

# QUANTIFICATION OF QUINOLIZIDINE ALKALOIDS IN LUPIN SEEDS, LUPIN-BASED INGREDIENTS AND FOODS

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## ABSTRACT

Since lupin proteins are more and more used to replace animal proteins and other plants ingredients in foods such as bakery products, imitation dairy and meat products, and beverages, there is an increasing risk of an undesired exposition to quinolizidine alkaloids, bitter compounds produced by lupin plants as a defence against predators that have shown to possess acute oral toxicity mainly related to neurological effects. In order to estimate the current exposition of the EU population, quinolizidine alkaloids were quantified in 3 seed samples, 3 protein isolates and 18 foods, either models or commercially available. Our data indicate that all food samples respect the maximum limit of 200 mg/kg fixed by the Health Authorities of Australia, New Zealand, Great Britain, and France. Two samples of *L. albus* and *L. angustifolius* exceeded 200 mg/kg. The contents are particularly low in lupin protein isolates and in foods containing these ingredients, indicating that their use is a very effective tool for keeping low the alkaloid daily intake.

## KEY WORDS

GC-MS analyses, *Lupinus albus*, lupanine, quinolizidine alkaloids

## INTRODUCTION

In recent years lupin seed appears particularly promising as a source of innovative ingredients having high protein content (34-43% of dry matter) and an acceptable composition of essential amino acids. Moreover lupin protein concentrates and isolates exhibit useful techno-functional properties (D'Agostina *et al.* 2006) allowing their use in the production of several food products, such as biscuits, pasta, and beverages. The quinolizidine alkaloids represent an important safety issue of lupin products. They are secondary metabolites synthesised by plants belonging to genus *Lupinus* and other species of the Fabaceae family, as a defence against predator, both herbivores and microorganisms (Wink, 1998). In mammals, the quinolizidine alkaloid intoxication is characterised by trembling, shaking, excitation and convulsion. The

Health Authorities of some countries (Great Britain, France, Australia and New Zealand) have decided to regulate the quinolizidine alkaloid content in lupin flours and foods fixing the maximum limit to 200 mg/kg (ANZFA, 2001; ACNFP, 1996; Bulletin, 1998).

## MATERIALS AND METHODS

### MATERIALS

Sparteine (purity > 99.5%) was purchased from Fluka (Sigma-Aldrich, St Louis, MO, USA); a sample of lupanine was provided by Prof. F. Sparatore (University of Genova, Italy) and a sample of 13 $\alpha$ -hydroxylupanine by Dr H. Reinhard (Swiss Federal Office of Public Health, Bern, Switzerland).

### SAMPLING

Different kinds of ingredients and foods were analysed for a total of 24 samples; they were protein isolates (food ingredients), model foods, and commercial foods. The lupin protein isolates (Table 1) had been prepared from the seeds of *Lupinus albus* cv. Ares or cv. Typtop, and of *L. angustifolius* cv. Boregine by the Fraunhofer Institut Verfahrenstechnik und Verpackung (Freising, Germany) in a pilot plant within the EU projects Healthy-Profood and Bioprofibre. The model foods (Fig. 1) had been prepared within the EU project Healthy-Profood. Some of them had been produced by Terrena LUPINGREDIENTS (Matrign-Ferchaud, France) using the flour of *L. albus* cv. Arés. They were three snacks, named A, B and C, two biscuits, named A and B, and a lupin beverage. Other samples had been produced by using lupin protein isolates (LPI and LUPI): biscuits C and D by Fraunhofer IVV by using LPI-E from cv. Arés, whereas the spaghetti samples A and B by the University of Thessaloniki by using LPI-E from cv. Typ Top. All the details of composition and production of model foods were provided in Bez *et al.* 2005, Seger *et al.* 2005 and Doxastakis *et al.* 2007. The commercial samples (Fig. 1) were five meat imitation products, provided by Dominae Trading Srl (Origgio, Varese, Italy), one sample of gluten-free pasta (labelled as 'with lupin concentrate'), one sample of lupin rusk (labelled as 'with lupin flour'), and a sample of 'lupini beans' i.e. pickled lupin seeds. These last three samples were purchased in a local supermarket.

