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Research Article

Evaluation of total quinolizidine alkaloids content in lupin flours, lupin-based ingredients, and foods

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Lupin proteins are gaining attention to replace animal proteins and other plants ingredients in several foods such as bakery products, imitation dairy and meat products, and beverages. One of the major safety issues of lupin-based foods is the presence of quinolizidine alkaloids (QAs), bitter compounds produced by lupin plants as a defense mechanism against predators. In mammals, QA intoxication is characterized by trembling, shaking, excitation, and convulsion. Lupanine and sparteine, the most common QAs, show acute oral toxicity due to neurological effects leading to the loss of motor co-ordination and muscular control. In this paper, 27 samples of lupin-based products, *i.e.*, flours, protein isolates, and food (either model or commercially available ones), were analyzed for evaluating the QA content using a method based on GC/MS. All the analyzed samples were safe since they respect the maximum limit of 200 mg/kg fixed by the Health Authorities of Australia, New Zealand, Great Britain, and France, that have regulated this topic. The QA contents were particularly low in protein isolates and in foods containing these ingredients, indicating that their use is a very effective tool for keeping low the daily intake of QAs.

Keywords: GC-MS analysis / Lupanine / *Lupinus albus* / *Lupinus angustifolius* / Quinolizidine alkaloids

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1 Introduction

In recent years, there has been a growing interest in innovative proteins such as plant proteins to broaden the range and variety of foods. Lupin seed appears particularly promising as a source of innovative ingredients having, on an average, a protein content similar to soybean (34–43% of dry matter) and an acceptable composition in essential amino acids [1]. Furthermore, lupin protein concentrates and isolates exhibit useful techno-functional properties [2, 3], allowing their use as ingredients in the production of several palatable food products, such as biscuits, pasta, and beverage [4–6]. Recent literature has indicated, in addition, some possible health benefits related to lupin protein consumption. The substitution in the diet of lupin protein for casein led to a reduction of total and low-density lipoprotein cholesterol levels in a rat [7] and in a pig model of hypercholesterolemia [8]. A pilot clinical trial based on a lupin beverage indicated that lupin protein may be effective in lowering cholesterol, blood pressure, glycaemia, and some inflammatory markers [9]. Otherwise, lupin proteins may give crossallergy with peanut proteins and for this reason the European Commission has recently decided to include lupin in the list of food allergens whose declaration on the food label is mandatory (http://eur-lex.europa.eu/LexUriServ/site/en/oj/2006/l_368/l_36820061223en01100111.pdf).

The quinolizidine alkaloids (QAs) represent an important safety issue of lupin products. They are metabolites synthesized by lupin plants and other species of the Genisteae family as a defense mechanism against insects and herbivores [10]. In mammals, QA intoxication is characterized by trembling, shaking, excitation, and convulsion [11]. The majority of acute toxicity studies were performed on lupanine and sparteine, the most common QAs: both showed moderate acute oral toxicity due to neurological effects leading to the loss of motor co-ordination and muscular control [12]. Anagyrine and ammodendrine are also teratogenic at high concentrations [13]; the latter is actually a piperidine alkaloid, but it is generally analyzed and quantified together with QAs.

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Abbreviations: KI, Kovats Index; **LPI,** lupin protein isolate from *L. albus*; **LUPI,** lupin protein isolate from *L. angustifolius*; **QA,** quinolizidine alkaloids

Table 1. Main analytical features of the alkaloids detected in flours, lupin-based ingredients, and foods

