Alternative Strategies for the Development of High-Nutritional-Value Products from Cereals and Pulses

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This PhD thesis aimed at improving the nutritional value of cereals and pulses-based food through different strategies as: using the natural modifications occurring during germination; exploiting underutilized crops; recover and upcycling food industry by-products with the possible implementation of high-power low-frequency ultrasound technology (US).

Strategie alternative per lo sviluppo di prodotti alimentari ad alto valore nutrizionale da cereali e legumi

Questa tesi di dottorato ha riguardato il miglioramento del valore nutrizionale degli alimenti a base di cereali e legumi attraverso strategie diverse, quali: le naturali modificazioni che si verificano durante la germinazione; lo sfruttamento delle colture sottoutilizzate; il recupero e il riciclo dei sottoprodotti dell'industria alimentare con l’eventuale adozione degli ultrasuoni a bassa frequenza.

**Key words**: pulses; legumes; cereals; ultrasound; germination; sprouting; by-product; sidestream.

# **1. Introduction**

Consumers interest towards alternative products with high nutritional value is pushing the food industry to fulfil this demand by identifying and introducing new food resources. Lupin, a pulse crop with thrifty agronomic requirements, represents an excellent source for human nutrition being rich in proteins, lipids (mainly unsaturated fatty acids) and antioxidant compounds (Briceño Berru *et al*., 2021; Estivi *et al*., 2022a). Lupin utilisation is hampered by the limited number of studies on its composition and technological characteristics, as well as the lack of improved varieties suitable for cultivation in the main lupin cropping areas. Furthermore, despite their excellent nutritional composition, lupin seeds must be debittered before consumption to remove toxic alkaloids, a further limitation to their diffusion because debittering is a water-intensive and protracted process, which lasts up to six days (Estivi *et al*., 2022b).

Peas occupy a prominent place among vegetables due to their high content in protein (23–25%), digestible starch (50%), soluble sugars (5%), fibre, vitamins A and C, calcium and phosphorus (Sharma *et al*., 2013). However, industrial processing discards from 5 to 25% of the harvest as by-product (i.e., pods, husks and broken, dark or stained seeds; Conserve Italia Scarl., Italy), still rich in protein, dietary fibre, polyphenols and other molecules (e.g., peptides and lectins) with antioxidant or antimicrobial activities (Mateos-Aparacio *et al*., 2010). The high amount of protein has pushed the food industry to try to upcycle legume by-products by incorporating them into various kinds of food (e.g., high protein pasta, chips, hamburger patties, nuggets, beverages, baby food, imitation cheese, whipped toppings, soy milk and baked goods; Boye *et al.*, 2010). A popular way to exploit them is also the extraction of valuable fractions. Several techniques are employed to obtain protein concentrates and isolates from pea flour, including alkaline wet extraction/isoelectric precipitation (IEP) without or with ultrafiltration (Qiaoyun *et al*., 2017; Vogelsang-O'Dwyer *et al*., 2021), dry fractionation with possible tribo-electric separation (Wang *et al.*, 2015), salt extraction, micellization and gentle fractionation, or hybrid wet/dry approaches (Geerts *et al.*, 2017). The wet techniques can be coupled with high-power low-frequency ultrasound technology, whose capability to improve extraction yield and duration is well-documented (Estivi *et al.*, 2022c).

In recent years, alongside vegan and vegetarian products (Kumar *et al*., 2017), the demand for functional foods enriched with bioactive compounds of plant origin has gradually increased, driven by consumers awareness of the close relationship between nutrition and health. However, the deficiency of vitamin B12 intake emerged as a major risk in strictly vegetable-based diets, naturally devoid of it (Chamlagain *et al*., 2015). Moreover, the vast variety of commercial B12 supplements from algae, often containing the inactive pseudovitamin, contributes to mislead the consumers (van den Oever and Mayer, 2022). Fermentation with B12-producing *Propionibacterium freudenreichii* strains has proved to be a feasible strategy for *in-situ* enrichment of cereal and legume ingredients (Xie *et al*., 2021).

