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Development of legume-derived ingredients and foods enriched in bioactive compounds using different strategies [AGR/15]

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Estivi, L., Brandolini, A., Gasparini, A., & Hidalgo, A. (2023). Lupin as a source of bioactive antioxidant compounds for food products. *Molecules*, *28*, 7529. https://doi.org/10.3390/molecules28227529

Estivi, L., Buratti, S., Fusi, D., Benedetti, S., Rodríguez, G., Brandolini, A., & Hidalgo, A. (2022). Alkaloid content and taste profile assessed by electronic tongue of *Lupinus albus* seeds debittered by different methods. *J Food Compos Anal*, 104810. https://doi.org/10.1016/j.jfca.2022.104810

Estivi, L., Fusi, D., Brandolini, A., & Hidalgo, A. (2022). Effect of debittering with different solvents and ultrasound on carotenoids, tocopherols and phenolics of *Lupinus albus* seeds. *Antioxidants*, *11*, 2481. https://doi.org/10.3390/antiox11122481

Estivi, L., Grassi, S., Briceño-Berrú, L., Glorio-Paulet, P., Camarena, F., Hidalgo, A., & Brandolini, A. (2022). Free phenolic compounds, antioxidant capacity and FT-NIR survey of debittered *Lupinus mutabilis* seeds. *Processes*, *10*, 1637. https://doi.org/10.3390/pr10081637

Estivi, L., Pascual Chagman, G. J., Santa Cruz Olivos, J. E., Savasi, P., Brandolini, A., & Hidalgo, A. (2023). Changes in colour, tocopherols and carotenoids during the germination of lupin seeds. *J Food Compos Anal*, *124*, 105682. https://doi.org/10.1016/j.jfca.2023.105682

Under review:

Estivi, L., Brandolini, A., Catalano, A., Di Prima, R., & Hidalgo, A. (n.d.-a). Characterization of an industrial pea canning by-product and its protein concentrate obtained by optimized ultrasound-assisted extraction.

Estivi, L., Chamlagain, B., Edelmann, M., Varmanen, P., Hidalgo, A., & Piironen, V. (n.d.-b). By-product of canned green peas as a substrate for *Propionibacterium freudenreichii* fermentation to fortify bread with vitamin B12.

A Fabio "C." Pinci
—mentore

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Abstract

This PhD thesis is a comprehensive exploration aimed at elevating the nutritional profile of legume-derived ingredients and foods through different strategies, including exploiting the biodiversity of underutilized crops, leveraging natural modifications during germination, and repurposing food industry by-products with the integration of high-power low-frequency ultrasound technology. The research unfolds in two major segments: the first involves the investigation of lupin and the second concentrates on the recycling of a by-product from green pea canning. According to a preliminary literature review, lupin is an underutilized legume with an excellent nutritional profile; its integration in the diet could be advantageous, especially among vulnerable populations with inadequate intake of essential nutrients. Furthermore, according to numerous authors, ultrasonication has emerged as an up-and-coming technology, capable of improving many unit operations in food processing, such as extraction, homogenization, filtration, dehydration, freezing, thawing, and sanitization.

In the initial phase, the study focused on Andean lupin (*Lupinus mutabilis*): a screening was conducted across 33 ecotypes, plus 5 controls belonging to the Mediterranean species, to assess free phenolic compounds and explore the potential of FT-NIR for evaluating antioxidant properties. Additionally, an optimized debittering method was proposed and compared with traditional ones for impact on residual alkaloids, taste profile (by e-tongue), and antioxidants. Germination was also explored as a means to enhance lipophilic antioxidants. The results unveiled the remarkable free phenolic content of *L. mutabilis* (294.98–1342.98 mg/kg dry matter; DM), still present after the necessary removal of alkaloids, surpassing those of the *albus*, *angustifolius* and *luteus* species. Notably, the models based on FT-NIR spectra showed good retrieval for free phenolics, carotenoids, tocols, and antioxidant capacity compared to conventional methods, thus paving the way for a rapid, reliable, and non-destructive characterization of lupin seeds. The proposed debittering method, employing sodium chloride or citric acid solutions, shortened the traditional treatment (from 5 days to 45 h), reduced water consumption (from 100 L/kg to 45), and better preserved the antioxidants in lupin seeds, especially free phenolics, more prone to leaching. The e-tongue effectively differentiated samples treated with the same solvent, forming distinct clusters, with bitter and umami being the main tastes. Germination led to a considerable increase in carotenoid (12-fold) and vitamin E (from not quantifiable to 74.8 mg/kg DM) contents, representing a simple and effective treatment to improve lupin nutritional value.

The latter part of the thesis focused on recycling an industrially-generated canning by-product from green peas. Ultrasound-assisted protein extraction, by alkaline solubilization and isoelectric precipitation, was optimized, showcasing the effectiveness of ultrasound technology, which tripled recovery (from 21.5% to 66.6%) and reduced treatment duration fourfold (from 4 h to 1 h), when compared to the magnetic stirring method. The extract obtained had a protein content of 74.9 g/100 DM and microbial contamination below the guideline values; however, some protein degradation was also observed, likely due to pre-sampling fermentation. The by- product was then explored as a substrate for *Propionibacterium freudenreichii* fermentation to synthesize vitamin B12. The fermentation process yielded substantial amounts of vitamin (1374–1535 ng/g DM), and the resultant material was incorporated into bread (32.0–52.3 ng/g of vitamin), aiming to address the deficiency of B12 in strictly plant-based diets. The fortified product contained 32.0–52.3 ng/g B12, thus from 40 to 70 g of bread would provide the recommended daily intake. This section of the research highlights the potential for upcycling by-products to enhance the nutritional value of food while contributing to waste reduction.

The study fills critical knowledge gaps regarding lupin bioactive compounds and also introduces sustainable approaches for by-product utilization. The optimized ultrasound-assisted protein extraction from the pea by-product demonstrates a significant breakthrough, providing the food industry with practical solutions to enhance nutritional content and create value-added products. Future research should further explore the integration of the proposed ingredients into food preparations, assessing nutrient bioaccessibility by *in vitro* digestion and their potential impact on health and nutritional status by cell culture. Furthermore, the consumers' acceptability of foods and ingredients should be appraised. The outcomes of this research are poised to promote advancements in the production of healthier, sustainable, and value-added food products.

1

Introduction

The contents of this chapter were partially published in:

Estivi, L., Brandolini, A., Condezo-Hoyos, L., & Hidalgo, A. (2022). Impact of low-frequency ultrasound technology on physical, chemical and technological properties of cereals and pseudocereals. *Ultrason Sonochem*, *86*, 106044. https://doi.org/10.1016/j.ultsonch.2022.106044

Estivi, L., Brandolini, A., Gasparini, A., & Hidalgo, A. (2023). Lupin as a source of bioactive antioxidant compounds for food products. *Molecules*, *28*, 7529. https://doi.org/10.3390/molecules28227529

Current global food consumption is stuck in a paradox: while 770 million people starve, 13.6% of global population is obese, with the highest incidence in Oceania, North America and Europe (27–28%; FAO, 2022). Moreover, among human activities the food chain has the greatest impact on the environment. Facing the future overpopulation of 9.6 billion people by 2050, deep changes in food supply are needed, including a transition from animal to plant-derived protein sources, a trend already underway given the growing number of vegetarians and vegans (Willett et al., 2019).

Legumes are often referred to as inexpensive and thrifty crops whose seeds provide protein, dietary fibre and micro-nutrients besides calories, especially in the cereal-based diets of food deficit countries, where subsistence agriculture is practised (Thavarajah et al., 2019). Pulses are acknowledged as the main plant source of protein, whose amino acid composition is complementary to cereal proteins, poor in lysine but rich in sulphur-containing amino acids. Thanks to their nutritional profile, several health benefits are associated with pulses consumption, making legumes a relevant component of diet guidelines and recommendations. However, consumers' resistance in modifying their purchasing habits and the perceived "poor convenience", due to long-lasting cooking, still hinder legume consumption (Amoah et al., 2023; Hughes et al., 2022; Thavarajah et al., 2019). The development of user-friendly legume-derived ingredients and foods thus appears crucial in facilitating legume integration into current diets. However, an up-to-date

food industry cannot ignore the emerging issue of sustainability and should adopt a holistic approach, taking advantage of strategies such as: exploiting the biodiversity of underutilized legumes; modifying seeds composition by germination; recovering and reusing food by-products; applying efficient new technologies throughout the whole manufacturing process.

1.1 Lupin: an underutilized crop

Lupins are annual plants belonging to the Fabaceae family, genus *Lupinus*. More than 400 species are known (Reinhard et al., 2006), originating from the Mediterranean region, North Africa and North and South America. Four *Lupinus* species, i.e., white lupin (*Lupinus albus* L.), yellow lupin (*Lupinus luteus* L.), blue or narrow leaf lupin (*Lupinus angustifolius* L.) and Andean lupin (*Lupinus mutabilis* Sweet.) are widely grown as green manure, forage and food. *L. angustifolius*, *L. albus* and *L. luteus* (all from the Mediterranean region) are the most widespread species worldwide, while *L. mutabilis* (a.k.a. chocho or tarwi) is cultivated mainly in South America. According to the Food and Agriculture Organization (FAO), in 2021 the lupin cropped area was almost 1 million hectares worldwide, for a total production of 1.385 million metric tons; Australia is by far the most major lupin producer, with 865619 t (about 63% of world production), followed by Poland (221390 t), the Russian Federation (69723 t), Morocco (56856 t), Germany (53400 t), Chile (37049 t), Greece (15830 t), Peru (15790 t), France (15130 t) and South Africa (9876 t; fao.org/faostat; last accessed on 23 September 2023). Lupins are considered catch crops because they can grow in a wide range of soil and climate conditions, where other crops often fail, and because, like other legumes, they fix the atmospheric nitrogen in the soil through a symbiotic relationship with *Rhizobium* bacteria, and the fixed nitrogen becomes available also for subsequent crops. The fruit of the lupins is a pod that splits open along two seams and contains several seeds that may be eaten as snacks, in salads or side dishes, while their flour is used in the preparation of many foods, like vegetable milk, cheese and meat, fermented products, mayonnaise, chips, noodles, pasta, baked goods, etc. (Albuja-Vaca et al., 2019; Al-Saedi et al., 2020; Güémes-Vera et al., 2008; Jayasena, Khu, & Nasar-Abbas, 2010; Jayasena, Leung, & Nasar-Abbas, 2010; Özcan et al., 2021; Yaver & Bilgiçli, 2021). The flour does not contain gluten-forming proteins and is suitable for the manufacturing of gluten-free foods for people with coeliac disease. Furthermore, from the seeds, it is possible to extract an oil rich in monounsaturated and polyunsaturated fatty acids (Pascual-Chagman et al., 2021; Rodríguez et al., 2022) as well as protein isolates with outstanding physical, technological and functional properties (Alu'datt et al., 2017; Lo et al., 2021; Paraskevopoulou et al., 2010). This excellent composition confers to lupin-based foods many health benefits (Arnoldi et al., 2015), thanks to anticancer, antimicrobial, antidiabetic, antihypertensive,

antioxidant, anti-inflammatory and antimicrobial activities (Ishaq et al., 2022; Okagu et al., 2021).

The proximate composition of lupin seeds is reported in Table 1.1. They are characterized by high protein content, which may vary from 28 to 48 g/100 g depending on species, variety, growing conditions and soil types (Kohajdová et al., 2011; Ruiz-López et al., 2019); the lowest values are found in *L. angustifolius* and the highest in *L. mutabilis* (Briceño Berru et al., 2021; Carvajal-Larenas et al., 2016; Czubinski et al., 2019; Musco et al., 2017). The main storage proteins are globulins (80–90%), which are classified into four groups: α-conglutin (11S globulin), β-conglutin (7S globulin), γ-conglutin (7S basic globulin) and δ-conglutin (2S sulphur-rich albumin; Foley et al., 2011; Prusinski, 2017). Prolamins and glutelins are scarce (Gulewicz et al., 2008). Lysine, isoleucine, leucine, phenylalanine and tyrosine are in proportions appropriate for adults (FAO/WHO/ONU, 1985), while methionine and cysteine (sulphur-containing amino acids), valine and tryptophan are the principal limiting compounds (Sujak et al., 2006; Vogelsang-O'Dwyer et al., 2000). Lupin seeds contain high levels of dietary fibre (Martínez-Villaluenga et al., 2006; Musco et al., 2017), both before and after the removal of the hulls (a common step before milling). In fact, the flour on average contains 41.5 g/100 g dietary fibre (about 75% insoluble), which mainly consists of nonstarch polysaccharides (79% cellulose, 14% hemicellulose and 7% lignin) located in the endosperm cell walls (Ciesiołka et al., 2005). Lipid content in *L. albus*, *L. luteus*, and *L. angustifolius* seeds ranges from 4.6 to 13.5 g/100 g (Boschin & Arnoldi, 2011; Erbaş et al., 2005; Ruiz-López et al., 2019; Siger et al., 2023), while the *L. mutabilis* seeds have a higher concentration: Ruiz-López et al. (2019) determined a 14.7 g/100 g lipid content, while Briceño Berru et al. (2021) and Carvajal-Larenas (2019) recorded even higher values (on average 16.1 and 18.9 g/100 g, respectively), but some genotypes even reached 20 g/100 g (Carvalho et al., 2005). The oil extracted from lupin beans is characterized by scarce saturated fatty acids (10–27%) and abundant unsaturated fatty acids (78–87%), mainly oleic (25–63%), linoleic (13–57%) and linolenic acid (3–11%; Al-Amrousi et al., 2022; Boschin & Arnoldi, 2011; Musco et al., 2017; Sbihi et al., 2013; Siger et al., 2023; Yorgancilar & Bilgiçli, 2014), but maintains remarkably high oxidative stability (Czubiński & Siger, 2023; Multari et al., 2019; Rodríguez et al., 2022). Lupin seeds contain very little starch (5–12%), while nonstarch polysaccharides are abundant. Cellulose, hemicellulose and pectin are found in the hulls, while the oligosaccharides are predominant in the cotyledons (Prusinski, 2017). The total content of seed oligosaccharides (mainly stachyose and raffinose) varies depending on species, cultivar and environment: for example, in *L. albus* is 53.0 g/kg dry matter (DM; Mohamed & Rayas-Duarte, 1995) while in *L. mutabilis* is 41.5 g/kg DM (Czubinski et al., 2021). Lupin seeds are also a rich source of macro- (Ca, K, Na, and Mg) and micro-elements (Fe, Zn and Mn; Trugo et al., 2003; Zelalem & Chandravanshi, 2014), vitamins (thiamine, niacin and riboflavin) and phytochemicals with antioxidant capacity such as

Table 1.1. Lupin proximate composition (g/100 dry matter). Carbohydrates are determined by difference.

	Protein	Lipid	Ash	Dietary fibre		Carbohydrates	Starch
				Soluble	Insoluble		
L. albus							
Briceño Berru et al., 2021	29.9–32.2	7.6–9.5	3.3–3.5			45.7–49.7	
Erbaş et al., 2005	32.2	6.0	2.7	16.2			
Erbaş, 2010	41.3	9.9	2.6	14.6			2.8
Martínez-Villaluenga et al., 2006	30.6–37.4	11.3–14.6	3.6–3.8	3.6–5.2	30.8–34.2		2.8–3.3
Mavromatis et al., 2023	30.9–52.8			33.3–44.2			
Mohamed and Rayas-Duarte, 1995	38.0	10.0	4.0	34.0		48.0	3.0
Musco et al., 2017	30.9–45.4	7.04–10.6	4.4–6.8	4.7–8.4	34.9–47.4		
Sujak et al., 2006	44.7–48.2	4.5–6.0	4.3–4.9				
Yorgancilar and Bilgiçli, 2014	33–36	9.7–12	2.8–4.3				
L. luteus							
Briceño Berru et al., 2021	39.0	14.3	3.4			35.2	
Martínez-Villaluenga et al., 2006	36.8–37.9	8.5–8.8	3.1–5.0	3.2–4.9	28.8–31.1		4.0–4.5
Musco et al., 2017	32.2–36.2	5.2–5.9	4.0–6.6	9.8–11.6	39.2–41.5		
Sujak et al., 2006	35.1–37.6	10.4–12.6	3.7–4.1				
L. angustifolius							
Briceño Berru et al., 2021	29.2	5.2	3.2			51.3	
Musco et al., 2017	27.7–30.3	3.3–4.6	3.1–4.7	6.3–8.1	47.3–51.1		
Sujak et al., 2006	29.5–35.6	5.5–8.6	3.5–4.0				
L. mutabilis							
Briceño Berru et al., 2021	32.0–46.9	13.6–18.6	2.7–4.4			24.9–33.9	
Carvajal-Larenas et al., 2014	41.4	23.4	5.0			31.7	
Córdova-Ramos et al., 2020	47.4	16.2	4.8			23.0	
Czubinski et al., 2021	44.7	15.4				26.6	
Romero-Espinoza et al., 2020	39.9	15.5	3.4	7.1		33.9	

The raw fibre is reported across the two columns of soluble and insoluble fibre.

carotenoids, tocopherols and phenolic compounds (Brandolini et al., 2022; Briceño Berru et al., 2021; Czubinski et al., 2021).

Compared to other legumes, lupin seeds are low in antinutritional factors such as trypsin inhibitors, lectins and saponins (Martínez-Villaluenga et al., 2006). Phytic acid, a compound that reduces the bioavailability of some minerals through cation chelation, is scarce (0.25–0.62 g/100 g in white lupin seeds; Martínez-Villaluenga et al., 2006; Saastamoinen et al., 2013) and significantly inferior than in soybean (1.54–2.27%; Saastamoinen et al., 2013). The main antinutritional compounds in lupin seeds are the quinolizidine alkaloids (Cortés-Avendaño et al., 2020; El-Adawy et al., 2001; Jiménez-Martínez et al., 2001; Sujak et al., 2006), which have a bitter taste and are often toxic, so they must be removed before consumption. The existing varieties with genetically low alkaloid content (Kroc et al., 2019; Uauy et al., 1995) have low yields, are more susceptible to pests and diseases and over time are inclined to regain their bitterness (Cardoza et al., 2006; Uauy et al., 1995). In general, debittering is achieved by removing the alkaloids from whole seeds using water as a solvent. Nevertheless, the chemical composition of the seeds is altered because several water-soluble molecules are also washed away: minerals, carbohydrates and oligosaccharides decrease, while lipids and proteins increase as a consequence of concentration due to the elimination of the other compounds (Carvajal-Larenas et al., 2016; Córdova-Ramos et al., 2020; Erbaş, 2010). The traditional debittering method starts with hydration in water, followed by cooking and repeated washings for several days (Carvajal-Larenas et al., 2014; Erbaş, 2010). Besides disrupting cell walls and facilitating alkaloid removal, cooking blocks germination, coagulates proteins, inactivates enzymes and sanitizes the product (Carvajal-Larenas et al., 2013; Gross et al., 1983). To shorten processing time and save water, several improved debittering methods have been proposed. Generally, they include longer boiling times (Jiménez-Martínez et al., 2001), post-cooking washing with warm water (Guadagnucci Fontanari et al., 2012), different rinsing solutions (Jiménez-Martínez et al., 2001; Mohammed et al., 2017; Villacrés et al., 2020), germination (de Cortes Sánchez et al., 2005; Mohammed et al., 2017), fermentation (Jiménez-Martínez et al., 2007) or a combination of these methods (Erbaş, 2010; Jiménez-Martínez et al., 2010).

The main antioxidant of lupin (i.e., tocopherols, carotenoids and phenolic compounds) are sensitive to heat, oxidation or washout, so their content may vary greatly after debittering. Therefore, lupin antioxidants should be determined in plausible conditions of use after alkaloids removal, which is unavoidable even for some so-called *sweet* varieties. While lipophilic antioxidants appeared scarcely affected, or even concentrated, by debittering (Brandolini et al., 2022; Briceño Berru et al., 2021), with regards to phenolic compounds, the available studies provide very limited information; moreover, the soluble conjugated and insoluble bound fractions of phenolic compounds have barely been studied.

1.2 Germination

Germination is the sum of events that break the seed quiescence status and allow the embryonic axis to appear. As a first step, the seed hydrates so the pre-existent enzymes are activated and initiate the Krebs cycle, the endosperm reserves are mobilized, and the protein synthesis becomes intensive. Germination ends with cells elongation, which leads to root and shoot emission (Srivastava, 2002). The activation of dormant enzymes during germination leads to significant changes in biochemical and nutritional characteristics. The α- and β-amylases rapidly degrade the carbohydrates, with a consequent surge in reducing sugars, while other enzymes decompose cell walls and enhance the accessibility of internal nutrients (Olaerts & Courtin, 2018). Proteins are hydrolysed, increasing peptides and amino acids availability (Olaerts & Courtin, 2018). Various bioactive compounds, such as tocols, thiamine, riboflavin, folic acid, vitamin C and carotenoids, are bio-synthesized to generate the nutrients necessary for seedling growth (Aborus et al., 2018; Olaerts & Courtin, 2018). The metabolic activities fuel phenolic compounds biosynthesis (Benincasa et al., 2015; Hidalgo et al., 2019), while the degradation of cell walls leads to an increase of readily available free phenolic compounds (Van Hung et al., 2011). The abundant presence of bioactive molecules boosts the antioxidant capacity (Alvarez-Jubete et al., 2010). Furthermore, sprouting increases phytase activity, leading to phytic acid degradation and better micronutrients absorption in the gastrointestinal tract (Alvarez-Jubete et al., 2010; Gupta et al., 2015). Overall, controlled germination improves the nutritional composition of the kernels, but the enzymatic activity may have a negative impact on some technological properties of the flours (e.g., leavening), stressing the importance of a carefully controlled process to avoid excessive hydrolysis (Olaerts & Courtin, 2018).

In legumes, germination can be conveniently used a "green food engineering method" (Gan et al., 2017) to reduce antinutritional factors such as trypsin and amylase inhibitors, tannins, lectins (Anaemene & Fadupin, 2022; Savelkoul et al., 1992), oxalic acid (Anaemene & Fadupin, 2022), phytic acid (Anaemene & Fadupin, 2022; Dagnia et al., 1992; Mohammed et al., 2017; Satya et al., 2010) and oligosaccharides (Chilomer et al., 2013; El-Adawy, 2002; Vidal-Valverde et al., 2002). Table 1.2 summarizes the effect of germination on compounds with vitamin or antioxidant activity in different legumes. Generally, large variations are observed, even within the same type of legume, due to different conditions or ecotypes. B vitamins tend to increase, with the notable exception of thiamine, usually lower in germinated pulses, but whose loss can be limited adjusting the photoperiod (Prodanov et al., 1997). Total carotenoids and phenolic compounds often decrease after germination, while ascorbic acid and α-tocopherol always increase. Overall, limited information is available about how germination affects the individual carotenoids and whether any substantial increase in antioxidants occurs in germinated lupin.

Table 1.2. Effect of optimal germination conditions on compounds with vitamin or antioxidant activity in legumes.

Compound	Legume	Time (d)	Illumination	Variation	Method	Reference
Vitamin A	soybean	4	n	81%	HPLC	Plaza et al., 2003
Total carotenoids	mung bean	1	n	212%	Spectrophotometer	Alkaltham et al., 2020
Total carotenoids	pigeon pea	3	–	-12%	Spectrophotometer	Anaemene and Fadupin, 2022
Total carotenoids	fenugreek	4	n	482%	HPLC	El-Sebaiy and El-Mahdy, 1983
Total carotenoids	cowpea	4	n	-16%	Spectrophotometer	James et al., 2020
Total carotenoids	common bean	4	n	-57%	Spectrophotometer	James et al., 2020
Vitamin B1	pigeon pea	3	n	60%	HPLC	Chinma et al., 2022
Vitamin B1	chickpea	3	n	-38%	Microbiological assay	El-Adawy, 2002
Vitamin B1	soybean	4	n	328%	Spectrophotometer	Plaza et al., 2003
Vitamin B1	faba bean	6	n	-28%	HPLC	Prodanov et al., 1997
Vitamin B1	lentils	6	n	-(11–14)%	HPLC	Prodanov et al., 1997
Vitamin B1	common bean	6	n	-7%	HPLC	Vidal-Valverde et al., 2002
Vitamin B1	common bean	6	y	-8%	HPLC	Vidal-Valverde et al., 2002
Vitamin B1	pea	6	n	-4%	HPLC	Vidal-Valverde et al., 2002
Vitamin B1	pea	6	y	1%	HPLC	Vidal-Valverde et al., 2002
Vitamin B1	lentils	6	n	-4%	HPLC	Vidal-Valverde et al., 2002
Vitamin B1	lentils	6	y	-6%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	pigeon pea	3	n	134%	HPLC	Chinma et al., 2022
Vitamin B2	chickpea	3	n	16%	Microbiological assay	El-Adawy, 2002
Vitamin B2	soybean	4	n	1047%	Spectrophotometer	Plaza et al., 2003
Vitamin B2	lentils	6	n	38–320%	HPLC	Prodanov et al., 1997
Vitamin B2	faba bean	6	n	253%	HPLC	Prodanov et al., 1997
Vitamin B2	lentils	6	y	133%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	lentils	6	n	167%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	common bean	6	n	86%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	common bean	6	y	82%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	pea	6	n	135%	HPLC	Vidal-Valverde et al., 2002
Vitamin B2	pea	6	y	135%	HPLC	Vidal-Valverde et al., 2002

Photoperiod is indicated in hours, where available;
continued on the next page.

Compound	Legume	Time (d)	Illumination	Variation	Method	Reference
Vitamin B3	chickpea	3	n	-5%	Microbiological assay	El-Adawy, 2002
Vitamin B3	lentils	6	n	26–71%	HPLC	Prodanov et al., 1997
Vitamin B3	faba bean	6	n	72%	HPLC	Prodanov et al., 1997
Vitamin B6	pigeon pea	2	n	100%	HPLC	Chinma et al., 2022
Vitamin B6	chickpea	3	n	4%	Microbiological assay	El-Adawy, 2002
Vitamin B6	soybean	4	n	145%	Spectrophotometer	Plaza et al., 2003
Vitamin B9	soybean	4	16 h	251–273%	HPLC	Shohag et al., 2012
Vitamin B9	mung bean	4	16 h	304–336%	HPLC	Shohag et al., 2012
Vitamin C	pigeon pea	3	n	577%	Titration	Chinma et al., 2022
Vitamin C	blue lupin	6	n	213%	MECC	Fernandez-Orozco et al., 2006
Vitamin C	mung bean	7	n	388%	MECC	Fernandez-Orozco et al., 2008
Vitamin C	white lupin	9	n	766%	MECC	Frias et al., 2005
Vitamin C	soybean	4	n	218%	Titration	Plaza et al., 2003
Vitamin E	blue lupin	5	n	2767%	HPLC	Fernandez-Orozco et al., 2006
Vitamin E	mung bean	7	n	309%	HPLC	Fernandez-Orozco et al., 2008
Vitamin E	soybean	4	n	93–185%	HPLC	Fernandez-Orozco et al., 2008
Vitamin E	white lupin	9	n	1953%	HPLC	Frias et al., 2005
Vitamin E	soybean	4	n	564%	HPLC	Plaza et al., 2003
Phenolic acids	mung bean	1	n	30%	HPLC	Alkaltham et al., 2020
Phenolic acids	common bean	6	n	54%	HPLC	López-Amorós et al., 2006
Phenolic acids	common bean	6	y	511%	HPLC	López-Amorós et al., 2006
Phenolic acids	pea	6	n	-70%	HPLC	López-Amorós et al., 2006
Phenolic acids	pea	6	y	-62%	HPLC	López-Amorós et al., 2006
Phenolic acids	lentils	6	n	-69%	HPLC	López-Amorós et al., 2006
Phenolic acids	lentils	6	y	-45%	HPLC	López-Amorós et al., 2006
Phenolic acids	lentils	8	n	182%	HPLC	Świeca et al., 2012
Phenolic acids	lentils	8	12 h	265%	HPLC	Świeca et al., 2012
Flavonoids	mung bean	1	n	83%	HPLC	Alkaltham et al., 2020
Flavonoids	soybean (n=6)	1	n	-63–85%	HPLC	Lin and Lai, 2006
Flavonoids	soybean (n=7, black)	1	n	-12%–39%	HPLC	Lin and Lai, 2006
Flavonoids	lentils	8	n	-81%	HPLC	Świeca et al., 2012
Flavonoids	lentils	8	12 h	-80%	HPLC	Świeca et al., 2012

1.3 Recovery and upcycling of food by-products

In 2011, about 1.3 billion tonnes of food were wasted in the world, accounting for roughly one third of global food production (FAO, 2011). Currently, FAO (2019) defines *food loss and waste* (FLW) as

> "the decrease in quantity or quality of food along the food supply chain. [...] Food loss results from decisions and actions by suppliers. [...] Therefore it concerns all stages of the food supply chain up to, but excluding, the point where there is interaction with the final consumer and thus excludes retail, food service providers and consumers. Food waste is the result of purchasing decisions by consumers, or decisions by retailers and food service providers that affect consumer behaviour."

Food loss is currently preferably expressed on a relative basis to provide a more durable estimate. Roughly 14% of the current food production is *lost* on average, with the greatest share from roots, tubers and oil-bearing crops (25.5% of loss) followed by fruit and vegetables (21.6%), meat and animals products (12%), and cereals and pulses (8.7%; FAO, 2019). Around 17% of food is instead *wasted* at households (67%), food services (26%) and retailers (13%), corresponding to 931 million tonnes (Mt) in 2019 (UNEP, 2021). In the European Union (i.e., EU28) the 2011 FLW was estimated in 129.2 Mt, equivalent to 257 kg per capita; fruit and vegetables had an overall impact of 46%, being characterized by the highest *food loss* (33 Mt) and *food waste* (26.2 Mt). However, high uncertainty is associated with this kind of analysis: the coefficient of variation was 16% for *food loss*, while it spanned from 12% to 46% for *food waste* assessed at retail, household and food service (Caldeira et al., 2019).

FLW absorbs 31% of global food production and has shown no decreasing trend in the last decade. Besides the ethical issue, it poses a serious environmental footprint due to the unnecessary surplus of greenhouse gases emissions (4.4 billion tonnes of CO_2 equivalent), blue water exploitation (250 km^3) and occupied soil (1.4 billion hectares; Xue et al., 2017). Moreover, due to the large amount of organic matter, food waste presents a high chemical and biochemical oxygen demand, thus resulting hazardous and pollutant (Anal, 2017). Reducing the amount of FLW has become a priority since the definition by United Nations of the Sustainable Development Goal (SDG) 12.3, aimed at

> "halving per capita global food waste at the retail and consumer levels and reducing food loss along production and supply chains (including post-harvest losses) by 2030." (FAO, 2019)

The food diverted to feed, cropping or industrial purposes can be considered lost or wasted only if originally destined to human consumption. Inedible parts, although not considered food, cannot be deducted from *food loss*, which

is based on gross production and includes all post-harvest losses (FAO, 2019). Some processes inherently generate inedible residues by dividing raw materials into their components, as in the case of the pomace resulting from juice extraction. These materials (e.g., fruits and vegetables pomace, seeds or peels) could be considered as non-food side-streams, when disposed of, or source of food, whether they are re-utilized for food manufacturing. The European Directive 2008/98/EC (2008) clarifies this concept distinguishing *waste* (art. 3),

> "any substance or object which the holder discards or intends or is required to discard",

from *by-product* (art. 5),

> "a substance or object, resulting from a production process, the primary aim of which is not the production of that item, [...] if the following conditions are met:
>
> a) further use of the substance or object is certain;
> b) the substance or object can be used directly without any further processing other than normal industrial practice;
> c) the substance or object is produced as an integral part of a production process; and
> d) further use is lawful, i.e. the substance or object fulfils all relevant product, environmental and health protection requirements for the specific use and will not lead to overall adverse environmental or human health impacts."

The same Directive provides a general framework defining (art. 4)

> "a priority order in waste prevention and management legislation and policy:
>
> a) prevention;
> b) preparing for re-use;
> c) recycling;
> d) other recovery, (e.g. energy recovery); and
> e) disposal."

In agreement with the SDG 12.3, the Directive 2018/851/EC (2018) was recently adopted partially amending the Directive 2008/98/EC (2008) and highlighting the necessity for a "transition towards a circular economy" and a "prudent, efficient and rational utilization of natural resources".

Food by-products were traditionally destined to livestock feeding or, in the worst case, to incineration or landfill. The current trend aims at their re-use for human nutrition by two main strategies: the extraction of functional or bioactive fractions (e.g., dietary fibre, protein, phenolic compounds) or the direct addition in traditional formulations (Caponio et al., 2022; Socas-Rodríguez et al., 2021; Tassoni et al., 2020). Galanakis (2012) defined a general framework for the extraction and recovery of food components based on the following five stages.

- Macroscopic pre-treatment, aimed at preparing the matrix by regulation of the water content. Drying, centrifugation, compression, filtration or wetting are the typical treatments of this stage.
- Macro- micromolecules separation: interfering fractions are isolated by alcohol or isoelectric precipitation, or ultrafiltration.
- Extraction: target molecules are solubilized in a carrier (e.g., solvent, acid or alkali) exhibiting, ideally, the highest affinity and specificity. Ethanol is frequently used, but deep eutectic mixtures and supercritical fluids are gaining popularity offering a non-toxic, non-hazardous alternative to traditional apolar solvents (e.g., hexane).
- Isolation and purification: co-extracted impurities are removed by selective techniques (e.g., dialysis, reverse osmosis, nanofiltration, chromatography).
- Product formation by freeze- or spray-drying, emulsification, encapsulation, extrusion.

This structure is then tailored according to the by-product characteristics. Mild conditions (e.g., low temperatures, enzymatic hydrolysis, use of green solvents) and emerging technology (e.g., high-power ultrasound, micro- and nano-filtration, microwaves, pulsed electric fields, high voltage electrical discharge, electric-osmotic dewatering) are increasingly adopted to minimize the impact on sensitive extracts, improve the recovery, shorten treatments duration and, in some cases, to maintain the process economical (Ben-Othman et al., 2020; Galanakis, 2012). Novel technologies are often called "green technologies" thanks to their efficient consumption of energy and solvents. However, relevant entry barriers hinder their diffusion, including high costs of the facilities, advanced know-how, difficult scalability (Plazzotta & Manzocco, 2019). The complexity of the extraction processes may be pushed to the point of complete fractionation. For instance, in the model proposed by Banerjee et al. (2017), fruit processing wastes are separated into protein, oil, polyphenols, pectins, and the residual material is used as biofuel. A zero-waste approach is expensive, but the added-value of the extracts easily overcomes the costs when their purity allows the employment in the manufacturing of drugs, cosmetics or food supplements (Mazzutti et al., 2020).

The recycling of by-products into food must undergo a preliminary feasibility assessment: food waste should be available in sufficient amount, continuously and with constant composition (Plazzotta & Manzocco, 2019). By-products often display a high moisture content along with sugar, protein or lipid substrates, thus being prone to microbial degradation. Heavy metals, pesticides, toxins, pathogens, dioxins or PCBs easily contaminate food waste materials and could be further concentrated during processing (Socas-Rodríguez et al., 2021). Also for this reason, in the European Union by-products employed as ingredients must have a solid history of safe use, otherwise they come under the *novel foods* regulation, being authorized only after safety assessment (Reg. 2015/2283/EU, 2015). Finally, consumers' ac-

ceptability must be taken into account, considering that people generally have conservative food habits and distrust innovations; nonetheless, sustainability has become a driver of demand (Plazzotta & Manzocco, 2019).

Overall median *food loss* in the legume sector is concentrated in the post-harvest (10.4%) and processing (5.3%) stages, values far below of those estimated for vegetables, because pulses (i.e., legume dry seeds) are barely perishable. Conversely, about 26% of green legumes is lost during the post-harvest (faostat.org), of which from 5% to 25% due to industrial processing (i.e., canning, freezing or drying; Tassoni et al., 2020). Based on the production data summarized in Table 1.3, the greatest flows of by-products are associated with fresh beans and peas, so they are composed of pods, hulls (also indicated as husks or seed coats), leaves, stems and dark, spotted or broken seeds (Tassoni et al., 2020).

Table 1.3. Global (World) and European (EU27) gross production of legumes in 2021 expressed as millions tonnes (Mt) and relative shares (%; faostat.org).

	World (Mt)	World (%)	EU27 (Mt)	EU27 (%)
Soya beans †	371.69		2.71	
Peanuts †	53.93			
Total pulses	88.97	100	4.48	100
Beans	27.72	31		
Chick peas	15.87	18		
Peas	12.40	14	1.84	41
Cow peas	8.99	10	0.00	
Faba beans	5.96	7	1.12	25
Lentils	5.61	6		
Pigeon peas	5.48	6		
Other pulses	4.69	5	1.19	27
Lupins	1.38	2	0.32	7
Vetches	0.63	1		
Bambara beans	0.24			
Total fresh legumes	45.67		2.18	
Beans	23.41	51	1.08	50
Peas	20.53	45	0.86	39
Faba beans	1.73	4	0.24	11

† Reported under oilseed crops despite belonging to Fabaceae.

The recycling of these materials is highly focused on protein extraction, although the protein content ranges widely among hulls (5–14%), pods (10–14%), cotyledon parts (23–27%) and oilseed meals (34–55%), with a median value of 14% (Kamani et al., 2023). Dry fractionation is rarely applicable, due to moisture content, while alkaline solubilization followed by isoelectric precipitation is by far the preferred method. However, drastic pH changes are often detrimental for protein interfacial properties due to irreversible de-

naturation and aggregation, thus milder techniques like enzymatic hydrolysis and separation by membrane ultrafiltration may be employed. To improve the recovery, protein extraction is frequently assisted with microwaves, high-pressure homogenization (Kamani et al., 2023; Nartea et al., 2023; Tassoni et al., 2020) or high-power ultrasound (Estivi et al., 2022), a thriving technology extensively discussed in the following section.

Wet by-products are usually dried and milled to flour, to be used as an ingredient. Sometimes blanching is adopted to pre-gelatinize starch and reduce contaminant bacteria. The addition of legume by-products powder as bulking agent or source of fibre, sometimes in the purified form, has been extensively evaluated in bread, cake, extruded snacks, crackers, cookies, gluten-free pasta, noodles, low-fat chicken nuggets, soups and mayonnaise (Nartea et al., 2023). Besides solid by-products, since 2014 aquafaba (i.e., the cooking water of canned chickpeas) gained notoriety when vegan community discovered that its emulsifying properties could imitate egg white in whipped food preparations (Serventi, 2020). Aquafaba was successfully tested as egg replacer in sponge cake (Mustafa et al., 2018) and meringue (Stantiall et al., 2018). Another liquid by-product with peculiar properties is the washing water from lupin debittering. From it, lupanine and sparteine alkaloids can be purified and employed in the manufacturing of drugs (Esteves et al., 2020) or insecticides (Cely-Veloza et al., 2023).

The major drawbacks reported after foods supplementation with legume by-products are related to sensory acceptability due to unpleasant aspect, texture or flavour. Additionally, the presence of heat-stable antinutritional factors (i.e., phytic acid, trypsin inhibitors, tannins, saponins and oligosaccharides) must be acknowledged, albeit lactic fermentation is emerging as a novel strategy suitable for their reduction (Gobbetti et al., 2020; Nartea et al., 2023). In addition, fermentation releases phenolic aglycones from bound and conjugated fractions, improving their bioaccessibility (Gobbetti et al., 2020). This is particularly relevant because legume by-product are often richer in phenolics than cotyledons (Dueñas et al., 2004).

1.4 High-power ultrasound for food processing

Ultrasound is a mechanical wave with frequency higher than 20 kHz, beyond the audible frequency range of humans. It can be differentiated in three frequency ranges:

- 20 to 100 kHz, conventional high-power or low-frequency ultrasound (US, hereafter);
- 100 kHz to 2 MHz, sonochemistry range;
- 5 to 10 MHz, medical or analytical range (T. J. Mason & Peters, 2002).

Over the past twenty years, low-frequency ultrasound (US) has gained popularity in the food industry and today represents a versatile technology used

to improve yield and/or accelerate various unit operations such as extraction, homogenization, filtration, dehydration, freezing, thawing and sanitization (Awad et al., 2012; Bhargava et al., 2021; Mohammadi et al., 2021). The simplicity of use, the relatively low costs of the systems and their operation, and the reduction of treatment times, and therefore of energy costs, are the basis of the success of ultrasound (Bhargava et al., 2021; T. J. Mason & Cintas, 2002; T. J. Mason et al., 1996).

US can be employed to make changes in liquids, dispersions, solid and gaseous media, but in any case a liquid, thus incompressible, medium is necessary (Feng & Yang, 2011). In fact, US effects are mainly related to the cavitation phenomenon: during the negative pressure half-wave (rarefaction) the medium is stressed by tensile forces that increase the distance among molecules; once the cavitation threshold is reached, the liquid breaks down and empty bubbles form. When a cavity cannot tolerate the pressure of surrounding liquid, it implodes violently and energy is immediately released. Cavities collapse is far more rapid than heat diffusion, creating a localized hot spot while the liquid bulk remains cold (Suslick, 1990). Inside the cavities, temperatures of 1700–4700 °C and pressures of 180–300 MPa are reached (T. J. Mason & Peters, 2002). As temperature rises, cavitation threshold goes down due to drops in surface tension and viscosity, thus a liquid will start to cavitate at lower sound pressure. However, water vapour pressure will be higher and vapour-filled bubbles will form, reducing pressure difference and cushioning bubbles implosions (T. J. Mason & Lorimer, 2002). Very high tensile forces are needed for cavitation. Energy and intensity, along with medium viscosity, surface tension, vapour pressure, nature and concentration of dissolved gas, presence of solid particles, temperature, and pressure determine the extent of cavitation (Soria & Villamiel, 2010). Gas-filled bubbles or particulate produce weak spots in the liquid that allow the process to begin; alternatively, pre-generated cavitation bubbles can work as nuclei. The most common frequency range applied is 20–40 KHz, because cavitation is hardly achieved at high frequencies unless very great intensities are adopted (Suslick, 1990). The true mechanical power in the liquid is usually far lower than the nominal one; in addition, devices with different efficiency, geometry and transducers can be used and the liquid bulk volume may change as well. Therefore, to compare results it is necessary to experimentally evaluate the specific amount of energy per volume unit, an often-overlooked aspect (Guimarães et al., 2020). The volumetric power can be estimated by the following formula (Margulis & Margulis, 2003):

$$P = m\, C_p \frac{dT}{dt}$$

where m is the water mass (g), C_p its specific heat (4.186 $\mathrm{J\,g^{-1}\,{}^{\circ}C^{-1}}$) and $\frac{dT}{dt}$ the temperature increase ($\mathrm{{}^{\circ}C\,s^{-1}}$) during the first 90 s of sonication. Besides the mechanical effect, US generates free radicals through water homolytic

cleavage: $H_2O \longrightarrow H^{\bullet} + OH^{\bullet}$ (T. J. Mason & Peters, 2002; Suslick, 1990). However, free radicals are produced at the fastest rate from water at around 200 kHz (i.e., in the sonochemistry range; T. J. Mason & Peters, 2002), while at 20 kHz their development is minimal (Ashokkumar et al., 2008; T. J. Mason & Peters, 2002).

Several studies have suggested ultrasound as a useful technology to accelerate seed hydration and promote germination (Borsato et al., 2019; Ding et al., 2018; Kalita et al., 2021; Miano et al., 2017; Xia et al., 2020; Yaldagard et al., 2008). According to Miano et al. (2016) two different mechanisms are involved: a) direct, related to inertial flow and *sponge effect*, where the ultrasound-induced cavitation creates a compression-rarefaction turnover that squeezes and releases the matrix, pumping water through pre-existing pores; and b) indirect, related to the formation of new micro-channels because of physical damage to the tissues. However, low water activity does not allow cavitation, hence dry seeds ($a_w \approx 0.65$) undergo slow hydration (Miano et al., 2016). In fact, new cavities begin to form in the matrix only when an adequate a_w is reached, implying that US treatments are effective when applied on pre-soaked matrices (Chatchavanthatri et al., 2020). Generally, faster germination and higher success ratio are observed in ultrasound-treated seeds, primarily thanks to the improved hydration. In fact, cavitation causes fissures in the pericarp, augmenting the availability of water and oxygen (Ding et al., 2018; Yaldagard et al., 2008) and probably facilitates the mobilization of endosperm nutrients by disrupting cell membranes (Yaldagard et al., 2008). In addition, US-treated wheat seeds exhibit greater antioxidant activities of catalase, superoxide dismutase and glutathione reductase: apparently, reactive oxygen species (ROS) generated by cavitation elicit an antioxidant response able to enhance plant vigour (Y.-p. Chen et al., 2013).