No.	Alkaloid	T_r (min)	Lit. KI[a)]	Exp. KI[b)]	EI-MS
1	Sparteine	12.45	1785	1746	234 (M^+, 20), 193 (42), 137 (75), 98 (100), 84 (26)
2	Albine	14.95	1900	1887	232 (M^+, 18), 191 (100), 149 (40), 122 (25), 110 (62)
3	Angustifoline	18.89	2083	2111	234 (M^+, 1), 193 (100), 150 (27), 112 (77), 94 (12), 55 (45)
4	α-Isolupanine	19.10	2105	2123	248 (M^+, 50), 219 (5), 150 (50), 136 (100), 98 (30)
5	Lupanine	19.92	2165	2169	248 (M^+, 56), 219 (12), 150 (42), 149 (60), 136 (100)
6	Multiflorine	22.02	2310	2288	246 (M^+, 65), 217 (5), 148 (20), 134 (100), 110 (15)
7	13α-Hydroxylupanine	23.97	2400	2399	264 (M^+, 34), 246 (54), 165 (45), 152 (100), 134 (35)
8	13α-Angeloyloxylupanine	29.17	2733	2693	346 (M^+, 1), 246 (100), 148 (15), 134 (32), 112 (18), 55 (55)
9	13α-Tigloyloxylupanine	30.11	2753	2747	346 (M^+, 1), 246 (100), 148 (18), 134 (35), 112 (20), 55 (42)

a) Lit. KI: KI from literature [25].
b) Exp. KI: experimental KI (see Section 2.4).

Literature on human acute toxicity is restricted to a few poisoning cases [12, 14, 15]: general symptoms attributed to the ingestion of high levels of alkaloids are reported to include malaise, nausea, respiratory arrest, progressive weakness, and coma. There are, however, indications that QAs may have some favorable activities on insulin secretion and suggestions that they could be used in the treatment of type 2 diabetes [16].

The presence of QAs was a big concern in ancient ecotypes whose seeds had to be soaked in water for some days to become edible, whereas in the last decades sweet varieties containing reduced amounts of alkaloids have been obtained by breeding [12].

The Health Authorities of some countries (Great Britain [17], France [18], Australia, and New Zealand [12]) have decided to regulate QA content in lupin flours and foods, fixing a maximum limit of 200 mg/kg.

Considering the increasing interest of consumers for lupin foods in Europe and in other countries, the aim of this work is to assess the content of QAs of lupin ingredients and foods for human consumption. In part this was possible, thanks to the availability of numerous lupin ingredients and model foods produced within two projects (Healthy Profood and Bioprofibre) financed by the European Commission. The QAs were quantified by a method based on GC/MS (Table 1) using lupanine, the most common QA, as external standard.

2 Materials and methods

2.1 Materials

Hexane and dichloromethane were purchased from Baker (Deventer, The Netherlands), 36% hydrochloridic acid from Merck (Darmstadt, Germany), 25% ammonia from Carlo Erba (Rodano, Italy). *n*-Alkanes for the determination of the Kovats indices (KIs) were bought from Sigma–Aldrich (St. Louis, MO, USA): pentadecane (purity 99%), octadecane (99%), docosane (99%), tetracosane (99%), triacontane (99%), and hexatriacontane (98%). Sparteine (purity >99.5%) was purchased from Fluka (Sigma–Aldrich); a sample of lupanine was provided by Professor F. Sparatore (University of Genova, Italy) and a sample of 13-hydroxylupanine by Dr. H. Reinhard (Swiss Federal Office of Public Health, Bern, Switzerland).

2.2 Sampling

Different kinds of ingredients and foods were analyzed for a total of 24 samples; they were divided in three groups: protein isolates (food ingredients), model foods, and commercial foods. The lupin protein isolates (Table 2) had been prepared in a pilot plant from the seeds of *Lupinus albus* cv Arés or cv Typtop and of *L. angustifolius* cv Boregine by the Fraunhofer Institut für Verfahrenstechnik und Verpackung (Freising, Germany) within the EU projects Healthy Profood and Bioprofibre. The preparation of lupin protein isolate from *L. albus* (LPI), LPI-E, LPI-F, and lupin protein isolate from *L. angustifolius* (LUPI), LUPI-E, have been already described elsewhere [3], whereas the procedure for the preparation of LPI-M is still confidential. Table 2 also reports a comparison of the total QA content in the starting seeds.