In accordance with the PhD thesis project previously described (Estivi, 2021), this oral communication reports the main results of the following activities directed to:

A1) study the effect of controlled germination on lipophilic antioxidants (i.e., tocols and carotenoids) and colour in Andean lupin seeds;

A2) characterize the water-debittered seeds of 33 Andean ecotypes of *Lupinus mutabilis*, originating from different regions of Peru, for free phenolic compounds and perform a preliminary investigation of FT-NIR suitability as a fast, reliable and non-destructive approach to assess their antioxidant properties;

A3) develop and fine-tune a method for fast and water-efficient debittering of white lupin seeds;

A4) evaluate the impact of the debittering method on the antioxidants of white lupin seeds;

B1) develop and optimise an ultrasound-assisted alkaline extraction of protein to upcycle the by-product from canned green peas production (pea waste, hereafter);

B2) evaluate the pea waste as a culture medium for synthesizing vitamin B12 by *P. freudenreichii* fermentation and prepare a B12-rich bread with fermented material.

# **2. Materials and Methods**

A1) Two Andean lupin cultivars (*Lupinus mutabilis* Sweet) were analysed before and after germination in the dark for two, four and six days. Colour coordinates (*L*\*, *a*\*, *b*\*) were evaluated by a tristimulus colorimeter, while tocols and carotenoids were extracted by saponification (Panfili *et al*., 2003) and quantified by HPLC (Brandolini *et al*., 2022).

A2) 33 Andean ecotypes of *L. mutabilis* and five varieties belonging to *L. luteus*, *L. angustifolius* and *L. albus*, as controls, were analysed to assess their free phenolic compounds content by RP-HPLC (Brandolini *et al*., 2022). The acquisition of FT-NIR spectra was performed as well.

A3) Two lots of white lupin (*Lupinus albus*) seeds were used in different trials to optimize the experimental debittering method and to assess the influence of sonication (with and without the employment of US), solvent (water, solutions of salt or citric acid) and treatment time. The effectiveness of debittering was evaluated by extraction and titration of the residual alkaloids (von Baer *et al*., 1979) and evaluation of the bitterness by e-tongue (Marengo *et al*., 2016). Two debittering reference methods (Córdova Ramos *et al*., 2020; Villacrés *et al*., 2020) and four commercial lupin snacks were used as controls.

A4) The very same materials produced and analysed in the A3 activity were characterized for their content in tocopherols, carotenoids and phenolic compounds (soluble free, soluble conjugated and bound fractions) by HPLC as previously detailed.

B1) The by-products of the canned peas production line were sampled on three different days and stored at -20 °C. According to the Design of Experiment (DoE) technique, trials were carried out to fine tune ultrasound-assisted alkaline extraction of protein followed by isoelectric precipitation from thawed by-product. Protein concentrate, dried by-product and a commercial pea flour were characterized for protein content (Kjeldahl), water activity, colour, water and oil retention capacity, gelling capacity, foaming capacity and stability, emulsifying activity, protein pattern by SDS-PAGE, total bacterial count, total lactic acid bacteria, *Enterobacteriaceae*, moulds and yeasts.

B2) The dried pea waste described in the B1 activity was fermented with the following procedure, established during several preliminary trials: 500 g of a 15% batter were prepared mixing dried pea waste with MilliQ water; the pH was adjusted to 6 with 10 M NaOH and the batter was pasteurized at 70 °C for 20 min and then aseptically inoculated with *P. freudenreichii subsp. freudenreichii* 282 culture to reach a cells concentration of approximately 9 log CFU/g. The material was incubated at 30 °C for 72 h with constant shaking. Fermentation was performed in two independent repetitions. Six lots of bread were baked according to Edelmann *et al*. (2016), including two controls (with and without addition of non-fermented pea waste batter) and four enriched lots (with 15 and 20% for each of two batches of fermented batter). Vitamin B12 and organic acids were quantified as outlined by Chamlagain *et al*. (2015) and Xie *et al*. (2018), respectively. Propionibacteria, lactic acid bacteria and *Enterobacteriaceae* were enumerated by plate count in agarised YEL, MRS and VRBG, respectively. The baking loss was determined gravimetrically; bread volume and crumb texture were determined by Volscan and texture analyser (Stable Microsystems), respectively.

