet heat treatments, such as toasting, may reduce the QA content, in a range from -11% to -23%. As can be seen in Table 2 and Figure 2, the results indicate a larger decrease in the mean QA content, when toasting the dehulled lupins, both by autoclavation and steam cooking, in comparison to the whole lupin seeds. Presumably, the seed coat, which comprises about 15% of the seed weight, exerted a protective role here (Petterson, 2004). It was demonstrated by Goelema et al. (1998) that the transfer of heat and moisture during processing will be hindered by the presence of hulls. In terms of the toasted whole lupin flour, the steam cooking procedure gave rise to a highly variable change in QA concentration, in contrast to the autoclaved lupins. Indicating that high pressure toasting will result in a more reliable QA reduction. This is confirmed by the dehulled toasted lupin flour, for which autoclavation resulted in a larger decrease in the QA content. The variability in this case may be due to the variability inherent to the dehulling procedure. When zooming in on the individual quinolizidine alkaloids, an increase in 13-hydroxylupanine is observed for all of the toasting experiments. This may be due to the hydrolysis of the esterified quinolizidine alkaloids, e.g. 13-angeloyloxylupanine or 13-tigloyloxylupanine and cis- and trans-13α-cinnamoyloxylupanine, giving the respective organic acids and hydroxylupanines, in this case 13-hydroxylupanine as a transformation product (Table 1) (Aslanov et al., 1987). The HRMS data showed a decrease in the 13-angeloyloxylupanine and/or 13-tigloyloxylupanine concentration for all of the toasting experiments, as such supporting the hypothesis of hydrolysis of esterified quinolizidine alkaloids causing an increase of 13-hydroxylupanine. Toasted lupin flour may find applications in different specialty-baked items, like cakes and

386 waffles (van de Noort, 2017). While toasting of lupins is a value-added processing
387 methodology, that is claimed to improve the emulsifying properties of lupin flour, the impact
388 on the QA content of lupins seems to be limited.

389 **3.5. The fate of QAs during the production of sterilized lupin seeds**

390 An overall reduction of 63% in the total quinolizidine alkaloid content was obtained during the
391 sterilization production process (Table 2; Figure 2). The autoclaving unit process had the largest
392 effect on the total alkaloid concentration. The initial hydration step resulted in a minor change.
393 Notably, the concentration of all QAs except lupanine increased, with the 13-hydroxylupanine
394 concentration doubling after soaking the seeds. Here, the conversion of lupanine to 13-
395 hydroxylupanine in water seems possible. Erbas (2010) found that the concentration of α-
396 isolupanine increased at certain stages during the production process of a lupin snack, a similar
397 production process which did not include a sterilization step, and suggested isomer
398 transformations among the alkaloids. The cooking process decreased the QA concentration with
399 nearly 30%. However, this aqueous heat treatment gave rise to a large variability in the QA
400 concentrations over the different batches (RSD% = 18%; n = 6). It is known that boiling lupin
401 seeds will disrupt the cell walls, as such facilitate the alkaloids removal (Carvajal-Larenas et
402 al., 2013; Gross et al., 1983). The seeds were then heat treated in a lab-scale autoclave,
403 simulating the industrial sterilization process. The QA content further declined with 46%, yet
404 again with a large variability in the overall reduction (RSD% = 24%; n = 6). A mean QA
405 concentration of 0.24 ± 0.07 g L^{-1} was measured in the drained brine solution. Estivi et al. (2022)
406 evaluated different aqueous debittering procedures, and found that a 1% NaCl solution
407 improved the alkaloid removal of lupin seeds during the aqueous debittering process, whilst
408 reducing the water consumption and processing time, in comparison to distilled water. Clearly,
409 the application of a saline solution in the jars had a positive debittering effect. Sodium chloride
410 increases the porous microstructure of legume grains, and as such improves the water