The model foods (Table 3) were prepared within the EU project Healthy Profood. Some of them were produced by Terrena LUP'INGREDIENTS (Matrigné-Ferchaud, France) using the flour of *L. albus* cv Arés. They were three snacks,

Table 2. Total alkaloid content (expressed in mg/kg product) of lupin seeds and related lupin protein isolates (LPI and LUPI)

Lupin seeds and lupin protein isolates	Total alkaloids[a] (mg/kg product)
L. albus cv Arés	
Seed[b]	146 ± 9
LPI-E	9.0 ± 0.04
LPI-F	37.0 ± 0.5
L. albus cv Typtop	
Seed[b]	1247 ± 44
LPI-E	21.4 ± 0.65
LPI-F	66.5 ± 5.2
LPI-M	8.45 ± 0.63
L. angustifolius cv Boregine	
Seed[b]	1107 ± 102
LUPI-E	34.1 ± 3.05

a) Mean ± SD.
b) Estimated total alkaloids (see Section 2.5).

named A, B, and C [4], two biscuits, named A and B [4], and a lupin beverage [6]. Other samples were produced by using lupin protein isolates: biscuits C and D were manufactured by Fraunhofer IVV [4] by using LPI-E from cv Arés, whereas the spaghetti samples A and B were produced by the University of Thessaloniki by using LPI-E from cv Typtop [5].

The commercial samples (Table 4) were five lupin imitation meat products (entries 1 – 5), provided by Dominae Trading Srl. (Origgio, Varese, Italy), one sample of lupin pasta (labeled as "with lupin concentrate", entry 6), one sample of lupin rusk (labeled as "with lupin flour," entry 7), and a sample of "lupini beans", pickled lupin seeds (entry 8). The samples 6 – 8 were purchased from a local supermarket.

2.3 Experimental procedure

Each item was independently analyzed at least four times. Lupin seeds were dehulled by hand, ground in a house-hold mill (Braun, Germany), sieved through a 60 mesh screen, and then defatted with hexane (300 mL) for 6 h in a Soxhlet apparatus using cellulose extraction thimbles (123 mm × 45 mm ed; 43 mm id; Whatman International, Brentford, UK). Lupin protein isolates were directly analyzed without any previous treatment. All other samples were ground, lyophilized, and then defatted by suspending in hexane at room temperature overnight under stirring.

Each dried sample (1 g) was suspended in 8 mL of 0.1 N HCl and stirred at room temperature for 17 h; the mixture was centrifuged at 10 000 rpm for 50 min at 4°C, the supernatant was collected and the solids were washed twice with 5 mL of 0.1 N HCl. The gathered extracts were alkalinized with 5% NH_4OH to pH 10 – 11 and then applied to an Extrelut NT 20 column (Merck). After 20 min, the alkaloids were eluted with CH_2Cl_2 (4 × 20 mL) and the solvent was evaporated to dryness under vacuum. The residue was then diluted in an appropriate volume of dichloromethane and analyzed by GC-MS. As an example, the total ion chromatogram (TIC) of the lupin beverage is shown in Fig. 1.

2.4 GC-MS analyses

The analyses were performed on a Shimadzu QP-5000 GC/MS instrument, equipped with an autosampler (AOC20i, Shimadzu) and a capillary column AT™ – 1 ms (30 m × 0.25 mm id, 0.25 µm film, Supelco, Italy). The temperature program was: 150°C for 5 min, from 150 to 300°C at 5°C/min, and 300°C for 15 min. Analyses were performed in split mode (split ratio 1:25), the injection volume was 1 µL, the injection temperature 250°C, the interface temperature 300°C, and the acquisition scan ranged from 50 to 450 *m/z*. The source operated in EI mode at 70 eV. The analyses were performed in full-scan mode.

The KIs (Table 1) were determined by injection of a mixture of linear alkanes: pentadecane (C15), octadecane (C18), docosane (C22), tetracosane (C24), triacontane (C30), and hexatriacontane (C36) according to ref. [19].

