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Lupanine and Sparteine content after debittering process in *Lupinus mutabilis* Sweet grains, produced in the Andean region from Bolivia

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Abstract

Lupinus mutabilis Sweet grains, commonly known as Tarwi in the Bolivian highlands, are garnering increased attention as an emerging food source due to their elevated protein and fat content. Originally, these grains possess a bitter taste attributed to the presence of quinolizidine-type alkaloids, necessitating a debittering process. In this investigation, we assessed the most abundant alkaloids (sparteine and lupanine) in six Tarwi cultivars both before and after debittering, employing water and a sodium bicarbonate solution. The findings reveal that the debittering process effectively reduces alkaloid concentrations to permissible levels and may even confer health benefits. Additionally, a proximal analysis was carried and swift, straightforward qualitative method was devised to estimate quinolizidine alkaloids through the formation of an orange complex resulting from iodide-alkaloid ion interaction, that could be applied by the indigenous communities that produce this food.

Keywords: Andean food, Alkaloid, bitterness, Legumes, Protein, Quinolizidine

1. Introduction

"Lupinus mutabilis Sweet" is a legume that is mainly cultivated in the highlands of the Andes (South America) from Colombia to northern Argentina and Chile, but more prominently in Ecuador, Peru and Bolivia, traditionally known as tarwi, tauri, chocho, altramuz or Andean lupine [1,2]; The oldest records of this foodstuff date back to pre-Inca times (500 BC to 1000 AC). Paintings from the Tiwanaku culture depicting the tarwi plant have been found, and remains of tarwi seeds were also discovered in tombs from the Nazca culture [3]. There is currently growing interest in European countries because this food can be used as a source of protein, oil and biomass, as well as contributing to the improvement of marginal soils [1,4,5].

Tarwi grains initially have a bitter taste attributed to the presence and high concentration of quinolizidine-type alkaloids, which result from the L-lysine biosynthetic pathway, these alkaloids are formed through the cyclization of cadaverine units and are widely distributed in species of the genus Lupinus [6-8], because they are present in amounts ranging from 0.007 to 4.5 g of alkaloids per 100 g, with the highest concentrations being Lupanine, Sparteine, 13-Hydroxylupanine, and 4-Hydroxylupanine, but there are also alkaloids present in lower concentrations, such as Tetrahydrorombifoline, Angustifoline, 4,13-Dihydroxylupanine, and 13-Angeloiloxy-lupanine, among others [9].

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These alkaloids may interact with taste receptors known as TASTE 2, TAS2R or T2R, which constitute a subfamily of G protein-coupled receptors; Approximately 25 types of TAS2R are known, capable of detecting numerous chemically diverse compounds with variable specificity. They are genetically linked and associated with bitter perception, selectively expressed in taste receptor cells containing gustducin, an α-subunit of the G protein involved in bitter taste transduction [10-12].

Tarwi, despite initially having a bitter taste, is a highly significant food due to its unique virtues when the bitterness is removed it boasts a high percentage of protein, serves as an excellent source of essential amino acids, and contains unsaturated fats, oligosaccharides [9,13] and micronutrients [14]. Due to this, those cultivating this legume must carry out a de-bittering process, aiming not only to remove alkaloids but also other antinutrients like phytic acid, tannins, nitrates, and trypsin inhibitors; but the duration of the debittering process varies based on the tarwi ecotype [15]. The *Lupinus* genus offers various alternatives for debittering, including biological processes through germination and fermentation involving bacteria and fungi; chemical processes, primarily solvent extraction using hexane, alcohol, and basic solutions; and finally, traditional processes that rely solely on water as the removal agent. The drawback of the latter method is that it requires an extended de-bittering period, ranging from 3 to 6 days [9].

The tarwi producers estimate the residual concentration of alkaloids solely based on taste perception, identifying the presence or absence of a bitter taste. Therefore, the objective of this study is to quantify the initial and debittered samples through the concentration of the major alkaloids, sparteine and lupanine, in Bolivian tarwi grains. Additionally, the study proposes a quick and simple visual method for the qualitative estimation of quinolizidine alkaloids.