Currently, the most popular US application is improving extraction processes by enhancing yield (i.e., recovery) and abbreviating duration. In the presence of ultrasound, it is possible to adopt non-toxic green solvents (e.g., water, ethanol or deep eutectic solvents), which are safe, non-polluting and often cheaper than traditional counterparts (Shang et al., 2019; Vilkhu et al., 2008). The improvement in yields with ultrasonication is mainly due to the mechanical impact, which facilitates the diffusion of solutes by formation of pores and channels; disruption of cellular compartments; the micro-movement that renews the solvent layer in contact with the solutes, maintaining a high concentration gradient (Ashokkumar, 2015; Vilkhu et al., 2008). Combining ultrasound with other techniques (e.g., enzymatic pretreatment, supercritical fluids) allows the extraction of oil from seeds and their by-products in aqueous environment (Cravotto et al., 2004; Pingret et al., 2013). However, the recovery of hydrophilic nutraceuticals remains the most common use of US: numerous sources described its efficiency in extracting phenolic compounds from cereal industry by-products (Cengiz et al., 2021; L. Chen et al., 2018; X. Chen et al., 2020; Cherif et al., 2020; Das et al., 2017; Đordević & Antov,

2017; Jha et al., 2017; X. Luo et al., 2018; Muangrat et al., 2018; J. Wang et al., 2008; Zeng et al., 2019; Zheng et al., 2016). Giopato Viell et al. (2020) suggested that cavitation could release phenolic compounds from the insoluble bound fraction. US treatment of legume flours is instead mainly aimed at obtaining protein isolates through alkaline extraction and isoelectric precipitation (Byanju et al., 2020; Quintero-Quiroz et al., 2021; F. Wang et al., 2020). Over-treatment with ultrasound leads to poor performances: power excess lowers yields since cavitation bubbles hinder wave propagation, while too long treatments increase the quantity of suspended impurities worsening solvent penetration. In addition, dissipation of high power can be detrimental for heat-sensitive compounds, requiring the use of intermittent cycles. The extractive capacity may also be interpreted as the removal of anti-nutritional or toxic components: Mohammadi et al. (2021) and Yadav et al. (2021) documented a greater reduction of phytates in rice bran and millet flour, respectively, Navarro del Hierro et al. (2018) of saponins in quinoa, lentil, fenugreek, soy and lupin flours, and Miano et al. (2019) of quinolizidine alkaloids in lupin seeds.

Ultrasound can also improve and regulate the technological properties of food macromolecules. In protein, cavitation disrupts weak interactions (i.e., electrostatic bonds, hydrogen bonds, hydrophobic effect), thus leading to conformation changes in the secondary and tertiary structures. During ultrasound-induced denaturation, proteins unfold exposing the hydrophobic core, rich in phenylalanine, tyrosine, and tryptophan residues, and thus augmenting the surface hydrophobicity and improving the interfacial properties (Constantino & Garcia-Rojas, 2020; Jia et al., 2010; Jin et al., 2012; S. Li, Yang, Zhang, Ma, Liang, et al., 2016; S. Li, Yang, Zhang, Ma, Qu, et al., 2016; X. Li et al., 2018; Nazari et al., 2018; O'Sullivan et al., 2016; Ren et al., 2017, 2018; X. Yang et al., 2017, 2020; H. Zhang et al., 2020; K. Zhang et al., 2022; X. Zhang et al., 2021; Y. Zhang, Li, et al., 2018; Y. Zhang, Ma, Wang, Qu, Wali, & Zhou, 2015; C. Zhou et al., 2013). Disulphides are also affected, although far higher energy is required to reduce covalent S–S bonds (226 $kJ\,mol^{-1}$) than to disrupt weak interactions (13 $kJ\,mol^{-1}$; Ashokkumar et al., 2008; K. Zhang et al., 2022; Y. Zhang, Li, et al., 2018). This can be exploited for regulating the firmness of gluten (H. Zhang et al., 2020; Y. Zhang, Li, et al., 2018; Y. Zhang, Ma, Wang, Qu, Li, et al., 2015). The above described changes do not affect protein molecular weight, but can break down aggregates (K. Zhang et al., 2022). Nevertheless, overtreatment may result, on the opposite, in new intermolecular disulphide and hydrophobic interactions, forming larger aggregates (Constantino & Garcia-Rojas, 2020; O'Sullivan et al., 2016; Quintero-Quiroz et al., 2021; Vera et al., 2019; K. Zhang et al., 2022; C. Zhou et al., 2013). With regards to protein secondary structure, the relative number of α-helices decreases with sonication in favour of β-sheets and random coils (S. Li, Yang, Zhang, Ma, Liang, et al., 2016; X. Li et al., 2018; X. Yang et al., 2017; H. Zhang et al., 2020; K. Zhang et al., 2022; Y. Zhang, Ma, Wang, Qu, Li, et al., 2015; Y. Zhang, Ma, Wang,

Qu, Wali, & Zhou, 2015), while intramolecular β-sheets are converted to intermolecular (Y. Zhang, Ma, Wang, Qu, Li, et al., 2015).

In starch granules, ultrasound treatments induce pores, depressions and cracks because of the rapid water jets produced by the cavitation bubbles collapse. Ultrasonication breaks down waxy starches particles into smaller ones, but causes aggregation in amylose-rich starches (Boufi et al., 2018; Kang et al., 2016; C. Li et al., 2017). According to Kang et al. (2016) and C. Li et al. (2017), US first disrupts weak interactions and then cleaves covalent C-O-C α-1,6 glycosidic bonds (apparently less stable than α-1,4 bonds), thus leading to amylopectin debranching (Q.-Y. Yang et al., 2019). Generally, a decrease in gelatinization enthalpy is observed, due to the loss of amylopectin double helices, essential for granule integrity (Amini et al., 2015; Boufi et al., 2018; Cooke & Gidley, 1992; Huang et al., 2007; C. Li et al., 2017; Yu et al., 2013; X. Zhou et al., 2021). B-type starches, which have a hexagonal arrangement enclosing a cavity occupied by freezable water, are more susceptible to US-induced modification (Z. Luo et al., 2008; Pérez et al., 2009). These changes affect some of the pasting properties of the flours, as peak viscosity, breakdown, and setback, but not the pasting temperature (Hu et al., 2015; Huang et al., 2007; Isono et al., 1994; C. Li et al., 2017; M. Li et al., 2018; Y. Li et al., 2019; Z. Luo et al., 2008; Q.-Y. Yang et al., 2019; Zuo et al., 2009). According to C. Li et al. (2017) and Zuo et al. (2009), the decrease in viscosity is due to amylose and long linear amylopectin depolymerization. In fact, the partial hydrolysis of amylose results in a weaker network and smaller increase in viscosity during setback (W. R. Mason, 2009; Varavinit et al., 2003). Lastly, complete starch liquefaction can be achieved, which is useful for saccharification (Montalbo-Lomboy et al., 2010; Shewale & Pandit, 2009).

References

Aborus, N. E., Tumbas Šaponjac, V., J., Č.-B., Ćetković, G., Hidalgo, A., Vulić, J., & Šeregelj, V. (2018). Sprouted and freeze-dried wheat and oat seeds - phytochemical profile and *in vitro* biological activities. *Chem Biodivers*, *15*, e1800119. https://doi.org/10.1002/cbdv.201800119

Al-Amrousi, E. F., Badr, A. N., Abdel-Razek, A. G., Gromadzka, K., Drzewiecka, K., & Hassanein, M. M. M. (2022). A comprehensive study of lupin seed oils and the roasting effect on their chemical and biological activity. *Plants*, *11*, 2301. https://doi.org/10.3390/PLANTS11172301

Albuja-Vaca, D., Yépez, Y., Vernaza, M. G., & Navarrete, D. (2019). Gluten-free pasta: Development of a new formulation based on rice and lupine bean flour (*Lupinus mutabilis*) using a mixture-process design. *Food Sci Technol*, *40*, 408–414. https://doi.org/10.1590/FST.02319

Alkaltham, M. S., Salamatullah, A. M., Özcan, M. M., Uslu, N., & Hayat, K. (2020). The effects of germination and heating on bioactive properties, phenolic compounds and mineral contents of green gram seeds. *LWT - Food Sci Technol*, *134*, 110106. https://doi.org/10.1016/j.lwt.2020.110106

Al-Saedi, N., Agarwal, M., Ma, W., Islam, S., & Ren, Y. (2020). Proteomic characterisation of lupin (*Lupinus angustifolius*) milk as influenced by extraction techniques, seed coat and cultivars. *Molecules*, *25*, 1782. https://doi.org/10.3390/molecules25081782

Alu'datt, M. H., Rababah, T., Alhamad, M. N., Ereifej, K., Gammoh, S., Kubow, S., & Tawalbeh, D. (2017). Preparation of mayonnaise from extracted plant protein isolates of chickpea, broad bean and lupin flour: Chemical, physiochemical, nutritional and therapeutic properties. *J Food Sci Technol*, *54*, 1395–1405. https://doi.org/10.1007/S13197-017-2551-6

Alvarez-Jubete, L., Wijngaard, H., Arendt, E. K., & Gallagher, E. (2010). Polyphenol composition and *in vitro* antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chem*, *119*, 770–778. https://doi.org/10.1016/j.foodchem.2009.07.032

Amini, A. M., Razavi, S. M. A., & Mortazavi, S. A. (2015). Morphological, physicochemical, and viscoelastic properties of sonicated corn starch. *Carbohydr Polym*, *122*, 282–292. https://doi.org/10.1016/j.carbpol.2015.01.020

Amoah, I., Ascione, A., Muthanna, F. M. S., Feraco, A., Camajani, E., Gorini, S., Armani, A., Caprio, M., & Lombardo, M. (2023). Sustainable strategies for increasing legume consumption: Culinary and educational approaches. *Foods*, *12*, 2265. https://doi.org/10.3390/foods12112265

Anaemene, D., & Fadupin, G. (2022). Anti-nutrient reduction and nutrient retention capacity of fermentation, germination and combined germination-fermentation in legume processing. *Appl Food Res*, *2*, 100059. https://doi.org/10.1016/j.afres.2022.100059

Anal, A. K. (2017). Food processing by-products and their utilization: Introduction. In A. K. Anal (Ed.), *Food processing by-products and their utilization* (pp. 1–10). John Wiley & Sons. https://doi.org/10.1002/9781118432921

Arnoldi, A., Boschin, G., Zanoni, C., & Lammi, C. (2015). The health benefits of sweet lupin seed flours and isolated proteins. *J Funct Foods*, *18*, 550–563. https://doi.org/10.1016/J.JFF.2015.08.012

Ashokkumar, M. (2015). Applications of ultrasound in food and bioprocessing. *Ultrason Sonochem*, *25*, 17–23. https://doi.org/10.1016/j.ultsonch.2014.08.012

Ashokkumar, M., Kentish, D. S., Mawson, R., Simons, L., Vilkhu, K., & Versteeg, C. K. (2008). Modification of food ingredients by ultrasound to improve functionality: A preliminary study on a model system. *Innov Food Sci Emerg Technol*, *9*, 155–160. https://doi.org/10.1016/j.ifset.2007.05.005

Awad, T., Moharram, H., Shaltout, O., Asker, D., & Youssef, M. (2012). Applications of ultrasound in analysis, processing and quality control of food: A review. *Food Res Int*, *48*, 410–427. https://doi.org/10.1016/j.foodres.2012.05.004

Banerjee, J., Singh, R., Vijayaraghavan, R., MacFarlane, D., Patti, A. F., & Arora, A. a. (2017). Bioactives from fruit processing wastes: Green approaches to valuable chemicals. *Food Chem*, *225*, 10–22. https://doi.org/10.1016/j.foodchem.2016.12.093

Benincasa, P., Galieni, A., Manetta, A. C., Pace, R., Guiducci, M., Pisante, M., & Stagnari, F. (2015). Phenolic compounds in grains, sprouts and wheatgrass of hulled and non-hulled wheat species. *J Sci Food Agric*, *95*, 1795–1803. https://doi.org/10.1002/jsfa.6877

Ben-Othman, S., Jõudu, I., & Bhat, R. (2020). Bioactives from agri-food wastes: Present insights and future challenges. *Molecules*, *25*, 510. https://doi.org/10.3390/molecules25030510

Bhargava, N., Mor, R. S., Kumar, K., & Sharanagat, V. S. (2021). Advances in application of ultrasound in food processing: A review. *Ultrason Sonochem*, *70*, 105293. https://doi.org/10.1016/j.ultsonch.2020.105293

Borsato, V. M., Jorge, L. M. M., Mathias, A. L., & Jorge, R. M. M. (2019). Ultrasound assisted hydration improves the quality of the malt barley. *J Food Process Eng*, *42*, e13208. https://doi.org/10.1111/jfpe.13208

Boschin, G., & Arnoldi, A. (2011). Legumes are valuable sources of tocopherols. *Food Chem*, *127*, 1199–1203. https://doi.org/10.1016/j.foodchem.2011.01.124

Boufi, S., Haaj, S. B., Magnin, A., Pignon, F., Impéror-Clerc, M., & Mortha, G. (2018). Ultrasonic assisted production of starch nanoparticles: Structural characterization and mechanism of disintegration. *Ultrason Sonochem*, *41*, 327–336. https://doi.org/10.1016/j.ultsonch.2017.09.033

Brandolini, A., Glorio-Paulet, P., Estivi, L., Locatelli, N., Córdova-Ramos, J. S., & Hidalgo, A. (2022). Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) seeds processing. *J Food Compos Anal*, *106*, 104335. https://doi.org/10.1016/j.jfca.2021.104335

Briceño Berru, L., Glorio-Paulet, P., Basso, C., Scarafoni, A., Camarena, F., Hidalgo, A., & Brandolini, A. (2021). Chemical composition, tocopherol and carotenoid content of seeds from different Andean

lupin (*Lupinus mutabilis*) ecotypes. *Plant Food Hum Nutr*, *76*, 98–104. https://doi.org/10.1007/S11130-021-00880-0

Byanju, B., Rahman, M., & Hojilla-Evangelista, M. P. (2020). Effect of high-power sonication pretreatment on extraction and some physicochemical properties of proteins from chickpea, kidney bean, and soybean. *Int J Biol Macromol*, *145*, 712–721. https://doi.org/10.1016/j.ijbiomac.2019.12.118

Caldeira, C., De Laurentiis, V., Corrado, S., van Holsteijn, F., & Sala, S. (2019). Quantification of food waste per product group along the food supply chain in the European Union: A mass flow analysis. *Resour Conserv Recycl*, *149*, 479–488. https://doi.org/10.1016/j.resconrec.2019.06.011

Caponio, F., Piga, A., & Poiana, M. (2022). Valorization of food processing by-products. *Foods*, *11*, 3246. https://doi.org/10.3390/foods11203246

Cardoza, Y. J., Wang, S. F., Reidy-Crofts, J., & Edwards, O. R. (2006). Phloem alkaloid tolerance allows feeding on resistant *Lupinus angustifolius* by the aphid *Myzus persicae*. *J Chem Ecol*, *32*, 1965–1976. https://doi.org/10.1007/s10886-006-9121-0

Carvajal-Larenas, F. E. (2019). Nutritional, rheological and sensory evaluation of *Lupinus mutabilis* food products — A review. *Czech J Food Sci*, *37*, 301–311. https://doi.org/10.17221/4/2019-CJFS

Carvajal-Larenas, F. E., Linnemann, A. R., Nout, M. J. R., Koziol, M., & van Boekel, M. A. J. S. (2016). *Lupinus mutabilis*: Composition, uses, toxicology, and debittering. *Crit Rev Food Sci Nutr*, *56*, 1454–1487. https://doi.org/10.1080/10408398.2013.772089

Carvajal-Larenas, F. E., Nout, M. J. R., van Boekel, M. A. J. S., Koziol, M., & Linnemann, A. R. (2013). Modelling of the aqueous debittering process of *Lupinus mutabilis* Sweet. *LWT - Food Sci Technol*, *53*, 507–516. https://doi.org/10.1016/j.lwt.2013.03.017

Carvajal-Larenas, F. E., van Boekel, M. A. J. S., Koziol, M., Nout, M. J. R., & Linnemann, A. R. (2014). Effect of processing on the diffusion of alkaloids and quality of *Lupinus mutabilis* Sweet. *J Food Process Pres*, *38*, 1461–1471. https://doi.org/10.1111/jfpp.12105

Carvalho, I. S., Chaves, M., & Pinto Ricardo, C. (2005). Influence of water stress on the chemical composition of seeds of two lupins (*Lupinus albus* and *Lupinus mutabilis*). *J Agron Crop Sci*, *191*, 95–98. https://doi.org/10.1111/J.1439-037X.2004.00128.X

Cely-Veloza, W., Kato, M. J., & Coy-Barrera, E. (2023). Quinolizidine-type alkaloids: Chemodiversity, occurrence, and bioactivity. *ACS Omega*, *8*, 27862–27893. https://doi.org/10.1021/acsomega.3c02179

Cengiz, M. F., Babacan, U., Akinci, E., Kesci, S. T., & Kaba, A. (2021). Extraction of phenolic acids from ancient wheat bran samples by ultrasound application. *J Chem Technol Biotechnol*, *96*, 134–141. https://doi.org/10.1002/jctb.6519

Chatchavanthatri, N., Junyusen, T., Moolkaew, P., Arjharn, W., & Junyusen, P. (2020). Effect of soaking and sprouting treatment on germination rate of paddy. *E3S Web of Conferences*, *187*, 04016. https://doi.org/10.1051/e3sconf/202018704016

Chen, L., Yang, M., Mou, H., & Kong, Q. (2018). Ultrasound-assisted extraction and characterization of anthocyanins from purple corn bran. *J Food Process Preserv*, *42*, e13377. https://doi.org/10.1111/jfpp.13377

Chen, X., Li, X., Zhu, X., Wang, G., Zhuang, K., Wang, Y., & Ding, W. (2020). Optimization of extrusion and ultrasound-assisted extraction of phenolic compounds from Jizi439 black wheat bran. *Processes*, *8*, 1153. https://doi.org/10.3390/pr8091153

Chen, Y.-p., Liu, Q., Yue, X.-z., Meng, Z.-w., & Liang, J. (2013). Ultrasonic vibration seeds showed improved resistance to cadmium and lead in wheat seedling. *Environ Sci Pollut Res*, *20*, 4807–4816. https://doi.org/10.1007/s11356-012-1411-1

Cherif, M. M., Grigorakis, S., Halahlah, A., Loupassaki, S., & Makris, D. P. (2020). High-efficiency extraction of phenolics from wheat waste biomass (bran) by combining deep eutectic solvent, ultrasound-assisted pretreatment and thermal treatment. *Environ Process*, *7*, 845–859. https://doi.org/10.1007/s40710-020-00449-0

Chilomer, K., Kasprowicz-Potocka, M., Gulewicz, P., & Frankiewicz, A. (2013). The influence of lupin seed germination on the chemical composition and standardized ileal digestibility of protein and amino acids in pigs. *J Anim Physiol Anim Nutr*, *97*, 639–646. https://doi.org/10.1111/j.1439-0396.2012.01304.x

Chinma, J. O., C. E. Abu, Adedeji, L. . C., O. E. an Aburime, Joseph, D. G., Agunloye, G. F., Adebo, J. A., Oyeyinka, S. A., Njobeh, P. B., & Adebo, O. A. (2022). Nutritional composition, bioactivity, starch characteristics, thermal and microstructural properties of germinated pigeon pea flour. *Food Biosci*, *49*, 101900. https://doi.org/10.1016/j.fbio.2022.101900

Ciesiołka, D., Gulewicz, P., Martinez-Villaluenga, C., Pilarski, R., Bednarczyk, M., & Gulewicz, K. (2005). Products and biopreparations from alkaloid-rich lupin in animal nutrition and ecological agriculture. *Folia Biol*, *53*, 59–66. https://doi.org/10.3409/173491605775789443

Constantino, A. B. T., & Garcia-Rojas, E. E. (2020). Modifications of physicochemical and functional properties of amaranth protein isolate (*Amaranthus cruentus* BRS Alegria) treated with high-intensity ultrasound. *J Cereal Sci*, *95*, 103076. https://doi.org/10.1016/j.jcs.2020.103076

Cooke, D., & Gidley, M. J. (1992). Loss of crystalline and molecular order during starch gelatinisation: Origin of the enthalpic transition. *Carbohydr Res*, *227*, 103–112. https://doi.org/10.1016/0008-6215(92)85063-6

Córdova-Ramos, J. S., Glorio-Paulet, P., Camarena, F., Brandolini, A., & Hidalgo, A. (2020). Andean lupin (*Lupinus mutabilis* Sweet): Pro-

cessing effects on chemical composition, heat damage, and *in vitro* protein digestibility. *Cereal Chem*, *97*, 827–835. https://doi.org/10.1002/cche.10303

Cortés-Avendaño, P., Tarvainen, M., Suomela, J.-P., Glorio-Paulet, P., Yang, B., & Repo-Carrasco-Valencia, R. (2020). Profile and content of residual alkaloids in ten ecotypes of *Lupinus mutabilis* Sweet after aqueous debittering process. *Plant Food Hum Nutr*, *75*, 184–191. https://doi.org/10.1007/s11130-020-00799-y

Cravotto, G., Binello, A., Merizzi, G., & Avogadro, M. (2004). Improving solvent-free extraction of policosanol from rice bran by high-intensity ultrasound treatment. *Eur J Lipid Sci Technol*, *106*, 147–151. https://doi.org/10.1002/ejlt.200300914

Czubinski, J., Grygier, A., & Siger, A. (2021). *Lupinus mutabilis* seed composition and its comparison with other lupin species. *J Food Compos Anal*, *99*, 103875. https://doi.org/10.1016/j.jfca.2021.103875

Czubinski, J., Wroblewska, K., Czyzniejewski, M., Górnaś, P., Kachlicki, P., & Siger, A. (2019). Bioaccessibility of defatted lupin seed phenolic compounds in a standardized static *in vitro* digestion system. *Food Res Int*, *116*, 1126–1134. https://doi.org/10.1016/j.foodres.2018.09.057

Czubiński, J., & Siger, A. (2023). Physicochemical characteristics and technological properties of *Lupinus mutabilis* oil. *Eur J Lipid Sci Tech*, *125*, 2200200. https://doi.org/10.1002/EJLT.202200200

Dagnia, S. G., Petterson, D. S., Bell, R. R., & Flanagan, F. V. (1992). Germination alters the chemical composition and protein quality of lupin seeds. *J Sci Food Agric*, *60*, 419–423. https://doi.org/10.1002/jsfa.2740600403

Das, A. B., Goud, V. V., & Das, C. (2017). Extraction of phenolic compounds and anthocyanin from black and purple rice bran (*Oryza sativa* L.) using ultrasound: A comparative analysis and phytochemical profiling. *Ind Crops Prod*, *95*, 332–341. https://doi.org/10.1016/j.indcrop.2016.10.041

de Cortes Sánchez, M., Altares, P., Pedrosa, M. M., Burbano, C., Cuadrado, C., Goyoaga, C., Muzquiz, M., Jiménez-Martínez, C., & Dávila-Ortiz, G. (2005). Alkaloid variation during germination in different lupin species. *Food Chem*, *90*, 347–355. https://doi.org/10.1016/j.foodchem.2004.04.008

Ding, J., Hou, G. G., Dong, M., Xiong, S., Zhao, S., & Feng, H. (2018). Physicochemical properties of germinated dehulled rice flour and energy requirement in germination as affected by ultrasound treatment. *Ultrason Sonochem*, *41*, 484–491. https://doi.org/10.1016/j.ultsonch.2017.10.010

Directive 2008/98/EC of the European Parliament and of the Council of 19 november 2008 on waste and repealing certain Directives (2008).

Directive 2018/851/EC of the European Parliament and of the Council of 30 May 2018 amending Directive 2008/98/EC on waste (2018).

Đordević, T., & Antov, M. (2017). Ultrasound assisted extraction in aqueous two-phase system for the integrated extraction and separation of antioxidants from wheat chaff. *Sep Purif Technol*, *182*, 52–58. https://doi.org/10.1016/j.seppur.2017.03.025

Dueñas, M., Estrella, I., & Hernández, T. (2004). Occurrence of phenolic compounds in the seed coat and the cotyledon of peas (*Pisum sativum* L.) *Eur Food Res Technol*, *219*, 116–123. https://doi.org/10.1007/S00217-004-0938-X

El-Adawy, T. A. (2002). Nutritional composition and antinutritional factors of chickpeas (*Cicer arietinum* L.) undergoing different cooking methods and germination. *Plant Food Hum Nutr*, *57*, 83–97. https://doi.org/10.1023/A:1013189620528

El-Adawy, T. A., Rahma, E. H., El-Bedawey, A. A., & Gafar, A. F. (2001). Nutritional potential and functional properties of sweet and bitter lupin seed protein isolates. *Food Chem*, *74*, 455–462. https://doi.org/10.1016/S0308-8146(01)00163-7

El-Sebaiy, L. A., & El-Mahdy, A. R. (1983). Lipid changes during germination of fenugreek seeds (*Trigonella foenum-graecum*). *Food Chem*, *1983*, 309–319. https://doi.org/10.1016/0308-8146(83)90086-9

Erbaş, M. (2010). The effects of different debittering methods on the production of lupin bean snack from bitter *Lupinus albus* L. seeds. *J Food Qual*, *33*, 742–757. https://doi.org/10.1111/j.1745-4557.2010.00347.x

Erbaş, M., Certel, M., & Uslu, M. K. (2005). Some chemical properties of white lupin seeds (*Lupinus albus* L.) *Food Chem*, *89*, 341–345. https://doi.org/10.1016/j.foodchem.2004.02.040

Esteves, T., Mota, A. T., Barbeitos, C., Andrade, K., Afonso, C. A. M., & Castelo Ferreira, F. (2020). A study on lupin beans process wastewater nanofiltration treatment and lupanine recovery. *J Clean Prod*, *277*, 123349. https://doi.org/10.1016/j.jclepro.2020.123349

Estivi, L., Brandolini, A., Condezo-Hoyos, L., & Hidalgo, A. (2022). Impact of low-frequency ultrasound technology on physical, chemical and technological properties of cereals and pseudocereals. *Ultrason Sonochem*, *86*, 106044. https://doi.org/10.1016/j.ultsonch.2022.106044

FAO. (2011). *Global food losses and food waste — Extent, causes and prevention*. Rome, IT. https://www.fao.org/3/i2697e/i2697e.pdf

FAO. (2019). *The state of food and agriculture 2019. Moving forward on food loss and waste reduction*. Rome, IT. http://www.fao.org/3/ca6030en/ca6030en.pdf

FAO. (2022). *Statistical yearbook — World food and agriculture 2022*. Rome, IT. https://doi.org/10.4060/cc2211en

FAO/WHO/ONU. (1985). *Energy and protein requirements. Report of a Joint FAO/WHO/ONU Expert Consultation*. Geneva, CH.

Feng, H., & Yang, W. (2011). Ultrasound processing. In H. Q. Zhang, G. V. Barbosa-Cánovas, V. M. Balasubramaniam, C. P. Dunne, D. F. Farkas, & J. T. C. Yuan (Eds.), *Nonthermal processing technologies for food* (pp. 135–154). John Wiley & Sons, Wiley-Blackwell; IFT Press. https://doi.org/10.1002/9780470958360

Fernandez-Orozco, R., Frias, J., Zielinski, H., Piskula, M. K., Kozlowska, H., & Vidal-Valverde, C. (2008). Kinetic study of the antioxidant compounds and antioxidant capacity during germination of *Vigna radiata* cv. emmerald, *Glycine max* cv. jutro and *Glycine max* cv. merit. *Food Chem*, *111*, 622–630. https://doi.org/10.1016/j.foodchem.2008.04.028

Fernandez-Orozco, R., Piskula, M. K., Zielinski, H., Kozlowska, H., Frias, J., & Vidal-Valverde, C. (2006). Germination as a process to improve the antioxidant capacity of *Lupinus angustifolius* L. var. Zapaton. *Eur Food Res Technol*, *223*, 495–502. https://doi.org/10.1007/s00217-005-0229-1

Foley, R. C., Gao, L. L., Spriggs, A., Soo, L. Y. C., Goggin, D. E., Smith, P. M. C., Atkins, C. A., & Singh, K. B. (2011). Identification and characterisation of seed storage protein transcripts from *Lupinus angustifolius*. *BMC Plant Biol*, *11*, 1–15. https://doi.org/10.1186/1471-2229-11-59

Frias, J., Miranda, M. L., Doblado, R., & Vidal-Valverde, C. (2005). Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. Multolupa. *Food Chem*, *92*, 211–220. https://doi.org/10.1016/j.foodchem.2004.06.049.

Galanakis, C. M. (2012). Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. *Trends Food Sci Tech*, *26*, 68–87. https://doi.org/10.1016/j.tifs.2012.03.003

Gan, R. Y., Lui, W. Y., Wu, K., Chan, C. L., Dai, S. H., Sui, Z. Q., & Corke, H. (2017). Bioactive compounds and bioactivities of germinated edible seeds and sprouts: An updated review. *Trends Food Sci Technol*, *59*, 1–14. https://doi.org/10.1016/j.tifs.2016.11.010

Giopato Viell, F. L., Madeira, T. B., Nixdorf, S. L., Terezinha, S., Gomes, M., Bona, E., & Matsushita, M. (2020). Comparison between ultra-homogenisation and ultrasound for extraction of phenolic compounds from teff (*Eragrostis tef* (Zucc.)) *Int J Food Sci Technol*, *55*, 2700–2709. https://doi.org/10.1111/ijfs.14523

Gobbetti, M., De Angelis, M., Di Cagno, R., Polo, A., & Rizzello, C. G. (2020). The sourdough fermentation is the powerful process to exploit the potential of legumes, pseudo-cereals and milling by-products in baking industry. *Crit Rev Food Sci*, *60*, 2158–2173. https://doi.org/10.1080/10408398.2019.1631753

Gross, U., Galindo, R. G., & Schoeneberger, H. (1983). The development and acceptability of lupine (*Lupinus mutabilis*) products. *Plant Food Hum Nutr*, *32*, 155–164. https://doi.org/10.1007/bf01091336

Guadagnucci Fontanari, G., Batistuti, J. P., Da Cruz, R. J., Nascimento Saldiva, P. H., & Gomes Arêas, J. A. (2012). Cholesterol-lowering effect of whole lupin (*Lupinus albus*) seed and its protein isolate. *Food Chem*, *132*, 1521–1526. https://doi.org/10.1016/J.FOODCHEM.2011.11.145

Güémes-Vera, N., Peña-Bautista, R. J., Jiménez-Martínez, C., Dávila-Ortiz, G., & Calderón-Domínguez, G. (2008). Effective detoxification and decoloration of *Lupinus mutabilis* seed derivatives, and effect of these derivatives on bread quality and acceptance. *J Sci Food Agr*, *88*, 1135–1143. https://doi.org/10.1002/jsfa.3152

Guimarães, B., Polachini, T. C., Augusto, P. E. D., & Telis-Romero, J. (2020). Ultrasound-assisted hydration of wheat grains at different temperatures and power applied: Effect on acoustic field, water absorption and germination. *Chem Eng Process*, *155*, 108045. https://doi.org/10.1016/j.cep.2020.108045

Gulewicz, P., Martínez-Villaluenga, C., Frias, J., Ciesiołka, D., Gulewicz, K., & Vidal-Valverde, C. (2008). Effect of germination on the protein fraction composition of different lupin seeds. *Food Chem*, *107*, 830–844. https://doi.org/10.1016/J.FOODCHEM.2007.08.087

Gupta, R. K., Gangoliya, S. S., & Singh, N. K. (2015). Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J Food Sci Technol*, *52*, 676–684. https://doi.org/10.1007/s13197-013-0978-y

Hidalgo, A., Tumbas Šaponjac, V., Ćetković, G., Šeregelj, V., Čanadanović-Brunet, J., Chiosa, D., & Brandolini, A. (2019). Antioxidant properties and heat damage of water biscuits enriched with sprouted wheat and barley. *LWT - Food Sci Technol*, *114*, 108423. https://doi.org/10.1016/j.lwt.2019.108423

Hu, A., Jiao, S., Zheng, J., Li, L., Fan, Y., Chen, L., & Zhang, Z. (2015). Ultrasonic frequency effect on corn starch and its cavitation. *LWT - Food Sci Technol*, *60*, 941–947. https://doi.org/10.1016/j.lwt.2014.10.048

Huang, Q., Li, L., & Fu, X. (2007). Ultrasound effects on the structure and chemical reactivity of cornstarch granules. *Starke*, *59*, 371–378. https://doi.org/10.1002/star.200700614

Hughes, J., Pearson, E., & Grafenauer, S. (2022). Legumes—A comprehensive exploration of global food-based dietary guidelines and consumption. *Nutrients*, *14*, 3080. https://doi.org/10.3390/nu14153080

Ishaq, A. R., El-Nashar, H. A. S., Younis, T., Mangat, M. A., Shahzadi, M., Ul-Haq, A. S., & El-Shazly, M. (2022). Genus *Lupinus* (Fabaceae): A review of ethnobotanical, phytochemical and biological studies. *J*

Pharm Pharmacol, *74*, 1700–1717. https://doi.org/10.1093/JPP/RGAC058

Isono, Y., Kumagai, T., & Watanabe, T. (1994). Ultrasonic degradation of waxy rice starch. *Biosci Biotechnol Biochem*, *58*, 1799–1802. https://doi.org/10.1271/bbb.58.1799

James, S., Nwabueze, T. U., Ndife, J., Onwuka, G. I., & Usman, M. A. (2020). Influence of fermentation and germination on some bioactive components of selected lesser legumes indigenous to Nigeria. *J Agric Food Res*, *2*, 100086. https://doi.org/10.1016/j.jafr.2020.100086

Jayasena, V., Khu, W. S., & Nasar-Abbas, S. M. (2010). The development and sensory acceptability of lupin-based tofu. *J Food Quality*, *33*, 85–97. https://doi.org/10.1111/j.1745-4557.2009.00290.x

Jayasena, V., Leung, P. P. Y., & Nasar-Abbas, S. M. (2010). Effect of lupin flour substitution on the quality and sensory acceptability of instant noodles. *J Food Quality*, *33*, 709–727. https://doi.org/10.1111/j.1745-4557.2010.00353.x

Jha, P., Das, A. J., & Deka, S. C. (2017). Optimization of ultrasound and microwave assisted extractions of polyphenols from black rice (*Oryza sativa* cv. Poireton) husk. *J Food Sci Technol*, *54*, 3847–3858. https://doi.org/10.1007/s13197-017-2832-0

Jia, J., Ma, H., Zhao, W., Wang, Z., Tian, W., Luo, L., & He, R. (2010). The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. *Food Chem*, *119*, 336–342. https://doi.org/10.1016/j.foodchem.2009.06.036

Jiménez-Martínez, C., Hernández-Sánchez, H., Alvárez-Manilla, G., Robledo-Quintos, N., Martínez-Herreraand, J., & Dávila-Ortiz, G. (2001). Effect of aqueous and alkaline thermal treatments on chemical composition and oligosaccharide, alkaloid and tannin contents of *Lupinus campestris* seeds. *J Sci Food Agr*, *81*, 421–428. https://doi.org/10.1002/1097-0010(200103)81:4<421::AID-JSFA829>3.0.CO;2-U

Jiménez-Martínez, C., Hernández-Sánchez, H., & Dávila-Ortiz, G. (2007). Diminution of quinolizidine alkaloids, oligosaccharides and phenolic compounds from two species of *Lupinus* and soybean seeds by the effect of *Rhizopus oligosporus*. *J Sci Food Agr*, *87*, 1315–1322. https://doi.org/10.1002/JSFA.2851

Jiménez-Martínez, C., Mora-Escobedo, R., Cardador Martínez, A., Muzquiz, M., Martin Pedrosa, M., & Dávila-Ortiz, G. (2010). Effect of aqueous, acid, and alkaline thermal treatments on antinutritional factors content and protein quality in *Lupinus campestris* deed flour. *J Agr Food Chem*, *58*, 1741–1745. https://doi.org/10.1021/JF902688R

Jin, H.-l., Wang, J.-s., & Bian, K. (2012). Characteristics of enzymatic hydrolysis of the wheat gluten proteins treated by ultrasound wave. *Adv Mat Res*, *343-344*, 1015–1022. https://doi.org/10.4028/www.scientific.net/AMR.343-344.1015

Kalita, D., Jain, S., Srivastava, B., & Goud, V. V. (2021). Sono-hydro priming process (ultrasound modulated hydration): Modelling hydration kinetic during paddy germination. *Ultrason Sonochem*, *70*, 105321. https://doi.org/10.1016/j.ultsonch.2020.105321

Kamani, M. H., Neji, C., Fitzsimons, S. M., Fenelon, M. A., & Murphy, E. G. (2023). Unlocking the nutritional and functional potential of legume waste to produce protein ingredients. *Crit Rev Food Sci*. https://doi.org/10.1080/10408398.2023.2184322

Kang, N., Zuo, Y. J., Hilliou, L., Ashokkumar, M., & Hemar, Y. (2016). Viscosity and hydrodynamic radius relationship of high-power ultrasound depolymerised starch pastes with different amylose content. *Food Hydrocoll*, *52*, 183–191. https://doi.org/10.1016/j.foodhyd.2015.06.017

Kohajdová, Z., Karovičová, & Schmidt, Š. (2011). Lupin composition and possible use in bakery - A review. *Czech J Food Sci*, *29*, 203–211. https://doi.org/10.17221/252/2009-CJFS

Kroc, M., Czepiel, K., Wilczura, P., Mokrzycka, M., & Święcicki, W. (2019). Development and validation of a gene-targeted dCAPS marker for marker-assisted selection of low-alkaloid content in seeds of narrow-leafed lupin (*Lupinus angustifolius* L.) *Genes*, *10*, 428. https://doi.org/10.3390/GENES10060428

Li, C., Liu, W., Gu, Z., Fang, D., Hong, Y., Cheng, L., & Li, Z. (2017). Ultrasonic pretreatment improves the high-temperature liquefaction of corn starch at high concentrations. *Starke*, *69*, 1600002. https://doi.org/10.1002/star.201600002

Li, M., Li, J., & Zhu, C. (2018). Effect of ultrasound pretreatment on enzymolysis and physicochemical properties of corn starch. *Int J Biol Macromol*, *111*, 848–856. https://doi.org/10.1016/j.ijbiomac.2017.12.156

Li, S., Yang, X., Zhang, Y., Ma, H., Liang, Q., Qu, W., He, R., Cunshan Zhou, C., & Mahunu, G. K. (2016). Effects of ultrasound and ultrasound assisted alkaline pretreatments on the enzymolysis and structural characteristics of rice protein. *Ultrason Sonochem*, *31*, 20–28. https://doi.org/10.1016/j.ultsonch.2015.11.019

Li, S., Yang, X., Zhang, Y., Ma, H., Qu, W., Ye, X., Muatasim, R., & Oladejo, A. O. (2016). Enzymolysis kinetics and structural characteristics of rice protein with energy-gathered ultrasound and ultrasound assisted alkali pretreatments. *Ultrason Sonochem*, *31*, 85–92. https://doi.org/10.1016/j.ultsonch.2015.12.005

Li, X., Da, S., Li, C., Xue, F., & Zang, T. (2018). Effects of high-intensity ultrasound pretreatment with different levels of power output on the antioxidant properties of alcalase hydrolyzates from quinoa (*Chenopodium quinoa* Willd.) protein isolate. *Cereal Chem*, *95*, 518–526. https://doi.org/10.1002/cche.10055

Li, Y., Hu, A., Zheng, J., & Wang, X. (2019). Comparative studies on structure and physiochemical changes of millet starch under microwave and ultrasound at the same power. *Int J Biol Macromol*, *141*, 76–84. https://doi.org/10.1016/j.ijbiomac.2019.08.218

Lin, P.-Y., & Lai, H.-M. (2006). Bioactive compounds in legumes and their germinated products. *J Agric Food Chem*, *54*, 3807–3814. https://doi.org/10.1021/jf060002o

Lo, B., Kasapis, S., & Farahnaky, A. (2021). Lupin protein: Isolation and techno-functional properties, a review. *Food Hydrocoll*, *112*, 106318. https://doi.org/10.1016/J.FOODHYD.2020.106318

López-Amorós, M. L., Hernández, T., & Estrella, I. (2006). Effect of germination on legume phenolic compounds and their antioxidant activity. *J Food Compos Anal*, *19*, 277–283. https://doi.org/10.1016/j.jfca.2004.06.012

Luo, X., Cui, J., Zhang, H., Duan, Y., Zhang, D., Cai, M., & Chen, G. (2018). Ultrasound assisted extraction of polyphenolic compounds from red sorghum (*Sorghum bicolor* L.) bran and their biological activities and polyphenolic compositions. *Ind Crop Prod*, *112*, 296–304. https://doi.org/10.1016/j.indcrop.2017.12.019

Luo, Z., Fu, X., He, X., Luo, F., Gao, Q., & Yu, S. (2008). Effect of ultrasonic treatment on the physicochemical properties of maize starches differing in amylose content. *Starke*, *60*, 646–653. https://doi.org/10.1002/star.200800014

Margulis, M. A., & Margulis, I. M. (2003). Calorimetric method for measurement of acoustic power absorbed in a volume of a liquid. *Ultrason Sonochem*, *10*, 343–345. https://doi.org/10.1016/S1350-4177(03)00100-7

Martínez-Villaluenga, C., Frías, J., & Vidal-Valverde, C. (2006). Functional lupin seeds (*Lupinus albus* L. and *Lupinus luteus* L.) after extraction of α-galactosides. *Food Chem*, *98*, 291–299. https://doi.org/10.1016/j.foodchem.2005.05.074

Mason, T. J., & Cintas, P. (2002). Sonochemistry. In J. Clark & D. Macquarrie (Eds.), *Handbook of green chemistry and technology*. Blackwell Science. https://doi.org/10.1002/9780470988305.ch16

Mason, T. J., & Lorimer, J. P. (2002). *Applied sonochemistry: The uses of power ultrasound in chemistry and processing*. Wiley-VCH. https://doi.org/10.1002/352760054X

Mason, T. J., Paniwnyk, L., & Lorimer, J. P. (1996). The uses of ultrasound in food technology. *Ultrason Sonochem*, *3*, S253–260. https://doi.org/10.1016/S1350-4177(96)00034-X

Mason, T. J., & Peters, D. (2002). *Practical sonochemistry: Power ultrasound uses and applications*. Woodhead Publishing.

Mason, W. R. (2009). Starch use in foods. In J. BeMiller & R. Whistler (Eds.), *Starch: Chemistry and technology*. Elsevier. https://doi.org/10.1016/B978-0-12-746275-2.00020-3

Mavromatis, A., Nianiou-Obeidat, I., Polidoros, A., Parissi, Z., Tani, E., Irakli, M., Aliferis, K. A., Zafeiriou, I., Mylona, P. V., Sarri, E., Papadopoulou, E.-A., Rafail Tagiakas, R., Kougiteas, L., Kostoula, S., & Abraham, E. M. (2023). Characterization of lupin cultivars based on phenotypical, molecular and metabolomic analyses. *Agronomy*, *13*, 370. https://doi.org/10.3390/agronomy13020370

Mazzutti, S., Curi Pedrosa, R., & Salvador Ferreira, S. R. (2020). Green processes in foodomics. Supercritical fluid extraction of bioactives. *Ref Mod Food Sci*. https://doi.org/10.1016/B978-0-08-100596-5.22816-3

Miano, A. C., Ibarz, A., & Augusto, P. E. D. (2016). Mechanisms for improving mass transfer in food with ultrasound technology: Describing the phenomena in two model cases. *Ultrason Sonochem*, *29*, 413–419. https://doi.org/10.1016/j.ultsonch.2015.10.020

Miano, A. C., Ibarz, A., & Augusto, P. E. D. (2017). Ultrasound technology enhances the hydration of corn kernels without affecting their starch properties. *J Food Eng*, *197*, 34–43. https://doi.org/10.1016/j.jfoodeng.2016.10.024

Miano, A. C., Rojas, M. L., & Augusto, P. E. D. (2019). Using ultrasound for improving hydration and debittering of Andean lupin grains. *J Food Process Eng*, *42*, e13170. https://doi.org/10.1111/jfpe.13170

Mohamed, A. A., & Rayas-Duarte, P. (1995). Composition of *Lupinus albus*. *Cereal Chem*, *72*, 643–647.