Table 3. Total alkaloids (expressed in mg/kg food) of model foods containing lupin ingredients

Entry	Model food	Lupin ingredient	Lupin protein content (%)	Total alkaloids[a] (mg/kg food)
1	Lupin beverage[b]	8.8% lupin flour cv Arés	3.6	20.7 ± 1.4
2	Snack A[b]	40% lupin flour cv Arés	16.4	83.2 ± 0.9
3	Snack B[b]	40% lupin flour cv Arés	16.4	86.5 ± 1.1
4	Snack C[b]	40% lupin flour cv Arés	16.4	78.8 ± 0.9
5	Biscuit A[b]	7.5% lupin flour cv Arés	3.1	50.4 ± 0.9
6	Biscuit B[b]	15% lupin flour cv Arés	6.2	83.9 ± 1.8
7	Biscuit C	6.75% LPI-E cv Arés	6.1	<LOQ
8	Biscuit D	13.5% LPI-E cv Arés	12.1	3.12 ± 0.03
9	Spaghetti A	5% LPI-E cv Typtop	4.5	<LOQ
10	Spaghetti B	10% LPI-E cv Typtop	9	<LOQ

a) Mean ± SD.
b) Estimated total alkaloids (see Section 2.5).

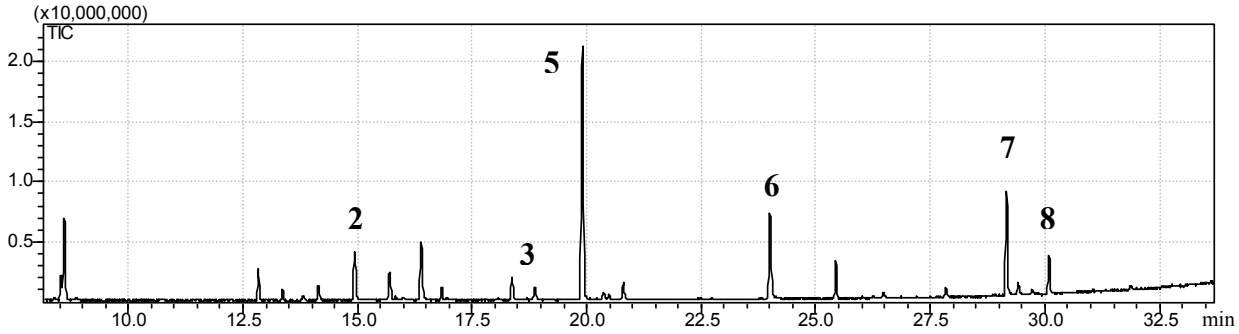

Figure 1. Total ion chromatogram of a GC-MS analysis in full-scan mode of a lupin beverage; 2 = albine; 3 = angustifoline; 5 = lupanine; 6 = 13α-hydroxylupanine; 7 = 13α-angeloyloxylupanine; 8 = 13α-tigloyloxylupanine.

2.5 Quantification

The alkaloid quantification was performed in full-scan mode by the external standard method, preparing two calibration curves for lupanine: the former in the range 100 – 900 ppm was used for the seeds and the latter in the range 2 – 100 ppm was used for ingredients and foods. Five solutions of lupanine at different concentrations were analyzed for each curve; to each solution a known amount of sparteine was added in order to check the response of the instrument. Over both ranges, linear relationships between peak area and concentration were observed: the regression coefficients (R^2) were always >0.99.

In standard solution, the LOD and LOQ values (S/N > 3) of sparteine were 1 and 3 ppm, respectively; whereas those of lupanine were 2 and 3 ppm, respectively. In the lupin flours, the LOD and LOQ values of lupanine were 1 and 2 mg/kg, respectively.

Since lupanine was the only standard in our hands in sufficient amount and purity, the single QA content was estimated by using lupanine calibration curves adjusted for the molecular weight of each QA. For this reason, the results were reported as "estimated total alkaloids" when beside lupanine, other QAs were detected (Tables 2 – 4). The same procedure was used by most published papers [20 – 24].