2. Materials and Method

2.1 Samples

Six cultivars of bitter white tarwi were collected from different tarwi producers in the Bolivian Altiplano (Table 1) between June amd July 2021, five cultivars from the department of La Paz, collected in Copacabana (TW1), Quiascapa (TW2), Carabuco (TW3), Escoma (TW4), Sahuiña (TW5); and one collected in Molles (TW6) in the department of Potosi.

2.2 Determination of proximal composition

The proximate composition was determined by the online methods described by “The Grain and Feed Trade Association” [16]. The proximate composition was determined by the online methods described by GAFTA. Moisture was determined by the GAFTA 2:1 method, fatty matter was determined with hexane by the Soxhlet method according to GAFTA 3:0, crude fiber was determined by the acid-base digestion method according to GAFTA 9:0, ash was determined by calcination according to GAFTA 11:0. The protein content was assessed using the Dumas combustion method with consumables and the nitrogen elemental analyzer NDA701 by Velp Scientifica (Usmate, Italy). Beginning with 100 mg of finely ground and homogenized samples enclosed in tin foil, they were introduced into the combustion reactor (1030ºC). In this process, organic matter underwent transformation into various gases. Water vapor and carbon dioxide were captured and expelled from the system, while NO_x gases were directed into the metallic copper reduction reactor (730ºC). This ensured that only N_2 could reach the thermal conductivity detector and was calculated using a EDTA nitrogen calibration curve and conversion factor for nitrogen of 6.25. Carbohydrate was calculated by the percentage difference 100 % - (%moisture + %protein + %fatty matter + %ash) and finally the average energy value was calculated by (4×%carbohydrate + 4×%protein + 9×%fatty matter).

2.3 Grain debittering process

The debittered process was formulated on the basis by [17,18]. The extraction agents used were water or a basic solution (0.5% $NaHCO_3$). To 50 g of non-debittered tarwi samples were weighed, to which a 1:5 ratio of water or alkaline solution (250 mL) was added. They were then soaked for 18 hours. After this period, the liquid was discarded, and the samples were cooked in a 1:3 ratio for 60 minutes at 86 ºC and at an altitude of 3600 meters above sea level (La Paz, Bolivia conditions). The resulting liquid was discarded, and the debittering process was carried out with water or alkaline solution in a 1:5 ratio at room temperature, with water changes every 6 hours, until the bitter taste of tarwi disappeared (5 days). After the debittenring, the seeds were dried at 35 ºC for 24 hours.

Table 1. Description of the tarwi cultivars evaluated in the present study

Place	Sample code	Coordinates	Altitude (m.a.s.l.)
Copacabana	TW1	-16.168301, -69.079964	3845
Quiascapa	TW2	-15.828896,-69.014006	3848
Carabuco	TW3	-15.780764, -69.037305	3859
Escoma	TW4	-15.696415, -69.174922	3830
Sahuiña	TW5	-16.202260, -69.101449	3846
Molles	TW6	-19.714952, -64.976847	3305

Table 2. Proximal composition of Bolivian tarwi cultivars

Constituents	Min - Max	Average
Moisture (%)	7.0 – 7.8	7.3 ± 0.3
Crude Protein (%)	39.2 – 41.3	40.1 ± 0.7
Fatty matter (%)	17.1 – 18.2	17.5 ± 0.6
Crude fiber (%)	9.9 – 10.1	10.0 ± 0.2
Ash (%)	3.9 – 4.2	4.0 ± 0.2
Carbohydrate (%)	28.5 – 31.5	29.02 ± 1.3
Energy (Kcal/100g)	441.3 – 443.4	442.4 ± 1.0

2.4 Alkaloids extraction

The method was slightly modified described by [19]. From dry matter (DM) with particles smaller than 0.5 mm; To 2 g of bitter sample or 5 g of debittered sample is homogenized with 25 mL of 0.5 M HCl in an ultrasound for 30 minutes at room temperature. Subsequently, it is centrifuged at 10000 rpm for 15 minutes, the solution is decanted, and NaOH 0.5 M is added until reaching a pH between 9-10. Later, it is washed with 20 mL of chloroform (3 times), and both phases are separated. The organic phase is dried and stored at -18 ºC for further analysis.