Mohammadi, F., Marti, A., Nayebzadeh, K., Hosseini, S. M., Tajdar-Oranj, B., & Jazaeri, S. (2021). Effect of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran. *Food Chem*, *334*, 127583. https://doi.org/10.1016/j.foodchem.2020.127583

Mohammed, M. A., Mohamed, E. A., Yagoub, A. E. A., Mohamed, A. R., & Babiker, E. E. (2017). Effect of processing methods on alkaloids, phytate, phenolics, antioxidants activity and minerals of newly developed lupin (*Lupinus albus* L.) cultivar. *J Food Process*, *41*, 1–9. https://doi.org/10.1111/jfpp.12960

Montalbo-Lomboy, M., Johnson, L., Khanal, S. K., van Leeuwen, J. H., & Grewell, D. (2010). Sonication of sugary-2 corn: A potential pretreatment to enhance sugar release. *Bioresour Technol*, *101*, 351–358. https://doi.org/10.1016/j.biortech.2009.07.075

Muangrat, R., Pongsirikul, I., & Blanco, P. H. (2018). Ultrasound assisted extraction of anthocyanins and total phenolic compounds from dried cob of purple waxy corn using response surface methodology. *J Food Process Preserv*, *42*, e13447. https://doi.org/10.1111/jfpp.13447

Multari, S., Marsol-Vall, A., Heponiemi, P., Suomela, J.-P., & Yang, B. (2019). Changes in the volatile profile, fatty acid composition and other markers of lipid oxidation of six different vegetable oils during short-term deep-frying. *Food Res Int*, *122*, 318–329. https://doi.org/10.1016/j.foodres.2019.04.026

Musco, N., Cutrignelli, M. I., Calabrò, S., Tudisco, R., Infascelli, F., Grazioli, R., Lo Presti, V., Gresta, F., & Chiofalo, B. (2017). Comparison of nutritional and antinutritional traits among different species (*Lupinus albus* L., *Lupinus luteus*, *Lupinus angustifolius* L.) and varieties of lupin seeds. *J Anim Physiol Anim Nutr*, *101*, 1227–1241. https://doi.org/10.1111/jpn.12643

Mustafa, R., He, Y., Shim, Y. Y., & Reaney, M. J. T. (2018). Aquafaba, wastewater from chickpea canning, functions as an egg replacer in sponge cake. *Int J Food Sci Tech*, *53*, 2247–2255. https://doi.org/10.1111/ijfs.13813

Nartea, A., Kuhalskaya, A., Fanesi, B., Lois Orhotohwo, O. L., Susek, K., Rocchetti, L., Di Vittori, V., Bitocchi, E., Pacetti, D., & Papa, R. (2023). Legume byproducts as ingredients for food applications: Preparation, nutrition, bioactivity, and techno-functional properties. *Compr Rev Food Sci Food Saf*, *22*, 1953–1985. https://doi.org/10.1111/1541-4337.13137

Navarro del Hierro, J., Herrera, T., García-Risco, M. R., Fornari, T., Reglero, G., & Martin, D. (2018). Ultrasound-assisted extraction and bioaccessibility of saponins from edible seeds: Quinoa, lentil, fenugreek, soybean and lupin. *Food Res Int*, *109*, 440–447. https://doi.org/10.1016/j.foodres.2018.04.058

Nazari, B., Mohammadifar, M. A., Shojaee-Aliabadi, S., Feizollahi, E., & Mirmoghtadaie, L. (2018). Effect of ultrasound treatments on functional properties and structure of millet protein concentrate. *Ultrason Sonochem*, *41*, 382–388. https://doi.org/10.1016/j.ultsonch.2017.10.002

Okagu, I. U., Ndefo, J. C., Aham, E. C., Obeme-Nmom, J. I., Agboinghale, P. E., Aguchem, R. N., Nechi, R. N., & Lammi, C. (2021). Lupin-derived bioactive peptides: Intestinal transport, bioavailability and health benefits. *Nutrients*, *13*, 3266. https://doi.org/10.3390/NU13093266

Olaerts, H., & Courtin, C. M. (2018). Impact of preharvest sprouting on endogenous hydrolases and technological quality of wheat and bread: A review. *Compr Rev Food Sci Food Saf*, *17*, 698–713. https://doi.org/10.1111/1541-4337.12347

O'Sullivan, J., Park, M., & Beevers, J. (2016). The effect of ultrasound upon the physicochemical and emulsifying properties of wheat and soy protein isolates. *J Cereal Sci*, *69*, 77–84. https://doi.org/10.1016/j.jcs.2016.02.013

Özcan, M. M., İpek, D., Ghafoor, K., Al Juhaimi, F., Uslu, N., Babiker, E. E., Mohamed Ahmed, I. A., & Alsawmahi, O. N. (2021). Physicochemical and sensory properties of chips produced using different lupin (*Lupinus albus* L.) flour formulations and cooking methods. *56*, 2780–2788. https://doi.org/10.1111/IJFS.14913

Paraskevopoulou, A., Provatidou, E., Tsotsiou, D., & Kiosseoglou, V. (2010). Dough rheology and baking performance of wheat flour—lupin protein isolate blends. *Food Res Int*, *43*, 1009–1016. https://doi.org/10.1016/J.FOODRES.2010.01.010

Pascual-Chagman, G., Santa Cruz Olivos, J., Hidalgo, A., Benavente, F., Pérez-Camino, M. C., Sotelo, A., Paucar-Menacho, L. M., & Encina-Zelada, C. R. (2021). *Lupinus mutabilis* oil obtained by expeller press: Yield, physicochemical characterization, antioxidant capacity, fatty acids and oxidative stability analyses. *Sci Agropecu*, *12*, 219–227. https://doi.org/10.17268/sci.agropecu.2021.025

Pérez, S., Baldwin, P. M., & Gallant, D. J. (2009). Structural features of starch granules I. In J. BeMiller & R. Whistler (Eds.), *Starch: Chemistry and technology*. Elsevier. https://doi.org/10.1016/B978-0-12-746275-2.00005-7

Pingret, D., Fabiano-Tixier, A.-S., & Chemat, F. (2013). Degradation during application of ultrasound in food processing: A review. *Food Control*, *31*, 593–606. https://doi.org/10.1016/j.foodcont.2012.11.039

Plaza, L., De Ancos, B., & Cano, P. (2003). Nutritional and health related compounds in sprouts and seeds of soybean (*Glycine max*), wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa*) treated by a new drying method. *Eur Food Res Technol*, *216*, 138–144. https://doi.org/10.1007/s00217-002-0640-9

Plazzotta, S., & Manzocco, L. (2019). Food waste valorization. In C. M. Galanakis (Ed.), *Saving food. Production, supply chain, food waste, and food consumption* (pp. 279–313). Academic Press. https://doi.org/10.1016/C2017-0-03480-8

Prodanov, M., Sierra, I., & Vidal-Valverde, C. (1997). Effect of germination on the thiamine, riboflavin and niacin contents in legumes. *Z Lebensm Unters Forsch A*, *205*, 48–52. https://doi.org/10.1007/s002170050122

Prusinski, J. (2017). White lupin (*Lupinus albus* L.) - nutritional and health values in human nutrition - a review. *Czech J Food Sci*, *35*, 95–105. https://doi.org/10.17221/114/2016-CJFS

Quintero-Quiroz, J., Celis-Torres, A., Ciro-Gómez, G., Torres, J., Corrales-García, L., & Rojas, J. (2021). Physicochemical properties and functional characteristics of ultrasound-assisted legume-protein isolates: A comparative study. *J Food Sci Technol*. https://doi.org/10.1007/s13197-021-05126-0

Reg. 2015/2283/EU of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No

1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (2015).

Reinhard, H., Rupp, H., Sager, F., Streule, M., & Zoller, O. (2006). Quinolizidine alkaloids and phomopsins in lupin seeds and lupin containing food. *J Chromatogr A*, *1112*, 353–360. https://doi.org/10.1016/j.chroma.2005.11.079

Ren, X., Liang, Q., & Ma, H. (2018). Effects of sweeping frequency ultrasound pretreatment on the hydrolysis of zein: Angiotensin-converting enzyme inhibitory activity and thermodynamics analysis. *J Food Sci Technol*, *55*, 4020–4027. https://doi.org/10.1007/s13197-018-3328-2

Ren, X., Zhang, X., Liang, Q., Hou, T., & Zhou, H. (2017). Effects of different working modes of ultrasound on structural characteristics of zein and ACE inhibitory activity of hydrolysates. *J Food Qual*. https://doi.org/10.1155/2017/7896037

Rodríguez, G., Aguirre, E., Córdova-Chang, A., Muñoz-Saenz, J. C., Baquerizo, M., Brandolini, A., Villanueva, E., & Hidalgo, A. (2022). Modification of the nutritional quality and oxidative stability of lupin (*Lupinus mutabilis* Sweet) and sacha inchi (*Plukenetia volubilis* L.) oil blends. *Molecules*, *27*, 7315. https://doi.org/10.3390/MOLECULES27217315

Romero-Espinoza, A. M., Serna-Saldivar, S. O., Vintimilla-Alvarez, M. C., Briones-García, M., & Lazo-Vélez, M. A. (2020). Effects of fermentation with probiotics on anti-nutritional factors and proximate composition of lupin (*Lupinus mutabilis* sweet). *LWT - Food Sci Technol*, *130*, 109658. https://doi.org/10.1016/j.lwt.2020.109658

Ruiz-López, M. A., Barrientos-Ramírez, L., García-López, P. M., Valdés-Miramontes, E. H., Zamora-Natera, J. F., Rodríguez-Macias, R., Salcedo-Pérez, E., & Bañuelos-Pineda, J. (2019). Nutritional and bioactive compounds in Mexican lupin beans species: A mini-review. *Nutrients*, *11*, 1785. https://doi.org/10.3390/nu11081785

Saastamoinen, M., Eurola, M., & Hietaniemi, V. (2013). The chemical quality of some legumes, peas, fava beans, blue and white lupins and soybeans cultivated in Finland. *J Agric Sci Technol*, *B3*, 92–100.

Satya, S., Kaushik, G., & Naik, S. N. (2010). Processing of food legumes: A boon to human nutrition. *Mediterr J Nutr Metab*, *3*, 183–195. https://doi.org/10.1007/s12349-010-0017-8

Savelkoul, F. H. M. G., Van Der Poel, A. F. B., & Tamminga, S. (1992). The presence and inactivation of trypsin inhibitors, tannins, lectins and amylase inhibitors in legume seeds during germination. A review. *Plant Food Hum Nutr*, *42*, 71–85. https://doi.org/10.1007/BF02196074

Sbihi, H. M., Nehdi, I. A., Tan, C. P., & Al-Resayes, S. I. (2013). Bitter and sweet lupin (*Lupinus albus* L.) seeds and seed oils: A comparison

study of their compositions and physicochemical properties. *Ind Crops Prod*, *49*, 573–579. https://doi.org/10.1016/j.indcrop.2013.05.020

Serventi, L. (2020). *Upcycling legume water: From wastewater to food ingredients*. Springer. https://doi.org/10.1007/978-3-030-42468-8

Shang, X., Dou, Y., Zhang, Y., Tan, J.-N., Liu, X., & Zhang, Z. (2019). Tailor-made natural deep eutectic solvents for green extraction of isoflavones from chickpea (*Cicer arietinum* L.) sprouts. *Ind Crop Prod*, *140*, 111724. https://doi.org/10.1016/j.indcrop.2019.111724

Shewale, S. D., & Pandit, A. B. (2009). Enzymatic production of glucose from different qualities of grain sorghum and application of ultrasound to enhance the yield. *Carbohydr Res*, *344*, 52–60. https://doi.org/10.1016/j.carres.2008.10.006

Shohag, M. J. I., Wei, Y., & Yang, X. (2012). Changes of folate and other potential health-promoting phytochemicals in legume seeds as affected by germination. *J Agric Food Chem*, *60*, 9137–9143. https://doi.org/10.1021/jf302403t

Siger, A., Grygier, A., & Czubinski, J. (2023). Comprehensive characteristic of lipid fraction as a distinguishing factor of three lupin seed species. *J Food Compos Anal*, *115*, 104945. https://doi.org/10.1016/J.JFCA.2022.104945

Socas-Rodríguez, B., Álvarez-Rivera, G., Valdés, A., Ibáñez, E., & Cifuentes, A. (2021). Food by-products and food wastes: Are they safe enough for their valorization? *Trends Food Sci Tech*, *114*, 133–147. https://doi.org/10.1016/j.tifs.2021.05.002

Soria, A. C., & Villamiel, M. (2010). Effect of ultrasound on the technological properties and bioactivity of food: A review. *Trends Food Sci Technol*, *21*, 323–331. https://doi.org/10.1016/j.tifs.2010.04.003

Srivastava, L. M. (2002). *Plant growth and development. Hormones and environment*. Academic Press. https://doi.org/10.1016/B978-012660570-9/50161-1

Stantiall, S. E., Dale, K. J., Calizo, F. S., & Serventi, L. (2018). Application of pulses cooking water as functional ingredients: The foaming and gelling abilities. *Eur Food Res Technol*, *244*, 97–104. https://doi.org/10.1007/s00217-017-2943-x

Sujak, A., Kotlarz, A., & Strobel, W. (2006). Compositional and nutritional evaluation of several lupin seeds. *Food Chem*, *98*, 711–719. https://doi.org/10.1016/J.FOODCHEM.2005.06.036

Suslick, K. S. (1990). Sonochemistry. *Science*, *247*, 1439–1445. https://doi.org/10.1126/science.247.4949.1439

Świeca, M., Gawlik-Dziki, U., Kowalczyk, D., & Złotek, U. (2012). Impact of germination time and type of illumination on the antioxidant compounds and antioxidant capacity of *Lens culinaris* sprouts. *Sci Hortic*, *140*, 87–95. https://doi.org/10.1016/j.scienta.2012.04.005

Tassoni, A., Tedeschi, T., Zurlini, C., Cigognini, I. M., Petrusan, J.-I., Rodríguez, Ó., Neri, S., Celli, A., Sisti, L., Cinelli, P., Signori, F., Tsatsos, G., Bondi, M., Verstringe, S., Bruggerman, G., & Corvini, P. F. X. (2020). State-of-the-art production chains for peas, beans and chickpeas—Valorization of agro-industrial residues and applications of derived extracts. *Molecules*, *25*, 1383. https://doi.org/10.3390/molecules25061383

Thavarajah, D., McSwain, M., Johnson, C. R., Kumar, S., & Thavarajah, P. (2019). Pulses, global health, and sustainability: Future trends. In W. J. Dahl (Ed.), *Health benefits of pulses* (pp. 1–17). Springer. https://doi.org/10.1007/978-3-030-12763-3

Trugo, L. C., von Baer, D., & von Baer, E. (2003). Lupin. In C. Benjamin (Ed.), *Encyclopedia of food sciences and nutrition* (pp. 3623–3629). Academic Press.

Uauy, R., Gattas, V., & Yañez, E. (1995). Sweet lupins in human nutrition. In A. P. Simopoulos (Ed.), *Plants in human nutrition* (pp. 75–88). Karger Publishers. https://doi.org/10.1159/000424466

UNEP. (2021). *Food waste index. Report 2021*. United Nations Environment Programme. Nairobi, KE. https://wedocs.unep.org/bitstream/handle/20.500.11822/35280/FoodWaste.pdf

Van Hung, P., Hatcher, D. W., & Barker, W. (2011). Phenolic acid composition of sprouted wheats by ultra-performance liquid chromatography (UPLC) and their antioxidant activities. *Food Chem*, *126*, 1896–1901. https://doi.org/10.1016/j.foodchem.2010.12.015

Varavinit, S., Shobsngob, S., Varanyanond, W., Chinachoti, P., & Naivikul, O. (2003). Effect of amylose content on gelatinization, retrogradation and pasting properties of flours from different cultivars of Thai rice. *Starke*, *55*, 410–415. https://doi.org/10.1002/star.200300185

Vera, A., Valenzuela, M. A., Yazdani-Pedram, M., Tapia, C., & Abugoch, L. (2019). Conformational and physicochemical properties of quinoa proteins affected by different conditions of high-intensity ultrasound treatments. *Ultrason Sonochem*, *51*, 186–196. https://doi.org/10.1016/j.ultsonch.2018.10.026

Vidal-Valverde, C., Frias, J., Sierra, I., Blazquez, I., Lambein, F., & Kuo, Y. H. (2002). New functional legume foods by germination: Effect on the nutritive value of beans, lentils and peas. *Eur Food Res Technol*, *215*, 472–477. https://doi.org/10.1007/s00217-002-0602-2

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry—A review. *Innov Food Sci Emerg Technol*, *9*, 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

Villacrés, E., Quelal, M. B., Fernandez, E., Garcìa, G., Cueva, G., & Rosell, C. M. (2020). Impact of debittering and fermentation processes on the antinutritional and antioxidant compounds in *Lupinus mutabilis*

Sweet. *LWT - Food Sci Technol*, *131*, 109745. https://doi.org/10.1016/j.lwt.2020.109745

Vogelsang-O'Dwyer, M., Bez, J., Petersen, I. L., Joehnke, M. S., Detzel, A., Busch, M., Krueger, M., Ispiryan, L., O'Mahony, J., Arendt, E. K., & Zannini, E. (2000). Techno-functional, nutritional and environmental performance of protein isolates from blue lupin and white lupin. *Foods*, *9*, 230. https://doi.org/10.3390/foods9020230

Wang, F., Zhang, Y., Xu, L., & Ma, H. (2020). An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. *LWT - Food Sci Technol*, *127*, 109348. https://doi.org/10.1016/j.lwt.2020.109348

Wang, J., Sun, B., Cao, Y., Tian, Y., & Li, X. (2008). Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. *Food Chem*, *106*, 804–810. https://doi.org/10.1016/j.foodchem.2007.06.062

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., Garnett, T., Tilman, D., DeClerck, F., Wood, A., Jonell, M., Clark, M., Gordon, L. J., Fanzo, J., Hawkes, C., Zurayk, R., Rivera, J. A., De Vries, W., Majele Sibanda, L., ... Murray, C. J. L. (2019). Food in the Anthropocene: The EAT–Lancet Commission on healthy diets from sustainable food systems. *Lancet*, *393*, 447–492. https://doi.org/10.1016/S0140-6736(18)31788-4

Xia, Q., Tao, H., Li, Y., Pan, D., Cao, J., Liu, L., Zhou, X., & Barba, F. J. (2020). Characterizing physicochemical, nutritional and quality attributes of wholegrain *Oryza sativa* L. subjected to high intensity ultrasound-stimulated pre-germination. *Food Control*, *108*, 106827. https://doi.org/10.1016/j.foodcont.2019.106827

Xue, L., Liu, G., Parfitt, J., Liu, X., Van Herpen, E., Stenmarck, A., O'Connor, C., Östergren, K., & Shengkui Cheng, S. (2017). Missing food, missing data? A critical review of global food losses and food waste data. *Environ Sci Technol*, *51*, 6618–6633. https://doi.org/10.1021/acs.est.7b00401

Yadav, S., Mishra, S., & Pradhan, R. C. (2021). Ultrasound-assisted hydration of finger millet (*Eleusine Coracana*) and its effects on starch isolates and antinutrients. *Ultrason Sonochem*, *73*, 105542. https://doi.org/10.1016/j.ultsonch.2021.105542

Yaldagard, M., Mortazavi, S. A., & Tabatabaie, F. (2008). Application of ultrasonic waves as a priming technique for accelerating and enhancing the germination of barley seed: Optimization of method by the Taguchi approach. *J Inst Brew*, *114*, 14–21. https://doi.org/10.1002/j.2050-0416.2008.tb00300.x

Yang, Q.-Y., Lu, X.-X., Chen, Y.-Z., Luo, Z.-G., & Xiao, Z.-G. (2019). Fine structure, crystalline and physicochemical properties of waxy corn

starch treated by ultrasound irradiation. *Ultrason Sonochem*, *51*, 350–358. https://doi.org/10.1016/j.ultsonch.2018.09.001

Yang, X., Li, Y., Li, S., Oladejo, A. O., Ruan, S., Wang, Y., Huang, S., & Ma, H. (2017). Effects of ultrasound pretreatment with different frequencies and working modes on the enzymolysis and the structure characterization of rice protein. *Ultrason Sonochem*, *38*, 19–28. https://doi.org/10.1016/j.ultsonch.2017.02.026

Yang, X., Wang, L., Zhang, F., & Ma, H. (2020). Effects of multi-mode S-type ultrasound pretreatment on the preparation of ACE inhibitory peptide from rice protein. *Food Chem*, *331*, 127216. https://doi.org/10.1016/j.foodchem.2020.127216

Yaver, E., & Bilgiçli, N. (2021). Effect of ultrasonicated lupin flour and resistant starch (type 4) on the physical and chemical properties of pasta. *Food Chem*, *357*, 129758. https://doi.org/10.1016/J.FOODCHEM.2021.129758

Yorgancilar, M., & Bilgiçli, N. (2014). Chemical and nutritional changes in bitter and sweet lupin seeds (*Lupinus albus* L.) during bulgur production. *J Food Sci Technol*, *51*, 1384–1389. https://doi.org/10.1007/S13197-012-0640-0

Yu, S., Zhang, Y., Ge, Y., Zhang, Y., Sun, T., Jiao, Y., & Zheng, X.-Q. (2013). Effects of ultrasound processing on the thermal and retrogradation properties of nonwaxy rice starch. *J Food Process Eng*, *36*, 793–802. https://doi.org/10.1111/jfpe.12048

Zelalem, K. A., & Chandravanshi, B. S. (2014). Levels of essential and non-essential elements in raw and processed *Lupinus albus* L. (white lupin, gibto) cultivated in Ethiopia. *African J Food Agric Nutr Dev*, *14*, 9215–9235. https://doi.org/10.18697/AJFAND.65.13525

Zeng, J., Dou, Y., Yan, N., Li, N., Zhang, H., & Tan, J.-N. (2019). Optimizing ultrasound-assisted deep eutectic solvent extraction of bioactive compounds from Chinese wild rice. *Molecules*, *24*, 2718. https://doi.org/10.3390/molecules24152718

Zhang, H., Chen, G., Liu, M., Mei, X., Yu, Q., & Kan, J. (2020). Effects of multi-frequency ultrasound on physicochemical properties, structural characteristics of gluten protein and the quality of noodle. *Ultrason Sonochem*, *67*, 105135. https://doi.org/10.1016/j.ultsonch.2020.105135

Zhang, K., Wen, Q., Li, T., Wang, Y., Zhang, Y., & Luo, D. (2022). Comparative study of the effects of ultrasonic power on the structure and functional properties of gliadin in wheat and green wheat. *J Food Sci*, *87*, 1020–1034. https://doi.org/10.1111/1750-3841.16050

Zhang, X., Zuo, Z., Ma, W., Yu, P., Li, T., & Wang, L. (2021). Assemble behavior of ultrasound-induced quinoa protein nanoparticles and their roles on rheological properties and stability of high internal

phase emulsions. *Food Hydrocoll*, *117*, 106748. https://doi.org/10.1016/j.foodhyd.2021.106748

Zhang, Y., Li, J., Ma, H., & Zhang, H. (2018). Mechanism study of multimode ultrasound pretreatment on the enzymolysis of wheat gluten. *J Sci Food Agric*, *98*, 1530–1538. https://doi.org/10.1002/jsfa.8624

Zhang, Y., Li, Y., Li, S., Zhang, H., & Ma, H. (2018). *In situ* monitoring of the effect of ultrasound on the sulfhydryl groups and disulfide bonds of wheat gluten. *Molecules*, *23*, 1376. https://doi.org/10.3390/molecules23061376

Zhang, Y., Ma, H., Wang, B., Qu, W., Li, Y., He, R., & Wali, A. (2015). Effects of ultrasound pretreatment on the enzymolysis and structural characterization of wheat gluten. *Food Biophys*, *10*, 385–395. https://doi.org/10.1007/s11483-015-9393-4

Zhang, Y., Ma, H., Wang, B., Qu, W., Wali, A., & Zhou, C. (2015). Relationships between the structure of wheat gluten and ACE inhibitory activity of hydrolysate: Stepwise multiple linear regression analysis. *J Sci Food Agric*, *96*, 3313–3320. https://doi.org/10.1002/jsfa.7509

Zheng, L.-L., Wen, G., Yuan, M.-Y., & Gao, F. (2016). Ultrasound-assisted extraction of total flavonoids from corn silk and their antioxidant activity. *J Chem*. https://doi.org/10.1155/2016/8768130

Zhou, C., Ma, H., Yu, X., Liu, B., Yagoub, A. E.-G. A., & Pan, Z. (2013). Pretreatment of defatted wheat germ proteins (by-products of flour mill industry) using ultrasonic horn and bath reactors: Effect on structure and preparation of ACE-inhibitory peptides. *Ultrason Sonochem*, *20*, 1390–1400. https://doi.org/10.1016/j.ultsonch.2013.04.005

Zhou, X., Wang, S., & Zhou, Y. (2021). Study on the structure and digestibility of high amylose Tartary buckwheat (*Fagopyrum tataricum* Gaertn.) starch-flavonoid prepared by different methods. *J Food Sci*, *86*, 1463–1474. https://doi.org/10.1111/1750-3841.15657

Zuo, J. Y., Knoerzer, K., Mawson, R., Kentish, S., & Ashokkumar, M. (2009). The pasting properties of sonicated waxy rice starch suspensions. *Ultrason Sonochem*, *16*, 462–468. https://doi.org/10.1016/j.ultsonch.2009.01.002

2
Aim

This PhD thesis aimed at developing legume-derived ingredients and foods enriched in bioactive compounds through different strategies, like: exploiting underutilized crops; taking advantage of the natural modifications occurring during germination; recovery and upcycling food industry by-products with the possible adoption of high-power low-frequency ultrasound technology.

In the first part of this research, lupin was chosen as an example of a underutilized legume and three studies were conducted to: evaluate the free phenolic compounds across 33 ecotypes of *Lupinus mutabilis*, kindly provided by the Universidad Nacional Agraria La Molina (Lima, Peru), and perform a preliminary investigation of FT-NIR suitability as a non-destructive approach to assess antioxidant properties; fine-tune an optimized debittering method evaluating its effect on antioxidants; assess germination as a process for improving the content in lipophilic antioxidants.

The second part of this thesis focused on the recycling of an industrially-generated by-product resulting from canning of green peas. Casalasco Società Agricola SPA kindly provided the material that was employed in two studies. The former aimed at optimizing an ultrasound-assisted method for protein extraction, while in the latter the by-product was assessed as a substrate for *Propionibacterium freudenreichii* fermentation to synthesize vitamin B12 and fortify bread. This task was accomplished during a period abroad of six months at the University of Helsinki.

3

Free phenolics and FT-NIR survey in *Lupinus mutabilis*

The contents of this chapter were published in:

Estivi, L., Grassi, S., Briceño-Berrú, L., Glorio-Paulet, P., Camarena, F., Hidalgo, A., & Brandolini, A. (2022). Free phenolic compounds, antioxidant capacity and FT-NIR survey of debittered *Lupinus mutabilis* seeds. *Processes*, *10*, 1637. https://doi.org/10.3390/pr10081637

Abstract

Lupinus mutabilis protein-rich seeds must be debittered before consumption. The aim of this research was to assess free phenolic compounds, antioxidant capacity and FT-NIR spectra of flours from debittered seeds of 33 Andean ecotypes of *L. mutabilis*, and five varieties belonging to *L. luteus*, *L. angustifolius* and *L. albus* as controls. The free phenolics were quantified by RP-HPLC, while the antioxidant capacity was evaluated spectrophotometrically through the Reducing Power, ABTS, FRAP and DPPH methods. The free phenolics of *L. mutabilis* were mostly (97–100%) flavonoids (genistein and genistein derivatives, apigenin, catechin and naringenin). Other compounds, detected in low quantities, were phenolic acids (cinnamic acid derivatives). The highest total free phenolic concentration was observed in H6 INIA BP (1343 mg/kg DM), followed by Chacas, Moteado beige, Huánuco and Lircay. The antioxidant capacity of the *L. mutabilis* ecotypes exceeded that of the controls and was correlated to flavonoids content. Additionally, 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.

3.1 Introduction

The growing interest of consumers for nutritional quality and the beneficial effects of foods on health is constantly stimulating the search for new raw materials with good macronutrients composition and rich in bioactive compounds. In this context, the rediscovery of traditional but often underutilized crops from different regions of the world is an appealing and realistic alternative. The Andean lupin (*L. mutabilis* Sweet), locally called tarwi or chocho, is a South American legume belonging to a genus which includes three species of worldwide agricultural interest, i.e., *L. albus*, *L. luteus* and *L. angustifolius* (Musco et al., 2017). The Andean lupin, cultivated for millennia in the temperate and cold climates of the Andes in South America, combines thrifty agronomic requirements with excellent nutritional properties, thus representing a promising resource for the preparation of high nutritional value food products.

Lupin seeds have a balanced composition, characterized by an excellent content of proteins, lipids and micronutrients (Bähr et al., 2014; Carvajal-Larenas et al., 2016; Ruiz-López et al., 2019), as well as by numerous bioactive compounds with antioxidant capacity, such as tocols (Boschin & Arnoldi, 2011; Briceño Berru et al., 2021; Czubinski et al., 2021), carotenoids (Briceño Berru et al., 2021; Czubinski et al., 2021) and phenolics (Brandolini et al., 2022; Czubinski et al., 2021; Grela et al., 2017). Particularly interesting are the phenolic compounds, primary antioxidants that stabilize free radicals protecting cell membranes, proteins, lipids and DNA from oxidative damage (Nimse & Pal, 2015). Lupins also contain quinolizidine alkaloids, bitter and toxic compounds which must be removed before consumption through a long debittering procedure that includes boiling and repeated water washes (Carvajal-Larenas et al., 2014; Córdova-Ramos, Glorio-Paulet, Camarena, et al., 2020; Jiménez-Martínez et al., 2010); to avoid this process, some breeding programmes are selecting genotypes characterized by low levels of alkaloids (Galek et al., 2017; Gulisano et al., 2019; Ruiz-López et al., 2019). Once debittered, lupin seeds have many potential applications as food for humans and animals, and as additives for cosmetic, pharmaceutical and medical industries (Gulisano et al., 2019; Sbihi et al., 2013).

Most of the published results on the phenolic composition and antioxidant capacity of lupins are about Mediterranean lupins, and refer to bitter seeds. However, the boiling and repeated washings needed for debittering induce a drastic reduction of water-soluble compounds, such as minerals, mono-, di- and oligosaccharides (Carvajal-Larenas et al., 2014; Córdova-Ramos, Glorio-Paulet, Camarena, et al., 2020; Jiménez-Martínez et al., 2010), and phenolics (Brandolini et al., 2022; Córdova-Ramos, Glorio-Paulet, Hidalgo, & Camarena,

2020; Villacrés et al., 2020). In fact, after debittering, a 96–97% reduction in total polyphenols across three *L. mutabilis* ecotypes was observed (Villacrés et al., 2020), while in three other Andean lupins an average of 76% phenolics decrease was noticed; flavonoids (-71%) were particularly susceptible, while phenolic acids (-57%) seemed to be more resilient (Brandolini et al., 2022).

The industry is looking for rapid, low-cost, eco-friendly but accurate methods for chemical composition determination. Among rapid measurement techniques, Near Infrared (NIR) spectroscopy was demonstrated to be a reliable approach for food product characterization. Indeed, NIR spectra, deriving from the interaction light-matter in the region of 12500–400 cm^{-1}, contain abundant information on the molecular interaction between functional groups.

The widespread industrial utilization of the Andean lupin is hampered by the limited number of studies analysing its composition and technological characteristics, as well as the lack of improved varieties suitable for cultivation in the main lupin cropping areas. A recent study characterized chemical composition, carotenoids and tocols of 33 Andean ecotypes of *Lupinus mutabilis*, originating from different eco-geographic regions of Peru (Briceño Berru et al., 2021). To identify the most suitable ecotypes for developing high nutritional value food products, the aim of this research was to improve the characterization of these very same materials by determining the free phenolic compounds and antioxidant capacity of the water-debittered seeds. A second aim was to perform a preliminary investigation of FT-NIR suitability as a fast, reliable and non-destructive approach to assess the antioxidant properties of lupins.

3.2 Materials and methods

3.2.1 Materials

The seeds of the 33 *Lupinus mutabilis* ecotypes analysed (Supplementary Table S.1), kindly provided by the Leguminous Program of the Universidad Nacional Agraria La Molina (Lima, Peru), were originally collected in different regions of the Peruvian Andes (Supplementary Figure S.1). One *Lupinus albus* from Peru (cv. Dulce 7), as well as two *L. albus* (cv. Ares and Multitalia), one *Lupinus angustifolius* (cv. Boregine) and one *Lupinus luteus* (cv. Percoz) from Italy were tested as controls (Supplementary Table S.1).

3.2.2 Debittering and milling

To remove the alkaloids, debittering was conducted according to Córdova-Ramos, Glorio-Paulet, Camarena, et al. (2020). The lupin beans were hydrated at room temperature for 12 h with a 1:6 (w/v) seeds:water ratio; boiled for 1 h (hydrated seeds:water 1:3 w/v), changing water after 30 min; soaked in water (cooked seeds:water 1:3 w/v) at room temperature for 5 days, substituting the water every day; strained and then dried at 50 °C in a

SW-10S dryer (Xinhang, Henan, China) for 18 h; and stored in the dark at room temperature until milling.

The debittered lupin grains were ground with a Grindomix GM 200 knife mill (Retsch GmbH, Germany) at 6000 rpm for 35 s. The whole meals were sifted through a 20.0 mesh (0.85 mm) sieve, vacuum-packed in high-density polyethylene bags and stored at 4 °C until the analyses.

3.2.3 Free phenolic compounds

The extraction of free phenolic compounds was carried out according to the procedure reported by Brandolini et al. (2022). Approximately 1 g of flour was weighed into capped centrifuge tubes and 15 mL of methanol 80% in water were added. After vortexing with a Wizard Advanced IR Vortex Mixer (Velp Scientifica, Usmate Velate, Italy) for about 45–60 s, the samples were placed in an ultrasonic bath for 10 min, vortexed again for few seconds and centrifuged at 16100 *g* for 10 min at 8 °C with a LISA centrifuge (AFI Groups, Château-Gontier, France). The supernatants, which contain the free phenolics, were recovered in a 250 mL flask and covered with foil to protect the content from light. The extraction was repeated two more times. The three extracts were merged, evaporated under vacuum using a Laborota 4000 Efficient rotavapor (Heidolph, Milan, Italy) for 40 min at 35 °C and completely dried by nitrogen flow for 1 min. The dry samples were re-suspended with 2 mL of methanol:chromatographic water 8:2 (v/v), vortexed and filtered on a 0.45 µm PTFE membrane (Diana Beck Scientific, Angera, Italy).

The reverse-phase HPLC analysis was performed according to Aborus et al. (2018) using column Adamas® C18-AQ 250 × 4.6 mm, 5 µm and a guard column C18 10 × 4.6 mm, 5 µm (SepaChrom SRL, Rho, Italy) thermostated at 30 °C. The system included the following: injector Rheodyne 7125 (VWR, Hitachi, Tokyo, Japan) mounting a 20 µL loop; pump L-2130 Elite LaChrom (VWR, Hitachi); column oven L-2300 Elite LaChrom (VWR, Hitachi); Diode Array Detector L-2450 Elite LaChrom (Merck, Hitachi, Tokyo, Japan); software EZChrom Client/Server v3.1.7). Elution was achieved using acetonitrile (A) and 1% (v/v) formic acid in water (B) mobile phases at 1 mL/min flow rate with the following gradient profile: 0–10 min, from 10% to 25% A with linear rise; 10–20 min linear rise to 60% A; 20–30 min linear rise to 70%; 10 min reverse to 10% A; 5 min of equilibration time.

The identity of the compound was confirmed by the congruence of retention times and UV/VIS spectra with those of pure authentic standards. The unidentified peaks were quantified using the calibration curve of the compound with a similar absorption spectrum and named as "phenolic derivative", as proposed by several authors (Brandolini et al., 2022; Campos & Markham, 2007; Dueñas et al., 2009; Magalhães, Taveira, et al., 2017; Zalewski et al., 2020); additionally, the derivatives of each compound were grouped together. For

phenolics quantification, the calibration curves of the identified phenolics were constructed using standards (Sigma-Aldrich) recorded at 280 nm for catechin (13.9–99.2 mg/L), genistein (27.5–110.0 mg/L), naringenin (2.3–9.0 mg/L), cinnamic acid (4.1–19.1 mg/L); at 320 nm for apigenin (1.0–20.0 mg/L); at 360 nm for diosmin (5.2–104.8 mg/L). Based on the calibration curve, the limit of detection was calculated as the intercept value of the regression line, plus three times the standard error of the estimate (Miller & Miller, 1998). The calibration curves were linear in the concentration intervals assessed, with the respectively following detection limits: 1.86, 1.52, 0.15, 0.19, 1.19 and 0.41 mg/L. The results are reported as mg/kg dry matter (DM).

3.2.4 Antioxidant capacity

Exactly 0.100 ± 0.010 g of flour was mixed in a Eppendorf tube with 1 mL of 80% methanol in water, vortexed, placed in an ultrasonic bath for 10 min, shaken for 20 min and centrifuged (4224 Centrifuge, ALC, Milan, Italy) at 15290 *g* for 10 min. After recovering the supernatant, the extraction was repeated and the two supernatants combined. The antioxidant capacity of debittered lupins was evaluated by the reducing power (RP) method, according to Oyaizu (1986), the ABTS method according to Re et al. (1999), the FRAP method and the DPPH method according to Yilmaz et al. (2015). The antioxidant capacity was expressed as millimoles Trolox equivalent (TE)/kg (DM). All the assays were carried out in three independent samples.

The antioxidant capacity of 20 of the 33 *Lupinus mutabilis* ecotypes here analysed, as well as that of the controls, was previously reported in Briceño Berru (2023).

3.2.5 Near infrared spectroscopy

The debittered lupin flours were analysed by a Fourier Transform (FT)-NIR spectrometer (MPA, Bruker Optics, Ettlingen, Germany) equipped with an integrating sphere module, with a 25 mm diameter window, working in diffuse reflectance mode and a RT-PbS external detector (non-linearity correction: cut-off 3350 cm^{-1}, efficiency: 0.9). Two 1 g aliquots of each sample were analysed by positioning the glass cups containing the sample on the measuring window. The spectra were collected in the range 12500 to 3600 cm^{-1} (resolution, 8 cm^{-1}; scanner velocity, 10 kHz; background, 32 scans; sample, 32 scans). OPUS software (v. 6.5, Bruker Optics, Ettlingen, Germany) was used for instrumental control and for spectra acquisition.

3.2.6 Statistical analysis

To assess the differences among Andean lupins, species or geographical area of origin for the traits analysed, a one-way analysis of variance (ANOVA) was performed. The geographical areas were: North (groups A + B), Centre North

(C + D), Centre South (E + F) and South (G + H). Normal distribution of the data was verified and, if needed, the log-transformation was applied. When significant differences were detected, Fisher's least significant differences (LSD) at $p \leq 0.05$ were calculated. Pearson's linear correlation between the total free phenolic acid content of lupins and their antioxidant capacity was also determined. All these analyses were performed using the Statgraphics® Centurion 18 statistical programme (StatPoint Technologies Inc., Warrengton, VA, USA). The average values, standard deviations and standard errors were computed using the Excel® program (Microsoft, Redmond, WA, USA).

Furthermore, Principal Component Analysis (PCA) was applied to explore sample distribution of debittered seeds, according to species or geographical area of origin, for the traits analysed in this work (free phenolics and antioxidant capacities) and in previous research (carotenoids and tocols) on the same samples (Briceño Berru et al., 2021), as well as to assess variable relationships (Wold et al., 1984). Regression models were developed by partial least squares (PLS), combining NIR spectral information (X) with the dependent information (Y), i.e., phenolic compounds and antioxidant capacity data. The spectral range was reduced to the most informative region (9000–3800 cm^{-1}) and transformed by different spectral pre-processing techniques (alone or in combination), i.e., no spectral pre-processing; standard normal variation (SNV); smoothing (Savitzky-Golay with filter width 15); first derivative (Savitzky-Golay with filter width 15); second derivative (Savitzky-Golay with filter width 15); and always followed by mean centring. In addition, a variable selection procedure was performed using interval Partial Least Square (i-PLS; Nørgaard et al., 2000), to identify the best subset of spectral variables for the elaboration of the PLS regression models. The model dimensionality, i.e., the number of latent variables (LV), was assessed by the venetian blind cross-validation strategy. Statistical parameters such as root mean error in calibration ($RMSE_{CAL}$) and cross-validation ($RMSE_{CV}$); root mean square percentage error in calibration ($RMSPE_{CAL}$) and cross-validation ($RMSPE_{CV}$); coefficient of determination of both calibration (R^2_{CAL}) and cross-validation (R^2_{CV}), and bias and ratio of performance to deviation (RPD) were used to assess model performance. The PCA and PLS models were developed with PLS Toolbox (Eigenvector, Manson, WA, USA) working under MATLAB environment (R2016b, The Mathworks, Natick, MA, USA).

3.3 Results and discussion

3.3.1 Phenolics composition and content

The phenolic composition of the analysed debittered Andean lupins is reported in Supplementary Table S.2, while one chromatogram is shown in the Supplementary Figure S.2 as an example. Seven compounds were detected: the flavonoids genistein; genistein derivative; apigenin derivative; catechin

derivative; diosmin derivative; naringenin derivative; and, among phenolic acid, cinnamic acid derivatives. The most abundant phenolics were the flavonoids, encompassing 97.0–100.0% of total phenolics, followed by the phenolic acids (not detected–2.1%). Genistein derivative (465 ± 25 mg/kg DM) and genistein (136 ± 10 mg/kg DM) represented 67.4% and 19.7% of total flavonoids, respectively. Four compounds (genistein and derivatives of genistein, apigenin and diosmin) were present across all Andean lupins, while others were below the detection limits in one (naringenin derivative) or more (catechin derivative, and cinnamic acid derivative) samples. The phenolics profile and concentration was similar to that described by Brandolini et al. (2022) for three debittered Andean lupin ecotypes, but lacking the vanillic acid, *p*-hydroxybenzoic acid and 2,4-hydroxybenzoic acid derivatives. Tian et al. (2024) assessed the flavonoids profile in the debittered seeds of ten of the *L. mutabilis* ecotypes here studied: total flavonoids ranged from 1252 to 2032 mg/kg DM, while the main compounds identified were the isoflavones methoxy-genistein (ranging from 20% to 54% of total flavonoids), genistein (11–27%) and methoxy-genistein-hexoside (4–20%).

The ANOVAs detected highly significant differences ($p \leq 0.001$) among ecotypes for all the traits analysed. The H6 INIA BP showed the highest total free phenolics concentration (1342.98 mg/kg DM), followed by Chacas (1290.67 mg/kg DM), Huánuco 2 (1226.32 mg/kg DM), Moteado beige (1208.69 mg/kg DM) and Lircay (1170.61 mg/kg DM), while the ecotypes CD Junín 7-2 (382.54 mg/kg DM) and Churibamba (294.98 mg/kg DM) had the lowest (Figure 3.1). Gálvez-Ranilla et al. (2009), studying the isoflavones of bitter seeds from six Andean lupins from the Legume and Cereal program of the Universidad Agraria of Lima, Peru, observed a broad variation, with one sample (H6, the source of the two H6 INIA ecotypes) having isoflavones and antioxidant capacity (DPPH method) far superior to the other *L. mutabilis*, including three accessions present in this research, SCG 22, Yunguyo and Andares (*sic*, probably a transcription error for Andenes).

The ANOVAs (not reported) showed the existence of highly significant differences ($p \leq 0.05$ to $p \leq 0.001$) between Andean lupins and the controls for all the phenolic compounds, except apigenin; hence, the differences were highly significant ($p \leq 0.001$) for total phenolics content too. The lupin controls, belonging to the *L. albus* and *L. angustifolius* species, had very low phenolics content (6.8–31.3 mg/kg DM) and completely different composition (Table 3.1), limited to apigenin derivatives and, for Dulce 7, to genistein and its derivative. On the other hand, the *L. luteus* control showed a phenolics content (398.7 mg/kg DM) in the lower range of the *L. mutabilis* tested and a comparable composition, rich in flavonoids. Several authors (Czubinski et al., 2019; Ferchichi et al., 2021; Magalhães, Taveira, et al., 2017; Siger et al., 2012) observed that non-debittered *L. luteus* seeds have higher phenolic acids and flavonoids content than *L. albus* and *L. angustifolius*. A higher total phenolic content in *L. mutabilis* is also shown from the results and the principal component analysis performed by Czubinski et al. (2021).

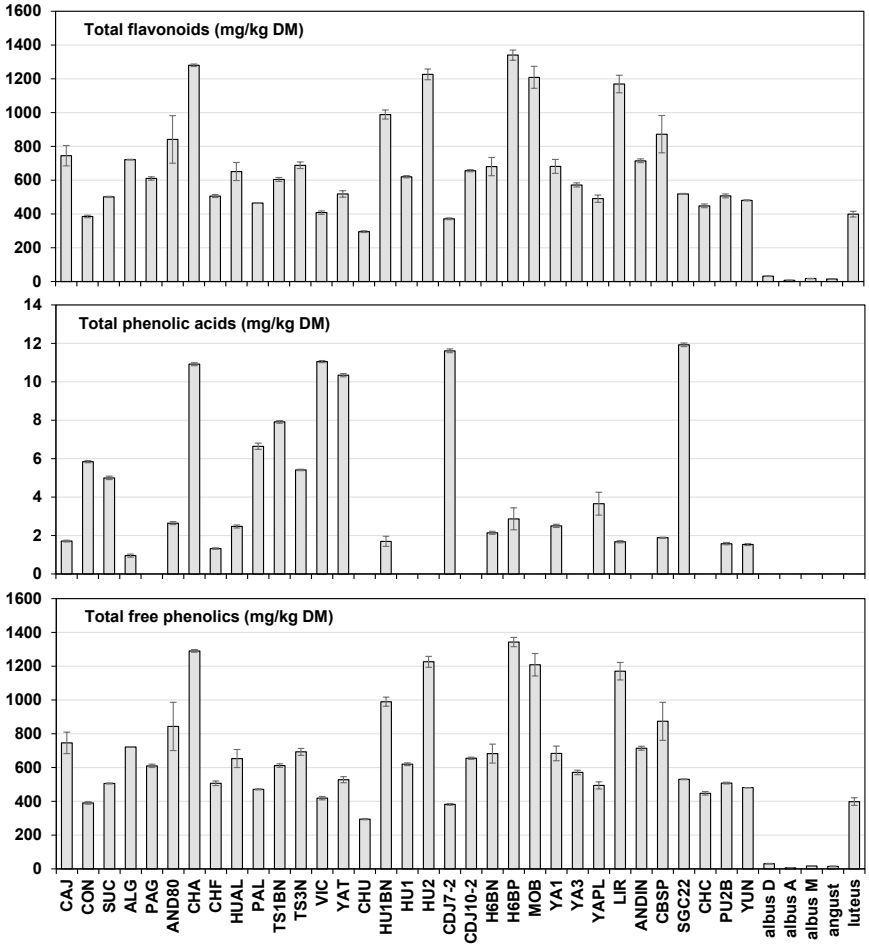

Figure 3.1. Total flavonoids, total phenolic acids and total free phenolics in debittered seeds of 33 *L. mutabilis*, three *L. albus*, one *L. angustifolius* and one *L. luteus*. The ecotype codes are defined in Supplementary Table S.1. Error bars represent standard deviation.