The recovery was estimated by spiking *L. albus* flour (cv Arés) with known amounts of sparteine and was always >92%. The precision of the method was evaluated by analyzing sparteine solutions within the same day (intraday with $n = 10$) and in different days (interday with $n = 5$), obtaining RSD% of 2 – 3 and 5 – 6%, respectively. Each sample was analyzed at least four times.

3 Results

3.1 Analytical method

The main steps in the preparation of analytical samples were: (i) defatting with hexane, since the presence of fat causes an increase in noise and a less resolution of peaks in GC-MS analyses (data not shown); (ii) extraction with aqueous HCl; (iii) basification with aqueous NH_3; and (iv) purification on Extrelut columns. The use of Extrelut columns was necessary, since the separatory funnel procedure suggested by other Authors [20] in our hands gave persistent emulsions both with dichloromethane and ethyl acetate, preventing an efficient alkaloid extraction.

The QAs were identified by comparison of the experimental KIs and mass spectral data (Table 1) with those reported in the literature [25]. Lupanine and 13-hydroxylupanine were confirmed also by spiking the samples with authentic standards. In total, the QAs detected in our samples were eight: albine, angustifoline, α-isolupanine, lupanine, multiflorine, 13α-hydroxylupanine, 13α-angeloyloxylupanine, and 13α-tigloyloxylupanine (reported here in the elution order). Their spectral data are listed in Table 1.

Table 4. Total alkaloids (expressed in mg/kg food) of commercially available lupin-based foods; lupin content and cultivar not available

Entry	Food	Total alkaloids[a] (mg/kg food)
1	Lupin tofu[b]	24.7 ± 0.8
2	Lupin steak[b]	55.9 ± 1.3
3	Lupin morsels[b]	56.3 ± 2.0
4	Lupin cutlet[b]	36.6 ± 1.4
5	Lupin steak with mushrooms[b]	14.7 ± 1.5
6	Lupin pasta (with lupin concentrate)	2.7 ± 0.1
7	Lupin rusk (with lupin flour)	4.2 ± 0.1
8	"Lupini beans"	<LOQ

a) Mean ± SD.
b) Estimated total alkaloids (see Section 2.5).

3.2 Quantification

Table 2 reports the estimated total QA content in three seed samples, two from *L. albus* and one from *L. angustifolius*, and the corresponding protein isolates.

Five alkaloids were detected in *L. albus* cv Arés (albine (16 mg/kg), lupanine (63 mg/kg), 13α-hydroxylupanine

(21 mg/kg), 13α-angeloyloxylupanine (33 mg/kg), and 13α-tigloyloxylupanine (13 mg/kg)) and in *L. albus* cv Typtop (albine (75 mg/kg), lupanine (959 mg/kg), multiflorine (32 mg/kg), 13α-hydroxylupanine (134 mg/kg), and 13α-angeloyloxylupanine (47 mg/kg)). Four alkaloids were detected in *L. angustifolius* cv Boregine (angustifoline (257 mg/kg), 13α-isolupanine (130 mg/kg), lupanine (552 mg/kg), and 13α-hydroxylupanine (168 mg/kg)). Among the teratogenic alkaloids, ammodendrine was detected only in traces, whereas anagyrine was never detected.

In all protein isolates only lupanine was detected. All these food ingredients respect the limit of 200 mg/kg, since lupanine concentration ranged from 8 to 66 mg/kg product, depending on the manufacturing process and the starting cultivar. The applied processes appear to be very efficient in removing the QAs, considering that the protein isolates LPI-E and LPI-F from the cv Typtop contain only 2.4- and 1.8-folds more lupanine, respectively, than the corresponding protein isolates from the cv Arés, although the former seeds contained about 8.5-folds more alkaloids than the latter. The lowest concentration of QAs was detected in LPI-M from cv Typtop, which indicates that the procedure applied for its preparation (still confidential) is particularly efficient in removing QAs.