2.5 Analysis of alkaloids by GC-MS

On the basis of Cortés-Avendaño et al. [20] description, some modifications were made. The extract is dissolved in chloroform and then filtered through a 0.45 µm pore size PTFE filter. The analysis was conducted using a gas chromatograph (GC2010 Plus) coupled with a mass spectrometer (QP 2020), both from Shimadzu, Japan. The injection volume was 1 µL, the column flow rate was 0.82 mL/min, the injector temperature was set at 280ºC, and the detector temperature at 300ºC. The temperature program initiated at 130ºC for 6 minutes, followed by an increase to 180ºC for 1.5 minutes, then to 200ºC for 12.5 minutes, and finally to 300ºC for 2 minutes, at a rate of 10ºC/min, totaling 22 minutes for the method.

The ionization temperature was set at 280ºC, and the interface temperature at 300ºC. Alkaloid verification was performed by comparing retention times with their standards and by comparing mass spectra with literature [21]. For the preparation of the calibration curves, patter solutions of 100 ppm were prepared in chloroform and diluted into solutions of 1, 5, 10, 20, and 25 ppm for (+)-sparteine (Apollo Scientific, Manchester, UK) and 1, 5, 10, 15, 25, and 50 ppm for (+)-Lupanine hydrochloride (Phytolab, Vestenbergsgreuth, Germany).

2.6 Qualitative analysis of quinolidizine alkaloids whith lugol

The lugol reagent was prepared by dissolving 0.2 g of potassium iodide in 5 mL of distilled water, then adding 0.02 g of metallic iodine slowly with constant stirring and making up to 10 mL in volume with distilled water. The method described by Galek et al. [22] was slightly modified, where 2 tarwi lentils (bitter or debbitering) were placed in a test tube and 3 mL of distilled water was added, the mixture was boiled in a water bath for 30 min. Once the time had elapsed, the tarwi lentil was removed and 2 to 3 drops of lugol reagent were added to the aqueous extract, where the formation of orange turbidity indicates the presence of alkaloids, while no change indicates that the sample is free or has a low concentration of alkaloids.

2.7 Statistical analysis

The results are reported as mean ± standard deviation. A post-hoc analysis using the Tukey test ($p<0.05$) was performed on the alkaloid content to assess differences between cultivars and determine which treatment means differed significantly. IBM SPSS 22.0 software from the United States was employed for this analysis.

3. Results and discussion

3.1 Proximal composition

Table 2 shows a breakdown of the proximal composition of the six tarwi cultivars. The results obtained show similar values to those of previous studies [9,23].

According to the results obtained, it can be seen that tarwi beans have a high protein value (≈ 40%), which is significantly higher than other Andean grain [13], legumes [24,25] and even surpasses that of one of the most commercially valuable grains, such as soybeans [26]. The fatty matter content of tarwi (≈ 17%) is slightly lower than that of soya beans, but higher than that of other legumes [24,27] and Andean grain [13]. While the fiber content of tarwi is also slightly higher compared to other legumes, seeds, grains, and pseudocereals [28].

3.2 Alkaloid content

The main alkaloids were identified through comparison of their retention time with the standards for sparteine (11.2 min) and lupanine (16.3 min), in addition to comparison of their mass spectra with literature [21].

The structure of lupanin and espartein show in Figure 1, and their concentration was quantified in six tarwi cultivars of Bolivian origin (Table 3), where lupanin was quantified in a range of 2117 - 3288 mg/100 g DM in the bitter grains, where TW1 (Copacabana) had the lowest concentration of lupanin while TW4 (Escoma) and TW2 (Quiascapa) had the highest concentration. It was possible to quantify that sparteine is present in a range of 44.8 - 232.7 mg/100 g DM, where the highest concentration was TW2 (Quiescapa) while the lowest concentration was TW1 (Copacabana), this variation in the concentration of alkaloids in the cultivars is mainly due to genetic and physiological characteristics, soil quality, temperature and drought stress among other factors [8,29].

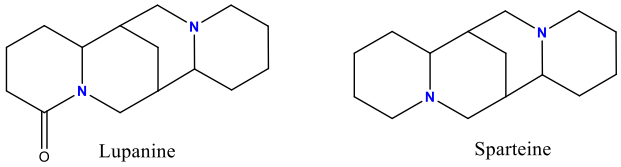

Figure 1. Structure of the most abundant alkaloids of L. mutabilis

Lupanine is the predominant alkaloid in most species of the *Lupinus* genus, as can be seen in Table 4. The second most abundant alkaloid in the *Lupinus* genus is sparteine. The content of these alkaloids in Bolivian-origin *L. Mutabilis* grains is comparable and even slightly lower than that of Peruvian origin and to *L. angustifolius* [30], but it is significantly higher compared, *L. campestris* (Martinez et al., 2003), *L. albus* and *L. luteus* [31].