# **3. Results and Discussion**

A1) Germination significantly affected all the examined characteristics (Estivi *et al*., 2022d). Luminosity (*L*\*) showed an uncertain pattern as the germination time increased, but minor reduction in *a*\* and relevant growth in *b*\* indicated yellow-greenish compounds formation. No tocotrienols were found and the most abundant tocol was γ-tocopherol. Although total tocols were almost unchanged during germination, α-tocopherol increased from 0.7 to 74.8 mg/kg dry matter (DM) after six days, while γ-tocopherol (Fig. 1) decreased. The γ homologue is the precursor of α-tocopherol, hence we suggested that germination triggered their conversion, improving 4.3-fold lupin flour biological activity. The most abundant carotenoid was lutein, but (α+β)-carotene, β-cryptoxanthin and zeaxanthin were identified as well. Total carotenoids increased 12-fold with germination time, from 1.8 to 22.3 mg/kg DM (Fig. 1).The (α+β)-carotene, precursor of vitamin A, showed the greatest growth rate; β-cryptoxanthin and zeaxanthin, originally below or close to the detection limit, exhibited detectable amounts after six days (1 and 1.5 mg/kg DM, respectively); lutein continued to prevail, with a final amount of 13.6 mg/kg DM. Germinating seeds in the dark proved to be a viable and effective technique to significantly improve the nutritional properties of Andean lupin.



**Figure 1** *Changes in tocopherols and carotenoids content in two Andean lupin accession, Altagracia (circles) and Cholo fuerte (triangles), during controlled germination up to 6th day. Error bars indicate the standard deviations.*

A2) The total free phenolics of *L. mutabilis* were mostly (85.5–99.6%) flavonoids (genistein and genistein derivatives, apigenin, catechin and naringenin). Other compounds, detected in low quantities, were phenylethanoids (tyrosol and tyrosol derivative) and phenolic acids (cinnamic acid derivatives). The total free phenolic concentration ranged from 340.8 (cv. Churibamba) to 1393.3 mg/kg DM (cv. H6 INIA BP) exceeding that of controls (6.8–31.3 mg/kg DM). A relationship between free phenolic compounds and spectral bands was established by FT-NIR, paving the way for a fast, reliable and non-destructive approach to lupin seeds characterization. Even after debittering, lupin flours maintained high free phenolic concentrations and antioxidant capacity.

A3) The sonication did not accelerate debittering, while the sodium chloride and citric acid solutions significantly shortened debittering time, reduced water consumption and decreased alkaloid content to commercial values (0.31–1.03 g/kg DM). Debittering with a 1% citric acid solution saved 88 h and 65 L water/kg dry lupin compared to the water control method, and 13 h and 31 L water/kg dry lupin compared to the salt solution control method. The electronic tongue grouped the experimental and commercial samples in well-defined clusters; bitter and umami tastes were the main factors, well correlated with alkaloid content. The proposed procedure, either with citric acid or sodium chloride, could be easily adopted by the industry to reduce time and costs of lupin debittering.

A4) The sonication decreased the content of carotenoids and soluble-free phenolics but did not influence tocopherols or soluble-conjugated and insoluble-bound phenolic compounds. Nevertheless, the debittered lupins showed interesting quantities of tocopherols (172.8–241.3 mg/kg DM), carotenoids (10.9–25.1 mg/kg DM), and soluble-free (106.9–361.1 mg/kg DM), soluble-conjugated (93.9–118.9 mg/kg DM), and insoluble-bound (59.2–156.7 mg/kg DM) phenolic compounds. Using citric acid or sodium chloride solution preserved in a better way the soluble-free phenolics likely due to the reduction in treatment times (Estivi *et al*., 2022e).