411 penetration and alkaloid leakage from the seeds to the brine solution (Sievwright & Shipe,
412 1986). The osmotic effect of NaCl may also accommodate the counter-current mass transport
413 from seed tissues to the brine solution, by assisting the disruption of phospholipid membranes
414 and cellular compartments (Hameed et al., 2021). Furthermore, the conservation of the lupin
415 seeds in this 2% NaCl brine solution could lead to a prolongation of debittering. The effect of
416 storage duration and water to seeds ratio on the alkaloid removal requires further investigation.
417 The hydrophilic character of lupin alkaloids explains the leaching into the boiling water and
418 brine solution, illustrated by log P values ranging between 0.6 and 2.5 (Table 1). These results
419 confirm the effectivity of the classic aqueous debittering procedure of lupin seeds. The EFSA
420 CONTAM panel concluded that an aqueous debittering process is the only food-grade
421 debittering process currently applied on a commercial scale, and that it is the only debittering
422 approach that can be applied at the household level (EFSA, 2019). In an overview paper
423 Carvajal-Larenas et al. (2016) compared multiple debittering approaches, including biological
424 processes, like microbial and fungal fermentation, and chemical and aqueous extractions. An
425 aqueous alkaloid extraction can remove up to 97% of the initial alkaloid content. However,
426 these water treatments are characterized by long processing times, about five to seven days, and
427 frequent water replenishment, leading to the consumption of large volumes of water (Carvajal-
428 Larenas et al. 2016). Rather than optimizing a debittering procedure for lupin seeds (Villacrés
429 et al., 2020), the objective of the current study was to quantify the impact of typical unit
430 processes on the QA content of lupin seeds, already in place in the food industry. It is clear that
431 the production of sterilized lupin seeds cannot assure a complete removal of the initial QA
432 content and that it doesn't have the same reducing potential as an aqueous debittering
433 procedure. Still, when the QA content of the raw material exceeds the 200 mg kg^{-1} dw limit
434 (ANZFA, 2001; Pilegaard & Gry, 2008), it may be a more energy and resource efficient

approach to decrease the alkaloid content of lupin seeds to commercial values, taking into account the variability introduced by the different unit processes.

3.6. The fate of QAs when processing complex multi ingredient matrices (e.g. lupin cookies)

Cookies, chips and pasta, were selected as model products for complex, multi-ingredient food matrices, relevant for lupins. The lab-scale production simulated commercial operative conditions and household cooking practices. As shown in Table 2 and Figure 3, the initial QA concentration in the cookie dough fortified with lupin flour was 0.27 ± 0.01 g kg^{-1} dw. The dry heat treatment reduced the total QA concentration with 15%. The largest reduction was observed for angustifoline with 38%, while the concentration of sparteine remained unaffected.

Initially a QA concentration of 1.19 ± 0.00 g kg^{-1} dw was obtained for the lupin chips dough. The lupin chips production, which assessed the impact of frying, resulted in an overall reduction of 19% in the total QA level. In analogy with lupin cookies, angustifoline was the most heat-labile compound, with a reduction of 39%, and sparteine the most heat stabile quinolizidine alkaloid. (-)-Sparteine is generally considered the most toxic quinolizidine alkaloid in lupins (ANZFA, 2001; EFSA, 2019). Deep-fat frying and baking are similar unit operations, in a way that they involve the transfer of heat into the food, and mass transfer of moisture from the food, and in the case of frying oil into the surface of the food. Only the heating media, namely oil and hot air, differ (Fellows, 2017).

The measured QA concentration in the lupin pasta dough was 0.32 ± 0.01 g kg^{-1} dw. The boiling process of the lupin pasta depleted the total QA concentration with 52%. Different components of the mass balance as measured and calculated for the pasta boiling experiment over two trial days are given in Table 3. Negligible differences between the amount of QAs going in and out can be seen for the two production days. It can be concluded that the loss of quinolizidine

alkaloids in the lupin pasta can be attributed entirely to the leaching of components into the pasta water, rather than an heat-induced transformation or matrix binding effect of compounds.

All QAs included in the HRMS analysis were removed during the pasta cooking experiment and sterilized lupins production process. Again, verifying the effectivity of aqueous extractions for QA removal. In contrast to baking and frying, which didn't generate any conclusive outcomes.

Table 3 Measured total QA concentrations (g kg^{-1} wet weight) in the lupin pasta before and after boiling, and in the pasta water. Absolute masses as used in the mass balance calculations for the lupin pasta.