## QUINOLIZIDINE ALKALOIDS EXTRACTION

Lupin seeds were dehulled by hand, ground in a house-hold mill (Braun, Germany), sieved through a 60 mesh screen, then defatted with hexane (Baker, Deventer, The Netherlands) for 6 h in a Soxhlet apparatus using cellulose extraction thimbles (123 mm x 43 mm i.d.; Whatman International, Brentford, UK). Lupin protein isolates (LPI) were directly analysed without any previous treatment. All other samples were ground, lyophilised and then defatted by suspending in hexane at room temperature over night under stirring. Each dried sample (1 g) was suspended in 8 mL of 0.1 N HCl (Merck, Darmstadt, Germany) 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 solid was washed again twice with 5 mL of 0.1 N HCl. The gathered extracts were alkalised with 5% NH<sub>4</sub>OH (Carlo Erba, Rodano, Italy) to pH 10-11 and then applied to an Extrelut NT 20 column (Merck, Darmstadt, Germany). After 20 min, the alkaloids were eluted with CH<sub>2</sub>Cl<sub>2</sub> (Baker, Deventer, The Netherlands) (4 x 20 mL) and the solvent evaporated to dryness under vacuum. The residue was then diluted in an appropriate volume of dichloromethane and analysed by GC-MS (Resta *et al.* 2008). Each sample was independently extracted at least three times.

## GC-MS ANALYSES

The analyses were performed on a Shimadzu QP-5000 GC/MS instrument equipped with an AOC20i autosampler (Shimadzu) and a 30 m x 0.25 mm i.d., 0.25 µm, AT-1ms capillary column (Supelco, Milan, Italy). The temperature program was: 150°C for 5 min, from 150°C to 300°C at 5°C/min, then 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, the acquisition from *m/z* 50 to 450. The source operated in EI mode at 70 eV. Each analysis was repeated at least four times.

## RESULTS AND DISCUSSION

### QUINOLIZIDINE ALKALOIDS 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 analysed 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 areas and concentrations were observed: the regression coefficients ( $R^2$ ) were always > 0.99. Since lupanine was the only standard in our hands in sufficient amount and purity, the concentration of the other alkaloids was

estimated by using the lupanine calibration curve adjusted for the molecular weight of each alkaloid. For this reason, the quantitative results reported have to be regarded as estimated concentrations. In standard solutions, the limit of detection (LOD) and limit of quantification (LOQ) values ( $S/N > 3$ ) of lupanine were 2 mg/L and 3 mg/L, respectively, and those of sparteine 1 mg/L and 3 mg/L, respectively, whereas in the lupin flour, the LOD and LOQ values of lupanine were 1 mg/kg and 2 mg/kg, respectively. The precision of the method was estimated by analysing sparteine solutions within the same day (intraday with  $n = 10$ ) and in different days (interday with  $n = 5$ ), obtaining relative standard deviations (RSD%) of 2-3% and 5-6%, respectively. The recovery was evaluated by spiking the flour of *L. albus* cv. Arés with known amounts of sparteine and is always > 92%.

Table 1 reports the alkaloid content of *L. albus* cv. Arés and cv. Typ Top, and *L. angustifolius* cv. Boregine, and the corresponding protein isolates. In *L. albus* cv. Arés seeds five alkaloids were detected: albine, lupanine, 13 $\alpha$ -hydroxylupanine, 13 $\alpha$ -angeloyloxylupanine and 13 $\alpha$ -tigloyloxylupanine; in cv. Typ Top: albine, multiflorine, 13 $\alpha$ -hydroxylupanine, 13 $\alpha$ -angeloyloxylupanine were detected. Four alkaloids were detected in *L. angustifolius* cv. Boregine: angustifoline, 13 $\alpha$ -isolupanine, lupanine and 13 $\alpha$ -hydroxylupanine. In all isolates, lupanine was the only alkaloid and its content was below the limit of 200 mg/kg; thus the applied process appears to be very efficient in removing the alkaloids considering the high alkaloid content of cv. Typ Top and Boregine seeds.