In non-debittered seed samples, Gálvez-Ranilla et al. (2009) noticed that their *L. albus* and *L. angustifolius* controls had very low or non-detectable concentrations of isoflavones, while apigenin and two *p*-coumaric derivatives were observed in *L. albus* (Karamać et al., 2018), and ferulic, sinapic and *p*-coumaric acids were recorded in *L. angustifolius* (Król et al., 2018). On the other hand, from bitter seeds of the three Mediterranean lupin species, Lampart-Szczapa et al. (2003) recovered protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, *p*-coumaric and ferulic acids, while Siger et al. (2012)

Table 3.1. Average values (mean ± standard error) of free phenolics content (mg/kg DM) and antioxidant capacity (mmol TE/kg DM) in debittered seeds of 33 *L. mutabilis*, three *L. albus*, one *L. angustifolius* and one *L. luteus*. Ranges of values are reported in parentheses.

	L. mutabilis	*L. albus*	*L. angustifolius*	*L. luteus*
Genistein	135.78 ± 10.49 (38.03–352.12)	2.21 ± 0.01 (nd–13.26)	nd	40.39 ± 3.89
Genistein der	464.69 ± 25.30 (219.57–1062.84)	0.82 ± 0.01 (nd–4.95)	nd	230.00 ± 13.68
Apigenin der	13.02 ± 1.01 (2.70–27.99)	6.19 ± 0.79 (6.79–17.31)	14.82 ± 1.47	27.82 ± 0.39
Catechin der	6.11 ± 1.06 (nd–36.49)	nd	nd	nd
Diosmin der	64.69 ± 4.35 (7.23–168.19)	nd	nd	98.43 ± 5.47
Naringenin der	5.36 ± 0.31 (nd–11.58)	nd	nd	2.03 ± 0.07
Cinnamic acid der	3.49 ± 0.47 (nd–11.92)	nd	nd	nd
Σ free phenolics	693.16 ± 34.74 (294.98–1342.98)	9.23 ± 0.79 (6.79–31.27)	14.82 ± 1.47	398.68 ± 15.84
Reducing power	2.53 ± 0.15 (1.09–6.68)	1.03 ± 0.19 (1.44–3.10)	0.89 ± 0.02	4.01 ± 0.53
ABTS	40.17 ± 1.74 (17.25–74.27)	7.41 ± 3.03 (2.36–21.98)	9.23 ± 0.49	31.34 ± 0.80
DPPH	2.63 ± 0.14 (1.06–6.47)	0.34 ± 0.06 (0.25–1.40)	0.50 ± 0.02	0.86 ± 0.07
FRAP	18.41 ± 0.74 (9.48–30.90)	2.30 ± 0.06 (3.90–5.21)	5.08 ± 0.11	12.44 ± 0.04

der, derivative; nd, not detected, i.e., below the detection limit.

identified apigenin along with gallic, protocatechuic, *p*-hydroxybenzoic, caffeic and *p*-coumaric acids. Further examples of the great intra- and inter-species heterogeneity come from Czubinski et al. (2019), who found only apigenin derivatives in the three species *L. albus*, *L. angustifolius* and *L. luteus* (382.4–569.7, 784.0–1027.3 and 1380.2–1417.7 mg/kg DM, respectively), and from Kalogeropoulos et al. (2010) who, conversely, reported the prevalence of phenolic acids on flavonoids in *L. albus* (14.1 and 8.2 mg/kg DM, respectively).

The broad differences observed between, and within, species were correctly attributed to the effects of genetic selection and long-term adaptation towards eco-geographical areas with different edaphic, climatic and pathogenic characteristics (Ferchichi et al., 2021). Additionally, debittering is often unavoidable for sweet varieties as well (Villacrés et al., 2020), and this process entails an additional source of variability due to different methods and conditions.

3.3.2 Antioxidant capacity and its correlation with phenolics content

The antioxidant capacity of the *L. mutabilis* ecotypes, measured by reducing power, DPPH, ABTS and FRAP, is presented in Figure 3.2. The ANOVAs highlighted the presence of significant differences ($p \leq 0.001$) among samples for all methods. Andenes 80, H6 INIA BN, H6 INIA BP, Moteado beige and Lircay were the ecotypes with the highest values across most tests; conversely, Congona and Churibamba displayed the lowest results.

The ABTS average result for *L. mutabilis* (40.2 mmol TE/kg DM) is in the same order of magnitude of non-debittered *L. albus* and *L. angustifolius* analysed by Martínez-Villaluenga et al. (47.2–71.4 mmol TE/kg DM; 2009); some ecotypes (e.g., Andenes 80, Puno 2 blanquita, Andenes INIA and Lircay) reached the highest values, 1.5–1.8-fold the mean.

The antioxidant capacity of the controls was significantly different and inferior to that of the Andean lupins in three out of four tests (DPPH, ABTS and FRAP). Among the European lupins, *L. luteus* showed the highest values (Table 3.1). Interestingly, the debittered Andean lupin results were similar or only slightly inferior to those recorded in bitter beans of the Mediterranean lupins. In fact, ABTS and FRAP antioxidant capacities of 53–123 µmol TE/g DM and 9.4–12.4 µmol Fe^{2+}/g DM were recorded in *L. albus* (Karamać et al., 2018); 87–152 µmol TE/g extract (ABTS) and 0.108–0.235 µmol Fe^{2+}/g extract (RP) were observed in *L. angustifolius* (Król et al., 2018); and DPPH values of 3.51–6.58 µmol TE/g DM in *L. albus*, 6.89–7.54 in *L. angustifolius* and 8.12–9.03 in *L. luteus* were detected (Siger et al., 2012). Generally, a decrease in antioxidant activity is evident after debittering, as documented by several authors (Córdova-Ramos, Glorio-Paulet, Hidalgo, & Camarena, 2020; Mohammed et al., 2017; Villacrés et al., 2020), and is most likely due to soluble phenolics thermal degradation and washout (Khan et al., 2015; Villacrés et al., 2020). In fact, the bound fraction and the lipophilic antioxidants appear unaffected or even concentrated by the removal of other components (Brandolini et al., 2022; Briceño Berru et al., 2021), while quinolizidine alkaloids do not exhibit antioxidant activities (Chan & Hui, 2000; Hassine et al., 2021; Magalhães, Fernandes, et al., 2017).

The linear correlations between antioxidant capacity and individual phenolics (Table 3.2) were mostly significant, with the exceptions of diosmin derivative and cinnamic acid derivative, which did not present any correlation. The results of the four antioxidant capacity tests were always significantly correlated ($p \leq 0.01$ and $p \leq 0.001$) to flavonoids and total phenolics concentrations, while phenolic acids, scarcely present in the debittered Andean lupins, did not show significant correlation. Hassine et al. (2021) found significant, very high, Pearson's correlation coefficients between ABTS and total phenolics or phenolic acids (0.948 and 0.905, respectively).

The results of the PCA performed on the phenolic contents (flavonoids, phenolic acids, tocols, carotenoids and total free phenolics) and the antioxidant capacity (reducing power, DPPH, ABTS and FRAP) are shown in

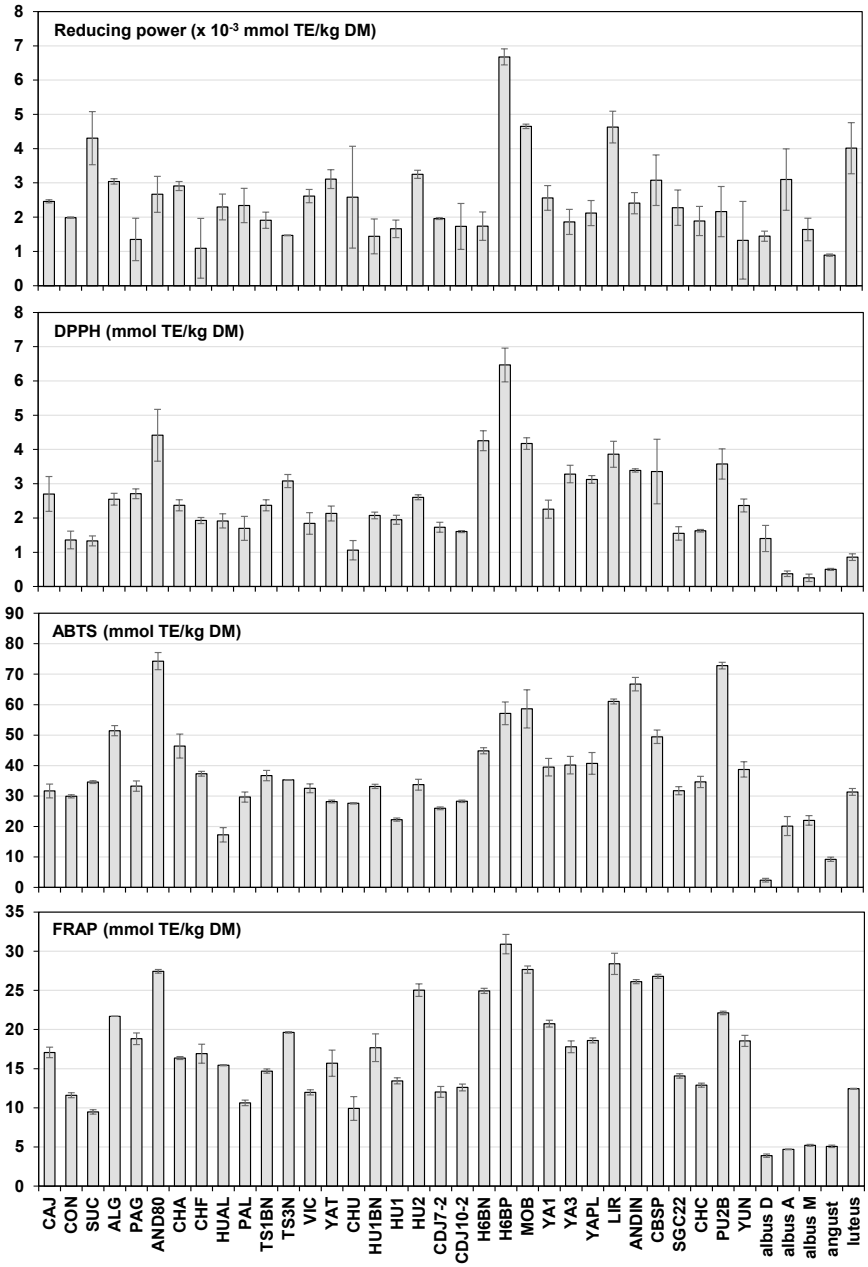

Figure 3.2. Antioxidant capacity (reducing power, DPPH, ABTS and FRAP methods) in debittered seeds of 33 *L. mutabilis*, three *L. albus*, one *L. angustifolius* and one *L. luteus*. The ecotype codes are defined in Supplementary Table S.1. Error bars represent standard deviation.

Table 3.2. Pearson's linear correlation coefficients ($n = 38$) among antioxidant capacity (RP, reducing power; ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power) and free phenolics content of all *Lupinus* samples.

	RP	ABTS	DPPH	FRAP
Genistein	0.35^{*}	0.52^{***}	0.55^{***}	0.69^{***}
Genistein der	0.59^{***}	0.60^{***}	0.76^{***}	0.80^{***}
Apigenin der	0.38^{*}	0.49^{**}	0.47^{**}	0.58^{***}
Catechin der	0.05	0.42^{**}	0.47^{**}	0.45**
Diosmin der	0.16	0.21	0.17	0.32
Naringenin der	0.10	0.45^{**}	0.34^{*}	0.35*
Cinnamic acid der	0.04	−0.04	−0.05	−0.11
Flavonoids	0.61^{***}	0.45^{**}	0.63^{***}	0.71^{***}
Phenolic acids	0.01	−0.25	−0.27	−0.40*
Total phenolics	0.63^{***}	0.48^{**}	0.64^{***}	0.71^{***}

der, derivative; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

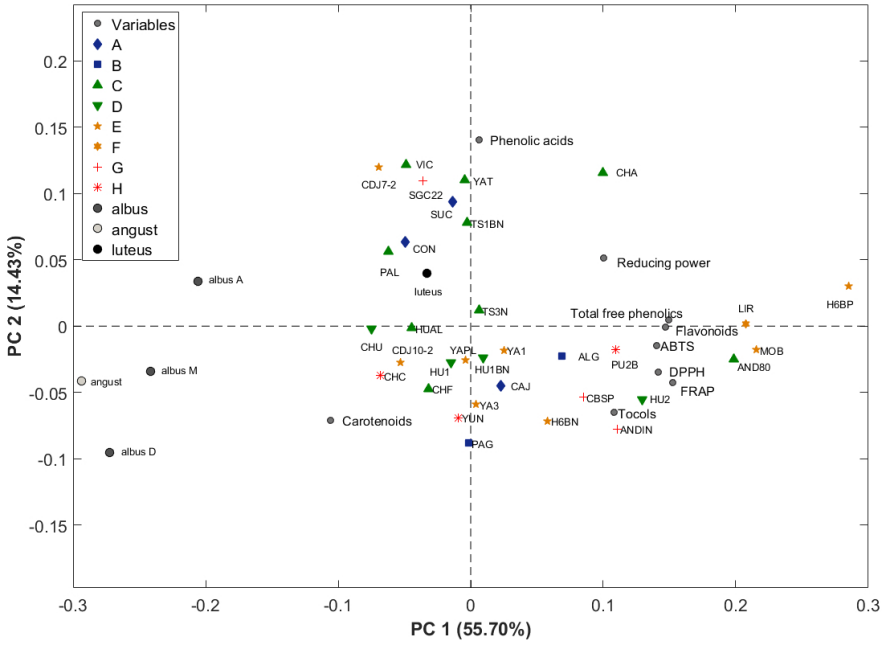

Figure 3.3. Biplot of the principal component analysis performed on phenolic contents (flavonoids, phenolic acids and total free phenolics), tocols, carotenoids, and antioxidant capacity (reducing power, DPPH, ABTS and FRAP) in debittered seeds of 33 *L. mutabilis*, three *L. albus*, one *L. angustifolius*, and one *L. luteus*. The ecotype codes are defined in Supplementary Table S.1. Carotenoids and tocols data are from a previous study (Briceño Berru et al., 2021).

Figure 3.3. The biplot confirmed that *L. albus* and *L. angustifolius* differ from the Andean lupins for all of the phenolic compounds and the antioxidant capacity, whereas *L. luteus* showed phenolic contents and antioxidant capacity comparable to those of *L. mutabilis*. Indeed, the *L. albus* and *L. angustifolius* controls assumed highly negative PC1 scores (< -0.2), thus positioning far away from the other samples. On the opposite side of the PC1 axis it was possible to notice the positioning of the ecotypes with the highest total free phenolics concentration, i.e., H6 INIA BP, Lircay, Moteado beige, Chacas, Huánuco 2 and Andenes 80. This result is linked to a higher carotenoids content in *L. albus* and *L. angustifolius* coupled to lower flavonoids and phenolic acids contents, as also confirmed by the negative loadings for carotenoids and positive loadings for the other compounds. The variable loadings confirmed the existing relationship between antioxidant capacity and tocols, flavonoids and total phenolics, as their loadings were near DPPH, ABTS and FRAP, indicating that they are related and contribute similarly to samples distribution.

3.3.3 Geographical origin

The ANOVAs conducted after grouping the Andean lupins in geographical areas, *viz* North, Centre-North, Centre-South and South, did not highlight any significant difference for phenolic content among ecotypes of the different regions. This result was corroborated by the PCA (Figure 3.3), as no sample grouping was observed according to the origin. On the other hand, ABTS, DPPH and FRAP antioxidant capacities were significantly different between groups at $p \leq 0.05$ and RP was significant at $p \leq 0.089$. On average, the ecotypes coming from the Centre-South and South of Peru showed higher antioxidant capacity than those from the Centre-North and North areas (Table 3.3).

Since no geographical diversities were found for polyphenols content, these differences may be due to other antioxidant compounds present in Andean lupins, such as tocols, carotenoids, etc. In fact, Briceño Berru et al. (2021), in the very same ecotypes, observed greater concentration of β-tocopherol in samples coming from the Centre-South and South regions of Peru, as well as of lutein and total carotenoids in samples from the South, and attributed them to the selection pressure exerted by the harsher climatic conditions of those environments.

3.3.4 Near infrared spectra and regression models

All the species showed similar FT-NIR spectra before (Figure 3.4a) and after the SNV transformation (Figure 3.4b). An absorption band around 8550–8300 cm^{-1} could be attributed to the second overtone of $-CH_2$, and the relatively wide peak at 6900 cm^{-1} can be assigned to the O–H stretch first overtone of hydroxyl groups (Tomar et al., 2021). Around 5785 cm^{-1}

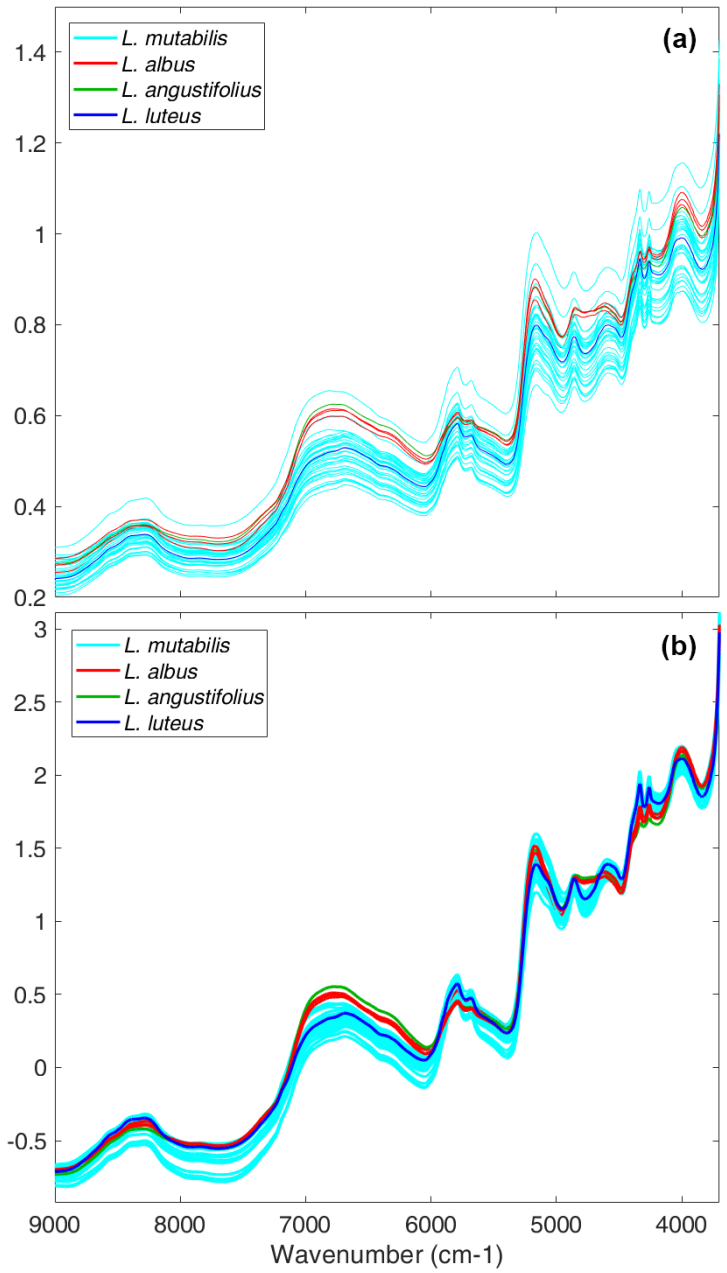

Figure 3.4. FT-NIR spectra collected for the 33 *L. mutabilis* ecotypes, three *L. albus* (cv. Dulce 7, Ares and Multitalia), one *L. angustifolius* (cv. Boregine) and one *L. luteus* (cv. Percoz): (a) raw spectra; (b) FT-NIR spectra after the SNV transformation.

Table 3.3. Mean value (± standard error) of free phenolics (mg/kg DM) and antioxidant capacity (mmol TE/kg DM) of 33 *L. mutabilis* from different regions of Peru. RP, reducing power; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power.

	North	Centre-North	Centre-South	South
N° ecotypes	5	13	9	6
Flavonoids	592.34 ± 67.65	699.20 ± 84.36	796.46 ± 116.43	589.92 ± 167.02
Phenolic acids	2.70 ± 1.15	4.65 ± 1.19	2.72 ± 1.20	2.82 ± 4.54
Total phenolics	595.04 ± 66.71	703.85 ± 84.32	799.18 ± 115.91	592.74 ± 67.91
RP	2.63 ± 0.50^{ab}	2.26 ± 0.19^{b}	3.10 ± 0.59^{a}	2.19 ± 0.58^{b}
ABTS	36.17 ± 3.90^{bc}	34.95 ± 3.84^{c}	44.02 ± 4.24^{ab}	49.02 ± 17.27^{a}
DPPH	2.13 ± 0.32^{b}	2.27 ± 0.22^{b}	3.42 ± 0.50^{a}	2.64 ± 0.92^{ab}
FRAP	15.73 ± 2.27^{b}	16.52 ± 1.42^{b}	21.52 ± 2.29^{a}	20.09 ± 5.93^{ab}

Values in the same row with different letters are significantly different ($p \leq 0.05$) following Fisher's LSD test.

and 5680 cm^{-1} were present the signals related to the first overtone of C–H stretching (Can et al., 2018), while the peak near 5200 cm^{-1} could be attributed to O–H bending/stretching of polysaccharides, converging with those of water; the absorption at 4332 cm^{-1} may be linked to C–H bending (Tomar et al., 2021). The only remarkable difference among the FT-NIR spectra of the different species was in the 4800–4600 cm^{-1} region, where sharp peaks at 4850 cm^{-1} and 4660 cm^{-1} were observed for the Andean lupins and *L. luteus*. This region was previously recognized as the most relevant for the assessment of phenolic contents in faba beans (Wang et al., 2014), and in tannins and flavonoids (Goodchild et al., 1998) due to the asymmetric C–O–O stretches (Zhang et al., 2017). Hence, the differences detected in this region confirmed the lower total phenolics content of *L. albus* and *L. angustifolius*.

The PCA performed with the FT-NIR spectra again split the *L. albus* and *L. angustifolius* samples from the other lupins along the PC1 (Figure 3.5a), linked to PC1 positive loadings of the region 4850 cm^{-1} and 4660 cm^{-1} (Figure 3.5b), where sharp peaks were observed in the FT-NIR spectra collected for Andean lupins and *L. luteus*. Their differentiation from the other lupins is confirmed by their high total carotenoid values, coupled to low tocols, flavonoids and total phenolic acids contents, antioxidant capacities, and b^* colour coordinate. Similarly, four Andean lupins (Andenes INIA, Lircay, Altagracia and Puno 2 blanquita) formed a small cluster in the top right quadrant (Figure 3.5a); their location is linked to high positive loadings, for both PC1 and PC2, in the region 5000–5500 cm^{-1} (Figure 3.5b), characterized by a peak near 5200 cm^{-1} related to O–H bending/stretching of polysaccharides, converging with those of water. The four ecotypes were always among the top scorers for total tocols content, ABTS and FRAP

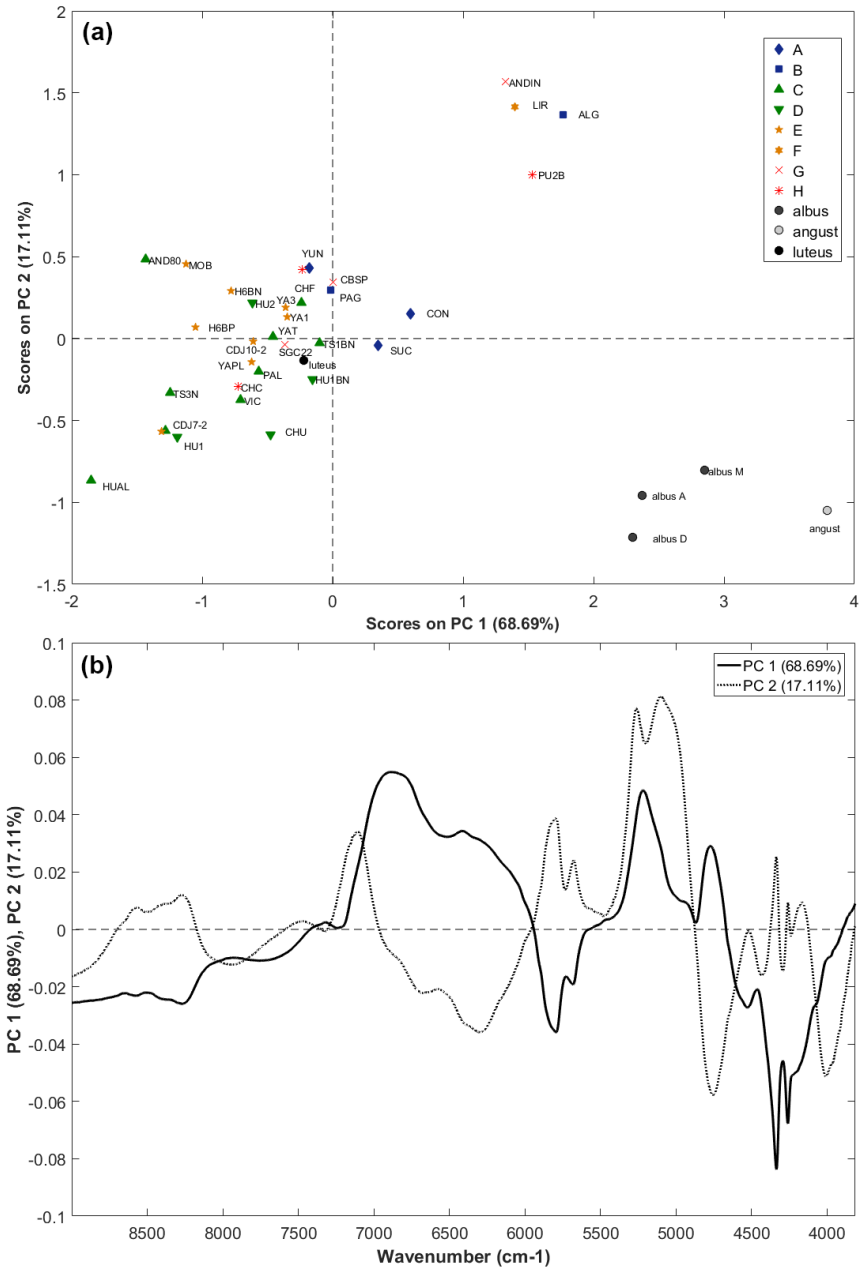

Figure 3.5. Principal component analysis performed on the FT-NIR spectra of debittered seeds of 33 *L. mutabilis*, three *L. albus*, one *L. angustifolius* and one *L. luteus*; the ecotype codes are defined in Supplementary Table S.1. (a) PC1 *vs* PC2 scores plot. (b) PC1 and PC2 loadings plot.

antioxidant capacity of the methanolic extracts and total free phenolics content, and b^* colour coordinate. Finally, the isolated Huallanca ecotype (Figure 3.5a, in the bottom left quadrant) was characterized by the lowest total carotenoids content and ABTS antioxidant capacity of the methanolic extracts.

The relationship between phenolic content and FT-NIR spectra was further investigated by the development of i-PLS regression models. The best models were obtained after SNV transformation for total phenolics (R^2_{CV} of 0.84, $RMSE_{CV}$ of 112.40 mg/kg DM), flavonoids (R^2_{CV} of 0.83, $RMSE_{CV}$ of 116.80 mg/kg DM), carotenoids (R^2_{CV} of 0.86, $RMSE_{CV}$ of 0.51 mg/kg DM), tocols (R^2_{CV} of 0.86, $RMSE_{CV}$ of 23.80 mg/kg DM) and antioxidant capacity as FRAP (R^2_{CV} of 0.76, $RMSE_{CV}$ of 2.89 mmol TE/kg DM); more details are reported in Table 3.4. The spectral regions selected by the i-PLS algorithm confirmed the relevance of the absorption bands related to phenolics presence. Similar results are reported by Tomar et al. (2021) for the prediction of phenolics (range 0.04–0.21 g/100 g) in pearl millet (*Pennisetum glaucum* L.), with standard error in cross validation of 0.0199 g/100 g. Carbas et al. (2020) reached higher performance in predicting phenolics and flavonoids (RPD of 5.20 and 5.18, respectively), but poorer performance for FRAP prediction (RPD of 2.02). Their regression models, developed for total phenolics and flavonoids, however, had a range of variability not comparable to those examined in this research, impeding any comparison of the results, together with a lower number of samples used for calibration ($n = 21$).

Even if the numerosity of the analysed samples is not substantial for model validation in prediction, its performance in cross-validation was evaluated in terms of retrieval, in respect to conventional method results. The average retrieval of the regression models was 100.0, 100.7, 105.1, 99.6 and 92.4% for total phenolics, flavonoids, carotenoids, tocols and antioxidant capacity, respectively. This means that on average the PLS models retrieve the compounds content as the reference method for total phenolics, flavonoids and tocols. The PLS model developed for carotenoids tends to overestimate the presence of compounds in the sample; instead, the model to predict FRAP, on average, underestimates the antioxidant capacity, in respect to the one measured by the colorimetric assay.

3.4 Conclusions

Even after a drastic debittering process that included boiling and recurrent washing, the Andean lupins maintained good free phenolic compounds concentrations, ranging from 340.81 to 1393.32 mg/kg DM. The flavonoids represented about 95% of the total free phenolics. The *L. albus* and *L. angustifolius* used as controls had different phenols composition and content, while the *L. luteus* had similar profile but inferior values. Accordingly, the antioxidant capacity of the *L. mutabilis* ecotypes exceeded that of the controls and was

Table 3.4. Prediction performance of PLS regression models.

Parameter	Range	Variables	LVs	CAL				CV				
				R^2	RMSE	RMSPE	Bias	R^2	RMSE	RMSPE	Bias	RPD
Total phenolics (mg/kg DM)	300.00–1393.32	6823–6711, 6245–6133, 4509–4397, 4278–4050	7	0.92	78.5	12.1	4.55×10^{-12}	0.84	112.4	17.4	1.11×10^{-2}	3
Flavonoids (mg/kg DM)	295.00–1340.11	6823–6711, 6245–6133, 4509–4397, 4278–4050	7	0.92	81	13.3	3.75×10^{-12}	0.83	116.8	19.1	7.20×10^{-2}	2.8
Carotenoids (mg/kg DM)	0.68–7.14	4586–4494	3	0.89	0.42	21.2	8.88×10^{-16}	0.86	0.51	25.8	4.33×10^{-2}	2.4
Tocols (mg/kg DM)	103.22–378.22	6245–6017	3	0.90	18.2	6.4	1.71×10^{-13}	0.86	23.8	8.4	9.72×10^{-2}	2.5
FRAP (mmol TE/kg DM)	3.90–30.90	9000–3800	2	0.80	2.56	14.2	-3.55×10^{-12}	0.76	2.89	16	1.77×10^{-2}	2.4

CAL, calibration;
CV, cross-validation;
R^2, coefficient of determination;
$RMSE_{CAL}$, root mean square error in calibration;
$RMSPE_{CAL}$, root mean square percentage error in calibration;
$RMSE_{CV}$, root mean error in cross validation;
$RMSPE_{CV}$, root mean square percentage error in cross-validation;
RPD, ratio of performance to deviation.
Carotenoids and tocols data are from a previous study (Briceño Berru et al., 2021).

largely correlated to flavonoids concentration. Five ecotypes (H6 INIA BP, Chacas, Moteado beige, Huánuco 2 and Lircay) showed excellent phenolics concentration and antioxidant capacity. The results demonstrate that after debittering, Andean lupin seeds are still rich in phenolic compounds with high antioxidant value, and as such are very promising for the preparation of high nutritional value food products for human consumption. Additionally, the FT-NIR results demonstrated the relationship between the free phenolic compounds and the spectral bands, thus paving the way for a fast, reliable and non-destructive approach to lupin seed characterization for phenolics content and antioxidant capacity. To this end, an increase of the lupin seed dataset would be critical to strengthen the prediction reliability of the regression models.

References

Aborus, N. E., Tumbas Šaponjac, V., J., Č.-B., Ćetković, G., Hidalgo, A., Vulić, J., & Šeregelj, V. (2018). Sprouted and freeze-dried wheat and oat seeds - phytochemical profile and *in vitro* biological activities. *Chem Biodivers*, *15*, e1800119. https://doi.org/10.1002/cbdv.201800119

Bähr, M., Fechner, A., Hasenkopf, K., Mittermaier, S., & Jahreis, G. (2014). Chemical composition of dehulled seeds of selected lupin cultivars in comparison to pea and soya bean. *LWT - Food Sci Technol*, *59*, 587–590. https://doi.org/10.1016/j.lwt.2014.05.026

Boschin, G., & Arnoldi, A. (2011). Legumes are valuable sources of tocopherols. *Food Chem*, *127*, 1199–1203. https://doi.org/10.1016/j.foodchem.2011.01.124

Brandolini, A., Glorio-Paulet, P., Estivi, L., Locatelli, N., Córdova-Ramos, J. S., & Hidalgo, A. (2022). Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) seeds processing. *J Food Compos Anal*, *106*, 104335. https://doi.org/10.1016/j.jfca.2021.104335

Briceño Berru, L. (2023). *Caracterización fisicoquímica, tocoles, carotenoides y capacidad antioxidante de 20 genotipos de tarwi (Lupinus mutabilis Sweet)* [PhD thesis]. Universidad Nacional Agraria La Molina.

Briceño Berru, L., Glorio-Paulet, P., Basso, C., Scarafoni, A., Camarena, F., Hidalgo, A., & Brandolini, A. (2021). Chemical composition, tocopherol and carotenoid content of seeds from different Andean lupin (*Lupinus mutabilis*) ecotypes. *Plant Food Hum Nutr*, *76*, 98–104. https://doi.org/10.1007/S11130-021-00880-0

Campos, M. d. G., & Markham, K. R. (2007). *Structure information from HPLC and on-line measured absorption spectra: Flavones, flavonols and phenolic acids*. https://doi.org/10.14195/978-989-26-0480-0

Can, A., Ayvaz, H., Pala, Ç. U., Condelli, N., Galgano, F., & Tolve, R. (2018). The potential of near and mid-infrared spectroscopy for rapid quantification of oleuropein, total phenolics, total flavonoids and antioxidant activity in olive tree (*Olea europaea*) leaves. *J Food Meas Charact*, *12*, 2747–2757. https://doi.org/10.1007/s11694-018-9892-3

Carbas, B., Machado, N., Oppolzer, D., Queiroz, M., Brites, C., Rosa, E. A., & Barros, A. I. (2020). Prediction of phytochemical composition, *in vitro* antioxidant activity and individual phenolic compounds of common beans using MIR and NIR spectroscopy. *Food Bioprocess Technol*, *13*, 962–977. https://doi.org/10.1007/s11947-020-02457-2

Carvajal-Larenas, F. E., Linnemann, A. R., Nout, M. J. R., Koziol, M., & van Boekel, M. A. J. S. (2016). *Lupinus mutabilis*: Composition, uses, toxicology, and debittering. *Crit Rev Food Sci Nutr*, *56*, 1454–1487. https://doi.org/10.1080/10408398.2013.772089

Carvajal-Larenas, F. E., van Boekel, M. A. J. S., Koziol, M., Nout, M. J. R., & Linnemann, A. R. (2014). Effect of processing on the diffusion of alkaloids and quality of *Lupinus mutabilis* Sweet. *J Food Process Pres*, *38*, 1461–1471. https://doi.org/10.1111/jfpp.12105

Chan, Y. T., & Hui, S. (2000). *In-vitro* assay as screening tools for determination of the antioxidant activities of quinolizidine (lupin) alkaloids. *Proceedings of the International Congress on Traditional Medicine 22–24 April 2000*.

Córdova-Ramos, J. S., Glorio-Paulet, P., Camarena, F., Brandolini, A., & Hidalgo, A. (2020). Andean lupin (*Lupinus mutabilis* Sweet): Processing effects on chemical composition, heat damage, and *in vitro* protein digestibility. *Cereal Chem*, *97*, 827–835. https://doi.org/10.1002/cche.10303

Córdova-Ramos, J. S., Glorio-Paulet, P., Hidalgo, A., & Camarena, F. (2020). Effect of technological process on antioxidant capacity and total phenolic content of Andean lupine (*Lupinus mutabilis* Sweet). *Sci Agropecu*, *11*, 157–165. https://doi.org/10.17268/sci.agropecu.2020.02.02

Czubinski, J., Grygier, A., & Siger, A. (2021). *Lupinus mutabilis* seed composition and its comparison with other lupin species. *J Food Compos Anal*, *99*, 103875. https://doi.org/10.1016/j.jfca.2021.103875

Czubinski, J., Wroblewska, K., Czyzniejewski, M., Górnaś, P., Kachlicki, P., & Siger, A. (2019). Bioaccessibility of defatted lupin seed phenolic compounds in a standardized static *in vitro* digestion system. *Food Res Int*, *116*, 1126–1134. https://doi.org/10.1016/j.foodres.2018.09.057

Dueñas, M., Hernández, T., Estrella, I., & Fernández, D. (2009). Germination as a process to increase the polyphenol content and antioxidant activity of lupin seeds (*Lupinus angustifolius* L.). *Food Chem*, *117*, 599–607. https://doi.org/10.1016/j.foodchem.2009.04.051

Ferchichi, N., Toukabri, W., Vrhovsek, U., Nouairi, I., Angeli, A., Masuero, D., Mhamdi, R., & Trabelsi, D. (2021). Proximate composition, lipid and phenolic profiles, and antioxidant activity of different ecotypes of *Lupinus albus*, *Lupinus luteus* and *Lupinus angustifolius*. *J Food Meas Charact*, *15*, 1241–1257. https://doi.org/10.1007/s11694-020-00722-8

Galek, R., Sawicka-Sienkiewicz, E., Zalewski, D., Stawiński, S., & Spychała, K. (2017). Searching for low alkaloid forms in the Andean lupin (*Lupinus mutabilis*) collection. *Czech J Genet Plant Breed*, *53*, 55–62. https://doi.org/10.17221/71/2016-CJGPB

Gálvez-Ranilla, L., Genovese, M. I., & Lajolo, F. M. (2009). Isoflavones and antioxidant capacity of Peruvian and Brazilian lupin cultivars. *J Food Compos Anal*, *22*, 397–404. https://doi.org/10.1016/j.jfca.2008.06.011

Goodchild, A. V., El Haramein, F. J., Abd El Moneim, A., Makkar, H. P. S., & Williams, P. C. (1998). Prediction of phenolics and tannins in forage legumes by near infrared reflectance. *J Near Infrared Spectrosc*, *6*, 175–181. https://doi.org/10.1255/jnirs.134

Grela, E., Kiczorowska, B., Samolińska, W., Matras, J., Kiczorowski, P., Rybiński, W., & Hanczakowska, E. (2017). Chemical composition of leguminous seeds: Part I—Content of basic nutrients, amino acids, phytochemical compounds, and antioxidant activity. *Eur Food Res Technol*, *243*, 1385–1395. https://doi.org/10.1007/s00217-017-2849-7

Gulisano, A., Alves, S., Neves Martins, J., & Trindade, L. M. (2019). Genetics and breeding of *Lupinus mutabilis*: An emerging protein crop. *Front Plant Sci*, *10*, 1385. https://doi.org/10.3389/fpls.2019.01385

Hassine, A. B., Rocchetti, G., Zhang, L., Senizza, B., Zengin, G., Mahomoodally, M. F., Ben-Attia, M., Rouphael, Y., Lucini, L., & El-Bok, S. (2021). Untargeted phytochemical profile, antioxidant capacity and enzyme inhibitory activity of cultivated and wild lupin seeds from Tunisia. *Molecules*, *26*, 3452. https://doi.org/10.3390/molecules26113452

Jiménez-Martínez, C., Mora-Escobedo, R., Cardador Martínez, A., Muzquiz, M., Martin Pedrosa, M., & Dávila-Ortiz, G. (2010). Effect of aqueous, acid, and alkaline thermal treatments on antinutritional factors content and protein quality in *Lupinus campestris* deed flour. *J Agr Food Chem*, *58*, 1741–1745. https://doi.org/10.1021/JF902688R

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V. T., Hassapidou, M., & Andrikopoulos, N. K. (2010). Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chem*, *121*, 682–690. https://doi.org/10.1016/j.foodchem.2010.01.005

Karamać, M., Orak, H. H., Amarowicz, R., Orak, A., & Piekoszewski, W. (2018). Phenolic contents and antioxidant capacities of wild and

cultivated white lupin (*Lupinus albus* L.) seeds. *Food Chem*, *258*, 1–7. https://doi.org/10.1016/j.foodchem.2018.03.041

Khan, M. K., Karnpanit, W., Nasar-Abbas, S. M., Huma, Z., & Jayasena, V. (2015). Phytochemical composition and bioactivities of lupin: A review. *Int J Food Sci Technol*, *50*, 2004–2012. https://doi.org/10.1111/ijfs.12796

Król, A., Amarowicz, R., & Weidner, S. (2018). Content of phenolic compounds and antioxidant properties in seeds of sweet and bitter cultivars of lupine (*Lupinus angustifolius*). *Nat Prod Commun*, *13*, 1341–1344. https://doi.org/10.1177/1934578X1801301027

Lampart-Szczapa, E., Korczak, J., Nogala-Kalucka, M., & Zawirska-Wojtasiak, R. (2003). Antioxidant properties of lupin seed products. *Food Chem*, *83*, 279–285. https://doi.org/10.1016/S0308-8146(03)00091-8

Magalhães, S. C. Q., Fernandes, F., Cabrita, A. R. J., Fonseca, A. J. M., Valentão, P., & Andrade, P. B. (2017). Alkaloids in the valorization of European *Lupinus* spp. seeds crop. *Ind Crops Prod*, *95*, 286–295. https://doi.org/10.1016/j.indcrop.2016.10.033

Magalhães, S. C. Q., Taveira, M., Cabrita, A. R., Fonseca, A. J., Valentão, P., & Andrade, P. B. (2017). European marketable grain legume seeds: Further insight into phenolic compounds profiles. *Food Chem*, *215*, 177–184. https://doi.org/10.1016/j.foodchem.2016.07.152

Martínez-Villaluenga, C., Zielinski, H., Frias, J., Piskuła, M. K., Kozłowska, H., & Vidal-Valverde, C. (2009). Antioxidant capacity and polyphenolic content of high-protein lupin products. *Food Chem*, *112*, 84–88. https://doi.org/10.1016/j.foodchem.2008.05.040

Miller, J. C., & Miller, J. N. (1998). *Statistics for Analytical Chemistry, 2nd ed.* Ellis Horwood Publishers.