	Concentration QAs (g kg^{-1} ww)		**Mass: pasta (g)**		**Mass: water (g)**		**Mass: QAs in pasta (mg)**		**Mass: QAs in boiling water (mg)**	
	Day 1	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**	**Day 1**	**Day 2**
Before boiling	0.28	0.28	100	100	1000	1000	28	28	0	0
After boiling	0.05	0.05	264	248	636	652	12	14	16	14

Similar (limited) changes in the total QA concentration were observed for both (dry) heat treatments, i.e. baking and frying, indicating that the depletion of QAs in these cases are due to a thermal degradation or heat-induced transformation of QAs, also providing evidence on the thermal stability of QAs. Clearly, numerous processing factors, including initial QA concentrations, product formulations and time/temperature profiles, may have influenced the QA reductions. Further research is recommended to investigate these time-temperature relations and the opportunities for thermal unit operations to produce commercially safe lupin-based foods. For example, lupins are being used as a raw material for the production of coffee substitutes, for which the beans are subjected to a roasting procedure to mimic the roasted coffee aroma. The extent of QA reductions when roasting lupins for a longer time period remains

unclear. Mostafa et al. (2021) suggest the soaking of bitter lupin seeds prior to roasting to assure a larger safety margin. Other alkaloids, e.g. caffeine, have been demonstrated to be stabile upon roasting (Ludwig et al., 2014).

With regard to the boiling of pasta, the QA removal may very well be modified by processing factors in the preparation of the dry pasta, e.g. variation in the pasta shapes, recipe changes and drying time/temperature or the at-home preparation of pasta, e.g. the water to pasta ratio and boiling time (Cano-Sancho et al., 2013).

In the presented study, only a limited number of unit processes and complex food matrices relevant for lupin seeds were included. For instance the production of lupin-based meat alternatives would have been an interesting addition (RIVM, 2015).

It should be emphasized that it is not advised here that consumers should be responsible for reducing QAs to safe levels by carrying out certain cooking practices. It has been established by the BfR (2017) that the limited cases of quinolizidine alkaloids intoxication due to the consumption of lupins, were caused by an insufficient debittering of lupin seeds by consumers.

4. Conclusion

This lab-scale study provides both qualitative and quantitative evidence on the fate of quinolizidine alkaloids during the processing of lupins and lupin-containing foods. The QA concentration, even in the so-called 'sweet' lupins, is subject to variability and may exceed the 200 mg kg^{-1} dw limit (ANZFA, 2001; Pilegaard & Gry, 2008). Hence, this study aimed to answer the question whether processing conditions, as currently applied in the food industry, are reliable to obtain safe QA levels in foods.

Toasting and dehulling lupin seeds had limited effect on the quinolizidine alkaloid concentration. However, the latter demonstrated that quinolizidine alkaloids are primarily located in the cotyledons of lupins seeds rather than the seed coats. The production process of

503 sterilized lupin beans depleted the QA content by over 60%, which is a more energy- and
504 resource efficient methodology than the aqueous debittering process. Furthermore, the boiling
505 of pasta confirmed that QA loss can be attributed entirely to the leaching of compounds into
506 the boiling water, rather than to a heat-induced degradation. Other heat treatments, including
507 baking (dry) and frying (oil), had limited effect on the alkaloid concentration, indicating that
508 QAs are heat resistant molecules. It can be concluded that the various unit processes, typically
509 applied in the food industry, can impact the depletion of quinolizidine alkaloids. It is
510 recommended to include this aspect in the process of product development in the search for
511 innovative, plant-based protein rich foods. Likewise, these results are relevant for risk managers
512 in case the consumption of these lupin-based foods increases, as it will be important to set limits
513 to protect consumers from too high exposures to quinolizidine alkaloids.

514

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518

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667

668 Figure Captions

669 Figure 1

670 Evolution of the mean concentration (g kg^{-1} dw) of five quinolizidine alkaloids (QA) starting
671 from whole lupin seeds into different fractions obtained when toasting and/or dehulling lupin
672 seeds. QA concentration in whole lupin seed flour is used as a proxy for QA concentration in
673 the raw material. AC: autoclaved and ST: steam cooked.

674 Figure 2

675 Evolution of the mean concentration (g kg^{-1} dw) of five quinolizidine alkaloids (QA) starting
676 from whole lupin seeds into different fractions obtained when producing sterilized jarred lupins.
677 QA concentration in whole lupin seed flour is used as proxy for QA concentration in the raw
678 material.

679 Figure 3

680 Evolution of the mean concentration (g kg^{-1} dw) of five quinolizidine alkaloids when processing
681 complex multi-ingredient matrices, including cookies, lupin chips and lupin pasta.

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