B1) The optimised extraction conditions were: ratio water/by-product, 20 mL/g; pH, 11; amplitude, 80 μm; time, 2 x 30 min; on/off cycle, 5/5 s; temperature, 25 °C. Table 1 compares the efficiency of ultrasound-assisted extraction *vs.* magnetic stirring and reports the characterisation of commercial pea flour, dried pea waste and protein concentrate. The extraction yield of the optimized process (66.6%) was comparable with that (62.6-76.7%) reported by Stone *et al*. (2015) from defatted pea flour with magnetic stirring only but was inferior to those (82.6% with ultrasonication and 60% with magnetic stirring) of Wang *et al*. (2020), possibly due to leaching of low molecular weight peptides formed during pre-sampling fermentation. This was confirmed by total microbial count (7.32 log CFU/g) and presence of smeared bands in the SDS-PAGE. Overall, the ultrasonication increased 3-fold the protein recovery yield, reducing to 1/4 the extraction time, at the cost of a lower purity of the concentrate. Prestes Fallavena *et al*. (2022) and Thirunavookarasu *et al*. (2022) collected numerous evidence related to protein glycation mediated by high-power ultrasound and to the formation of complexes between protein (sonication-denatured) and simple sugars, oligosaccharides or polysaccharides, including pectins. Thus, it can be hypothesized that the polysaccharides from the abundant soluble fibre of the pods formed soluble adducts with protein, reducing the purity of the concentrate.

B2) To allow the growth of *Propionibacterium,* pea waste was pasteurized to reduce lactic acid bacteria and enterobacteria competition, and the excess of lactic acid was neutralized. In preliminary trials was observed that insufficient batter shaking led to accumulation of 40-42% vitamin as pseudo-B12; in fact, oxygen availability is crucial in the synthesis of 5,6-dimethylbenzimidazole, the lower ligand distinguishing the active form of B12 (Chamlagain *et al*., 2018). In Table 2 the main results are summarized. The fermented batters reached an average B12 amount of 208.9 ng/g fresh weight (FW) approximately equivalent to 1390 ng/g DM, a high value compared to the results reported by Xie *et al*. (2021) for eleven fermented batters from cereals and legume flours: 51–742 (mean 301) ng/g DM. Such a high B12 content made it possible to reach the remarkable average amounts of 35.8 and 51.7 ng/g FW in 15 and 20%-enriched breads. Given a recommend daily intake equal to 2–2.4 μg (Chamlagain *et al*., 2018), about 40 to 70 g/d enriched bread would provide enough vitamin. Enriching the dough caused moderate loss in bread volume and increase in crumb hardness but did not affect the overall quality in a substantial way.

**Table 1** *Comparison of ultrasound-assisted extraction vs. magnetic stirring and characterisation of commercial pea flour, dried pea waste and protein concentrate.*

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Magnetic stirring** | **Ultrasonication** |  |
| **Time** | 2 x 2 h | 2 x 30 min |  |
| **Yield (%)** | 21.5±0.9 | 66.6±1.6 |  |
| **Protein (g/100 g)** | 86.4±0.3 | 68.4±0.2 |  |
|  | **Pea flour** | **Dried by-product** | **Protein concentrate** |
| **Protein (g/100 g DM)** | 25.09b ± 0.08 | 24.71b ± 0.71 | 74.86a ± 0.32 |
| **aw** | 0.530ᵃ ± 0.004 | 0.320ᶜ ± 0.002 | 0.510ᵇ ± 0.002 |
| **Colour coordinates** |  |  |  |
| ***L*\*** | 86.50ᵃ ± 0.30 | 54.47ᵇ ± 0.83 | 40.43ᶜ ± 0.35 |
| ***a*\*** | -9.63ᵇ ± 0.21 | -4.10ᵃ ± 0.46 | -3.50ᵃ ± 0.30 |
| ***b*\*** | 16.90ᵇ ± 0.35 | 19.17ᵃ ± 0.50 | 15.43ᶜ ± 0.55 |
| **Water holding capacity (g H2O/g)** | 1.23ᶜ ± 0.34 | 4.19ᵃ ± 0.28 | 2.13ᵇ ± 0.22 |
| **Oil holding capacity (g oil/g)** | 1.57ᵇ ± 0.08 | 2.24ᵃ ± 0.16 | 1.68ᵇ ± 0.22 |
| **Gelling capacity (g/100 mL)** | 21.52ᵇ ± 0.28 | 16.09ᶜ ± 0.99 | 28.67ᵃ ± 1.62 |
| **Foaming capacity (%)** | 69.18ᵃ ± 1.39 | 21.59ᶜ ± 1.36 | 28.46ᵇ ± 1.39 |
| **Foam stability (%)** | 49.55ᵃ ± 0.01 | 15.21ᵇ ± 2.08 | 6.38ᶜ ± 1.39 |
| **Emulsifying activity (%)** | 63.26 ± 1.15 | 62.20 ± 1.28 | 60.89 ± 1.07 |