Mohammed, M. A., Mohamed, E. A., Yagoub, A. E. A., Mohamed, A. R., & Babiker, E. E. (2017). Effect of processing methods on alkaloids, phytate, phenolics, antioxidants activity and minerals of newly developed lupin (*Lupinus albus* L.) cultivar. *J Food Process*, *41*, 1–9. https://doi.org/10.1111/jfpp.12960

Musco, N., Cutrignelli, M. I., Calabrò, S., Tudisco, R., Infascelli, F., Grazioli, R., Lo Presti, V., Gresta, F., & Chiofalo, B. (2017). Comparison of nutritional and antinutritional traits among different species (*Lupinus albus* L., *Lupinus luteus*, *Lupinus angustifolius* L.) and varieties of lupin seeds. *J Anim Physiol Anim Nutr*, *101*, 1227–1241. https://doi.org/10.1111/jpn.12643

Nimse, S., & Pal, D. (2015). Free radicals, natural antioxidants and their reaction mechanisms. *RSC Adv*, *6*, 27986. https://doi.org/10.1039/C4RA13315C

Nørgaard, L., Saudland, A., Wagner, J., Nielsen, J. P., Munck, L., & Engelsen, S. B. (2000). Interval partial least-squares regression (iPLS): A comparative chemometric study with an example from near-infrared

spectroscopy. *Appl Spectrosc*, *54*, 413–419. https://doi.org/10.1366/0003702001949500

Oyaizu, M. (1986). Studies on products of browning reaction–antioxidant activities of products of browning reaction prepared from glucoamine. *Jpn J Nutr Diet*, *44*, 307–315. https://doi.org/10.5264/eiyogakuzashi.44.307

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*, *26*, 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Ruiz-López, M. A., Barrientos-Ramírez, L., García-López, P. M., Valdés-Miramontes, E. H., Zamora-Natera, J. F., Rodríguez-Macias, R., Salcedo-Pérez, E., & Bañuelos-Pineda, J. (2019). Nutritional and bioactive compounds in Mexican lupin beans species: A mini-review. *Nutrients*, *11*, 1785. https://doi.org/10.3390/nu11081785

Sbihi, H. M., Nehdi, I. A., Tan, C. P., & Al-Resayes, S. I. (2013). Bitter and sweet lupin (*Lupinus albus* L.) seeds and seed oils: A comparison study of their compositions and physicochemical properties. *Ind Crops Prod*, *49*, 573–579. https://doi.org/10.1016/j.indcrop.2013.05.020

Siger, A., Czubinski, J., Kachlicki, P., Dwiecki, K., Lampart-Szczapa, E., & Nogala-Kalucka, M. (2012). Antioxidant activity and phenolic content in three lupin species. *J Food Compos Anal*, *25*, 190–197. https://doi.org/10.1016/j.jfca.2011.10.002

Tian, Y., Cortés-Avendaño, P., Yang, B., Glorio-Paulet, P., Repo-Carrasco-Valencia, R., & Suomela, J.-P. (2024). Flavonoid diversity in bitter and debittered seeds of Andean lupin (*Lupinus mutabilis* Sweet). *Food Chem*, *442*, 138411. https://doi.org/10.1016/j.foodchem.2024.138411

Tomar, M., Bhardwaj, R., Kumar, M., Singh, S. P., Krishnan, V., Kansal, R., Verma, R., Yadav, V. K., Ahlawat, S. P., Rana, J. C., Satyavathi, T., Praveen, S., & Sachdev, A. (2021). Development of NIR spectroscopy-based prediction models for nutritional profiling of pearl millet (*Pennisetum glaucum* (L.)) R.Br: A chemometrics approach. *LWT - Food Sci Technol*, *149*, 111813. https://doi.org/10.1016/j.lwt.2021.111813

Villacrés, E., Quelal, M. B., Fernandez, E., Garcìa, G., Cueva, G., & Rosell, C. M. (2020). Impact of debittering and fermentation processes on the antinutritional and antioxidant compounds in *Lupinus mutabilis* Sweet. *LWT - Food Sci Technol*, *131*, 109745. https://doi.org/10.1016/j.lwt.2020.109745

Wang, J., Liu, H., & Ren, G. (2014). Near-infrared spectroscopy (NIRS) evaluation and regional analysis of Chinese faba bean (*Vicia faba* L.) *Crop J*, *2*, 28–37. https://doi.org/10.1016/j.cj.2013.10.001

Wold, S., Ruhe, A., Wold, H., & Dunn, W. J. (1984). The collinearity problem in linear regression. The partial least squares (PLS) approach to

generalized inverses. *J Sci Stat Comp*, *5*, 735–743. https://doi.org/10.1137/0905052

Yilmaz, V. A., Brandolini, A., & Hidalgo, A. (2015). Phenolic acids and antioxidant activity of wild, feral and domesticated diploid wheats. *J Cereal Sci*, *64*, 168–175. https://doi.org/10.1016/J.JCS.2015.05.005

Zalewski, K., Nitkiewicz, B., Stolarski, M., Amarowicz, R., & Okorskid, A. (2020). The influence of exogenous methyl jasmonate on the germination and, content and composition of flavonoids in extracts from seedlings of yellow and narrow-leafed lupine. *J Food Compos Anal*, *87*, 103398. https://doi.org/10.1016/j.jfca.2019.103398

Zhang, K., Zhou, L., Brady, M., Xu, F., Yu, J., & Wang, D. (2017). Fast analysis of high heating value and elemental compositions of sorghum biomass using near-infrared spectroscopy. *Energy*, *118*, 1353–1360. https://doi.org/10.1016/j.energy.2016.11.015

4

Effect of optimized debittering on lupin alkaloids and antioxidants

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Abstract

Lupin seeds are rich in proteins, lipids and bioactive compounds, but before consumption must be debittered to remove toxic alkaloids. Traditionally, debittering is a water-intensive and protracted process, lasting up to six days. To develop a more efficient procedure, different washing solutions (0.5% and 1% NaCl or citric acid), with or without the use of ultrasound, were applied on *Lupinus albus* seeds and compared to two control methods, the first with water and the second with a sodium chloride solution. 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 dry matter). 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. The sonication, when it was significant, led to a large loss of bioactive compounds, which was most likely due to its extraction capability. The seeds that were debittered without ultrasound presented high concentrations 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) phenolics. The soluble-free fraction showed the greatest loss after a prolonged treatment. Overall, debittering with citric acid or NaCl preserved the highest concentration of antioxidant compounds by shortening the treatment time, thus preventing extensive leaching.

4.1 Introduction

Lupin seeds are a promising food resource for their high content of proteins, unsaturated lipids and bioactive compounds (Brandolini et al., 2022; Briceño Berru et al., 2021). They can be consumed as snack or side dish; otherwise, lupin flour may be used to prepare sauces, vegetable milk, cheese and meat, fermented products (tofu, tempeh, etc.), pasta and noodles, baked goods (cakes, biscuits, tortillas, muffins and bread), etc. (Albuja-Vaca et al., 2019; Al-Saedi et al., 2020; Güémes-Vera et al., 2008; Jayasena, Khu, & Nasar-Abbas, 2010; Jayasena, Leung, & Nasar-Abbas, 2010; Özcan et al., 2021). Furthermore, lupin seeds are a good source of oil (Guadagnucci Fontanari et al., 2018; Pascual-Chagman et al., 2021) as well as of protein isolates (Lo et al., 2021) that, besides the intrinsic nutritional value, have physical and functional properties comparable to soybean and boast interesting technological properties like water and oil absorption, emulsifying, foaming, gelling and stabilizing capacity (Alu'datt et al., 2017; Duranti et al., 2008; Lampart-Szczapa et al., 2006; Lo et al., 2021; Paraskevopoulou et al., 2010; Pollard et al., 2002). Lupin flours lack gluten-forming proteins hence they can be used in gluten-free foods for people suffering from coeliac disease.

The greatest obstacle to lupin utilization in human and animal nutrition is the high content of bitter (and often toxic) quinolizidine alkaloids, which grant the plant protection against pathogens and pests. To reduce alkaloid content and make the seeds suitable for consumption, appropriate technological processes have been developed. In parallel, varieties with low alkaloid levels have been selected (Kroc et al., 2019; Muzquiz et al., 1994; Uauy et al., 1995). This last strategy, however, is still of limited effectiveness since the so-called

"sweet" varieties are less productive, more susceptible to pests and diseases, and tend to reacquire their bitterness over time (Uauy et al., 1995).

The most widespread debittering methods rely on extracting the alkaloids from whole seeds using water as solvent. The traditional debittering method, widely adopted by the industry (Taverniti, 2019), starts with preliminary hydration, followed by cooking and repeated washings for several days (Carvajal-Larenas et al., 2014; Erbaş, 2010). Besides disrupting cell walls and facilitating alkaloids removal, cooking blocks germination, coagulates proteins, inactivates enzymes and sanitizes the product (Carvajal-Larenas et al., 2013; Gross et al., 1983).

However, debittering triggers changes in the chemical composition of the seeds because many water-soluble molecules are washed away. Compared to non-debittered seeds, the contents in minerals, fibre, carbohydrates and oligosaccharides decrease during the process; conversely, protein and lipid levels increase for a concentration effect due to the removal of the other compounds (Carvajal-Larenas et al., 2016; Córdova-Ramos, Glorio-Paulet, Camarena, et al., 2020; Erbaş, 2010). Debittering also affects the bioactive composition of the seeds, as it can remove or degrade part of the antioxidant compounds (Córdova-Ramos, Glorio-Paulet, Hidalgo, & Camarena, 2020; Villacrés, Quelal, et al., 2020) and reduce the nutritional value of lupin-based foods. Hence the necessity to develop debittering processes that are able to shorten the treatment time, reduce the water consumption and minimize the bioactive compound loss.

Several improved debittering methods have been proposed. Generally, they include longer boiling times (Jiménez-Martínez et al., 2001), post-cooking washing with warm water (Guadagnucci Fontanari et al., 2012), different rinsing solutions (Jiménez-Martínez et al., 2001; Mohammed et al., 2017; Villacrés, Álvarez, & Rossell, 2020), germination (de Cortes Sánchez et al., 2005; Mohammed et al., 2017), fermentation (Jiménez-Martínez et al., 2007) or their combination (Erbaş, 2010; Jiménez-Martínez et al., 2010). Recently, low frequency ultrasound (US) has been tested with promising results on lupin seeds hydration and debittering (Miano et al., 2019; Yaver & Bilgiçli, 2021). The US technology is already employed by the food processing industry to accelerate many operations, such as compounds extraction and products homogenization, filtration, dehydration, freezing, thawing and sanitization (Bhargava et al., 2021; Mohammadi et al., 2021; Schmidt et al., 2019). Furthermore, US is very effective in improving the yield and reducing the duration of micro- and macromolecules extraction (Estivi et al., 2022).

The aim of this work was therefore to improve the debittering process of *Lupinus albus* seeds using different solvents (water and solutions with 0.5% or 1.0% of NaCl or citric acid), either with or without ultrasound assistance, and to compare the proposed method with control methods with respect to their effect on the content of antioxidant bioactive compounds (i.e., carotenoids, tocopherols, and phenolics).

4.2 Materials and methods

4.2.1 Materials

Two different lots (Lot 1 and Lot 2) of *Lupinus albus* seeds from Chile, purchased from Colombo Legumi e Frutta Secca (Modica, Italy), were analysed. Four commercial debittered *L. albus* snacks (C1 and C2, without liquid in sealed plastic boxes; C3, in brine in sealed plastic bag; C4, without liquid under vacuum bag) were collected from local (Milan, Italy) supermarkets. All the reagents were purchased from Sigma-Aldrich (Burlington, USA).

4.2.2 Seeds debittering

Two trials were performed to assess

1. the influence of sonication (with or without) and solvent (water, NaCl 1% or citric acid 1%) on alkaloid content: the seeds of Lot 1 were debittered applying the experimental method for two different soaking times (28.5 h and 45 h, respectively);
2. the effect of solvent (water, NaCl 0.5%, NaCl 1%, citric acid 0.5% or citric acid 1%) and soaking time (45, 57 or 69 h) on alkaloid content and taste profile: the seeds of Lot 2 were debittered by the experimental method, without sonication. Seeds debittered by the control methods, as well as four commercial samples, were also considered.

Control method with water—100 g of seeds were debittered according to Erbaş (2010), with the minor modifications made by Córdova-Ramos, Glorio-Paulet, Camarena, et al. (2020). After a hydration phase (1:6 w/v seeds:water ratio) for 12 h at room temperature, the seeds were cooked in boiling water (hydrated seeds:water 1:3 w/v) for 1 h, changing the water after 30 min, and finally washed by water soaking (cooked seeds:water 1:3 w/v) for 5 days at room temperature, replacing the water every 12 h.

Control method with sodium chloride solution—100 g of seeds were debittered as described by Villacrés, Álvarez, and Rossell (2020). After an 8 h hydration at 80 °C (seeds:water with NaCl 0.5%, ratio 1:3 w/v), the solution was replaced and the seeds were cooked for 1 h at 91 °C (1:3 w/v), renewing the solvent halfway through cooking. Five washes were then carried out using water with NaCl 0.5% at 35 °C up to 28 h (first and second wash: 1:15 w/v, 3 h each; third wash: 1:5 w/v, 16 h; fourth wash: 1:7.5 w/v, 3 h; fifth wash: 1:5 w/v, 3 h). Two final washes with water at 18 °C (seeds:water 1:5 w/v), the first lasting 18 h and the second 3 h, were performed to remove the excess of salinity.

Experimental method—The experimental debittering method proposed in this study (Figure 4.1) was developed and fine-tuned after several preliminary trials (not shown). Five different debittering solutions were tested: distilled water, NaCl 0.5% and 1%, citric acid 0.5% and 1%. Additionally, two different

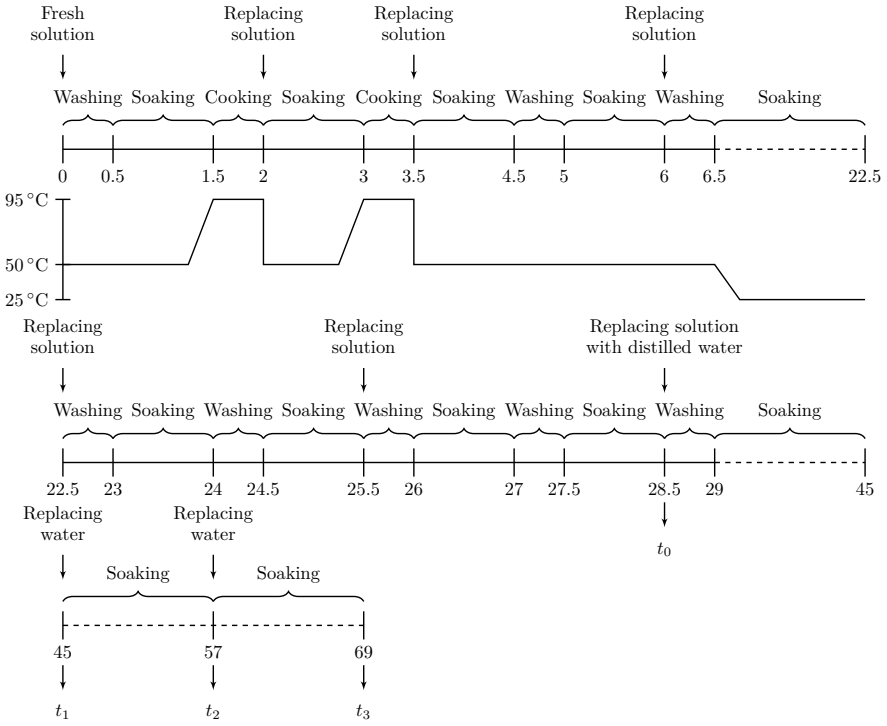


Figure 4.1. Time sequence (in hours) of the different phases of the proposed debittering process.

treatments were tested: without and with ultrasound. The UP400St US homogenizer (Hielscher Ultrasonics GmbH, Teltow, Germany), operating at 24 kHz and mounting a 14 mm diameter probe, was used to treat the seeds during washing (60% amplitude) and cooking (100% amplitude). The net power of the probe, determined by the calorimetric method as reported by Margulis and Margulis (2003) sonicating distilled water at the tested combinations of temperature and amplitude, was equal to 65.0 ± 3.3 W at 50 °C and 60% amplitude, and to 26.9 ± 1.7 W at 95 °C and 100% amplitude.

After an initial washing phase of 30 min at 50 °C (Figure 4.1), performed either without or with US, 100 g seeds were soaked for 1 h and then cooked for 30 min at 95 °C, without or with US. Soaking and cooking steps were repeated after replacing the solvent. Afterwards, the solvent was changed again and a series of soakings at room temperature/washings for 30 min at 50 °C (without or with US) was carried out, renewing the solvent according to the timeline in (Figure 4.1). After the last washing (i.e., $t_0 = 28.5$ h), from 0 to 3 soakings with distilled water were carried out for 12 h, to complete the debittering and remove salt or citric acid from the seeds. A dry seeds:solvent 1:5 (w/v) ratio was always employed.

For the Lot 1 seeds, the final rinsing was done at 25.5 h to interrupt the process after 28.5 h. For Lot 2, samples were taken at the end of the first ($t_1 = 45$ h), second ($t_2 = 57$ h) and third ($t_3 = 69$ h) final soaking.

The debittered seeds from trial 1 were dried 8 h at 60 °C in a Venticell 55 ventilated oven (MMM-Group, Planegg/München, Germany); the seeds from trial 2 were lyophilized under vacuum (10^{-2} mbar) in an Edwards 304 freeze dryer (Atlas Copco, Stockholm, Sweden). The dried seeds were ground with an MDI 204 disc mill (Buhler, Uzwil, Switzerland) and stored at −20 °C until the time of analysis.

4.2.3 Seeds characteristics

The 100-seeds weight was determined in triplicate with an analytical scale (E154, Gibertini Elettronica SRL, Novate Milanese, Italy). Seed size (major diameter, minor diameter and thickness) was measured on 100 seeds using a CD-15DC calliper (Mitutoyo Italiana SRL, Lainate, Italy). The moisture was determined gravimetrically according to the 925.10 method (AOAC, 2000).

4.2.4 Alkaloid content

The alkaloids quantification was performed in triplicate by colorimetric titration as described by von Baer et al. (1979) with minor modifications. Briefly, exactly 0.6 g of sample were weighed in a mortar, 0.6 mL of 15% potassium hydroxide and 1.8 g of basic aluminium oxide were added, and the mix was manually homogenized. The sample was then transferred to a 25 mL glass tube with a screw cap, 10 mL of chloroform were added, and the content was shaken with a TX4 Digital IR Vortex Mixer (VELP Scientifica, Usmate Velate, Italy) for 1 min. After sedimentation, the supernatant containing the alkaloids was filtered through paper and collected in a 100 mL flat-bottomed boiling flask; the chloroform extraction was repeated several times, until 1 mL of the most recent extract, dried with nitrogen and re-suspended in 5 drops of 0.01 N sulphuric acid, had a negative reaction with 4 drops of Dragendorff's reagent. The merged extracts were evaporated under vacuum using a Laborota 4000 Efficient rotavapor (Heidolph, Schwabach, Germany) at 35 °C for about 8 min. For titration, 5 mL of 0.01 N sulphuric acid were added and the flask was gently mixed. The excess of acid was titrated in the presence of 4 drops of methyl red with 0.01 N sodium hydroxide by means of a 10 mL burette. The alkaloid content, expressed as g/kg dry matter (DM), was calculated with the Equation 4.1, considering that 1 mL of 0.01 N sulphuric acid equals 2.48 mg of lupanine (INEN, 2005).

An empirical evaluation of the residual bitterness was also performed in all the trials by tasting the seeds.

$$\text{Alkaloid (g/kg DM)} = \frac{2.48 \cdot [5 - \text{V}_{\text{NaOH}}\ \text{(mL)}]}{\text{weight (g)} \cdot [100 - \text{moisture (\%)}]} \tag{4.1}$$

4.2.5 Electronic tongue

E-tongue measurements were carried out by Taste-Sensing System SA 402B (Intelligent Sensor Technology Co., Atsugi City, Japan). The system consists of sensors whose surface is attached with artificial lipid membranes having different response properties to chemical compounds on the basis of their taste. Five detecting sensors (CA0; CT0; C00; AAE; AE1) and two reference electrodes were used, separated in two arrays according to membrane charge.

The extracts were prepared according to Marengo et al. (2016), with some modifications. Exactly 3 g sample were weighed into a 500 mL centrifuge bottle, 100 mL of KCl 10 mM were added and the mixture was homogenized with a Ultra-Turrax T25 (IKA, Königswinter, Germany) at 15000 rpm for 2 min. The homogenizer probe was washed with 20 mL of 10 mM KCl, collected in the bottle to obtain a sample/solvent ratio of 1:40 (w/v). The tube was centrifuged with a Sorvall RC-5B Plus Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) at 6085 g for 5 min at room temperature. The supernatant was filtered on paper and analysed.

The detecting sensors and the reference electrodes were first dipped into the reference solution (30 mM KCl and 0.3 mM tartaric acid); the electric potential was measured for each sensor and defined as Vr. Then the sensors were dipped for 30 s into the sample solution and for each sensor the measured potential was defined as Vs. For each sensor the "relative value" (Rv) is the difference between the potential of the sample and the reference solution ($Vs - Vr$). The sensors were rinsed with fresh reference solution for 6 s and then dipped into the reference solution again. The new potential was defined as Vr'. From the difference between the potential of the reference solution after and before sample measurement ($Vr' - Vr$), the "CPA value" ($CPAv$; i.e., Change of membrane Potential caused by Absorption) was detected. Before starting a new measurement cycle, the sensors were rinsed for 90 s with a washing solution (ethanol 30%) and then for 180 s with the reference solution.

Each sample was evaluated in triplicate and the "taste values" were calculated multiplying sensor outputs by appropriate coefficients based on Weber-Fechner's law, which provides the intensity of sensation considering the sensor properties for tastes (Kobayashi et al., 2010). In particular, the "taste values" were estimated as follows:

$$\text{Sourness} = 0.3316\ Rv_{(CA0)}$$

$$\text{Saltiness} = -0.252\ Rv_{(CT0)}$$

$$\text{Bitterness} = -0.140\ Rv_{(C00)} + 0.084\ Rv_{(CT0)}$$

$$\text{Aftertaste–bitterness} = -0.210\ CPAv_{(C00)}$$

$$\text{Astringency} = 0.1575\ Rv_{(AE1)} + 0.1575\ Rv_{(CT0)}$$

$$\text{Aftertaste–astringency} = -0.252\ CPAv_{(AE1)}$$

$$\text{Umami} = -0.1575\ Rv_{(AAE)}$$

4.2.6 Carotenoids and tocopherols

Carotenoids and tocopherols were extracted by thermal saponification and quantified by normal-phase HPLC as outlined by Brandolini et al. (2022). Briefly, 2 g sample were saponified for 45 min at 70 °C, under nitrogen, with 5 mL ethanolic pyrogallol 60 g/L, 2 mL ethanol 95%, 2 mL sodium chloride 10 g/L and 2 mL potassium hydroxide 600 g/L. After saponification, the samples were cooled in an ice bath and 15 mL sodium chloride 10 g/L were added. The suspension was liquid-liquid partitioned twice with 15 mL hexane:ethyl acetate 9:1 (v/v). The organic layer was collected, evaporated under vacuum (Laborota 4000 Efficient) and dried with nitrogen flux; the residue was dissolved in 2 mL hexane:2-propanol 99:1 (v/v) and filtered through a 0.45 µm PTFE membrane (Millipore, Carrigtwohill Co., Cork, Ireland).

For carotenoids separation, the filtered solution was injected in a HPLC system operating with: injector Rheodyne 7125 (VWR, Hitachi, Tokyo, Japan) mounting a 50 µL loop; mobile phase, hexane:2-propanol 95:5 (v/v); isocratic flow rate, 1.5 mL/min; pump L-2130 Elite LaChrom (Hitachi, Tokyo, Japan); Alltima Silica column 250 × 4.6 mm, 5 µm and Alltima Silica guard column 7.5 × 4.6 mm, 5 µm (Alltech Associates Inc., Deerfield, USA). The carotenoids were detected at 445 nm by a 2996 PhotoDiode Array Detector (Waters Chromatography Division, Millipore, Milford, MA, USA) set in the range of 200–650 nm and connected to the Empower 2 software (Waters).

For tocopherols separation, the filtered solution was injected in a HPLC system operating with: injector Rheodyne 7125 (VWR, Hitachi) mounting a 50 µL loop; mobile phase, hexane:ethyl acetate:acetic acid 97.3:1.8:0.9 (v/v/v); isocratic flow rate, 1.6 mL/min; pump L-2130 Elite LaChrom (Hitachi); Alltima Silica column, 250 × 4.6 mm, 5 µm and Alltima Silica guard column 7.5 × 4.6 mm, 5 µm (Alltech Associates Inc.); fluorimetric detector 821-FP Intelligent Spectrofluorometer (Jasco, Tokyo, Japan) set at excitation-emission wavelengths of 290 nm and 330 nm, respectively, and connected to the Empower 2 software through the e-SAT/IN module (Waters).

For carotenoid and tocopherol quantification, calibration curves were built using standards of lutein (Fluka, St. Louis, MO, USA), β-carotene (Sigma, St. Louis, MO, USA), zeaxanthin and β-cryptoxanthin (Extrasynthese, Genay, France), α-tocopherol (Fluka BioChemika, Buchs, Switzerland), β-tocopherol, γ-tocopherol, and δ-tocopherol (Supelco, Bellefonte, PA, USA). The calibration curves are reported in the Supplementary Table S.3. Based on the calibration curve, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the intercept value of the regression line plus 3 times (LOD) or 10 times (LOQ) the standard error of the estimate (Miller & Miller, 2010). The tests were performed in duplicate and the results are expressed as mg/kg DM.

4.2.7 Phenolic compounds

The analysis of soluble-free, soluble-conjugated and insoluble-bound phenolic compounds was performed by reverse-phase HPLC as outlined by Brandolini et al. (2022). Exactly 1 g of sample was extracted three times with 15 mL of methanol 80% in water. After centrifugation (LISA, AFI Groups, Château-Gontier, France) at 11200 g, the pooled supernatants (containing the soluble-free or the soluble-conjugated fractions) and the sediment (containing the insoluble-bound fraction) were recovered. For the free phenolics determination, the supernatant was evaporated under vacuum at 35 °C for 50 min with a Laborota 4000 Efficient rotary evaporator, dried under nitrogen flux, re-suspended in 2 mL of methanol:water 8:2 (v/v), and filtered with a 0.45 µm PTFE membrane (Millipore). For the soluble-conjugated phenolics, the supernatant was partially evaporated under vacuum at 35 °C for 18 min. Then, the samples were hydrolysed under nitrogen and continuous shaking with 15 mL of 4 M NaOH for 4 h at room temperature, were brought to pH 1.5–2 with 6 M HCl, and then were extracted twice with 20 mL of diethyl ether:ethyl acetate 1:1 (v/v). The extracts were clarified with anhydrous sodium sulphate, filtered through 110 µm glass fibre (Whatman, Maidstone, England), evaporated under vacuum at 35 °C for 5 min, re-suspended in 2 mL of methanol:water 1:1 (v/v), and filtered with a 0.45 µm PTFE membrane. For the analysis of the insoluble-bound phenolic compounds, the sediment was hydrolysed and then extracted as outlined above for the soluble-conjugated phenolics.

The RP-HPLC analysis of the extracts and the peaks identification were performed as previously described in the Section 3.2.3. The calibration curves of the identified phenolics were constructed using standards (Sigma-Aldrich, St. Louis, MO, USA) recorded at 280 nm for catechin (13.9–99.2 mg/L), genistein (27.5–110.0 mg/L) and naringenin (2.3–9.0 mg/L); at 320 nm for apigenin (1.0–20.0 mg/L) and sinapic acid (0.5–21.4 mg/L); at 360 nm for diosmin (5.2–104.8 mg/L). The quantification was performed twice and the results are expressed as mg/kg DM.

4.2.8 Statistical analysis

To compare the morphological characteristics of the two lots of lupin seeds, the data were processed by means of a t-test. To compare the samples within each test the data underwent one-way analysis of variance (ANOVA), while to evaluate the effect of treatment (T), solvent (S) and time (t) or the effect of time and solvent, the data were processed by three- and two-way ANOVAs. The normal distribution of the data was always verified; the inverse transformation of the square root was only necessary for the alkaloid content in the test about the different debittering methods. When significant differences ($p \leq 0.05$) were detected, Fisher's LSD test at 95% significance level was applied. All analyses were performed with the Statgraphics®

Centurion 18 statistical programme (StatPoint Technologies Inc., Warrengton, VA, USA). Means, standard deviations and standard errors were computed using the Excel® software (Microsoft, Redmond, WA, USA). The electronic tongue data was processed using the XLSTAT statistical and data analysis software (Addinsoft, New York, USA). Principal Component Analysis (PCA) was applied as an exploratory tool to uncover in a reduced space the data structure and the relationships between objects and variables, thanks to graphical outputs (i.e., score plot, loading plot and bi-plot; Wold et al., 1987).

4.3 Results and discussion

4.3.1 Morphological characteristics of the seeds

The morphological characteristics of the two *Lupinus albus* lots are presented in Table 4.1. The Lot 2 seeds were significantly bigger and heavier ($p \leq 0.05$) than those of Lot 1. In both batches, the average weight of a seed was higher than those (0.151–0.479 g) reported by several authors (Annicchiarico et al., 2014; Julier et al., 1993, 1995; Mera et al., 2006), but still fell within the range (0.100–1.000 g) described by Huyghe (1997), who stressed the existence of extreme intraspecific variability both for weight and size. Additionally, cropping year and climatic conditions influence these parameters even within the same variety (Annicchiarico et al., 2014).

Table 4.1. Morphological characteristics, alkaloid content (mean ± standard deviation) and results of the *t*-test for the two lots of *Lupinus albus* seeds used in the analyses.

	Lot 1	Lot 2
100-seeds weight (g)	56.84 ± 0.83^{b}	74.75 ± 0.90^{a}
Major diameter (mm)	13.30 ± 0.57^{b}	14.28 ± 0.64^{a}
Minor diameter (mm)	12.37 ± 0.27^{b}	13.60 ± 0.51^{a}
Thickness (mm)	5.04 ± 0.39^{b}	5.32 ± 0.35^{a}
Alkaloid content (g/kg DM)	20.35 ± 0.17^{b}	20.92 ± 0.01^{a}

Different letters indicate significant differences between samples according to the t-test ($p \leq 0.05$).

4.3.2 Effect of debittering method on alkaloid content

The alkaloid content in the untreated seeds of Lot 1 and Lot 2 was 20.35±0.17 and 20.92 ± 0.01 g/kg DM respectively (Table 4.1); the difference between the two lots was low but statistically significant ($p \leq 0.05$). These results are coherent with those of Muzquiz et al. (1994), who recorded a range between 17.0 and 26.9 g/kg among 28 bitter *L. albus*.

The results of trial 1 are summarized in Figure 4.2, which shows the residual alkaloid concentration after debittering with deionized water, NaCl 1% or citric acid 1% solutions, with or without ultrasonication (US), and after two different soaking times. The ANOVA (Supplementary Table S.4) evidenced a highly significant effect ($p \leq 0.001$) of solvent and time, but the sonication did not significantly reduce the alkaloid content; the interaction between solvent and time was also significant, while those including the US treatment were not. Hence, US did not improve at all the debittering process. The LSD test demonstrated that citric acid produced a stronger debittering effect (residual alkaloids: 0.80 ± 0.09 g/kg DM) than NaCl (1.37 ± 0.15 g/kg DM), and that water was the worst solvent (5.42 ± 0.24 g/kg DM).

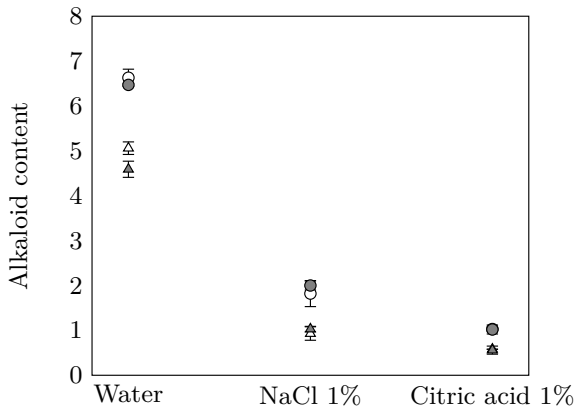


Figure 4.2. Alkaloid content (g/kg DM) of *Lupinus albus* seeds after debittering for 28.5 h (circles) or 45 h (triangles), without (empty) or with (filled) ultrasound (US), and with different solvents (water, NaCl 1% and citric acid 1%). Error bars indicate the standard deviations.

Sonication did not improve the removal of alkaloids, contrary to the report of Miano et al. (2019), probably because the seeds were cooked before the US treatment: cooking denatures cellular membranes and breaks cell compartments of the seed, performing the same function of sonication. A positive US effect may occur at the very beginning of the treatment, fostering seed hydration and formation of pores and canals, but the relatively long times required for debittering probably masked it. However, the preliminary trials demonstrated that US treatment was not enough to replace the cooking step: even if US accelerates alkaloids removal during the early debittering stages, it does not constitute a process improvement for practical purposes. Moreover, Miano et al. (2019), under their specific ultrasound protocol, did not reach a satisfactory debittering as their residual alkaloids score was 15.1 g/kg DM.

The positive debittering effect of sodium chloride is attributable to the increase in the microporosity of the seeds, which facilitates the solvent penetration and the alkaloids leakage (Sievwright & Shipe, 1986). Furthermore, the

osmotic effect of sodium chloride may favour the disorganization of phospholipid membranes and cellular compartments (Hameed et al., 2021) as well as foster mass transport from seed tissues to the saline solution (Casp Vanaclocha & Abril Requena, 2003). The better debittering capacity of saline solutions compared to water was likewise reported by Villacrés, Álvarez, and Rossell (2020). These results also agree with Jiménez-Martínez et al. (2010) and Karara (1987), who demonstrated greater debittering efficiency using, respectively, 0.1 M acetic acid and 0.1% citric acid solutions instead of pure water.

With the notable exceptions of caffeine and ephedrine, alkaloids are soluble in non-polar solvents only when they are in the form of conjugated bases, while the affinity is inverted for the corresponding conjugated acids and their salts (Kukula-Koch & Widelski, 2017; Ortiz & Mukherjee, 1982). This explains both the positive removal effect of citric acid solutions and the limited capacity of water alone. Lupanine, the main alkaloid in the seeds of *Lupinus* spp. (Boschin et al., 2008; Otterbach et al., 2019), having a pKa of 9.2 (Wink & Mende, 1987), exhibits higher solubility in acidic aqueous solutions than in water, being present in its protonated form, lupanine-(1+). Furthermore, the formation of salts with the carboxylic acids is commonly used as a carrier for water-insoluble conjugated bases with pharmaceutical activity (Bharate, 2021). An alternative explanation could come from Giel-Pietraszuk et al. (2007), who observed that a slight excess of hydrogen ions (pH 6.2) is sufficient to hydrolyse the C–N bond of the δ-lactam, converting part of the lupanine into the corresponding acyclic molecule (lupanic acid), whose fate during debittering is however unknown. In this case, the pH of the citric acid solutions (2.2–2.4), far lower than 6.2, might have shifted the equilibrium towards lupanic acid, richer in polar groups and hence probably more water-soluble and easier to wash away than lupanine.

The results of trial 2, assessing the alkaloid content of the Lot 2 seeds, untreated and treated with distilled water, NaCl 0.5% and 1%, citric acid 0.5% and 1%, and applying three debittering times (45 h; 57 h; 67 h), are shown in Table 4.2. Additionally, the results obtained with the control methods, as well as the concentration in four commercial ready-to-eat lupin snacks (C1–C4), are reported. Among the samples debittered according to the experimental method, those treated with the citric acid solutions gave the best results in terms of operating times, since in only 45 h the sample with citric acid 1% reached an alkaloid level (0.56 ± 0.09 g/kg DM) not significantly different ($p \leq 0.05$) from those of the C1 and C2 commercial samples. When the treatment time was extended, performing additional soakings, the value further decreased to 0.31 ± 0.09 g/kg DM, comparable to the C3 commercial snack. It must be stressed that lupin snacks are generally stored in 6% NaCl brine until packaging (Erbaş, 2010), and sometimes even after (e.g., the C3 sample), leading to a prolongation of debittering.

The 1% solutions were significantly more efficient in removing the alkaloids than the 0.5% solutions. In fact, with NaCl 1% a residual amount

Table 4.2. Alkaloid content of *Lupinus albus* seeds (Lot 2) before and after debittering by the experimental method, the control methods with water (Erbaş, 2010) or with NaCl solution (Villacrés, Álvarez, & Rossell, 2020), and of four commercial samples (C1–C4).

Alkaloid content (g/kg DM)					Time (h)	Water (L/kg)
Untreated	20.92 ± 0.01^{a}					
Experimental methods						
Water	NaCl 0.5%	NaCl 1%	Citric acid 0.5%	Citric acid 1%		
4.59 ± 0.18^{b}	1.75 ± 0.15^{c}	1.03 ± 0.06^{def}	1.11 ± 0.06^{de}	0.56 ± 0.09^{c}	45	35
4.48 ± 0.03^{b}	1.16 ± 0.12^{cd}	0.72 ± 0.15^{ghi}	0.78 ± 0.01^{fgh}	0.37 ± 0.06^{cd}	57	40
3.62 ± 0.15^{b}	0.97 ± 0.09^{defg}	0.81 ± 0.03^{efgh}	0.62 ± 0.01^{hi}	0.31 ± 0.09^{defg}	69	45
Control methods						
Water	0.95 ± 0.12^{defg}				133	100
NaCl 0.5%	0.66 ± 0.01^{hi}				58	66
Commercial snack samples						
C1	0.62 ± 0.01^{hi}					
C2	0.56 ± 0.09^{i}					
C3	0.35 ± 0.09^{j}					
C4	0.99 ± 0.06^{defg}					

Different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$)

of 0.72 ± 0.15 mg/kg DM in the seeds was reached after 57 h, while with NaCl 0.5% the concentration was 1.16 ± 0.12 g/kg DM. Similarly, after 45 h the sample debittered with citric acid 1% achieved 0.56 ± 0.09 g/kg DM, while that treated with citric acid 0.5% still showed 1.11 ± 0.06 g/kg DM. At equal solute concentration and time, citric acid had a significantly greater debittering power than sodium chloride. Water was the solvent with the poorest performance, as already shown in the previous analyses: it is noteworthy that not even after three final soakings (69 h of treatment) the seeds reached an alkaloid content suitable for consumption. Based on these results, further trials were attempted to fine-tune the experimental method, when using only water, by increasing the seed:solvent ratio and/or soaking time; however, no satisfactory results were achieved.

The above-detailed results are further validated by the two-way ANOVA computed only on the data of the seeds treated with the experimental method, considering the time and the solvent as factors. The two-way ANOVA (Supplementary Table S.5) showed that both factors and their interaction were significant: the LSD test highlighted significant differences among all soaking times as well as among solvents, except for NaCl 1% and citric acid 0.5%, equally effective.

The seeds debittered according to the control method with water achieved a residual alkaloid value of 0.95 ± 0.12 g/kg DM only after 133 h and 100 L of water/kg dry seeds. The NaCl solution control method reduced the alkaloid content to 0.66 ± 0.01 g/kg DM after 58 h, consuming 66 L of water/kg dry seeds. This result is similar to those of the samples treated with NaCl 1% and citric acid 0.5% for 57 h (40 L of water consumed per kg of dry seeds), as well as with citric acid 1% for 45 h (35 L water/kg dry seed); however, the experimental method saved time and water, thus reducing the volume of toxic effluents to be disposed of or to be treated to recover lupanine, a building block in the synthesis of pharmaceutical molecules (Esteves et al., 2020). Additionally, the seeds debittered with citric acid 1% for 57 h attained an even lower alkaloid concentration (0.37 ± 0.06 g/kg DM), not statistically different from the minimum observed in commercial snacks (C3 sample); treatments up to 69 h are not necessary, because they did not further reduce the alkaloid content. It must be remembered that, according to the scores recorded in commercial snacks (0.35–0.99 g/kg DM) and in seeds debittered with the control procedures, alkaloid concentration below 1.00 g/kg DM are considerable suitable for consumption. Interestingly, this value was also the lowest threshold for bitterness perception when tasting the seeds.

4.3.3 Effect of debittering method on electronic tongue profile

Figure 4.3a displays the PCA bi-plot of e-tongue data in combination with alkaloid content collected on the four commercial ready-to-eat lupin snacks and on the seeds untreated and treated with control and experimental meth-

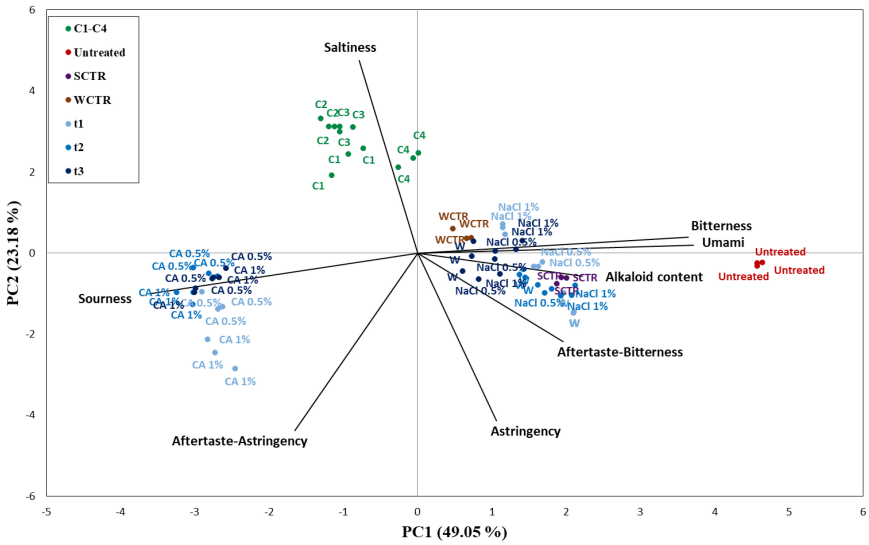
ods. The sample distribution in the plane defined by the first two principal components (PC1 and PC2), explaining 72.23% of the total variance (49.05% for PC1 and 23.18% for PC2), shows that the untreated control sample, located in the positive part of PC1, was characterized by the alkaloid content and its taste was perceived more bitter and umami than the samples treated with water (W) or debittered by the water control method (WCTR), the NaCl solution control method (SCTR), and the 0.5% and 1% NaCl solutions. On the opposite side of PC1, the seeds treated with 0.5% or 1% citric acid (CA) solutions were characterized by sourness and perceived less bitter than the other seeds. All the commercial samples (C1–C4), clustered in the positive part of PC2, were characterized by saltiness: although they came from several companies using different processes, their sodium chloride content was high and ranged between 6.3 and 9.9 g/100 g DM, as declared on their nutritional labels. It is noteworthy that lupins debittered with NaCl solutions (control and experimental methods) were located far from the commercial samples, and close to the samples treated with water, confirming the effectiveness of the final washing and soaking steps in salt removal.

Overall, the e-tongue showed a good ability to discriminate samples according to their debittering treatments and to cluster samples treated with the same solvent; on the other hand, there was no clear trend in the positioning of samples prepared with the same method and solvent, but for different times (t_1; t_2; t_3).

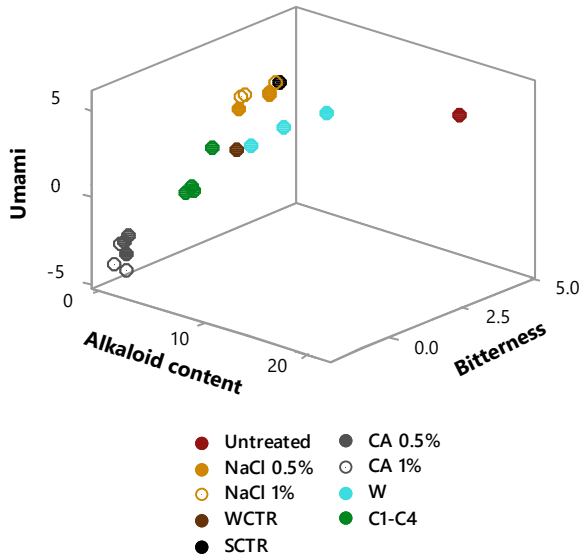
Considering the distribution of the variables in the plane defined by PC1 and PC2, the alkaloid content, placed to the right of PC1, was highly correlated to bitter and umami tastes, which were consequently identified as the main e-tongue variables, useful to discriminate samples on the basis of their debittering treatments.

To better visualize the relationship between the alkaloid content and the two main e-tongue variables (i.e., bitterness and umami), the data collected from all the analysed samples (average values) were depicted in the three-dimensional scatter plot (Figure 4.3b). The untreated control sample with the maximum alkaloid content was characterized by high "taste values" for bitterness and umami, while the samples debittered with citric acid achieved a low alkaloid concentration and were perceived as the least bitter and umami. All the other debittered samples showed a good relationship between the three considered variables; in addition, samples debittered with the same solvent were close in the plot and were characterized by the same taste note.

The decrease in the umami taste following the debittering treatments can be explained by considering that umami is an indicator of the presence of amino acids, nucleotides and peptides (W. Wang et al., 2020). In lupin seeds, umami is probably linked to the presence of glutamic acid, a molecule responsible for this specific taste (Bellisle, 1999). Glutamic acid is characterized by a carboxylic group able of assuming an additional negative charge on the side chain, so it is extremely soluble in aqueous and polar solvents,



(a) PCA bi-plot



(b) 3D-plot of the residual content of alkaloids and of bitter and umami variables

Figure 4.3. Results of electronic tongue analysis of the *Lupinus albus* samples (Lot 2) before (Untreated) and after debittering by control method with water (WCTR; Erbaş, 2010), control method with NaCl solution (SCTR; Villacrés, Álvarez, & Rossell, 2020), experimental method (different solvents), and of four commercial samples (C1–C4).

and thus easily solubilized and removed during debittering. Furthermore, since quinolizidine alkaloids are derived from lysine and are characterized by a quinolizidine nucleus containing a nitrogen atom (Frick et al., 2017), they can probably be revealed by the e-tongue umami sensor (Hwang et al., 2020).

4.3.4 Effect of sonication and solvents on antioxidant compounds

4.3.4.1 Carotenoids and tocopherols

Table 4.3 shows the carotenoids and tocopherols content of the Lot 1 seeds that were debittered with solutions of NaCl 1% for 45 h or citric acid 1% for 28.5 h, either without or with sonication, and with the water control method for comparison. The durations of the treatments were chosen based on the minimum time that was required to reach the same level of residual alkaloids (about 1 g alkaloids/kg DM), as previously reported (Figure 4.2). The total antioxidants contents are visualized in Figure 4.4.