Quinolidizine alkaloids (Lupanine and Sparteine) are biosynthesised mainly as a plant defence mechanism, but because of their toxicity they are considered as an anti-nutrient for humans [32,33]. Previous studies show different processes for debittering *L. mutabilis* grains using aqueous methods [13,20], saline [34], fermentation [15,34] and Resin-Based [35], In addition, it was found that the debittering process can improve the levels of proteins, certain amino acids, fatty acids and tocopherols. However, this process leads to a decrease in the concentration of anti-nutrients such as alkaloids, tannins, nitrates and phytic acid, as well as physical properties such as hardness and brightness. It also results in a reduction of some chemical properties, such as antioxidant capacity and polyphenol concentration [36-38], such as flavones and isoflavones [39].

In this current research, de-bittering was conducted using both water and a 0.5% $NaHCO_3$ solution, as illustrated in Table 3. In both cases, the presence of sparteine was not detected. Debittering with water resulted in a lupanine quantification of 0.16 to 13.1 mg/100g dry matter, reducing lupanine concentration by 99.7% to 99.9%. Meanwhile, samples debittered with $NaHCO_3$ solution showed slightly lower values, ranging from 0.002 to 1.04 mg/100g lupanine, reducing lupanine concentration by over 99.96%. These findings corroborate what has been described by Jiménez-Martínez et al. [40] which showed that the alkaline $NaHCO_3$ solution was more efficient at debittering the grains from *L. campestris.*

Regulations in countries such as France, Great Britain, Australia and New Zealand require a maximum of 200 mg alkaloids per 100 g of lupin products, but also the ANZFA (Australian and New Zealand Food Authority) suggests a dose of 0.35 g lupin alkaloids per kg body weight, i.e. 20 mg lupin alkaloids per day that a 60 kg person could tolerate without adverse effects [8]. Adhering to these recommendations, de-bittered tarwi is suitable for human consumption as it falls within the recommended permissible ranges. Furthermore, the residual alkaloid concentration could be beneficial for health, such as its demonstrated anti-diabetic effect, as it is proven to reduce blood glucose and insulin levels [41,42].

Table 3. Concentration of lupanine and sparteine in bitter and debittered grains of L. mutabilis

	Bitter (mg/100g)		**Debittered with water (mg/100g)**		**Debittered with $NaHCO_3$ (mg/100g)**	
Samples	**Spartein**	**Lupanine**	**Spartein**	**Lupanine**	**Spartein**	**Lupanine**
TW1	$44{,}8 \pm 2.1^{e}$	$2117 \pm 56.8^{b,A}$	ND	$0{,}16 \pm 0.05^{c,B}$	ND	$0{,}08 \pm 0.02^{c,B}$
TW2	$232{,}7 \pm 9.8^{a}$	$3209 \pm 97.5^{a,A}$	ND	$5{,}61 \pm 0.12^{b,B}$	ND	$0{,}16 \pm 0.03^{c,C}$
TW3	$182{,}9 \pm 9.5^{b}$	$2484 \pm 75.8^{b.A}$	ND	$4{,}21 \pm 0.16^{b,B}$	ND	$< 0.002^{d,C}$
TW4	$63{,}8 \pm 3.7^{e}$	$3288 \pm 88.5^{a,A}$	ND	$13{,}1 \pm 0.87^{a,B}$	ND	$0{,}75 \pm 0.02^{b,C}$
TW5	82.7 ± 6.6^{d}	2301 ± 75.1^{b}	ND	$6.71 \pm 0.13^{b,B}$	ND	$0.25 \pm 0.03^{c,C}$
TW6	$133{,}1 \pm 4.6^{c}$	2907 ± 95.1^{a}	ND	$5{,}75 \pm 0.25^{b,B}$	ND	$1{,}04 \pm 0.04^{a,C}$

Different lowercase letter in each column indicates significant difference between cultivars; different capital letter in each row indicates significant difference between treatment ($p<0.05$). ND: no detected;