All food products analysed in this work respected the limit of 200 mg/kg (Fig. 1), however there was a big difference between foods containing lupin flour and those containing lupin protein isolates (biscuits C and D, spaghetti A and B). In model foods (Fig. 1), i.e. in lupin beverage, in snack A, B and, C, and in biscuit A, and B, prepared with the flour of *L. albus* cv. Arés, the quantified alkaloids were the same present in the flour.

Comparing the alkaloid contents of snacks and biscuits, it seems that they do not seem to be directly proportional to the lupin flour percentage, suggesting that the extrusion process applied to the snack preparation might have partially destroyed these metabolites. In biscuits C and D, and in spaghetti A and B, prepared using lupin protein isolates, the quantification of lupanine, the only alkaloid detected, was possible only when the percentage of lupin protein isolates was bigger than 13%. All products respect the limit of 200 mg/kg (Fig. 1) in agreement to what was reported for lupin products available on the Swiss market (Reinhard *et al.* 2006).

In conclusion, considering the increasing consumption of lupin products by vegetarians and 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 quinolizidine alkaloids in lupin flours and foods. This research confirms that either the correct selection of lupin varieties or the use of an appropriate technology are crucially important and that lupin protein isolates are an excellent tool for the production of safe lupin products.

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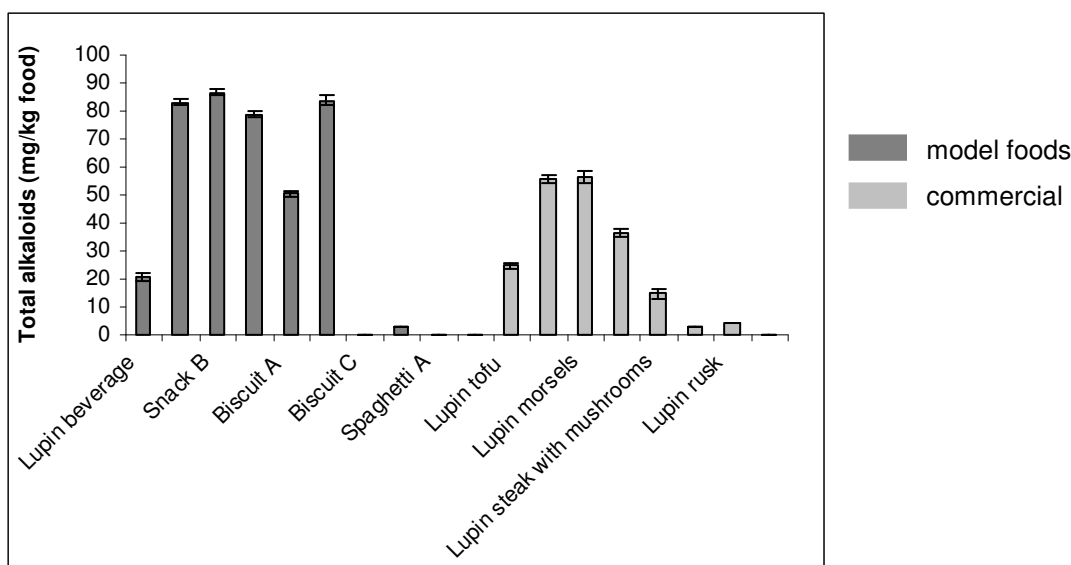
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**Table 1.** Total alkaloid content expressed in mg/kg product of lupin seeds and related lupin protein isolates, LPI-E and LUPI-E; values are reported as mean ± S.D. (n ≥ 3).

Lupin seeds and lupin isolates	Total alkaloids (mg/kg)
<i>L. albus</i> cv. Arés	
seed	146 ± 9
LPI-E	9 ± 0.04
<i>L. albus</i> cv. Typ Top	
seed	1247 ± 44
LPI-E	21.4 ± 0.65
<i>L. angustifolius</i> cv. Boregine	
seed	1107 ± 102
LUPI-E	34.1 ± 3.05



**Fig 1.** Total alkaloids expressed in mg/kg food of model and commercial foods; each value is expressed as mean ± S.D. (n ≥ 3).