The total carotenoids content varied from 10.9 to 13.2 mg/kg DM, values superior to those (1.9–5.3 mg/kg) recorded by Briceño Berru et al. (2021) in water-debittered seeds of *Lupinus albus*. In the literature, scarce information on carotenoids content exists and often refers to bitter seeds. The most abundant carotenoid in the treated seeds was lutein (6.8–8.7 mg/kg DM; Table 4.3), as also reported for bitter and debittered *L. albus* samples by Briceño Berru et al. (2021), and for bitter seeds of *L. luteus* by Fernández-Marín et al. (2014) and *L. mutabilis* by S. Wang et al. (2008). However, other authors (El-Difrawi & Hudson, 1979; S. Wang et al., 2008) found that zeaxanthin was the main carotenoid in bitter seeds of *L. albus*, *L. luteus*, *L. angustifolius*, and *L. mutabilis*.

The two-way ANOVA (Supplementary Table S.6) showed that both the treatment and the solvent influenced the total carotenoids content, while their interaction was not significant. Sonication led to values that were inferior to those of the non-sonicated seeds, but the differences were minimal. The one-way ANOVA (not shown) and the LSD test (Table 4.3) evidenced that the seeds debittered with citric acid 1% and US showed the lowest content of all of the identified carotenoids, bar β-cryptoxanthin; the other treatments preserved the total carotenoids slightly better.

Only tocopherols were recovered from the debittered *L. albus* seeds; as observed also by Boschin and Arnoldi (2011), Fernández-Marín et al. (2014) and Briceño Berru et al. (2021), no tocotrienol was identified. Total tocopherol contents were 195.4 mg/kg DM after the control treatment and 172.8–192.0 mg/kg DM after the experimental treatments. These concentrations were in the range (135.9–222.7 mg/kg DM) of the debittered samples analysed by Briceño Berru et al. (2021) and were superior to those of the bitter *L. albus* seeds (107.0–153.0 mg/kg DM) reported by Annicchiarico et al. (2014). The most abundant compound was γ-tocopherol, which represented over 99% of the total tocopherols. Its concentration (169.0–188.7 mg/kg DM) was lower

Table 4.3. Carotenoids, tocopherols and phenolic compounds (mg/kg DM; mean ± std deviation) in *L. albus* seeds (Lot 1) debittered by the control method with water (H_2O Ctrl; Erbaş, 2010) and the experimental method with and without ultrasound (US).

	H_2O Ctrl	NaCl 1%	NaCl 1% US	Citric acid 1%	Citric acid 1% US
Carotenoids					
(α+β)-carotene	2.25 ± 0.36^{ab}	2.44 ± 0.09^{ab}	2.41 ± 0.05^{ab}	2.49 ± 0.07^{a}	2.02 ± 0.02^{b}
β-criptoxanthin	0.18 ± 0.02^{c}	0.11 ± 0.01^{d}	0.10 ± 0.01^{d}	0.42 ± 0.01^{b}	0.50 ± 0.03^{a}
Lutein	7.91 ± 0.86^{ab}	8.74 ± 0.47^{a}	8.35 ± 0.03^{a}	8.05 ± 0.27^{a}	6.83 ± 0.03^{b}
Zeaxanthin	1.86 ± 0.14^{ab}	1.95 ± 0.10^{a}	1.94 ± 0.01^{ab}	1.74 ± 0.05^{b}	1.50 ± 0.01^{c}
Tocopherols					
α-tocopherol	0.45 ± 0.10^{b}	0.46 ± 0.04^{ab}	0.56 ± 0.03^{a}	0.50 ± 0.01^{ab}	0.44 ± 0.01^{b}
β-tocopherol	1.36 ± 0.51^{b}	1.01 ± 0.17^{b}	0.85 ± 0.25^{b}	2.64 ± 0.02^{a}	2.09 ± 0.15^{a}
γ-tocopherol	191.58 ± 27.96	188.73 ± 17.08	175.82 ± 0.06	182.93 ± 3.22	169.04 ± 3.38
δ-tocopherol	1.99 ± 0.40	1.77 ± 0.06	1.61 ± 0.12	1.56 ± 0.52	1.24 ± 0.08
Soluble-free phenolics					
Apigenin der	30.53 ± 0.08^{e}	101.90 ± 1.08^{c}	92.08 ± 1.97^{d}	163.96 ± 3.38^{a}	134.99 ± 0.88^{b}
Diosmin der	nd	nd	nd	3.94 ± 0.06^{a}	3.24 ± 0.01^{b}
Genistein der	20.88 ± 0.46^{e}	66.12 ± 0.03^{c}	54.33 ± 1.21^{d}	103.21 ± 0.27^{b}	117.92 ± 1.94^{a}
Naringenin der	nd	nd	nd	4.11 ± 0.14^{a}	3.58 ± 0.01^{b}
Sinapic acid der	120.60 ± 0.18^{a}	107.49 ± 0.48^{a}	89.30 ± 1.57^{b}	85.91 ± 7.16^{b}	66.40 ± 12.07^{c}
Soluble-conjugated phenolics					
Apigenin der	39.49 ± 3.02^{a}	0.52 ± 0.10^{b}	0.54 ± 0.02^{b}	nd	nd
Catechin der	0.41 ± 0.04^{c}	nd	nd	3.54 ± 0.11^{a}	2.53 ± 0.05^{b}
Genistein	nd	nd	nd	1.55 ± 0.06^{a}	0.44 ± 0.05^{b}
Genistein der	33.63 ± 2.58^{c}	82.80 ± 10.78^{b}	97.76 ± 13.13^{ab}	111.76 ± 4.82^{a}	99.15 ± 1.71^{ab}
Naringenin der	5.46 ± 0.39^{a}	1.97 ± 0.73^{b}	1.83 ± 0.45^{b}	2.08 ± 0.20^{b}	1.25 ± 0.03^{b}
Insoluble-bound phenolics					
Apigenin der	1.77 ± 0.13^{b}	0.84 ± 0.22^{b}	0.90 ± 0.09^{b}	6.01 ± 0.82^{a}	5.91 ± 0.12^{a}
Catechin der	26.24 ± 0.53^{a}	28.25 ± 0.98^{a}	26.50 ± 2.06^{a}	8.74 ± 0.95^{b}	8.38 ± 0.97^{b}
Genistein der	40.41 ± 6.07^{b}	53.71 ± 4.37^{a}	52.50 ± 3.45^{a}	29.23 ± 4.23^{b}	34.76 ± 3.64^{b}
Naringenin der	14.27 ± 0.57	18.39 ± 3.39	19.02 ± 1.15	15.25 ± 1.69	14.63 ± 1.04

der, derivative; nd, not detected; different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

than that (201–516 mg/kg DM) reported by Martínez-Villaluenga et al. (2006) for bitter *L. albus* seeds, but was similar to that (131.0–218.4 mg/kg DM) detected in debittered seeds by Briceño Berru et al. (2021), and superior to the results (61.2–130.0 and 73.6 mg/kg) reported for bitter seeds of *L. albus* by Boschin and Arnoldi (2011) and Lampart-Szczapa et al. (2003), respectively.

The two-way ANOVA (Supplementary Table S.6) did not show significant effects of the treatment, the solvent, or their interaction on tocopherol content, except for α-tocopherol (interaction) and β-tocopherol (treatment and solvent). In fact, β-tocopherol was slightly more abundant after debittering without the use of US and employing the citric acid solution.

The detrimental effect of ultrasound on carotenoids in the presence of citric acid could be hastily attributed to the production of radicals from the homolytic cleavage of water (Mason & Peters, 2002; Suslick, 1990). In fact, carotenoids are degraded by reactive oxygen species undergoing unspecific chain-breaking events (Sun et al., 2020). However, the frequency here employed (24 kHz) results in minimal development of hydroxyl radicals (Ashokkumar et al., 2008; Mason & Peters, 2002). Most likely, citric acid intensified the ultrasound probe erosion (Feng & Yang, 2011), releasing metal ions that catalyse the formation of superoxide anion (Kim & Min, 2008). In addition, carotenoids quench singlet oxygen with reaction rates two orders of magnitude higher than α-tocopherol (Kim & Min, 2008): this may explain why the decrease was significant for carotenoids and not for tocopherols.

Most of the literature deals with non-debittered seeds; nevertheless, the debittered samples studied here had similar or, in some cases, higher tocopherol and carotenoid contents. Debittering did not seem to cause a drastic loss of lipophilic antioxidants, which are insoluble in aqueous solutions and, therefore, are not washed away. On the contrary, the leaching of alkaloids and other water-soluble compounds may concentrate tocopherols and carotenoids, as observed by Brandolini et al. (2022) and Briceño Berru et al. (2021).

4.3.4.2 Phenolic compounds

Table 4.3 reports also the free, conjugated, and bound phenolic compounds content of the Lot 1 seeds after debittering with solutions of NaCl 1%, for 45 h, or citric acid 1%, for 28.5 h, either without or with sonication. Most of the peaks that were detected in the chromatograms did not correspond to the retention times of the standards, apart from genistein. Therefore, they were recorded, according to the similarity of their spectra, as derivatives of sinapic acid and of the flavonoids genistein, naringenin, diosmin, apigenin and catechin. Apigenin derivatives were found by Karamać et al. (2018) and Siger et al. (2012) in different lupin species, and by Czubinski et al. (2021) in *L. mutabilis*, while Kalogeropoulos et al. (2010) identified small amounts of sinapic acid (0.4 mg/kg DM), catechin (1.9 mg/kg DM) and genistein (1.6 mg/kg DM) in *L. albus*.

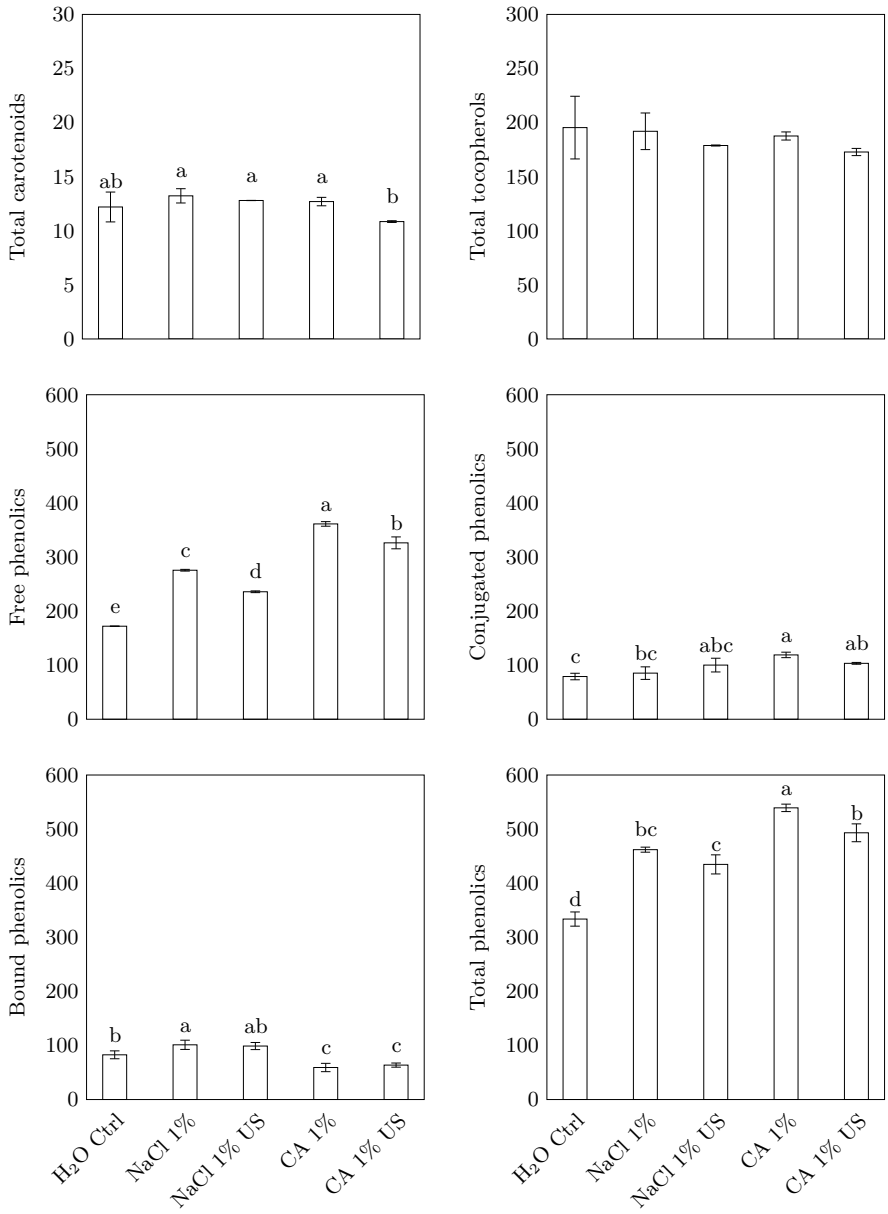


Figure 4.4. Total carotenoids, tocopherols and phenolics compounds (mg/kg DM) in *Lupinus albus* seeds (Lot 1) debittered by the control method with water (H_2O Ctrl; Erbaş, 2010) and by the experimental method using 1% NaCl (for 45 h) or citric acid (CA; for 28.5 h) solutions either without or with ultrasound (US). Error bars show the standard deviation, while different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

The overall total polyphenols (Figure 4.4) of the seeds debittered with the experimental method varied between 434.8 and 539.3 mg/kg DM; the soluble-free fraction was the most abundant and represented 54.2–67.0% of the total, while the soluble-conjugated and the insoluble-bound fractions were 18.5–23.0% and 11.0–22.8%, respectively. For *L. albus* species, the only literature data available deals with non-hydrolysed methanol extracts, which are akin to the soluble-free fraction, generally in non-debittered seeds. The samples that were debittered with the experimental method showed lower quantities of free polyphenols (235.7–361.1 mg/kg DM) than those (418.5–446.7 mg/kg DM and 537.4 mg/kg DM) that were reported for non-debittered *L. albus* seeds by Siger et al. (2012) and Kalogeropoulos et al. (2010), respectively. The soluble-conjugated and insoluble bound phenolic compounds contents, instead, were 85.3–118.9 mg/kg DM and 59.2–101.2 mg/kg DM, respectively.

The two-way ANOVA (Supplementary Table S.7) showed that, for the free phenolic compounds, the effects of the treatment (T), the solvent (S) and their interaction (T × S) were significant ($p \leq 0.05$), except for the genistein derivatives (T not significant), and the sinapic acid derivatives and the total free phenols (T × S not significant). The LSD test indicated that the ultrasound treatment, when it was significant, determined a lower free phenols content, which is attributable to its enhanced extraction capacity (Estivi et al., 2022; Vilkhu et al., 2008). The 1% citric acid and the NaCl solutions led to average residual soluble-free phenolic contents of 343.6 and 255.6 mg/kg DM, respectively. In fact, soaking the seeds in NaCl 1% solution required 16 h more than in citric acid 1% to reach the same alkaloid content (about 1 g/kg DM), leading to a greater leakage of the soluble phenolics.

The ANOVA showed a significant effect of the solvent on the total content of conjugated phenolic compounds, but not on the individual compounds: overall, the citric acid solution induced minor losses compared to the NaCl solution, probably for the same reason that has been described for the free fraction. On the other hand, the solvent effect on the insoluble-bound fraction was significant for the total content and all individual compounds, except for the naringenin derivatives; the samples that were treated with the citric acid solution lost more bound phenolics, except for the apigenin derivatives that were present, however, in limited quantities, and, of course, naringenin.

4.3.5 Effect of debittering methods on antioxidant compounds

4.3.5.1 Carotenoids and tocopherols

Table 4.4 reports the carotenoid and tocopherol values in the Lot 2 seeds, which were debittered by the control methods with water or with NaCl solution, and the experimental method using different solvents and soaking times. The total antioxidants contents are visualized in Figure 4.5.

The samples showed a total carotenoid content of 16.37–25.08 mg/kg DM. The highest concentrations were detected in the lupins that were treated with

Table 4.4. Carotenoids, tocopherols and phenolic compounds content (mg/kg DM; mean ± standard deviation) in *Lupinus albus* seeds (Lot 2) debittered by the control methods with water (H_2O Ctrl; Erbaş, 2010) or with 0.5% NaCl (NaCl Ctrl; Villacrés, Álvarez, & Rossell, 2020) and the experimental method with different solvents.

	H_2O Ctrl	NaCl Ctrl	NaCl 1% 45 h	NaCl 1% 57 h	Citric acid 1% 45 h	Citric acid 1% 57 h
Carotenoids						
(α+β)-carotene	7.86 ± 0.23^{c}	5.30 ± 0.22^{e}	7.10 ± 0.04^{d}	6.89 ± 0.03^{d}	12.01 ± 0.15^{a}	9.63 ± 0.06^{b}
β-criptoxanthin	1.58 ± 0.04^{c}	1.12 ± 0.19^{d}	1.29 ± 0.01^{d}	1.21 ± 0.03^{d}	3.05 ± 0.05^{a}	2.60 ± 0.06^{b}
Lutein	9.33 ± 0.09^{a}	8.06 ± 0.04^{b}	9.17 ± 0.19^{a}	9.35 ± 0.06^{a}	7.97 ± 0.02^{b}	7.70 ± 0.05^{c}
Zeaxanthin	2.30 ± 0.06^{a}	1.90 ± 0.04^{c}	2.07 ± 0.03^{b}	1.89 ± 0.01^{c}	2.06 ± 0.03^{b}	1.85 ± 0.03^{c}
Tocopherols						
α-tocopherol	0.26 ± 0.08^{b}	0.47 ± 0.03^{a}	0.51 ± 0.01^{a}	0.50 ± 0.01^{a}	0.29 ± 0.02^{b}	0.31 ± 0.02^{b}
β-tocopherol	1.13 ± 0.10^{ab}	1.30 ± 0.16^{a}	0.56 ± 0.01^{d}	0.52 ± 0.01^{d}	0.99 ± 0.02^{bc}	0.99 ± 0.07^{c}
γ-tocopherol	237.77 ± 4.34^{a}	197.33 ± 3.07^{cd}	230.53 ± 2.66^{a}	214.27 ± 2.65^{b}	208.35 ± 6.91^{bc}	196.79 ± 6.33^{d}
δ-tocopherol	2.09 ± 0.02^{bc}	1.98 ± 0.01^{cd}	1.82 ± 0.08^{d}	2.26 ± 0.03^{b}	2.45 ± 0.09^{a}	2.23 ± 0.15^{b}
Soluble-free phenolics						
Apigenin der*	49.83 ± 1.41^{e}	96.66 ± 1.67^{d}	163.25 ± 2.72^{b}	128.73 ± 1.68^{c}	207.93 ± 2.26^{a}	165.97 ± 2.79^{b}
Diosmin der	1.31 ± 0.04^{f}	1.63 ± 0.04^{e}	3.62 ± 0.05^{b}	2.76 ± 0.05^{c}	4.81 ± 0.07^{a}	2.43 ± 0.05^{d}
Naringenin der	2.58 ± 0.04^{a}	2.27 ± 0.03^{b}	2.37 ± 0.01^{b}	2.27 ± 0.03^{b}	1.73 ± 0.06^{c}	1.16 ± 0.05^{d}
Sinapic der	53.14 ± 0.33^{c}	50.43 ± 0.48^{c}	134.15 ± 4.20^{a}	82.60 ± 1.86^{b}	32.10 ± 1.56^{d}	12.76 ± 0.34^{e}
Soluble-conjugated phenolics						
Catechin der	nd	2.22 ± 0.06^{b}	1.54 ± 0.03^{d}	0.88 ± 0.03^{e}	4.06 ± 0.05^{a}	1.98 ± 0.07^{c}
Genistein	2.68 ± 0.02^{e}	3.71 ± 0.02^{c}	2.56 ± 0.03^{f}	3.15 ± 0.03^{d}	4.17 ± 0.02^{a}	3.97 ± 0.03^{b}
Genistein der	73.04 ± 2.59^{c}	65.63 ± 1.14^{d}	57.01 ± 2.01^{e}	63.22 ± 2.86^{d}	105.62 ± 1.60^{a}	81.18 ± 1.60^{b}
Naringenin der	6.36 ± 0.09^{a}	2.68 ± 0.05^{e}	2.80 ± 0.13^{de}	3.01 ± 0.19^{d}	4.87 ± 0.11^{b}	3.61 ± 0.06^{c}
Insoluble-bound phenolics						
Apigenin der	0.15 ± 0.04^{d}	0.30 ± 0.03^{c}	0.49 ± 0.06^{b}	0.42 ± 0.01^{b}	1.35 ± 0.01^{a}	1.27 ± 0.01^{a}
Catechin der	23.94 ± 0.91^{b}	28.57 ± 0.97^{a}	27.39 ± 0.13^{a}	27.37 ± 0.05^{a}	11.70 ± 0.06^{c}	11.63 ± 0.06^{c}
Genistein	2.13 ± 0.02^{a}	1.06 ± 0.03^{d}	1.51 ± 0.09^{c}	1.75 ± 0.09^{b}	1.77 ± 0.04^{b}	1.52 ± 0.01^{c}
Genistein der	78.23 ± 2.76^{c}	94.50 ± 2.87^{b}	112.91 ± 1.43^{a}	83.44 ± 5.57^{c}	83.91 ± 4.85^{c}	93.91 ± 4.19^{b}
Naringenin der	10.89 ± 0.91^{c}	13.24 ± 1.01^{ab}	14.42 ± 1.48^{a}	11.42 ± 0.84^{bc}	11.44 ± 0.22^{bc}	11.66 ± 0.15^{bc}

der, derivative; nd, not detected; different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

citric acid 1% for 45 h (25.08 mg/kg DM). The most abundant compound was lutein, except for the samples treated with citric acid, where (α+β)-carotene was predominant. Contrary to the Lot 1 lupins, the seeds debittered with NaCl 1% presented a lower percentage of total carotenoids than those treated with citric acid 1%. Overall, prolonged treatment times and, secondarily, high temperatures influenced the debittering results; the control method with salt induced the highest carotenoids loss, followed by the 57 h and 45 h treatments with 1% NaCl, the control method with water and the 57 h and 45 h treatments with citric acid 1%.

Again, no tocotrienols were identified and the most abundant tocopherol was γ-tocopherol (over 98%), with values in the range of 200.3–241.3 mg/kg DM, which was higher than those found in the debittered seeds of *L. albus* by Briceño Berru et al. (2021; 131.0–218.4 mg/kg DM) and by Annicchiarico et al. (2014; 107.0–153.0 mg/kg DM). The seeds that were treated with NaCl 1% for 45 h or with the traditional method showed significantly higher tocopherols content than the other samples. The control method with water limits the exposure to high temperatures to one hour, so this may have better preserved the tocopherols from thermal degradation; vice versa the lowest content was found in the seeds that were debittered with the control method with 0.5% NaCl, which involves 8 h of hydration at 80 °C and 1 h of cooking.

Among the samples that were treated with the experimental method, those debittered with NaCl 1% retained a significantly higher share of tocopherols compared to those treated with citric acid 1% for the same amount of time. Within solvents, the seeds that were debittered for 45 h showed a higher concentration of tocopherols than those that were treated for 57 h. Hence, prolonging the soaking time increases the tocopherols loss.

4.3.5.2 Phenolic compounds

Table 4.4 reports the free, conjugated and bound phenolic compounds of the Lot 2 seeds that were debittered with the control methods (water or salt solution) and the experimental method. The total phenol content (Figure 4.5) varied broadly (304.3–524.0 mg/kg DM) as a consequence of the very different treatment conditions. As already observed for the Lot 1 seeds, only genistein was identified with the corresponding retention time and spectrum, while the detected flavonoid derivatives included genistein, naringenin, diosmin, apigenin, and catechin; the only phenolic acid derivative recorded was sinapic acid. Differently from the Lot 1 seeds, the genistein derivatives were absent among the free phenolics, and so were the apigenin derivatives in the soluble-conjugated phenolics, while genistein was observed in the bound fraction. The total free phenolic content (106.9–303.4 mg/kg DM) was lower than the values (418.5–446.7 and 537.4 mg/kg DM) reported in non-debittered seeds by Siger et al. (2012) and Kalogeropoulos et al. (2010), respectively. The contents of soluble-conjugated and insoluble-bound phe-

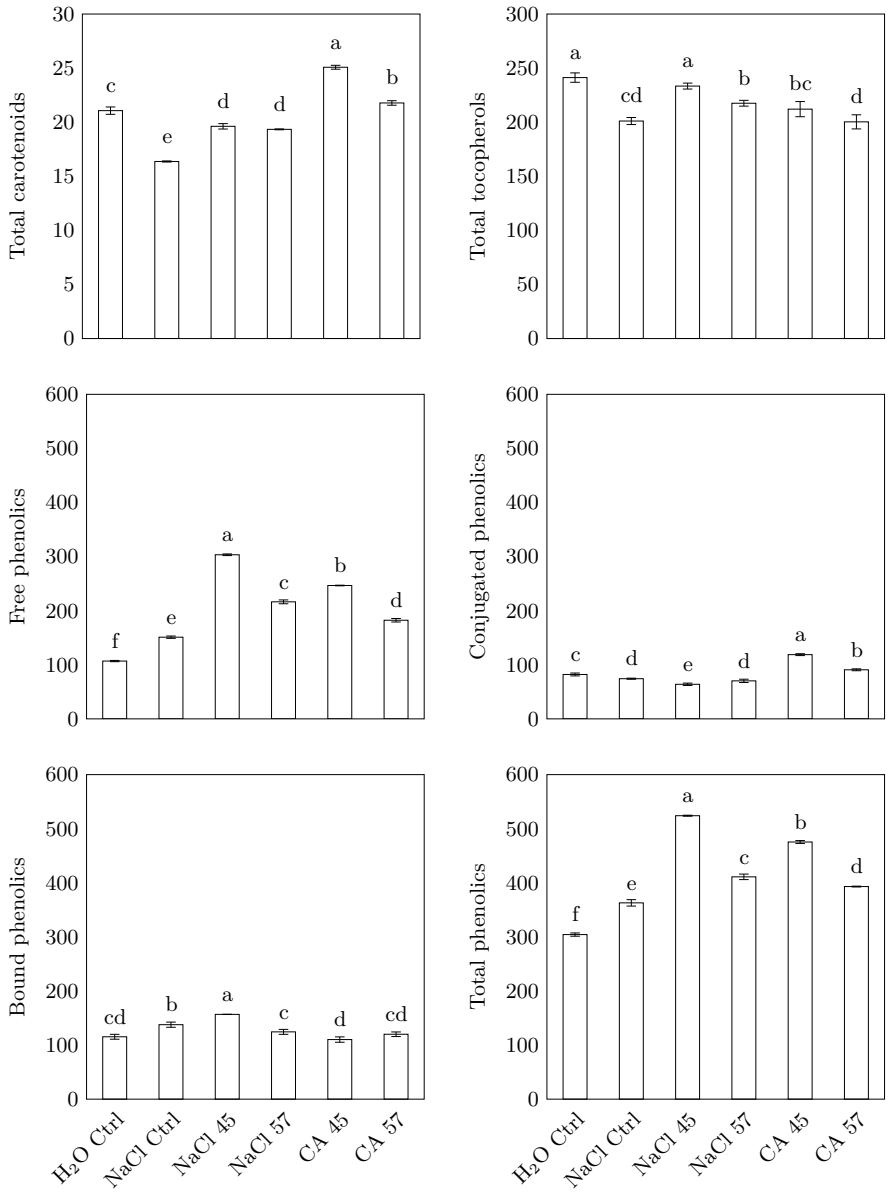


Figure 4.5. Total carotenoids, tocopherols and phenolic compounds (mg/kg DM) in *Lupinus albus* seeds (Lot 2) debittered by the control method with water (H_2O Ctrl; Erbaş, 2010) or with NaCl solution (NaCl Ctrl; Villacrés, Álvarez, & Rossell, 2020), and by the experimental method using 1% NaCl or citric acid (CA) solutions for 45 or 57 h. Error bars show the standard deviation, while different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

nolic compounds were 74.2–118.7 mg/kg DM and 110.2–156.7 mg/kg DM, respectively.

The LSD test revealed significant differences in the total polyphenol content among the debittering treatments (Figure 4.5). In particular, the treatments that better preserved the phenols were the shortest ones (45 h), with either 1% NaCl (524.0 mg/kg DM) or citric acid (475.5 mg/kg DM). The control treatment with water induced the greatest phenolic loss (residual content, 304.3 mg/kg DM), followed by the salt control method (362.9 mg/kg DM). The same ranking among treatments was recorded for the soluble-free fraction, which constituted the majority of the total phenolics and varied between 106.9 (traditional method) and 303.4 mg/kg DM (NaCl 1% solution for 45 h). Hence, the soaking time seems to be the main cause of the leaching of soluble-free phenolics.

Overall, even after debittering, the lupin seeds preserved a significant content of phenolic compounds, which are positively correlated to the antioxidant capacity (Córdova-Ramos, Glorio-Paulet, Hidalgo, & Camarena, 2020). The citric acid solutions better preserved the conjugated phenols, especially using the shortest soaking time, while the NaCl solutions behaved poorly. The contrary was instead evident for the bound phenols: all of the samples treated with salt solutions, either by the control method or by the experimental protocol, showed significantly higher levels of insoluble bound polyphenols (124.4–156.7 mg/kg DM) compared to the samples that were treated with the traditional method (115.3 mg/kg DM) or with citric acid (110.2–120.0 mg/kg DM). The treatment with NaCl 1% for 45 h retained the greatest amount of bounds phenols.

4.4 Conclusions

According to the results, the sonication did not accelerate lupin seeds debittering, while sodium chloride and citric acid solutions following the proposed experimental procedure significantly shortened debittering time, limited water consumption and reduced alkaloid content to a concentrations range (0.31–1.03 g/kg DM) comparable to that observed in commercial snacks (0.35–0.99 g/kg DM). The e-tongue discriminated the samples, placing those treated with the same solvent in well-defined clusters; bitter and umami tastes were the main factors characterizing samples according to their debittering treatments.

The sonication decreased the content of carotenoids and soluble-free phenolics, but did not influence the tocopherols or the soluble-conjugated and insoluble-bound phenolics. 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. However, the soluble-free fraction was the most suscep-

tible to increased treatment time. The use of citric acid or sodium chloride, thanks to the shortened debittering duration, better preserved the antioxidant compounds in the seeds.

Future investigations may take advantage of these results assessing the bioaccessibility of bioactive nutrients in ready-to-eat products from lupin flour.

References

Albuja-Vaca, D., Yépez, Y., Vernaza, M. G., & Navarrete, D. (2019). Gluten-free pasta: Development of a new formulation based on rice and lupine bean flour (*Lupinus mutabilis*) using a mixture-process design. *Food Sci Technol*, *40*, 408–414. https://doi.org/10.1590/FST.02319

Al-Saedi, N., Agarwal, M., Ma, W., Islam, S., & Ren, Y. (2020). Proteomic characterisation of lupin (*Lupinus angustifolius*) milk as influenced by extraction techniques, seed coat and cultivars. *Molecules*, *25*, 1782. https://doi.org/10.3390/molecules25081782

Alu'datt, M. H., Rababah, T., Alhamad, M. N., Ereifej, K., Gammoh, S., Kubow, S., & Tawalbeh, D. (2017). Preparation of mayonnaise from extracted plant protein isolates of chickpea, broad bean and lupin flour: Chemical, physiochemical, nutritional and therapeutic properties. *J Food Sci Technol*, *54*, 1395–1405. https://doi.org/10.1007/S13197-017-2551-6

Annicchiarico, P., Manunza, P., Arnoldi, A., & Boschin, G. (2014). Quality of *Lupinus albus* L. (white lupin) seed: Extent of genotypic and environmental effects. *J Agric Food Chem*, *62*, 6539–6545. https://doi.org/10.1021/jf405615k

AOAC. (2000). *Official Methods of Analysis of AOAC International, 17th ed.* Association of Official Analytical Chemists International.

Ashokkumar, M., Kentish, D. S., Mawson, R., Simons, L., Vilkhu, K., & Versteeg, C. K. (2008). Modification of food ingredients by ultrasound to improve functionality: A preliminary study on a model system. *Innov Food Sci Emerg Technol*, *9*, 155–160. https://doi.org/10.1016/j.ifset.2007.05.005

Bellisle, F. (1999). Glutamate and the umami taste: Sensory, metabolic, nutritional and behavioural considerations. A review of the literature published in the last 10 years. *Neurosci Biobehav Rev*, *23*, 423–438. https://doi.org/10.1016/S0149-7634(98)00043-8

Bharate, S. S. (2021). Carboxylic acid counterions in FDA-approved pharmaceutical salts. *Pharmaceut Res*, *38*, 1307–1326. https://doi.org/10.1007/s11095-021-03080-2

Bhargava, N., Mor, R. S., Kumar, K., & Sharanagat, V. S. (2021). Advances in application of ultrasound in food processing: A review. *Ultrason*

Sonochem, *70*, 105293. https://doi.org/10.1016/j.ultsonch.2020.105293

Boschin, G., Annicchiarico, P., Resta, D., D'Agostina, A., & Arnoldi, A. (2008). Quinolizidine alkaloids in seeds of lupin genotypes of different origins. *J Agric Food Chem*, *56*, 3657–3663. https://doi.org/10.1021/jf7037218

Boschin, G., & Arnoldi, A. (2011). Legumes are valuable sources of tocopherols. *Food Chem*, *127*, 1199–1203. https://doi.org/10.1016/j.foodchem.2011.01.124

Brandolini, A., Glorio-Paulet, P., Estivi, L., Locatelli, N., Córdova-Ramos, J. S., & Hidalgo, A. (2022). Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) seeds processing. *J Food Compos Anal*, *106*, 104335. https://doi.org/10.1016/j.jfca.2021.104335

Briceño Berru, L., Glorio-Paulet, P., Basso, C., Scarafoni, A., Camarena, F., Hidalgo, A., & Brandolini, A. (2021). Chemical composition, tocopherol and carotenoid content of seeds from different Andean lupin (*Lupinus mutabilis*) ecotypes. *Plant Food Hum Nutr*, *76*, 98–104. https://doi.org/10.1007/S11130-021-00880-0

Carvajal-Larenas, F. E., Linnemann, A. R., Nout, M. J. R., Koziol, M., & van Boekel, M. A. J. S. (2016). *Lupinus mutabilis*: Composition, uses, toxicology, and debittering. *Crit Rev Food Sci Nutr*, *56*, 1454–1487. https://doi.org/10.1080/10408398.2013.772089

Carvajal-Larenas, F. E., Nout, M. J. R., van Boekel, M. A. J. S., Koziol, M., & Linnemann, A. R. (2013). Modelling of the aqueous debittering process of *Lupinus mutabilis* Sweet. *LWT - Food Sci Technol*, *53*, 507–516. https://doi.org/10.1016/j.lwt.2013.03.017

Carvajal-Larenas, F. E., van Boekel, M. A. J. S., Koziol, M., Nout, M. J. R., & Linnemann, A. R. (2014). Effect of processing on the diffusion of alkaloids and quality of *Lupinus mutabilis* Sweet. *J Food Process Pres*, *38*, 1461–1471. https://doi.org/10.1111/jfpp.12105

Casp Vanaclocha, A., & Abril Requena, J. (2003). *Procesos de conservación de alimentos*. Ediciones Mundi-Prensa.

Córdova-Ramos, J. S., Glorio-Paulet, P., Camarena, F., Brandolini, A., & Hidalgo, A. (2020). Andean lupin (*Lupinus mutabilis* Sweet): Processing effects on chemical composition, heat damage, and *in vitro* protein digestibility. *Cereal Chem*, *97*, 827–835. https://doi.org/10.1002/cche.10303

Córdova-Ramos, J. S., Glorio-Paulet, P., Hidalgo, A., & Camarena, F. (2020). Effect of technological process on antioxidant capacity and total phenolic content of Andean lupine (*Lupinus mutabilis* Sweet). *Sci Agropecu*, *11*, 157–165. https://doi.org/10.17268/sci.agropecu.2020.02.02

Czubinski, J., Grygier, A., & Siger, A. (2021). *Lupinus mutabilis* seed composition and its comparison with other lupin species. *J Food Compos Anal*, *99*, 103875. https://doi.org/10.1016/j.jfca.2021.103875

de Cortes Sánchez, M., Altares, P., Pedrosa, M. M., Burbano, C., Cuadrado, C., Goyoaga, C., Muzquiz, M., Jiménez-Martínez, C., & Dávila-Ortiz, G. (2005). Alkaloid variation during germination in different lupin species. *Food Chem*, *90*, 347–355. https://doi.org/10.1016/j.foodchem.2004.04.008

Duranti, M., Consonni, A., Magni, C., Scarafoni, A., & Sessa, F. (2008). The major proteins of lupin seed: Characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends Food Sci Technol*, *19*, 624–633. https://doi.org/10.1016/j.tifs.2008.07.002

El-Difrawi, E. A., & Hudson, B. J. F. (1979). Identification and estimation of carotenoids in the seeds of four *Lupinus* species. *J Sci Food Agric*, *30*, 1168–1170. https://doi.org/10.1002/jsfa.2740301209

Erbaş, M. (2010). The effects of different debittering methods on the production of lupin bean snack from bitter *Lupinus albus* L. seeds. *J Food Qual*, *33*, 742–757. https://doi.org/10.1111/j.1745-4557.2010.00347.x

Esteves, T., Mota, A. T., Barbeitos, C., Andrade, K., Afonso, C. A. M., & Castelo Ferreira, F. (2020). A study on lupin beans process wastewater nanofiltration treatment and lupanine recovery. *J Clean Prod*, *277*, 123349. https://doi.org/10.1016/j.jclepro.2020.123349

Estivi, L., Brandolini, A., Condezo-Hoyos, L., & Hidalgo, A. (2022). Impact of low-frequency ultrasound technology on physical, chemical and technological properties of cereals and pseudocereals. *Ultrason Sonochem*, *86*, 106044. https://doi.org/10.1016/j.ultsonch.2022.106044

Feng, H., & Yang, W. (2011). Ultrasound processing. In H. Q. Zhang, G. V. Barbosa-Cánovas, V. M. Balasubramaniam, C. P. Dunne, D. F. Farkas, & J. T. C. Yuan (Eds.), *Nonthermal processing technologies for food* (pp. 135–154). John Wiley & Sons, Wiley-Blackwell; IFT Press. https://doi.org/10.1002/9780470958360

Fernández-Marín, B., Milla, R., Martín-Robles, N., Arc, E., Kranner, I., Becerril, J. M., & García-Plazaola, J. I. (2014). Side-effects of domestication: Cultivated legume seeds contain similar tocopherols and fatty acids but less carotenoids than their wild counterparts. *BMC Plant Biol*, *14*, 1599. https://doi.org/10.1186/s12870-014-0385-1

Frick, K. M., Kamphuis, L. G., Siddique, K. H., Singh, K. B., & Foley, R. C. (2017). Quinolizidine alkaloid biosynthesis in lupins and prospects for grain quality improvement. *Front Plant Sci*, *8*, 87. https://doi.org/10.3389/fpls.2017.00087

Giel-Pietraszuk, M., Gdaniec, Z., Brukwicki, T., & Barciszewski, J. (2007). Molecular mechanism of high pressure action on lupanine. *J Mol Struct*, *826*, 120–125. https://doi.org/10.1016/j.molstruc.2006.04.038

Gross, U., Galindo, R. G., & Schoeneberger, H. (1983). The development and acceptability of lupine (*Lupinus mutabilis*) products. *Plant Food Hum Nutr*, *32*, 155–164. https://doi.org/10.1007/bf01091336

Guadagnucci Fontanari, G., Kobelnik, M., Marques, M. R., Arêas, J. A. G., Franzin, B. T., Pastre, I. A., & Fertonani, F. L. (2018). Thermal and kinetic studies of white lupin (*Lupinus albus*) oil. *J Therm Anal Calorim*, *131*, 775–782. https://doi.org/10.1007/s10973-017-6468-0

Guadagnucci Fontanari, G., Martins, J. M., Kobelnik, M., Pastre, I. A., Arêas, J. A. G., & Batistuti, J. P. (2012). Thermal studies on protein isolates of white lupin seeds *Lupinus albus* L. *J Therm Anal Calorim*, *108*, 141–148. https://doi.org/10.1007/s10973-011-1898-6

Güémes-Vera, N., Peña-Bautista, R. J., Jiménez-Martínez, C., Dávila-Ortiz, G., & Calderón-Domínguez, G. (2008). Effective detoxification and decoloration of *Lupinus mutabilis* seed derivatives, and effect of these derivatives on bread quality and acceptance. *J Sci Food Agr*, *88*, 1135–1143. https://doi.org/10.1002/jsfa.3152

Hameed, A., Ahmed, M. Z., Hussain, T., Aziz, I., Ahmad, N., Gul, B., & Nielsen, B. L. (2021). Effects of salinity stress on chloroplast structure and function. *Cells*, *10*, 2023. https://doi.org/10.3390/cells10082023

Huyghe, C. (1997). White lupin (*Lupinus albus* L.) *Field Crops Res*, *53*, 147–160. https://doi.org/10.1016/S0378-4290(97)00028-2

Hwang, Y. H., Ismail, I., & Joo, S. T. (2020). Identification of umami taste in *sous-vide* beef by chemical analyses, equivalent umami concentration, and electronic tongue system. *Foods*, *9*, 251. https://doi.org/10.3390/foods9030251

INEN. (2005). *Norma Técnica Ecuatoriana NTE INEN 2 390:2004. Leguminosas. Grano desamargado de chocho. Requisitos.* Instituto Ecuatoriano de Normalizacíon. Quito, EC. https://www.normalizacion.gob.ec/buzon/normas/2390.pdf

Jayasena, V., Khu, W. S., & Nasar-Abbas, S. M. (2010). The development and sensory acceptability of lupin-based tofu. *J Food Quality*, *33*, 85–97. https://doi.org/10.1111/j.1745-4557.2009.00290.x

Jayasena, V., Leung, P. P. Y., & Nasar-Abbas, S. M. (2010). Effect of lupin flour substitution on the quality and sensory acceptability of instant noodles. *J Food Quality*, *33*, 709–727. https://doi.org/10.1111/j.1745-4557.2010.00353.x

Jiménez-Martínez, C., Hernández-Sánchez, H., Alvárez-Manilla, G., Robledo-Quintos, N., Martínez-Herreraand, J., & Dávila-Ortiz, G. (2001). Effect of aqueous and alkaline thermal treatments on chemical composition and oligosaccharide, alkaloid and tannin contents of *Lupinus campestris* seeds. *J Sci Food Agr*, *81*, 421–428. https://doi.org/10.1002/1097-0010(200103)81:4<421::AID-JSFA829>3.0.CO;2-U

Jiménez-Martínez, C., Hernández-Sánchez, H., & Dávila-Ortiz, G. (2007). Diminution of quinolizidine alkaloids, oligosaccharides and phenolic

compounds from two species of *Lupinus* and soybean seeds by the effect of *Rhizopus oligosporus*. *J Sci Food Agr*, *87*, 1315–1322. https://doi.org/10.1002/JSFA.2851

Jiménez-Martínez, C., Mora-Escobedo, R., Cardador Martínez, A., Muzquiz, M., Martin Pedrosa, M., & Dávila-Ortiz, G. (2010). Effect of aqueous, acid, and alkaline thermal treatments on antinutritional factors content and protein quality in *Lupinus campestris* deed flour. *J Agr Food Chem*, *58*, 1741–1745. https://doi.org/10.1021/JF902688R

Julier, B., Huyghe, C., Papineau, G. F. J., J. an Milford, Day, J. M., Billot, C., & Mangin, P. (1993). Seed yield and yield stability of determinate and indeterminate autumn-sown white lupins (*Lupinus albus*) grown at different locations in France and the UK. *J Agric Sci*, *121*, 177–186. https://doi.org/10.1017/S0021859600077030

Julier, B., Huyghe, C., Papineau, J., Billot, C., & Deroo, C. (1995). Genetic and environmental variation in architecture and yield components in determinate white lupin (*Lupinus albus* L.) *Euphytica*, *81*, 171–179. https://doi.org/10.1007/BF00025430

Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V. T., Hassapidou, M., & Andrikopoulos, N. K. (2010). Nutritional evaluation and bioactive microconstituents (phytosterols, tocopherols, polyphenols, triterpenic acids) in cooked dry legumes usually consumed in the Mediterranean countries. *Food Chem*, *121*, 682–690. https://doi.org/10.1016/j.foodchem.2010.01.005

Karamać, M., Orak, H. H., Amarowicz, R., Orak, A., & Piekoszewski, W. (2018). Phenolic contents and antioxidant capacities of wild and cultivated white lupin (*Lupinus albus* L.) seeds. *Food Chem*, *258*, 1–7. https://doi.org/10.1016/j.foodchem.2018.03.041

Karara, H. A. (1987). An efficient method for the extraction of alkaloids from bitter lupin seed. *Fat Sci Technol*, *89*, 442–446. https://doi.org/10.1002/lipi.19870891107

Kim, H. J., & Min, D. B. (2008). Chemistry of lipid oxidation. In C. C. Akoh & D. B. Min (Eds.), *Food lipids: Chemistry, nutrition, and biotechnology 3rd ed.* (pp. 299–320). CRC Press.