Table 4. Comparison of the concentration of lupanine and sparteine with other grain of the *Lupinus* genus

	Bitter (mg/100g)		**Debittered (mg/100g)**		
Lupinus species	**Esparteina**	**Lupanina**	**Esparteina**	**Lupanina**	**Reference**
Lupinus mutabilis, Bolivia	44.8 – 233	2117 – 3288	–	0.16 – 13.1	This study
Lupinus mutabilis, Peru	196 – 890	2504 – 5231	1 – 2	1 – 3	[20]
Lupinus angustifolius	2.16 – 6.32	27.8 – 5181	–	–	[30]
Lupinus campestris	–	120	–	–	[43]
Lupinus albus	7.0	3.04 – 3000	–	–	[31,44]
Lupinus luteus	12.0 – 23.4	0.64 – 3.7	–	–	[31]

3.3 Qualitative analysis

The qualitative technique to detect high contractions of quinolidizine alkaloids, is quick and simple where from a few drops of lugol to the *Lupinus* extract, as can be seen in Figure 2, if an orange colouration appears it shows the presence of alkaloids, while if nothing occurs it indicates the absence of alkaloids.

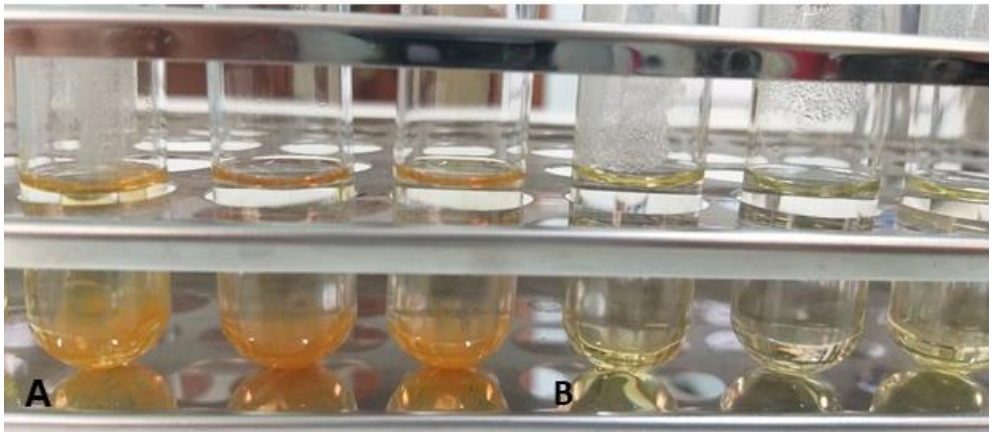

Figure 2. Lugol alkaloid test on aqueous extracts *of L. mutabbilis* A) bitter samples B) debittered samples

The development of this *in situ* technique could be very useful for producers to determine whether or not their samples still retain a bitter taste by applying the lugol reagent with the wastewater from the debittering process. Bitter taste can indicate toxicity and alert to the presence of toxic compounds, although not all compounds fall into this category. Alkaloids are generally known for their toxicity, as they are naturally occurring chemicals containing nitrogen atoms and are characterised by their bitterness [45].

4. Conclusions

Through this research, the aim is to revalorize the consumption of tarwi (*L. mutabilis*). The influence of aqueous and alkaline de-bittering processes on the content of sparteine and lupanine in six Bolivian-origin tarwi cultivars was investigated. Both debittering methods entirely reduced the sparteine content and brought down the lupanine concentration to permissible levels, thereby addressing food safety concerns related to quinolizidine alkaloids. This approach allows for the full utilization of the benefits of this valuable Andean food.

The qualitative method using Lugol's solution for the rapid detection of quinolizidine alkaloids can be implemented for any *Lupinus* variety. Additionally, it proves highly beneficial in rural communities, where residents and producers can easily determine if their products have been de-bittered through a simple and *in situ* process. This technique, unlike more expensive methods such as gas chromatography coupled with mass spectrometry (GC/MS) or high-performance liquid chromatography (HPLC), stands out for its versatility and low cost.

Compliance with Ethics Requirements. The authors declare that they comply with the Ethics requirements of the journal. The authors declare that they have no conflicts of interest and that all procedures involving human or animal subjects (if any) comply with specific regulations and standards.

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