The results of model foods are reported in Table 3. Some of them (entries 1 – 6) were prepared with the flour from *L. albus* cv Arés and, as expected, all the alkaloids quantified in the flour were detected also in these model foods. All these samples respect the limit of 200 mg/kg, ranging from 20 to 86 mg/kg food. Comparing the snacks with the biscuits A and B, it appears that the QA levels do not seem to be directly proportional to the lupin flour percentage, suggesting that the extrusion process applied for the snack preparation might have partly destroyed these metabolites. Biscuits C and D and the spaghetti samples were prepared using the lupin protein isolates. Only lupanine was detected in these samples and it was quantifiable only when the percentage of the protein isolate in foods was bigger than 13%.

Table 4 reports, instead, the results of some commercial products. The GC-MS analyses of the five imitation meat products (entries 1 – 5) showed four QAs, albine, 13α-hydroxylupanine, lupanine, and 13α-angeloyloxylupanine, the total QA content ranging from 14 to 56 mg/kg. Lupin pasta and rusk (entries 6 and 7) contained only lupanine at a very low concentration. No quantifiable QAs were detected in the "lupini beans".

4 Discussion

In the last decades, the diffusion of sweet varieties of lupin has enlarged enormously the possibility to include lupin ingredients in the formulation of complex food products, from bakery products to imitation dairy and meat products. The consequence could be a growing risk of human exposure to QAs. For this reason the Health Authorities of several countries have decided to fix the limit of 200 mg/kg for the QAs in lupin flours and food products [12, 17, 18].

All the food products investigated in this paper completely fulfill this legislation, although their QA contents vary in a very large range. In particular, the results of the analysis of the model foods of Table 3 indicate that there is a big difference between foods containing lupin flour (entries 1 – 6) and those containing lupin protein isolates (entries 7 – 10). This has relevant consequences on the possible daily exposure to alkaloids: for example, the intake of QAs derived from the consumption of 100 g of biscuit D is only 0.3 mg, whereas that derived from the consumption of 100 g of biscuit B is 8.4 mg. This is even more impressive considering that in the former case 100 g of biscuits correspond to 12.1 g of pure lupin protein, the nutraceutical component [7 – 9], whereas in the latter case only to 6.2 g.

Data reported in Table 2 permit to assess whether and how much the processes for preparing lupin protein isolates are efficient in reducing QA concentration. All the protein isolates are safe even when they were produced from the seeds containing up to 1100 – 1200 mg/kg product of QAs. This permits to affirm that a correct technological process permits to reduce significantly the QA concentration. A similar observation was made also by El-Adawy *et al.* [26] in 2001. They prepared two lupin protein isolates in laboratory starting either from sweet or bitter lupin seeds, obtaining in both cases, isolates containing negligible amounts of QAs [26].

As for commercial lupin-based foods, the only literature data are those of Reinhard *et al.* [27], regarding several products available on the Swiss market: they are in good agreement with ours.

In the Mediterranean countries, there is an ancient tradition of eating lupin seeds as a snack ("lupini beans"). In the past, they used to be seeds of bitter cultivars, which were cooked and washed in water for several days, in order to improve the palatability and to eliminate the alkaloids. Recent literature reports two cases of anticholinergic toxicity associated with the ingestion of "lupini beans" [14, 15], but both were related to inappropriate household behaviors. In the former, the patient consumed some raw beans; in the latter, in a domestic preparation, the patient tried to shorten the cooking time of the raw beans in order to maintain their "pleasant" bitter taste [15]. In contrast to these facts, our data showed that commercial "lupini beans" are completely safe (Table 4).

In conclusion, considering the increasing consumption of lupin products by vegetarians and by subjects interested to their nutraceutical properties, it appears justified that some Health Authorities have decided to fix a maximum limit of 200 mg/kg for QAs in lupin flours and foods.

This paper confirms that commercial and model foods fulfill this limit. This safety issue can be managed properly

by a careful selection of lupin varieties with low QA content and by the use of an appropriate technology for manufacturing the protein isolates, being these ingredients a very potent tool for assuring the safety of lupin products.

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