Kobayashi, Y., Habara, M., Ikezazki, H., Chen, R., Naito, Y., & Toko, K. (2010). Advanced taste sensors based on artificial lipids with global selectivity to basic taste qualities and high correlation to sensory scores. *Sensors*, *10*, 3411–3443. https://doi.org/10.3390/s100403411

Kroc, M., Czepiel, K., Wilczura, P., Mokrzycka, M., & Święcicki, W. (2019). Development and validation of a gene-targeted dCAPS marker for marker-assisted selection of low-alkaloid content in seeds of narrow-leafed lupin (*Lupinus angustifolius* L.) *Genes*, *10*, 428. https://doi.org/10.3390/GENES10060428

Kukula-Koch, W. A., & Widelski, J. (2017). Alkaloids. In S. Badal & R. Delgoda (Eds.), *Pharmacognosy: Fundamentals, applications and*

strategies. (pp. 163–198). Academic Press. https://doi.org/10.1016/B978-0-12-802104-0.00009-3

Lampart-Szczapa, E., Konieczny, P., Nogala-Kałucka, M., Walczak, S., Kossowska, I., & Malinowska, M. (2006). Some functional properties of lupin proteins modified by lactic fermentation and extrusion. *Food Chem*, *96*, 290–296. https://doi.org/10.1016/j.foodchem.2005.02.031

Lampart-Szczapa, E., Korczak, J., Nogala-Kalucka, M., & Zawirska-Wojtasiak, R. (2003). Antioxidant properties of lupin seed products. *Food Chem*, *83*, 279–285. https://doi.org/10.1016/S0308-8146(03)00091-8

Lo, B., Kasapis, S., & Farahnaky, A. (2021). Lupin protein: Isolation and techno-functional properties, a review. *Food Hydrocoll*, *112*, 106318. https://doi.org/10.1016/J.FOODHYD.2020.106318

Marengo, M., Baffour, L. C., Buratti, S., Benedetti, S., Saalia, F. K., Carpen, A., Manful, J., Johnson, P. N. T., Barbiroli, A., Bonomi, F., Pagani, A., Marti, A., & Iametti, S. (2016). Defining the overall quality of cowpea-enriched rice-based breakfast cereals. *Cereal Chem*, *94*, 151–157. https://doi.org/10.1094/CCHEM-04-16-0092-FI

Margulis, M. A., & Margulis, I. M. (2003). Calorimetric method for measurement of acoustic power absorbed in a volume of a liquid. *Ultrason Sonochem*, *10*, 343–345. https://doi.org/10.1016/S1350-4177(03)00100-7

Martínez-Villaluenga, C., Frías, J., & Vidal-Valverde, C. (2006). Functional lupin seeds (*Lupinus albus* L. and *Lupinus luteus* L.) after extraction of α-galactosides. *Food Chem*, *98*, 291–299. https://doi.org/10.1016/j.foodchem.2005.05.074

Mason, T. J., & Peters, D. (2002). *Practical sonochemistry: Power ultrasound uses and applications*. Woodhead Publishing.

Mera, M., Beltran, L., Miranda, H., & Rouanet, J. L. (2006). Strong heritability across years and sites for pod wall proportion and specific weight in *Lupinus albus* and genotypic correlation with other pod and seed attributes. *Plant Breed*, *125*, 161–166. https://doi.org/10.1111/j.1439-0523.2006.01174.x

Miano, A. C., Rojas, M. L., & Augusto, P. E. D. (2019). Using ultrasound for improving hydration and debittering of Andean lupin grains. *J Food Process Eng*, *42*, e13170. https://doi.org/10.1111/jfpe.13170

Miller, J. N., & Miller, J. C. (2010). *Statistics and chemometrics for analytical chemistry, 6th ed.* Pearson Education Limited.

Mohammadi, F., Marti, A., Nayebzadeh, K., Hosseini, S. M., Tajdar-Oranj, B., & Jazaeri, S. (2021). Effect of washing, soaking and pH in combination with ultrasound on enzymatic rancidity, phytic acid, heavy metals and coliforms of rice bran. *Food Chem*, *334*, 127583. https://doi.org/10.1016/j.foodchem.2020.127583

Mohammed, M. A., Mohamed, E. A., Yagoub, A. E. A., Mohamed, A. R., & Babiker, E. E. (2017). Effect of processing methods on alkaloids,

phytate, phenolics, antioxidants activity and minerals of newly developed lupin (*Lupinus albus* L.) cultivar. *J Food Process*, *41*, 1–9. https://doi.org/10.1111/jfpp.12960

Muzquiz, M., Cuadrado, C., Ayet, G., de la Cuadra, C., Burbano, C., & Osagie, A. (1994). Variation of alkaloid components of lupin seeds in 49 genotypes of *Lupinus albus* from different countries and locations. *J Agric Food Chem*, *42*, 1447–1450. https://doi.org/10.1021/jf00043a011

Ortiz, J. G. F., & Mukherjee, K. D. (1982). Extraction of alkaloids and oil from bitter lupin seed. *J Am Oil Chem Soc*, *59*, 241–244. https://doi.org/10.1007/BF02582186

Otterbach, S. L., Yang, T., Kato, L., Janfelt, C., & Geu-Flores, F. (2019). Quinolizidine alkaloids are transported to seeds of bitter narrow-leafed lupin. *J Exp Bot*, *70*, 5799–5808. https://doi.org/10.1093/jxb/erz334

Özcan, M. M., İpek, D., Ghafoor, K., Al Juhaimi, F., Uslu, N., Babiker, E. E., Mohamed Ahmed, I. A., & Alsawmahi, O. N. (2021). Physicochemical and sensory properties of chips produced using different lupin (*Lupinus albus* L.) flour formulations and cooking methods. *56*, 2780–2788. https://doi.org/10.1111/IJFS.14913

Paraskevopoulou, A., Provatidou, E., Tsotsiou, D., & Kiosseoglou, V. (2010). Dough rheology and baking performance of wheat flour—lupin protein isolate blends. *Food Res Int*, *43*, 1009–1016. https://doi.org/10.1016/J.FOODRES.2010.01.010

Pascual-Chagman, G., Santa Cruz Olivos, J., Hidalgo, A., Benavente, F., Pérez-Camino, M. C., Sotelo, A., Paucar-Menacho, L. M., & Encina-Zelada, C. R. (2021). *Lupinus mutabilis* oil obtained by expeller press: Yield, physicochemical characterization, antioxidant capacity, fatty acids and oxidative stability analyses. *Sci Agropecu*, *12*, 219–227. https://doi.org/10.17268/sci.agropecu.2021.025

Pollard, N. J., Stoddard, F. L., Popineau, Y., Wrigley, C. W., & MacRitchie, F. (2002). Lupin flours as additives: Dough mixing, breadmaking, emulsifying, and foaming. *Cereal Chem*, *79*, 662–669. https://doi.org/10.1094/CCHEM.2002.79.5.662

Schmidt, M., Zannini, E., & Arendt, E. K. (2019). Screening of post-harvest decontamination methods for cereal grains and their impact on grain quality and technological performance. *Eur Food Res Technol*, *245*, 1061–1074. https://doi.org/10.1007/s00217-018-3210-5

Sievwright, C. A., & Shipe, W. F. (1986). Effect of storage conditions and chemical treatments on firmness, *in vitro* protein digestibility, condensed tannins, phytic acid and divalent cations of cooked black beans (*Phaseolus vulgaris*). *J Food Sci*, *51*, 982–987. https://doi.org/10.1111/j.1365-2621.1986.tb11214.x

Siger, A., Czubinski, J., Kachlicki, P., Dwiecki, K., Lampart-Szczapa, E., & Nogala-Kalucka, M. (2012). Antioxidant activity and phenolic content in three lupin species. *J Food Compos Anal*, *25*, 190–197. https://doi.org/10.1016/j.jfca.2011.10.002

Sun, T., Tadmor, Y., & Li, L. (2020). Pathways for carotenoid biosynthesis, degradation, and storage. In M. Rodríguez-Concepción & R. Welsch (Eds.), *Plant and food carotenoids. Methods in molecular biology* (pp. 3–23, Vol. 2083). Humana. https://doi.org/10.1007/978-1-4939-9952-1_1

Suslick, K. S. (1990). Sonochemistry. *Science*, *247*, 1439–1445. https://doi.org/10.1126/science.247.4949.1439

Taverniti, N. (2019). *Digestione anaerobica di reflui da lavorazione dei lupini: potenzialità e pretrattamenti [Anaerobic digestion of lupins processing sludge: Potentials and pretreatments]* [Master thesis]. Polytechnic University of Milan. https://www.politesi.polimi.it/bitstream/10589/148684/3/Tesi%20Taverniti_Nicola.pdf

Uauy, R., Gattas, V., & Yañez, E. (1995). Sweet lupins in human nutrition. In A. P. Simopoulos (Ed.), *Plants in human nutrition* (pp. 75–88). Karger Publishers. https://doi.org/10.1159/000424466

Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry—A review. *Innov Food Sci Emerg Technol*, *9*, 161–169. https://doi.org/10.1016/j.ifset.2007.04.014

Villacrés, E., Álvarez, J., & Rossell, C. (2020). Effects of two debittering processes on the alkaloid content and quality characteristics of lupin (*Lupinus mutabilis* Sweet). *J Sci Food Agric*, *100*, 2166–2175. https://doi.org/10.1002/jsfa.10240

Villacrés, E., Quelal, M. B., Fernandez, E., Garcìa, G., Cueva, G., & Rosell, C. M. (2020). Impact of debittering and fermentation processes on the antinutritional and antioxidant compounds in *Lupinus mutabilis* Sweet. *LWT - Food Sci Technol*, *131*, 109745. https://doi.org/10.1016/j.lwt.2020.109745

von Baer, D., Reimerdes, E. H., & Feldheim, W. (1979). Methoden zur bestimmung der chinolizidinalkaloide in *Lupinus mutabilis*. *Z. für Lebensm.-Unters. und Forsch*, *169*, 27–31. https://doi.org/10.1007/BF01353410

Wang, S., Errington, S., & Yap, H. H. (2008). Studies on carotenoids from lupin seeds. In J. A. Palta & J. B. Berger (Eds.), *Proceedings of the 12th International Lupin Conference "Lupins for Health and Wealth", Fremantle, Western Australia, 14–18 September 2008*. International Lupin Association.

Wang, W., Zhou, X., & Liu, Y. (2020). Characterization and evaluation of umami taste: A review. *Trac Trends Anal Chem*, *127*, 115876. https://doi.org/10.1016/j.trac.2020.115876

Wink, M., & Mende, P. (1987). Uptake of lupanine by alkaloid-storing epidermal cells of *Lupinus polyphyllus*. *Planta Med*, *53*, 465–469. https://doi.org/10.1055/s-2006-962774

Wold, S., Esbensen, K., & Geladi, P. (1987). Principal component analysis. *Chemom Intell Lab Syst*, *2*, 37–52. https://doi.org/10.1016/0169-7439(87)80084-9

Yaver, E., & Bilgiçli, N. (2021). Effect of ultrasonicated lupin flour and resistant starch (type 4) on the physical and chemical properties of pasta. *Food Chem*, *357*, 129758. https://doi.org/10.1016/J.FOODCHEM.2021.129758

5

Changes in lipophilic antioxidants during lupin germination

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Abstract

This study investigated the impact of controlled germination on colour, carotenoids and tocopherols of Andean lupin (*Lupinus mutabilis* Sweet) seeds. Two Andean lupin ecotypes, Cholo fuerte and Altagracia, were germinated in the dark for two, four and six days; colour coordinates (L^*, a^*, b^*) were determined by colorimeter, while carotenoids and tocopherols were quantified by HPLC. The germination significantly modified the characteristics examined. The luminosity decreased with increasing germination time, while only minor changes were recorded for a^* and b^*. The sum of all carotenoids increased up to 12-fold during germination, with lutein always being the most abundant compound. The predominant tocol was γ-tocopherol; although total tocol content was almost unchanged, α-tocopherol increased from not-quantifiable up to 74.8 mg/kg dry matter, improving the vitamin E activity, while γ-tocopherol decreased progressively. The results demonstrate that germination promises to be a cost-effective approach to improve the nutritional properties of lupin seeds.

5.1 Introduction

Lupins are appreciated for the composition of their seeds, rich in proteins (32–55 g/100 g dry matter; DM), lipids (6–25 g/100 g DM) and minerals (2.4–5.2 g ash/100 g DM); their carbohydrates content (26–48 g/100 g DM; Bähr et al., 2014; Carvajal-Larenas et al., 2016) is inferior to other Faboideae. *Lupinus mutabilis* Sweet, the Andean lupin, is cropped in cool mountain areas (2000–3800 m asl) of South America (Suomela et al., 2022). Its diffusion in temperate regions is limited because its production is lower than those of *L. albus*, *L. luteus* and *L. angustifolius*, possibly due to poor adaptation to new environments. The protein content is comparable to the other cropped lupin species, but the Andean lupin displays a lipids content (13.0–24.6 g/100 g DM; Briceño Berru et al., 2021; Carvajal-Larenas et al., 2016) higher than those of *L. albus* (on average, 169–193%), *L. angustifolius* (300–316%) and *L. luteus* (113–344%).

The flour obtained by milling the seeds is used to enrich many products, such as cakes, snacks, burgers, biscuits, baby food and plant-based products, as well as a soy alternative in vegan milk and meat (Cremer, 1983; Güémes-Vera et al., 2008; Ruales et al., 1988). In fact, lupin isolates and protein concentrates have sensory, physical and functional properties (absorption of water and fats, stabilizing capacity, emulsifying and foaming activities, and gelling capacity) very similar to those of soy (Doxastakis, 2000; Gueguen & Cerletti, 1994; Moure et al., 2006).

Seed germination plays a fundamental role in the catabolism of storage compounds and anabolism of new molecules with positive nutritional properties, including those with antioxidant properties such as carotenoids and tocols. Germination is an inexpensive process to improve lupin flour quality by increasing antioxidant capacity, vitamin C and E activities (Fernandez-Orozco et al., 2006; Frias et al., 2005), reducing antinutritional factors like phytic acid (Dagnia et al., 1992; Mohammed et al., 2017) and oligosaccharides (Chilomer et al., 2013; Kaczmarska et al., 2017), and even by enhancing its flavour (Kaczmarska et al., 2018). Several studies report significant improvements in carotenoid and tocol contents in cereals (Aborus et al., 2018; Yang et al., 2001; Ziegler et al., 2016) as well as in different legumes, such as chickpeas (Khattak et al., 2008), soy and green mung bean (Mastropasqua et al., 2020), soy (Gu et al., 2017), mung bean (Alkaltham et al., 2020), green lentils, kidney beans and black beans (Riddoch et al., 1998), and 50 different species of Fabaceae (Fernández-Marín et al., 2017). Actually, Gan et al. (2017) defined germination as a "green food engineering method", because tocopherol content and antioxidant capacity increase in seeds of diverse plant species, and even a short germination leads to a significant improvement in nutritional quality, contributing to reduce the risk of malnutrition and certain chronic diseases. Unfortunately, lupins are also rich in bitter and/or toxic alkaloids that must be removed before consumption, but there is scant

information on the effect of germination on these compounds. In fact, some articles state that after four days of germination the total alkaloid content is not significantly altered in *L. albus* and *L. luteus* (de la Cuadra et al., 1994) or slightly decreases in *L. luteus* and *L. angustifolius* (Chilomer et al., 2010), while de Cortes Sánchez et al. (2005) detected, after nine days of germination, a total alkaloid increase, from 2.36 to 3.03 g/100 g, in *L. albus* and no changes, but a minimum after three days, in *L. angustifolius*.

Therefore, the aim of this research was to study the effect of germination in the dark up to six days on colour and content of tocopherols and carotenoids of seeds from two ecotypes of Andean lupin, Cholo fuerte and Altagracia.

5.2 Materials and methods

5.2.1 Materials

Two *Lupinus mutabilis* ecotypes (i.e., genetically distinct geographic populations) from different regions of Peru (Altagracia, from La Libertad, and Cholo fuerte, from Ancash), collected in November 2018, were kindly provided by the Programa de Leguminosas y Oleaginosas of the Universidad Nacional Agraria la Molina, Lima, Peru.

5.2.2 Methods

The seeds were disinfected with 0.1% sodium hypochlorite for 2 min, rinsed three times with distilled water and immersed in water at room temperature (19–21 °C) for 12 h. After draining, the seeds (200 g) were transferred to sterile stainless steel trays (26.5 × 35.5 cm), incubated in the dark for 2, 4 and 6 days at 15 ± 1 °C in an A-3920 Achieva germinator (Seedburo Equipment Company, Des Plaines, IL, USA), and sprayed with water for 8 s six times a day. The germinated seeds were dried for 12 h at 50 °C, ground with a grinder (Braun, Kronberg im Taunus, Germany) for 30 s and passed through a 1 mm sieve. The flour samples were then placed in heat-sealed vacuum bags and stored at −20 °C.

The trials were carried out in triplicate. The following analyses were performed on the non-germinated seeds (control or 0 days) and on the 2-days, 4-days and 6-days germinated seeds.

5.2.3 Colour

The colour analysis, performed using a Chroma Meter CR-II tristimulus colorimeter (Minolta Italia SPA, Milan, Italy), using the standard-white reflector plate and illuminant C, led to the determination of the coordinates L^* (luminosity), a^* (green–red) and b^* (blue–yellow).

5.2.4 Carotenoids and tocopherols

The moisture was determined gravimetrically according to method 44-15.02 (AACC, 2000).

Carotenoids and tocopherols were determined as outlined by Brandolini et al. (2022) and previously described in the Section 4.2.6. The tests were performed on three independent samples and the results are expressed as mg/kg DM.

5.2.5 Statistical analysis

To evaluate the effect of the different treatments and lupin ecotypes, the data were processed by two-way analysis of variance (ANOVA), considering germination time and ecotype as factors. When significant differences ($p \leq 0.05$) were found, Fisher's least significant difference (LSD) was computed at 95% significance level. All analyses were performed using Statgraphics® Centurion 18 statistical programme (StatPoint Technologies Inc., Warrengton, VA, USA). Means and standard deviations were computed using the Excel® software (Microsoft, Redmond, WA, USA).

5.3 Results and discussion

5.3.1 Analysis of variance

The two-way ANOVA (Supplementary Table S.8) evidenced that germination time (G) had a highly significant effect on colour and carotenoid content; ecotype (E) and G × E interaction, even when significant, were far less relevant. On the other hand, germination time influenced all three tocopherols, while the ecotype was significant for γ-, δ-tocopherol and total tocopherols.

5.3.2 Colour

The average values of the L^*, a^* and b^* coordinates of the Cholo fuerte and Altagracia seeds are presented in Table 5.1. At day 0 (non germinated) the Altagracia seeds were slightly less luminous than the Cholo fuerte ones, but the difference disappeared during germination. The L^* of both ecotypes increased significantly after two days of germination and slowly decreased afterwards. The a^* component (green–red), instead, showed a progressive shift towards a green hue, while the b^* component (blue–yellow), after an initial slight decrease, moved towards the yellow tinge. Hence, the increasingly darker and yellow–green colour of the flour samples (Figure 5.1). Not many studies on lupin colour are present in literature. The *L. albus* flours studied by Mohamed and Rayas-Duarte (1995) showed comparable values ($L^* = 82.8$, $a^* = -2.0$, $b^* = 21.3$), while those of *L. angustifolius* analysed by Rumiyati et al. (2015) were more luminous ($L^* = 90.6$, $a^* = 1.3$, $b^* = 28.5$). Briceño Berru et al.

(2021) studied 33 different *L. mutabilis* ecotypes and recorded luminosities ranging from 67.2 in dark-hulled samples to 87.5 in white-hulled samples, while a^* ranged between 0.3 and −3.4 and b^* varied between 22.0 and 33.3.

Table 5.1. Colour coordinates of the two *L. mutabilis* ecotypes during germination.

	Days	L^*	a^*	b^*
Cholo fuerte	0	80.9 ± 0.4^{c}	-2.4 ± 0.1^{a}	27.7 ± 0.2^{b}
	2	84.5 ± 0.4^{a}	-3.6 ± 0.1^{b}	26.5 ± 0.4^{c}
	4	83.3 ± 0.7^{b}	-4.8 ± 0.2^{c}	28.8 ± 0.6^{a}
	6	79.8 ± 0.7^{d}	-5.4 ± 0.4^{d}	32.3 ± 0.9^{a}
Altagracia	0	76.5 ± 0.7^{c}	-0.7 ± 0.3^{a}	28.9 ± 1.0^{b}
	2	84.9 ± 1.0^{a}	-4.1 ± 0.4^{b}	26.9 ± 1.1^{c}
	4	84.3 ± 0.7^{a}	-5.8 ± 0.1^{c}	29.4 ± 0.7^{b}
	6	79.4 ± 0.9^{b}	-6.0 ± 0.2^{c}	32.6 ± 1.1^{a}

Different letters in the column indicate significant differences among samples at $p \leq 0.05$ according to LSD multiple range test.

5.3.3 Carotenoids

Figure 5.2 depicts the changes in carotenoid composition and content upon germination, whose variations are visualized in Figure 5.3 by overlaying chromatograms of Altagracia samples. For all compounds, and for their total content, an increasing trend of the values during germination is evident. As mentioned above, the results of the two Andean lupins were not significantly different. Lutein was the most abundant carotenoid both before (1.5 mg/kg DM) and during germination (13.6 mg/kg DM after six days), followed by (α+β)-carotene and zeaxanthin; β-cryptoxanthin, absent in non-germinated seeds, increased up to 1 mg/kg DM after six days. The greatest percentual growth after six days occurred for (α+β)-carotene (3000%, i.e., from 0.2 to 6.2 mg/kg DM) and β-cryptoxanthin (not computable, going from not detectable to 1.0 mg/kg DM). Similar results were recorded during germination in the dark of soybean by Gu et al. (2017), who observed a hundred times increase of β-carotene content after four days; in *Vigna radiata* by Alkaltham et al. (2020), who recorded a 210% augment in total carotenoids after 21 h; and in chickpeas by Khattak et al. (2008), who found a 78% surge in total carotenoids after four days. The positive effect of germination on carotenoids content was evident also in cereals: Hidalgo et al. (2019) recorded that germinated wheat and barley seeds reached relevant concentrations (82.6–119.7 mg/kg DM); lutein was always the predominant carotenoid, but the (α+β)-carotene underwent sizeable increases.

According to Rodríguez-Villalón et al. (2009), the methylerythritol 4-phosphate pathway produces the precursors for carotenoids synthesis in seeds

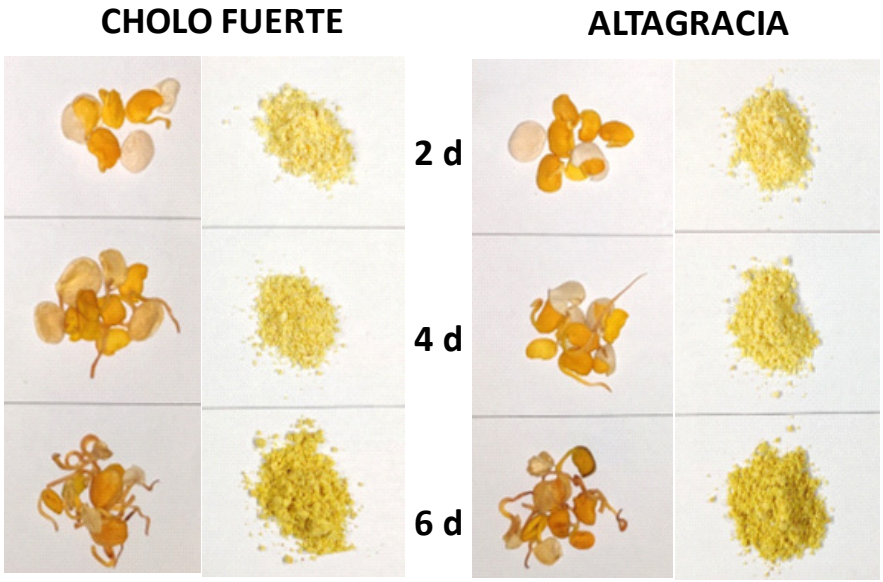


Figure 5.1. Seeds and flour samples of the *L. mutabilis* ecotypes Cholo fuerte and Altagracia during the germination.

germinated in the dark; additionally, the biosynthesis of a carotenoids key precursor, geranylgeranyl diphosphate (DellaPenna & Pogson, 2006), is fully operational during germination (Beck et al., 2013). Hence, when aetioplasts differentiate into chloroplasts in the absence of light, there is an accumulation of α-carotene, β-carotene, lutein and violaxanthin, whereas chlorophylls are synthesized only after exposure to the light.

5.3.4 Tocopherols

The evolution of tocopherols in the two Andean lupin ecotypes is depicted in Figure 5.3 and Figure 5.4. In the non-germinated seeds, Cholo fuerte showed a higher total tocopherols content than Altagracia (225 ± 25 and 166 ± 23 mg/kg DM, respectively), with a clear prevalence (95–99%) of γ-tocopherol, followed by the δ- and α- homologues. These values, as well as γ-tocopherol prevalence, agree with the ranges indicated by Briceño Berru et al. (2021) and Brandolini et al. (2022) in non-debittered Andean lupins, i.e., 172.1–249.8 and 228–231 mg/kg DM, respectively. During germination, the changes in individual tocopherols were similar in both ecotypes. On average, α-tocopherol increased from detectable but not-quantifiable amount (0.7 mg/kg DM) to 74.8 mg/kg DM, while γ-tocopherol decreased from 189.7 to 127.8 as the germination proceeded. The variations in δ-tocopherol, even when significant, were almost negligible given its scarce amount. Overall, the

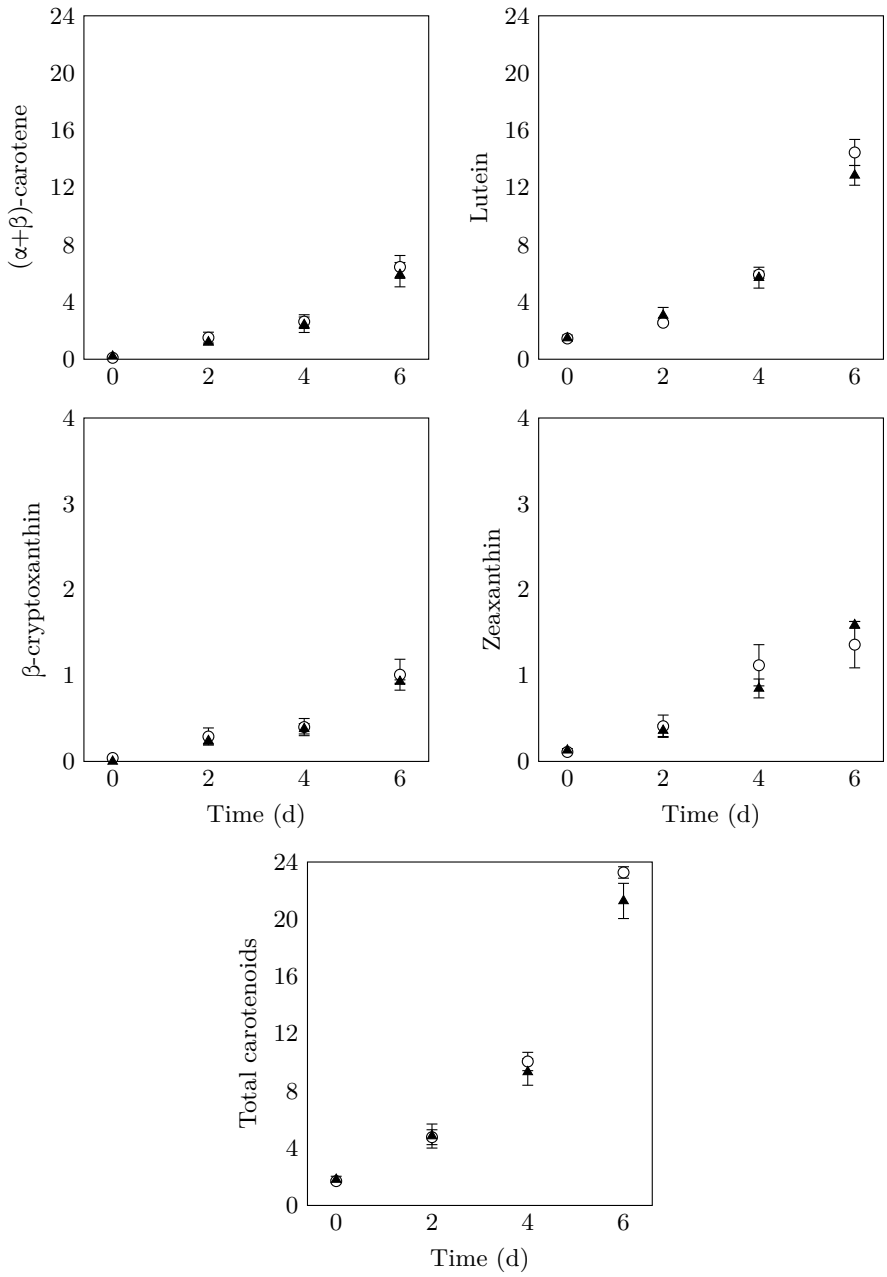


Figure 5.2. (α+β)-carotene, lutein, β-cryptoxanthin, zeaxanthin and total carotenoid content (mg/kg DM) in *L. mutabilis* seeds of Cholo fuerte (triangles) and Altagracia (circles) during the germination.

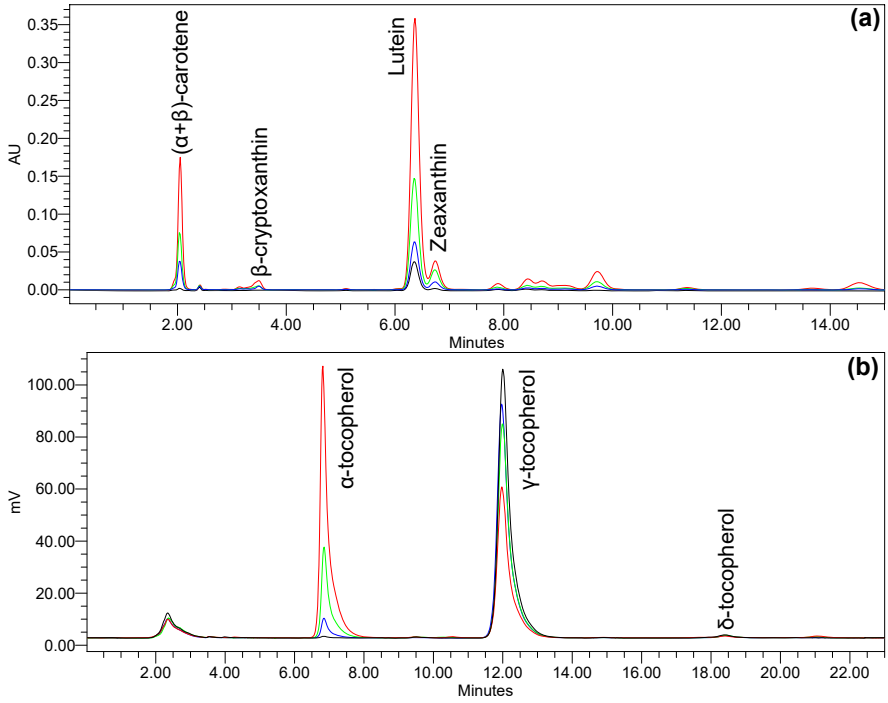


Figure 5.3. Overlaid chromatograms for carotenoids (a) and tocopherols (b) in Altagracia seeds before (black) and after 2 (blue), 4 (green) and 6 (red) days of germination.

total tocopherols quantity did not change substantially during germination, passing on average from 195.7, before germination, to 200.4 mg/kg DM, after six days. No tocotrienols were detected either before or after germination. Likewise, Frias et al. (2005) observed in *L. albus* a 29% γ-tocopherol decrease (from 201 to 143 mg/kg DM) after six days of germination, a variation comparable to that reported here (33%), while α-tocopherol increased from 1.9 to 23 mg/kg DM. The same trend was noticed by Fernandez-Orozco et al. (2006) germinating *L. angustifolius* for six days: γ-tocopherol went from 126.7 to 32.5 mg/kg DM, whereas α-tocopherol moved from 3 to 86 mg/kg DM. Similarly, working with *L. albus*, *Glycine max* and *Vigna radiata*, Gan et al. (2017) reported significant changes in α- and γ-tocopherol, accompanied by an increase in antioxidant capacity due to the improved α-tocopherol content.

The increase in α-tocopherol, coupled with a decrease in γ-tocopherol, is a phenomenon observed in several germinating legumes: peas (Fordham et al., 1975), green mung beans (Marero et al., 1991), lentils (Frias et al., 2002), soybean (Lee & Chang, 1993; Plaza et al., 2003); but also in cereals (wheat; Yang et al., 2001) and pseudocereals (tartary buckwheat; Zhou et al., 2015).

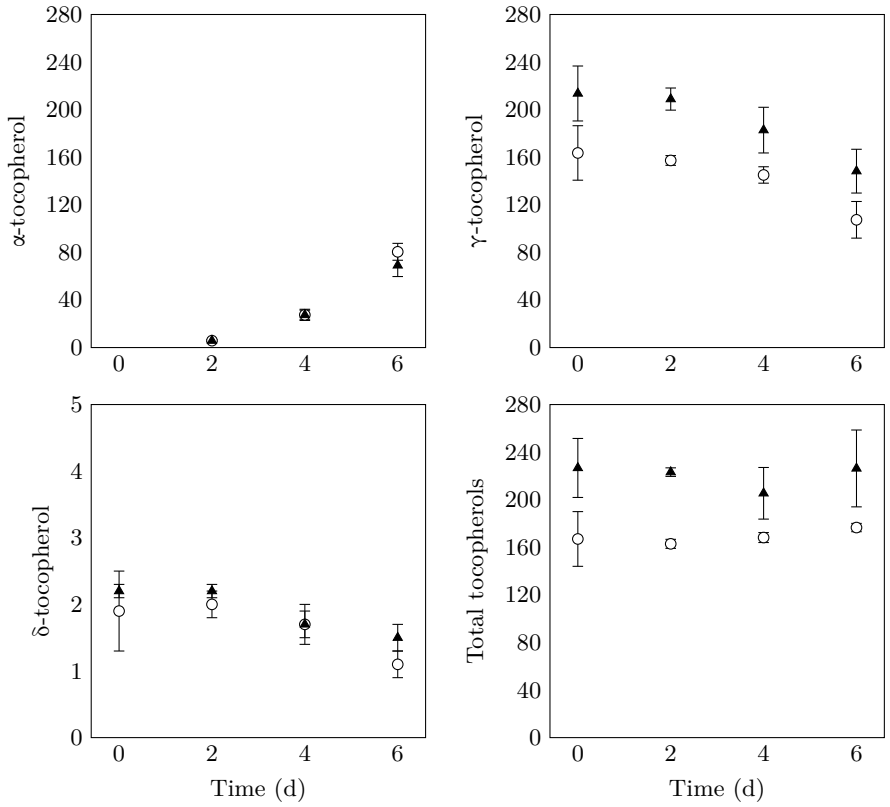


Figure 5.4. α-, γ- and δ-tocopherol and total tocopherols content (mg/kg DM) in *L. mutabilis* seeds of Cholo fuerte (triangles) and Altagracia (circles) during the germination.

The results clearly confirm that the decrease in γ-tocopherol (and, in minor measure, of δ-tocopherol) was compensated by an increase in the α-tocopherol homologue: therefore, it can be assumed that during germination a conversion between the two forms occurs. In fact, Jube and Borthakur (2006) report that γ-tocopherol is the precursor of α-tocopherol in the biosynthetic pathway. The conversion reaction is catalysed by the enzyme γ-tocopherol methyltransferase (γ-TMT, EC 2.1.1.95), which introduces a methylation in the –5 position using methionine as a donor of $-CH_3$: therefore, S-adenosyl-L-methionine + γ-tocopherol become S-adenosyl-L-homocysteine + α-tocopherol. The same enzyme catalyses the methylation of δ-tocopherol into β-tocopherol and the corresponding conversions among tocotrienols (Jube & Borthakur, 2006; enzyme.expasy.org). The α-tocopherol is the only tocol with proven efficacy in preventing the vitamin E deficiency disease (Azzi, 2019), thus its partial conversion from the more abundant γ-tocopherol dramatically

improves nutritional quality. Germination therefore represents a simple and effective way to improve the nutritional value of lupin. In soybean, Tavva et al. (2007), Dwiyanti et al. (2011) and Arun et al. (2014) studied the expression of the gene coding for γ-TMT and found that this enzyme is the factor limiting the conversion rate of γ-tocopherol to α-tocopherol.

In addition to its role as vitamin E, the α-tocopherol has also the greatest antioxidant activity: α-tocopherol > β-tocopherol and γ-tocopherol > δ-tocopherol (Kim & Min, 2008). Its lower phenolic hydrogen dissociation energy and reduction potential give α-tocopherol the greatest reaction rate with the peroxide radical: 1.5 and 3.4-fold higher than those of the γ and δ homologues (Kim & Min, 2008).

Even with a negligible change in the total tocopherols content (the variation was about +6%), the different behaviour and biological activities of the individual tocopherols influenced the nutritional properties of *L. mutabilis*. In addition, the lipophilic antioxidants are barely or non affected by the debittering treatment (Brandolini et al., 2022; Briceño Berru et al., 2021; Córdova-Ramos et al., 2020); therefore, after germination the debittering of bitter varieties will result in minor or no losses of *ex-novo* synthesized carotenoids and modified tocopherols.

5.4 Conclusions

During germination, the flour samples became darker and shifted towards yellow–green; additionally, carotenoid content increased by 12-fold, and a significant transition from γ- to α-tocopherol was observed. These findings indicate that germination is a simple and cost-effective approach to enhance provitamin A and vitamin E activities of the flour of Andean lupin. The increase in health awareness and the shift towards plant-based protein suggest that germinated lupin flour could become a valuable ingredient in the food industry either directly, in the case of sweet lupin varieties, or once the harmful alkaloids are efficiently removed.

References

AACC. (2000). *Approved Methods of Analysis 10th ed.* American Association of Cereal Chemists.

Aborus, N. E., Tumbas Šaponjac, V., J., Č.-B., Ćetković, G., Hidalgo, A., Vulić, J., & Šeregelj, V. (2018). Sprouted and freeze-dried wheat and oat seeds - phytochemical profile and *in vitro* biological activities. *Chem Biodivers*, *15*, e1800119. https://doi.org/10.1002/cbdv.201800119

Alkaltham, M. S., Salamatullah, A. M., Özcan, M. M., Uslu, N., & Hayat, K. (2020). The effects of germination and heating on bioactive properties, phenolic compounds and mineral contents of green gram seeds. *LWT - Food Sci Technol*, *134*, 110106. https://doi.org/10.1016/j.lwt.2020.110106

Arun, M., Subramanyam, K., Theboral, J., Sivanandhan, G., Rajesh, M., Dev, G. K., Jaganath, B., Manickavasagam, M., Girija, S., & Ganapathi, A. (2014). Transfer and targeted overexpression of γ-tocopherol methyltransferase (γ-TMT) gene using seedspecific promoter improves tocopherol composition in Indian soybean cultivars. *Appl Biochem Biotechnol*, *172*, 1763–1776. https://doi.org/10.1007/s12010-013-0645-9

Azzi, A. (2019). Tocopherols, tocotrienols and tocomonoenols: Many similar molecules but only one vitamin E. *Redox Biol*, *26*, 101259. https://doi.org/10.1016/j.redox.2019.101259

Bähr, M., Fechner, A., Hasenkopf, K., Mittermaier, S., & Jahreis, G. (2014). Chemical composition of dehulled seeds of selected lupin cultivars in comparison to pea and soya bean. *LWT - Food Sci Technol*, *59*, 587–590. https://doi.org/10.1016/j.lwt.2014.05.026

Beck, G., Coman, D., Herren, E., Ruiz-Sola, M. A., Rodríguez-Concepción, M., Gruissem, W., & Vranová, E. (2013). Characterization of the GGPP synthase gene family in *Arabidopsis thaliana*. *Plant Mol Biol*, *82*, 393–416. https://doi.org/10.1007/s11103-013-0070-z

Brandolini, A., Glorio-Paulet, P., Estivi, L., Locatelli, N., Córdova-Ramos, J. S., & Hidalgo, A. (2022). Tocopherols, carotenoids and phenolics changes during Andean lupin (*Lupinus mutabilis* Sweet) seeds processing. *J Food Compos Anal*, *106*, 104335. https://doi.org/10.1016/j.jfca.2021.104335

Briceño Berru, L., Glorio-Paulet, P., Basso, C., Scarafoni, A., Camarena, F., Hidalgo, A., & Brandolini, A. (2021). Chemical composition, tocopherol and carotenoid content of seeds from different Andean lupin (*Lupinus mutabilis*) ecotypes. *Plant Food Hum Nutr*, *76*, 98–104. https://doi.org/10.1007/S11130-021-00880-0

Carvajal-Larenas, F. E., Linnemann, A. R., Nout, M. J. R., Koziol, M., & van Boekel, M. A. J. S. (2016). *Lupinus mutabilis*: Composition, uses, toxicology, and debittering. *Crit Rev Food Sci Nutr*, *56*, 1454–1487. https://doi.org/10.1080/10408398.2013.772089

Chilomer, K., Kasprowicz-Potocka, M., Gulewicz, P., & Frankiewicz, A. (2013). The influence of lupin seed germination on the chemical composition and standardized ileal digestibility of protein and amino acids in pigs. *J Anim Physiol Anim Nutr*, *97*, 639–646. https://doi.org/10.1111/j.1439-0396.2012.01304.x

Chilomer, K., Zaleska, K., Ciesiolka, D., Gulewicz, P., Frankiewicz, A., & Gulewicz, K. (2010). Changes in the alkaloid, alpha-galactoside and

protein fractions content during germination of different lupin species. *Acta Soc Bot Pol*, *79*, 11–20.

Córdova-Ramos, J. S., Glorio-Paulet, P., Camarena, F., Brandolini, A., & Hidalgo, A. (2020). Andean lupin (*Lupinus mutabilis* Sweet): Processing effects on chemical composition, heat damage, and *in vitro* protein digestibility. *Cereal Chem*, *97*, 827–835. https://doi.org/10.1002/cche.10303

Cremer, H. D. (1983). Current aspects of legumes as a food constituent in Latin America with special emphasis on lupines: Introduction. *Plant Food Hum Nutr*, *32*, 95–100. https://doi.org/10.1007/BF01091329

Dagnia, S. G., Petterson, D. S., Bell, R. R., & Flanagan, F. V. (1992). Germination alters the chemical composition and protein quality of lupin seeds. *J Sci Food Agric*, *60*, 419–423. https://doi.org/10.1002/jsfa.2740600403

de la Cuadra, C., Muzquiz, M., Burbano, C., Ayet, G., Calvo, R., Osagie, A., & Cuadrado, C. (1994). Alkaloid, α-galactoside and phytic acid changes in germinating lupin seeds. *J Sci Food Agric.*, *66*, 357–364. https://doi.org/10.1002/jsfa.2740660313

de Cortes Sánchez, M., Altares, P., Pedrosa, M. M., Burbano, C., Cuadrado, C., Goyoaga, C., Muzquiz, M., Jiménez-Martínez, C., & Dávila-Ortiz, G. (2005). Alkaloid variation during germination in different lupin species. *Food Chem*, *90*, 347–355. https://doi.org/10.1016/j.foodchem.2004.04.008

DellaPenna, D., & Pogson, B. J. (2006). Vitamin synthesis in plants: Tocopherols and carotenoids. *Annu Rev Plant Biol*, *57*, 711–738. https://doi.org/10.1146/annurev.arplant.56.032604.144301

Doxastakis, G. (2000). Lupin seed proteins. In G. Doxastakis & V. Kiosseoglou (Eds.), *Novel macromolecules in food systems* (pp. 7–38). Elsevier Science B.V.

Dwiyanti, M. S., Yamada, T., Sato, M., Abe, J., & Kitamura, K. (2011). Genetic ariation of γ-tocopherol methyltransferase gene contributes to elevated α-tocopherol content in soybean seeds. *BMC Plant Biol*, *11*, 152. https://doi.org/10.1186/1471-2229-11-152

Fernández-Marín, B., Míguez, F., Méndez-Fernández, L., Agut, A., Becerril, J. M., García-Plazaola, J. I., Kranner, I., & Colville, L. (2017). Seed carotenoid and tocochromanol composition of wild Fabaceae species is shaped by phylogeny and ecological factors. *Front Plant Sci*, *8*, 1428. https://doi.org/10.3389/fpls.2017.01428

Fernandez-Orozco, R., Piskula, M. K., Zielinski, H., Kozlowska, H., Frias, J., & Vidal-Valverde, C. (2006). Germination as a process to improve the antioxidant capacity of *Lupinus angustifolius* L. var. Zapaton. *Eur Food Res Technol*, *223*, 495–502. https://doi.org/10.1007/s00217-005-0229-1

Fordham, J. R., Wells, C. E., & Chen, L. H. (1975). Sprouting of seeds and nutrient composition of seeds and sprouts. *J Food Sci*, 552–556. https://doi.org/10.1111/j.1365-2621.1975.tb12526.x.

Frias, J., Fernández-Orozco, R., Zielinski, H., Piskula, M., Kozlowska, H., & Vidal-Valverde, C. (2002). Effect of germination on the content of vitamins C and E of lentils. *Pol J Food Nutr Sci*, *11*, 76–78.

Frias, J., Miranda, M. L., Doblado, R., & Vidal-Valverde, C. (2005). Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. Multolupa. *Food Chem*, *92*, 211–220. https://doi.org/10.1016/j.foodchem.2004.06.049.