**Table 2** *Pea waste batters and enriched breads characterisation: B12 levels, microbial counts, organic acids, baking loss, specific volume and crumb hardness.*

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Batter** | **Fermentation** | **B12** | **Propioni**- | **Lactic acid** | | **Acetic acid** | **Propionic acid** |
| **batches** | **time (h)** | **(ng/g)** | **bacteria** | **bacteria** | **(mg/g)** | | **(mg/g)** |
| 1 | 0 |  | 8.64±0.09 | 3.13±0.12 |  | |  |
| 1 | 72 | 211.3a±22.9 | 9.93±0.04 | 4.86±0.20 | 2.81±0.01 | | 4.05±0.01 |
| 2 | 0 |  | 8.63±0.04 | 3.76±0.07 |  | |  |
| 2 | 72 | 206.5a±25.8 | 9.98±0.01 | 5.31±0.51 | 2.78±0.01 | | 4.03±0.01 |
| **Bread** | **Enrichment** | **B12** | **Baking loss** | **Volume** | **Specific volume** | | **Hardness** |
| **batches** | **(%)** | **(ng/g)** | **(%)** | **(ml)** | **(ml/g)** | | **(N)** |
| Control | 0 |  | 13.3a ±0.5 | 381.9a±3.6 | 2.90a±0.03 | | 14.0bc±1.1 |
| Control-NF | 20 |  | 12.2d±0.7 | 379.9a±1.5 | 2.88b±0.03 | | 11.9 c±0.8 |
| 1a | 15 | 39*.*7b±0*.*5 | 12.3cd±0.3 | 379.6a±1.9 | 2.89b±0.02 | | 16.2ab±1.6 |
| 2a | 15 | 32*.*0b±4*.*1 | 13.0ab±0.2 | 367.2b±2.0 | 2.82c±0.01 | | 14.1bc±1.1 |
| 1b | 20 | 52*.*3a±6*.*3 | 12.5bcd±0.2 | 358.1c±3.2 | 2.73d±0.03 | | 17.9 a±2.0 |
| 2b | 20 | 51*.*1a±3*.*5 | 13.0abc±0.2 | 351.3d±2.1 | 2.69d±0.01 | | 16.3ab±0.6 |

# **4. Conclusions and Future Perspectives**

Lupin was confirmed as a promising source of antioxidants, whose composition can be further improved by germinating seeds, selecting the most promising accessions for cultivation, and adopting optimised debittering to limit leaching of valuable compounds. The proposed procedure, either with citric acid or sodium chloride, could easily be adopted by the industry to reduce time, water consumption and costs of lupin debittering. Further studies should consider the incorporation of germinated lupin flour in food. Two approaches were attempted for the first time to upcycle the pea canning by-product: i) protein extraction based on ultrasound technology was developed; ii) waste fermentation for synthesising B12 proved to be feasible, although microbial alteration was identified as a limit, emphasising the importance of in-line processing. The fermented material was successfully added to the dough to obtain a bread rich in vitamin B12 (35.7–51.7 ng/g), without affecting leavening and volume development.

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