Gan, R. Y., Lui, W. Y., Wu, K., Chan, C. L., Dai, S. H., Sui, Z. Q., & Corke, H. (2017). Bioactive compounds and bioactivities of germinated edible seeds and sprouts: An updated review. *Trends Food Sci Technol*, *59*, 1–14. https://doi.org/10.1016/j.tifs.2016.11.010

Gu, E.-J., Kim, D. W., Jang, G.-J., Song, S. H., Lee, J.-I., Lee, S. B., Kim, B.-M., Cho, Y., Lee, H.-J., & Kim, H.-J. (2017). Mass-based metabolomic analysis of soybean sprouts during germination. *Food Chem*, *217*, 311–319. https://doi.org/10.1016/j.foodchem.2016.08.113

Gueguen, J., & Cerletti, P. (1994). Proteins of some legume seeds: Soybean, pea, fababean and lupin. In B. J. F. Hudson (Ed.), *New and developing sources of food proteins* (pp. 145–193). Chapman & Hall. https://doi.org/10.1007/978-1-4615-2652-0_6

Güémes-Vera, N., Peña-Bautista, R. J., Jiménez-Martínez, C., Dávila-Ortiz, G., & Calderón-Domínguez, G. (2008). Effective detoxification and decoloration of *Lupinus mutabilis* seed derivatives, and effect of these derivatives on bread quality and acceptance. *J Sci Food Agr*, *88*, 1135–1143. https://doi.org/10.1002/jsfa.3152

Hidalgo, A., Tumbas Šaponjac, V., Ćetković, G., Šeregelj, V., Čanadanović-Brunet, J., Chiosa, D., & Brandolini, A. (2019). Antioxidant properties and heat damage of water biscuits enriched with sprouted wheat and barley. *LWT - Food Sci Technol*, *114*, 108423. https://doi.org/10.1016/j.lwt.2019.108423

Jube, S., & Borthakur, D. (2006). Recent advances in food biotechnology research. In Y. H. Hui (Ed.), *Food biochemistry and food processing* (pp. 35–69). Blackwell Publishing. https://doi.org/10.1002/9780470277577.ch3

Kaczmarska, K. T., Chandra-Hioe, M. V., Frank, D., & Arcot, J. (2018). Aroma characteristics of lupin and soybean after germination and effect of fermentation on lupin aroma. *LWT - Food Sci Technol*, *87*, 225–233. https://doi.org/10.1016/j.lwt.2017.08.080

Kaczmarska, K. T., Chandra-Hioe, M. V., Zabaras, D., Frank, D., & Arcot, J. (2017). Effect of germination and fermentation on carbohydrate composition of australian sweet lupin and soybean seeds and flours.

J Agric Food Chem, *65*, 10064–10073. https://doi.org/10.1021/acs.jafc.7b02986

Khattak, A. B., Zeb, A., & Bibi, N. (2008). Impact of germination time and type of illumination on carotenoid content, protein solubility and *in vitro* protein digestibility of chickpea (*Cicer arietinum* L.) sprouts. *Food Chem*, *109*, 797–801. https://doi.org/10.1016/j.foodchem.2008.01.046

Kim, H. J., & Min, D. B. (2008). Chemistry of lipid oxidation. In C. C. Akoh & D. B. Min (Eds.), *Food lipids: Chemistry, nutrition, and biotechnology 3rd ed.* (pp. 299–320). CRC Press.

Lee, I. B., & Chang, K. W. (1993). Changes in concentration of tocopherols and fatty acids during germination and maturation of soybean (*Glycine max*). *J Korean Agric Chem Soc*, *36*, 127–133.

Marero, L. M., Payumo, E. M., Aguinaldo, A. R., Homma, S., & Igarashi, O. (1991). Vitamin E constituents of weaning foods from germinated cereals and legumes. *J Food Sci*, *56*, 270–271. https://doi.org/10.1111/j.1365-2621.1991.tb08031.x

Mastropasqua, L., Dipierro, N., & Paciolla, C. (2020). Effects of darkness and light spectra on nutrients and pigments in radish, soybean, mung bean and pumpkin sprouts. *Antioxidants*, *9*, 558. https://doi.org/10.3390/antiox9060558

Mohamed, A. A., & Rayas-Duarte, P. (1995). Composition of *Lupinus albus*. *Cereal Chem*, *72*, 643–647.

Mohammed, M. A., Mohamed, E. A., Yagoub, A. E. A., Mohamed, A. R., & Babiker, E. E. (2017). Effect of processing methods on alkaloids, phytate, phenolics, antioxidants activity and minerals of newly developed lupin (*Lupinus albus* L.) cultivar. *J Food Process*, *41*, 1–9. https://doi.org/10.1111/jfpp.12960

Moure, A., Sineiro, J., Domínguez, H., & Parajó, J. C. (2006). Functionality of oilseed protein products: A review. *Food Res Int*, *39*, 945–963. https://doi.org/10.1016/j.foodres.2006.07.002

Plaza, L., De Ancos, B., & Cano, P. (2003). Nutritional and health related compounds in sprouts and seeds of soybean (*Glycine max*), wheat (*Triticum aestivum* L.) and alfalfa (*Medicago sativa*) treated by a new drying method. *Eur Food Res Technol*, *216*, 138–144. https://doi.org/10.1007/s00217-002-0640-9

Riddoch, C. H., Mills, C. F., & Duthie, G. G. (1998). An evaluation of germinating beans as a source of vitamin C in refugee foods. *Eur J Clin Nutr*, *52*, 115–118. https://doi.org/10.1038/sj.ejcn.1600524

Rodríguez-Villalón, A., Gas, E., & Rodríguez-Concepción, M. (2009). Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown *Arabidopsis* seedlings. *Plant J*, *60*, 424–435. https://doi.org/10.1111/j.1365-313X.2009.03966.x

Ruales, J., Pólit, P., & Nair, B. M. (1988). Nutritional quality of blended foods of rice, soy and lupins, processed by extrusion. *Food Chem*, *29*, 309–321. https://doi.org/10.1016/0308-8146(88)90046-5

Rumiyati, R., James, A. P., & Jayasena, V. (2015). Effects of lupin incorporation on the physical properties and stability of bioactive constituents in muffins. *Int J Food Sci Technol*, *50*, 103–110. https://doi.org/10.1111/ijfs.12688

Suomela, J.-P., Repo-Carrasco-Valencia, R., & Lutz, M. (2022). Andean native grains, quinoa, and lupin as sources of bioactive components. In R. Repo-Carrasco-Valencia & M. C. Tomás (Eds.), *Native crops in Latin America* (pp. 1–34). CRC Press Taylor & Francis Group. https://doi.org/10.1201/9781003087618-1

Tavva, V. S., Kim, Y.-H., Kagan, I. A., Dinkins, R. D., Kim, K.-H., & Collins, G. B. (2007). Increased α-tocopherol content in soybean seed overexpressing the *Perilla frutescens* γ-tocopherol methyltransferase gene. *Plant Cell Rep*, *26*, 61–70. https://doi.org/10.1007/s00299-006-0218-2

Yang, F., Basu, T. K., & Ooraikul, B. (2001). Studies on germination conditions and antioxidant contents of wheat grain. *Int J Food Sci Nutr*, *52*, 319–330. https://doi.org/10.1080/09637480120057567

Zhou, X., Hao, T., Zhou, Y., Tang, W., Xiao, Y., Meng, X., & Fang, X. (2015). Relationships between antioxidant compounds and antioxidant activities of tartary buckwheat during germination. *J Food Sci Technol*, *52*, 2458–2463. https://doi.org/10.1007/s13197-014-1290-1

Ziegler, J. U., Flockerzie, M., Longin, C. F. H., Würschum, T., Carle, R., & Schweiggert, R. M. (2016). Development of lipophilic antioxidants and chloroplasts during the sprouting of diverse *Triticum* spp. *J Agric Food Chem*, *64*, 913–922. https://doi.org/10.1021/acs.jafc.5b05474.

6

Optimization of protein extraction from pea by-product

The contents of this chapter are under review as:

Estivi, L., Brandolini, A., Catalano, A., Di Prima, R., & Hidalgo, A. (n.d.). Characterization of an industrial pea canning by-product and its protein concentrate obtained by optimized ultrasound-assisted extraction.

Abstract

This work aimed to optimize protein extraction from an industrial pea canning by-product by developing an ultrasound-assisted alkaline solubilization and isoelectric precipitation method. Proximate composition and microbial contamination (total bacterial count, total lactic acid bacteria, Enterobacteriaceae, yeasts and moulds) were assessed in the wet by-product. The protein concentrate obtained, the dry by-product and a commercial pea flour were assessed for protein content, water activity, colour and techno-functional properties. The optimal two-phase extraction conditions from the wet by-product were: liquid-solid ratio 20 mL/g, pH 11, ultrasound amplitude 80%, time 30 min. The extraction from the dry by-product was not feasible. Sonication reduced extraction time from 4 h (magnetic stirring method) to 1 h and increased extraction recovery three-fold (from 21.5% to 66.6%). The concentrate had a 74.8 g/100 g DM protein content. The SDS-PAGE revealed protein degradation in some specimens, probably because of fermentation and enzymatic hydrolysis before sampling. However, the microbial counts in the concentrate, mainly related to lactic acid bacteria, were always below the

guideline values. The results demonstrated that the pea canning by-product is a valuable raw material for protein recovery and that the proposed method allows its rapid and direct treatment, avoiding drying costs and microbial proliferation.

6.1 Introduction

Peas (*Pisum sativum* L.) are among the most cultivated legumes worldwide, with a global production of 12.4 million metric tons (Mt) of dry seeds (14% of all pulses, third after beans and chickpeas), and 20.5 Mt of fresh legumes (45%, second after beans). Canada, Russia, China and India produce 63% of dry peas, whereas China and India yield 77% of fresh production (faostat.org, 2021; last accessed 1 December 2023). Peas are valuable products for human consumption and occupy a prominent place among vegetables thanks to their high nutritional value, linked to their good protein content (20–25 g/100 g) and health-promoting compounds, such as amylose-rich, slowly-digestible starch (39.4–46.2 g/100 g), resistant starch (1.8–7.0 g/100 g), insoluble (19.3–23.1 g/100 g) and soluble fibre (3.9–8.0 g/100 g, mainly pectin), potassium, phosphorus, phenolic compounds and β-carotene (Lam et al., 2018; Wu et al., 2023).

Pea proteins are appreciated for their hypo-allergenicity and nutritional value, since their high lysine content makes them complementary to cereal proteins, which are instead rich in sulphur-containing amino acids (i.e., methionine and cysteine; Lam et al., 2018; Shanthakumar et al., 2022). As concentrate (>65 g protein/100 g) or isolate (>90 g protein/100 g), pea proteins have been employed in the preparation of baked goods, pasta, meat products, beverages and milk-like drinks (Boukid et al., 2021; Boye et al., 2010). However, a major drawback that hinders the further diffusion of pulses proteins is their beany off-flavour.

Besides nutritional enrichment and substitution of animal protein, pea proteins provide viscoelasticity, missing in gluten-free foods for coeliacs (Boukid et al., 2021) and, thanks to their interfacial properties, are also used as emulsifiers, thickeners, fat binders and gelling or bulking agents in food formulations. Such properties can be further improved by different techniques including, for example, high-pressure treatment, ultrasonication, hydrolysis, glycation, acylation, deamidation and enzymatic cross-linking (Shanthakumar et al., 2022). Proteins modification usually leads to conformational changes and, thus, better adsorption at the interface of food dispersions: when unfolding occurs, surface hydrophobicity increases, enhancing the protein ability to stabilize foams and emulsions (Xiong et al., 2018). In addition, pea proteins in heterogeneous mixtures are exploited as encapsulating material and in the manufacturing of edible films (Boukid et al., 2021; Shanthakumar et al., 2022).

Industrial processing of legumes discards from 5% to 25% of harvested material as waste, containing pods, hulls, leaves, stems and broken, dark

or stained seeds (Tassoni et al., 2020). These residues are rich in proteins, dietary fibre, polyphenols and other molecules (e.g., peptides) with antioxidant, antimicrobial and other beneficial activities (Belghith Fendri et al., 2016, 2022; Dueñas et al., 2004; Mateos-Aparicio et al., 2010). Currently, legume waste is mainly destined to biogas production or to livestock feed, but there is a growing interest in its upcycling for human consumption, especially by protein recovery. Dry extraction methods are scarcely applicable for this purpose, thus the waste is more often re-processed by wet extraction, usually exploiting alkaline solubilization followed by isoelectric precipitation (Kamani et al., 2023; Tassoni et al., 2020); nevertheless, this technique leads to loss of solubility and worsening of technological properties, due to denaturation and formation of insoluble aggregates (Boukid et al., 2021; Vogelsang-O'Dwyer et al., 2020). The ultrasound technology has gained interest in the food industry for its versatility, safety and low energy requirements; in fact, when coupled with wet extraction it increases recovery and shortens treatment time (Estivi et al., 2022; Tassoni et al., 2020). Ultrasonication has previously been studied for protein extraction from pea flour (F. Wang et al., 2020) or pea pods (Karabulut et al., 2023), but no attempts were made with pea by-products from industrial processing.

Therefore, the aims of this work were to characterize the industrial pea canning by-product collected over different years, to optimize an ultrasound-assisted method of alkaline extraction and isoelectric precipitation of protein, and to verify the quality of the protein concentrate thus obtained.

6.2 Materials and methods

6.2.1 Materials

The by-products of a canned pea production line were sampled at the Casalasco Società Agricola SPA plant in Gariga di Podenzano (PC, Italy). The amount of waste was computed as the percentage of raw material discarded, based on a six years history (2014–2016; 2020–2022). The samples, collected from 2013 to 2022, were characterized for their chemical composition ($n = 10$) and microbial contamination ($n = 5$). The by-products collected on three different days in 2021 were stored at $-20\,^{\circ}C$ and then either thawed at $4\,^{\circ}C$ for 16 h (wet by-product sample) or dried at $55\,^{\circ}C$ for 24 h (dry by-product sample) in a Venticell 55 oven (MMM Medcenter Einrichtungen GmbH, Planegg, Germany). The analyses were performed on the dried by-product, the protein concentrate obtained by an optimized ultrasound-assisted extraction from the thawed by-product, and a pea flour purchased at a local store (NaturaSì, Milan, Italy).

6.2.2 Optimization of the ultrasound-assisted protein extraction

Protein was extracted by solubilization in alkalized water and subsequent isoelectric precipitation. Briefly, the samples were suspended in distilled water according to the selected extraction ratio (see below), taking in consideration their moisture content. The by-product mixture was homogenized with an immersion blender (Hr1611/00, Philips, Amsterdam, Netherlands) for two 30 s cycles with a 30 s rest; the pH was adjusted with 1 M NaOH under constant stirring. Ultrasonication (experimental method) or magnetic stirring (control method) were used during the extraction. The ultrasonication was achieved with an Up400St homogenizer (Hielscher, Teltow Germany), mounting a 14 mm probe and set up with on/off cycles of 5/5 s. The temperature was maintained between 25 and 30 °C with an icy water bath. After solubilization, the samples were transferred into a 400 mL polyethylene bottle and centrifuged at 10800 *g* for 10 min with a Sorvall RC-5B Plus Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The sediment was extracted a second time, the supernatants were pooled, adjusted to pH 4.5 with 1 M HCl and centrifuged as detailed above. The sediment, corresponding to the protein extract, was dried as above described for the by-product in order to obtain the protein concentrate sample.

The ultrasonication extraction conditions were optimized in a series of preliminary experiments considering as response variable the extraction recovery (%), calculated with the Equation 6.1, where N is the total nitrogen content.

$$Recovery\ (\%) = \frac{weight_{concentrate}\ (g) \cdot N_{concentrate}\ (g/g)}{weight_{by-product}\ (g) \cdot N_{by-product}\ (g/g)} \cdot 100 \quad (6.1)$$

The preliminary *Experiments 1* and *2* were conducted using commercial pea flour, while the *Experiments 3*, *4* and *5* were performed using the thawed pea by-product.

Experiment 1—A fractional factorial design 2^{4-1} with three central points (Supplementary Table S.9) was performed to study the effects of liquid:solid ratio (10–20 mL/g), pH (8–11), amplitude (30–50%) and time (5–15 min) on extraction recovery. The choice of the factors and of their levels was based on a previous investigation dealing with pea flour (F. Wang et al., 2020).

Experiment 2—A full factorial design 2^2 with three central points, replicated twice by assigning a block to each repetition (Supplementary Table S.10), was applied to study the effect of amplitude (60–80%) and time (10–20 min) at a liquid:solid ratio of 20 mL/g and pH 11.

Experiment 3—A full factorial design 2^2 with three central points (Supplementary Table S.11), was performed considering as independent variables amplitude (40–80%) and time (5–15 min), with liquid:solid ratio of 20 mL/g and pH 11.

Experiment 4—The extraction time was set up by applying the steepest ascent method (Montgomery, 2019); four runs at a constant amplitude of 80%

were performed between 18.75 and 30.00 min, with 3.75 min increments for each run (Supplementary Table S.12). The trials were performed in duplicate.

Experiment 5—A rotatable Central Composite Design (CCD) with two factors and four central points (Supplementary Table S.13) was applied to optimize amplitude (70–90%) and time (25–35 min) conditions.

Finally, a comparison of the recovery and protein content achieved by the traditional magnetic stirring method (ratio = 20 mL/g, pH = 11, time = 2×2 h) and by the optimized ultrasound-assisted extraction method (ratio = 20 mL/g, pH = 11, amplitude = 80%, and time = 2×30 min) was performed.

6.2.3 Proximate composition

The moisture content was determined gravimetrically according to the 925.10 official method (AOAC, 2000). The water activity (a_w) was measured with an Aqua Lab Series 3 TE instrument (Decagon Devices, Inc., Pullman, WA, USA). Total nitrogen content was determined by the Kjeldahl method 920.87 (AOAC, 2000) and protein content was computed as $N \times 6.25$. Lipid content was assessed by the Soxhlet method 920.39C (AOAC, 2000), total crude fibre by method 962.09 (AOAC, 2000), ash by method 923.03 (AOAC, 2000), starch by ISO method 10520:1997 (ISO, 1997) and total carbohydrates by difference. The results are expressed as g/100 g dry matter (DM).

6.2.4 Colour

The colour of the different samples was evaluated in the CIElab L^* a^* b^* colour space using a Chroma Meter CR-II tristimulus colorimeter (Minolta Camera Co., Osaka, Japan) with a C standard illuminant. Three measurements were performed.

6.2.5 Techno-functional properties

Water absorption capacity (WHC) and oil absorption capacity (OHC) were appraised with the method described by Beuchat (1977) and Stamatie et al. (2021). For WHC, 0.5 g of sample and 5 mL of distilled water were added in a centrifuge tube, vortexed and agitated for 1 h at room temperature with a PTR-35 rotating shaker (Grant Instruments, Royston, UK). The samples were then centrifuged (LISA, AFI Groups, Château-Gontier, France) at 15000 *g* for 15 min at 15 °C and the supernatant was discarded. The same procedure was carried out for OHC determination by replacing the water with peanut oil. The Equation 6.2 was used to estimate the absorption capacities.

The least gelling concentration (LGC) was determined according to Sathe et al. (1982). Sample suspensions (2–16 g/100 mL) were prepared in test tubes and then heated to 90 °C for 1 h in a water bath. After cooling at

4 °C for 2 h, the tubes were inverted and the minimum concentration that produced a self-sustaining gel was recorded.

Foam capacity (FC) and foam stability (FS) were estimated as in Miller and Groninger (1976). In a polyethylene bottle, 0.2 g of sample and 20 mL of pH 7 phosphate buffer (Carlo Erba, Cornaredo, Italy) were homogenized with a UltraTurrax T25 (IKA, Königswinter, Germany) at 17500 rpm for 1 min cooling the mixture with a water bath. The foam volume right after homogenization and then after 30 min was measured to compute FC (Equation 6.3) and FS (Equation 6.4), respectively (F. Wang et al., 2020).

To determine the emulsifying capacity (EC), 0.1 g of sample were mixed into a 50 mL beaker with 10 mL of distilled water, stirring the mix until a homogeneous solution was obtained; then pH was adjusted to 4.5–5. Subsequently, 20 mL peanut oil were added at 1 mL/min rate under stirring at room temperature. After the formation of an emulsion, the sample was centrifuged at 447 g for 10 min at 12 °C, and the height of the emulsified layer (He; cm) and the total height of the liquid (Ht; cm) were measured (Bai et al., 2019; Stamatie et al., 2021). The emulsifying capacity was calculated with Equation 6.5.

$$WHC/OHC\ (g/g) = \frac{weight_{wet} - weight_{dry}\ (g)}{weight_{dry}\ (g)} \tag{6.2}$$

$$FC\ (\%) = \frac{foam\ volume}{15} \cdot 100 \tag{6.3}$$

$$FS\ (\%) = \frac{foam\ volume\ after\ 30\ min}{initial\ foam\ volume} \cdot 100 \tag{6.4}$$

$$EC\ (\%) = \frac{He}{Ht} \cdot 100 \tag{6.5}$$

6.2.6 SDS-PAGE

The glutenin fraction, extracted according to Singh et al. (1991), was fingerprinted by discontinuous SDS-PAGE electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS), following Morel (1994). A protein marker (Thermo Fisher Scientific, Waltham, MA, USA) with molecular weight bands ranging from 116 kDa to 14.4 kDa was used as a reference.

6.2.7 Microbiological analyses

The microbiological analyses were performed by plate count. Briefly, 10 g samples were homogenized in a stomacher (BagMixer 400, Interscience,

Puycapel, France) for 1 min with 90 mL sterile diluent (0.9% NaCl). Then, 1 mL of the 1:10 dilution was transferred into a test tube containing 9 mL diluent and several decimal serial dilutions were similarly prepared. The total bacterial count (TBC) was determined according to the ISO 4833-1:2013 method (ISO, 2013): 1 mL of the appropriate dilution was placed in a Petri dish and incorporated into Plate Count Agar. The plates were then incubated at 30 °C for 72 h. The total Enterobacteriaceae were determined by the ISO 21528-2:2017 method (ISO, 2017a) by incorporating 1 mL into Violet Red Bile Glucose Agar and incubating the plates at 37 °C for 24–48 h. The mesophilic lactic acid bacteria were determined by the ISO 15214:1998 method (ISO, 1998) by incorporating 1 mL into De Man Rogosa Sharpe Agar and incubating the plates at 30 °C for 72 h under microaerophilic conditions. Total moulds and yeasts were determined according to the ISO 21527-2:2008 method (ISO, 2008) by spreading 100 µL of each dilution on Malt Extract Agar supplemented with 0.01% chloramphenicol as selective agent. The plates were incubated at 25 °C for 96–120 h. The presence of *Salmonella* was investigated according to the ISO 6579-1:2017 method (ISO, 2017b).

The plates containing 10–200 colonies were scored, recording at least two values for each replicate. The analyses were performed on three independent replicates and the results are expressed as the decimal logarithm of colony forming units per gram of sample (log CFU/g).

6.2.8 Statistical analysis

After verifying the normal distribution of the data, the recoveries obtained in *Experiment 4* and the characteristics of commercial pea flour, dried pea by-product and protein concentrate were evaluated by One-Way Analysis of Variance (ANOVA). When significant differences ($p \leq 0.05$) were found, Fisher's least significant difference (LSD) was calculated at 95% significance level. To compare the performance of the traditional magnetic stirring method and the optimized ultrasound-assisted extraction method, the Student's t-test was applied. All analyses were performed using the statistical programme Statgraphics® Centurion 18 (Statpoint Technologies Inc., Warrenton VA, USA). Mean, standard deviation and coefficient of variation (CV, expressed as %) were computed using the Excel® software (Microsoft, Redmond, WA, USA). The statistical processing of the experimental designs was performed with the Design Expert 10 software (StatEase, Minneapolis, MN, USA).

6.3 Results and discussion

6.3.1 By-product characterization

The recorded percentage of waste over six years corresponded to $7.6 \pm 2.5\%$ (CV = 33%). Figure 6.1 presents the chemical composition and microbial

contamination of the canned peas by-product across different years. The moisture content was characterized by the lowest variability (CV = 6.9%), ranging from 76.5 to 90.7 g/100 g. The proximate composition, expressed on dry matter (DM), varied broadly for lipids and fibre (CV = 43%), ranging from 0.0 to 5.3 g/100 g DM and from 10.5 to 54.6 g/100 g DM, respectively, due to the different proportion of pods, leaves, hulls and seeds. The protein and ash contents ranged with a CV = 20%, from 15.5 to 30.9 g/100 g DM and from 2.8 to 5.7 g/100 g DM. These values agree with the composition reported by Belghith Fendri et al. (2016) and Mateos-Aparicio et al. (2010) for pea pods and by Hall et al. (2017) for pea cotyledons. The starch content varied between 10.5 and 21.6 g/100 g DM (CV = 28%).

The microbial contamination presented a wide fluctuation among the different samplings: the total bacterial count varied from 6.3 to 8.4 log CFU/g, the Enterobacteriaceae from 5.4 to 7.5 log CFU/g, the yeasts from 3.7 to 5.7 log CFU/g and the moulds from 4.4 to 5.7 log CFU/g; *Salmonella* was always absent.

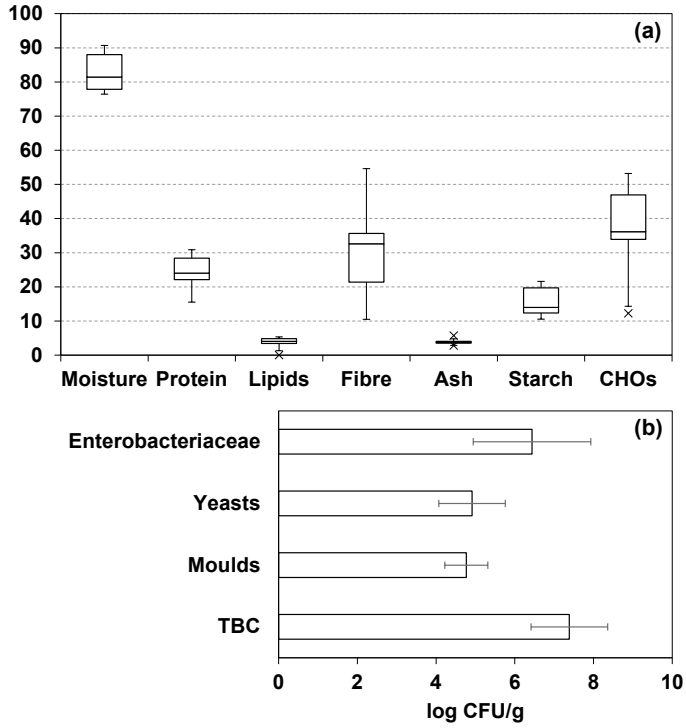
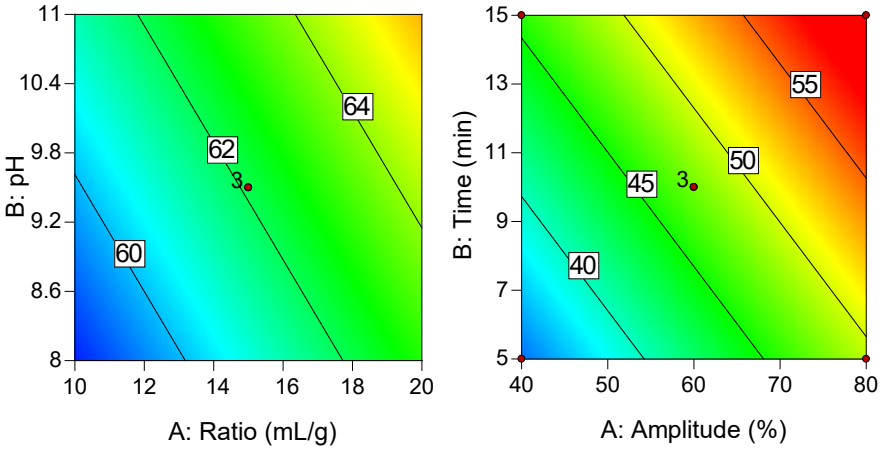


Figure 6.1. Proximate composition (a) and microbial counts (b) of pea canning by-product collected from 2013 to 2022. TBC, Total bacterial count.



(a) *Experiment 1* **(b)** *Experiment 3*

Figure 6.2. Contour plots of the experiments performed for protein extraction from commercial pea flour (a) and thawed pea by-product (b) by ultrasound-assisted method.

6.3.2 Optimization of the protein extraction process

6.3.2.1 Preliminary trials on commercial pea flour

The first extraction trial (*Experiment 1*), performed using commercial pea flour and following a fractional factorial design 2^{4-1} with three central points (Supplementary Table S.9), showed that only the liquid:solid ratio had a significant ($p \leq 0.01$) effect on the recovery (Supplementary Table S.14). The pH p-value (0.067) was just above the 0.05 threshold, suggesting the inclusion in the model of this parameter to verify its effect on the response variable (i.e., recovery). The chosen model was significant ($p \leq 0.001$), while the curvature was not ($p > 0.05$), and presented the coded Equation 6.6.

$$Recovery = 62.1 + 2.2\,ratio + 1.3\,pH \tag{6.6}$$

As clearly displayed in the contour plot (Figure 6.2a), greater recoveries were obtained with higher ratios and pH. Thus, for the following trials the extraction ratio was set at 20 mL/g and pH at 11. Being concentration gradient the driving force of extraction, diluting the solids in more solvent increases the quantity of extract; however, a plateau leading to negligible increases can be reached. In this case, ratios higher than 20 mL/g were considered unfeasible, because they would have reduced the amount of material treated. Furthermore, a trial performed at a 20 mL/g with pH values increasing from 8 to 11 confirmed the pH = 11 as the best choice.

Ultrasonication improves the extraction of various analytes, including protein, from food matrices (Estivi et al., 2022). Therefore, amplitude and time were further studied in *Experiment 2* according to a replicated full factorial 2^2 with three central points (Supplementary Table S.10), exploring higher factors levels. As reported in Supplementary Table S.14, the amplitude was the only significant factor and the model was defined by the coded Equation 6.7.

$$Recovery = 66.30 + 0.95\, amplitude \tag{6.7}$$

6.3.2.2 Fine tuning trials on by-product

Initially, protein extraction from the dry by-product was attempted. However, as soon as the sonication started, the dry by-product formed a viscous mixture with water, preventing it from flowing and causing hot spots (>60 °C). This might be related to gelation or swelling of damaged starch or starch pregelatinized during drying. In fact, although the drying temperature (55 °C) corresponded only to the pea starch gelatinization onset (53.6–59.5 °C; Sun et al., 2020; S. Wang & Copeland, 2012), the treatment lasted 24 h in the presence of an excess of water during the early stages. The starch gelatinization extent is temperature-dependent (Lund & Lorenz, 1984), but partially occurs over a long period even when the temperature is maintained around the onset (Pielichowski et al., 1998; Sablani et al., 2007). The gelatinization follows a first order kinetic and the reaction rate depends on the temperature according to the Arrhenius equation (Sablani et al., 2007); in addition, an alkaline pH facilitates starch swelling (Lam et al., 2018). It has to be remembered that as the fluid viscosity increases, the ultrasonic homogenizer needs to impart greater power to the probe to keep the amplitude constant. Therefore, a modest initial amount of swollen starch may have had an autocatalytic effect: greater viscosity corresponds to higher power, dissipated as heat, and consequently more gelatinization. This behaviour prevented a steady temperature control, necessary to limit the thermal denaturation of the proteins, as well as its reliable measurement, thus jeopardizing the possibility of correctly recording the experimental conditions. Increase of liquid:solid ratio and off-period of sonication cycles, and reduction of amplitude and coolant temperature were all unsuccessful, hence it was decided to treat the thawed by-product.

In *Experiment 3* the by-product was extracted by varying the amplitude and time factors according to a full factorial design 2^2 with three central points (Supplementary Table S.11). As shown in Supplementary Table S.15, the amplitude was the only significant factor ($p = 0.025$); however, the time was included in the model because its p-value (0.059) was just above the 0.05 significance level. The model was significant ($p \leq 0.05$), but the curvature was significant ($p = 0.02$) as well. The adjusted R^2 was 0.74 and the coded Equation 6.8 described the model. The direction of response maximization is evident in the contour plot shown in Figure 6.2b.

$$Recovery = 47.51 + 7.22\, amplitude + 5.42\, time \tag{6.8}$$

In *Experiment 4* the factors levels were explored following the steepest ascent path (Montgomery, 2019). The ratio of Equation 6.8 coefficients suggested a 0.75 incremental step, in coded terms, for treatment time and 1 incremental step for amplitude. However, increasing the amplitude would have brought the ultrasound probe to its operating limits in just two steps, also making it impossible to control the temperature. Therefore, the amplitude was kept constant at the highest previously explored value (80%) and only the treatment time was increased. The results (Supplementary Table S.12) evidenced higher extraction recovery after 26.25 min of treatment. This experiment identified a new region, probably close to the process optimum, which was further investigated with a Response Surface design by means of the CCD depicted in Supplementary Table S.13. The new model was not significant ($p > 0.05$; Supplementary Table S.15) but confirmed that the highest recoveries were obtained in a wide region whose central conditions were amplitude = 80% and time = 30 min. In this region, factor modifications led to negligible changes in recovery, therefore the central point was considered as the optimum: ratio = 20 mL/g, pH = 11, amplitude = 80% and time = 2×30 min.

Table 6.1. Recovery and protein content (mean ± standard deviation) in the extracts obtained from thawed pea-by product by magnetic stirring and the optimized ultrasound-assisted method.

	Treatment time	Recovery (%)	Protein (g/100 g DM)
Magnetic stirring	2 × 2 h	21.5 ± 0.9^{b}	91.5 ± 0.3^{a}
Ultrasonication	2 × 30 min	66.6 ± 1.6^{a}	74.9 ± 0.3^{b}

Different letters indicate significant differences among values in the same row according to the LSD test ($p \leq 0.05$).

Table 6.1 reports the recovery and protein content in the extracts obtained from thawed pea-by product by the magnetic stirring and by the optimized ultrasound-assisted method. The new method led to a recovery (66.6%) more than three times higher than the traditional magnetic stirring (21.5%), coupled with a drastic reduction in treatment time (one hour instead of four). Protein recovery from the by-product employing ultrasonication was comparable or inferior to those previously reported for pea flour extraction (62.6–76.7%) by Stone et al. (2015), using magnetic stirring, and by F. Wang et al. (2020; 82.6% with ultrasonication and 60% with magnetic stirring). However, lower recoveries were also reported for protein alkaline extraction from pea pods with (21.1%) and without (16.2%) ultrasound pre-treatment (Karabulut et al., 2023). Probably, the protein nitrogen was already present as soluble peptides due to pre-sampling fermentation and thus was lost during the wet extraction, leading to scarcer recovery. In addition, the granulometry (coarser than that of the pea flour) and the presence of other components

(e.g., cellulose, pectin and other polysaccharides) likely hindered protein diffusion to the solvent. An improvement in protein extraction by ultrasonication is reported for several vegetable matrices, including almond production residues (+480%), peanut flour (+136%), soy flour (+39%), sunflower seed cake (+317%), rice bran (+134%), rice syrup production residue (+100%) and quinoa flour (+140%; Jahan et al., 2022; Li et al., 2017; Quintero-Quiroz et al., 2022). Furthermore, a considerable reduction in treatment time is generally observed. The ultrasonication advantage in the extraction processes is universally attributed to the cavitation, which disrupts cellular compartments, facilitating the release of solutes, and renews the boundary layer of solvent, maintaining a high gradient of concentration (Estivi et al., 2022).

The protein content of the isolates produced from pea flour by alkaline extraction can reach 90 g/100 g (Boye et al., 2010). F. Wang et al. (2020) reported values of 81.5 and 87.5 g/100 g DM in isolates produced with agitation and ultrasonication, respectively. The highest protein concentration (Table 6.1) was observed in the extract obtained with magnetic stirring (91.5 g/100 g DM), while the optimized ultrasound method generated a less pure product (74.9 g/100 g DM). Karabulut et al. (2023) observed the same tendency in pea pods isolates, as they determined 54.3 g protein/100 g with ultrasound pre-treatment and 63.7 g/100 g without it, ascribing that to co-extraction of interfering components. According to Prestes Fallavena et al. (2022) and Thirunavookarasu et al. (2022) abundant evidence has been gathered on protein glycation mediated by high-power ultrasound and on the formation of complexes between denatured proteins and sugars, oligosaccharides or polysaccharides, including pectin. Protein-polysaccharide complexes are stabilized by electrostatic, hydrophobic interactions, van der Waals forces and hydrogen bonding (Thirunavookarasu et al., 2022), thus it can be hypothesized that the polysaccharides from the abundant soluble fibre of the pods formed soluble complexes with the proteins, reducing the purity of the concentrate. In fact, the sonication conditions, more drastic than those employed by F. Wang et al. (2020), the higher soluble fibre, especially the pectic polysaccharides from pods and hulls (Belghith-Fendri et al., 2018), and the presence of lectins, carbohydrates-binding proteins (Shi et al., 2018), may explain the lower purity of the extract. Additionally, the drying treatment could have reinforced or formed new protein-polysaccharides conjugates by the Maillard reaction, even at relatively low temperature (i.e., 60 °C; Tamnak et al., 2016), while the ultrasonication increased protein unfolding, promoting glycation (Ma et al., 2020). Finally, the high pH may have resulted in the contamination of the extract with swollen starch (Lam et al., 2018).

Dry fractionation of legumes desiccated in the field is more economical and sustainable to operate than wet extraction, although the protein-rich flour thus separated presents a concentration between 49% and 73%, lower than that achieved from alkaline extraction (Fernando, 2021). Nevertheless, the concentrate and the other co-products (starchy and fibrous fractions)

are obtained directly in the dehydrated form, facilitating their conservation. Conversely, the by-product evaluated in this research is conveyed to the collection point by hydraulic transport, before being drained by compression in a rotating drum. Therefore, to scale-up the process it should be treated as soon as collected, avoiding the drying energy costs.

6.3.3 Pea products characterization

The characteristics of commercial flour, dry by-product and protein concentrate are reported in Table 6.2. Protein content was significantly higher in the concentrate (74.9 ± 0.3 g/100 g DM) than in the commercial flour and the by-product (24.7–25.1 g/100 g DM). Water activity was low in all samples, although the by-product had the lowest one, probably due to the water-binding capacity of its high fibre content (36.1 g/100 g DM). The brighter green colour of the pea flour (Table 6.2 and Figure 6.3) is probably attributable to the drying conditions in the field (ambient temperature) *versus* those in the oven (55 °C) employed for the dry by-product and protein concentrate. In fact, the transition to a darker tinge can be a consequence of chlorophyll degradation into grey/brown pheophorbide or pheophytin (Zielinska et al., 2013). All samples showed an important yellow component (b^*), with the highest score in the by-product.

Significant differences in techno-functional properties, except emulsifying capacity, were detected. Generally, the wet extraction appeared detrimental for all the interfacial properties, probably due to formation of poorly soluble aggregates in the concentrate that prevailed over ultrasound-induced increase in surface hydrophobicity (Estivi et al., 2022; Fernando, 2021; Vogelsang-O'Dwyer et al., 2020; Xiong et al., 2018).

The concentrate WHC (2.13 g H_2O/g) was similar to those (2.06 and 2.4–2.6 g H_2O/g) reported for pea protein isolates by Kumar et al. (2022) and Stone et al. (2015), respectively, although higher values (3.2 and 3.0–

Figure 6.3. Images of the commercial pea flour (left), the dry by-product (centre), and the protein concentrate obtained by the optimized ultrasound-assisted method (right).

Table 6.2. Water activity, protein content, colour, techno-functional properties, and microbial count (mean ± standard deviation) of commercial pea flour, dry pea by-product and protein concentrate.

Parameters	Pea flour	Dry by-product	Concentrate
a_w	0.530 ± 0.004^{a}	0.320 ± 0.002^{c}	0.510 ± 0.002^{b}
Protein (g/100 g DM)	25.09 ± 0.08^{b}	24.71 ± 0.71^{b}	74.86 ± 0.32^{a}
Colour coordinates			
L^*	86.50 ± 0.30^{a}	54.47 ± 0.83^{b}	40.43 ± 0.35^{c}
a^*	-9.63 ± 0.21^{b}	-4.10 ± 0.46^{a}	-3.50 ± 0.30^{a}
b^*	16.90 ± 0.35^{b}	19.17 ± 0.50^{a}	15.43 ± 0.55^{c}
Techno-functional properties			
WHC (g H2O/g)	1.23 ± 0.34^{c}	4.19 ± 0.28^{a}	2.13 ± 0.22^{b}
OHC (g oil/g)	1.57 ± 0.08^{b}	2.24 ± 0.16^{a}	1.68 ± 0.22^{b}
LGC (g/100 mL)	10	15	10
Foaming capacity (%)	69.18 ± 1.39^{a}	21.59 ± 1.36^{c}	28.46 ± 1.39^{b}
Foam stability (%)	49.55 ± 0.01^{a}	15.21 ± 2.08^{b}	6.38 ± 1.39^{c}
Emulsifying capacity (%)	63.26 ± 1.15	62.20 ± 1.28	60.89 ± 1.07
Microbial counts (log CFU/g)			
Total bacterial count			5.46 ± 0.17
Total lactic acid bacteria			5.38 ± 0.12
Enterobacteriaceae			2.54 ± 0.11
Yeasts and moulds			3.32 ± 0.15

WHC, water holding capacity; OHC, oil holding capacity; LGC, least gelling concentration. Different letters indicate significant differences among values in the same row according to the LSD test ($p \leq 0.05$).

4.2 g H_2O/g) were described by Sareen et al. (2023) and F. Wang et al. (2020). The OHC (1.68 g oil/g) was like those (1.72 and 1.46 g oil/g) reported by Kumar et al. (2022) and Sareen et al. (2023). The by-product showed the greatest WHC and OHC, thanks to the higher content in dietary fibre. In fact, hemicellulose and pectin are hydrophilic and correlated to WHC, while cellulose also retains oil due to a certain hydrophobicity (He et al., 2023). The flour and the concentrate exhibited the same least gelling concentration (10 g/100 mL), as the lack of starch was probably compensated by the higher protein amount, while the by-product formed a strong gel only above 15 g/100 mL. The concentrate LGC was slightly lower, thus better, than those (11–13 and 12 g/100 mL) determined by F. Wang et al. (2020) and Kumar et al. (2022), suggesting that the hypothesized aggregates became soluble in the presence of heat. The protein concentrate displayed some foam capacity (28%) but poor stability (6%), both inferior to those of the flour. Foam-forming proteins have, ideally, low molecular weight, high surface hydrophobicity, good solubility, low net charge and proneness to denaturation (Barac et al., 2010). Poor foaming properties were previously detected in

pea protein isolate, especially at pH 4.5 (Kumar et al., 2022; Sareen et al., 2023), since as the pH increases to 7 the proteins net charge rises as well, improving unfolding, flexibility and, therefore, foaming (Barac et al., 2010). Furthermore, the SDS- PAGE (Figure 6.4) highlighted the presence of albumins only in the commercial flour. Albumins are rapidly adsorbed at the air-water interface and have strong protein-protein interactions (Kornet et al., 2022) resulting in a stiff and dense interfacial layer, and contributing to the greater foaming capacity and stability of the flour (Barac et al., 2010).

6.3.4 SDS-PAGE

The SDS-PAGE gel extraction and separation were performed to detect possible effects of the different treatments on pea proteins. The fingerprint patterns of glutenin extracts of commercial flour, dry by-product and protein concentrate are shown in Figure 6.4. The concentrate and the dry by-product showed overall the same protein pattern of the commercial flour. The presence of convicilin and legumin was detected near the 66.2 kDa band, while vicilin and legumin α were found at 45 kDa and 35 kDa, respectively. Finally, at approximately 25 kDa the presence of legumin β was observed. Albumin, the thin band around 14.4 kDa (Chang et al., 2022; Gao et al., 2020), was found only in the commercial flour sample. However, smeared bands in the dry by-product and concentrate lanes were also evident (Figure 6.4) and are proof of protein degradation, probably because of the wet by-product fermentation before collection, with consequent enzymatic hydrolysis. In fact, the processing waste was very humid and was exposed to summer temperatures for an unknown period before sampling. Schlegel et al. (2021) observed that the combination of fermentation and enzymatic hydrolysis of lupin protein isolate resulted in the breakdown of large polypeptides into shorter low molecular weight peptides. Furthermore, Lee et al. (2018) noted that the formation or disappearance of protein bands within an SDS-PAGE was clearly affected by fermentation: up to 9 h fermentation no changes were noticed, indicating that the protein subunits were still intact, but starting from 12 h the intensity of the 7S band and the 11S globulins decreased and new bands with lower molecular weight appeared; after 18 h, the 7S globulin and the 11S globulin acid subunit largely disappeared, while the base subunit of 11S globulin was much weaker. Finally, smeared bands were previously observed in pea protein isolate conjugated with pectin by Maillard reaction (Tamnak et al., 2016), as a consequence of the wide range of glycated proteins molecular weights. This complementary explanation better matches with the protein concentrate, because the by-product was dried before milling and therefore contact between proteins and polysaccharides was less likely. Smeared bands in pea pods isolates, more evident in those obtained with alkaline solubilization, were also reported by Karabulut et al. (2023).

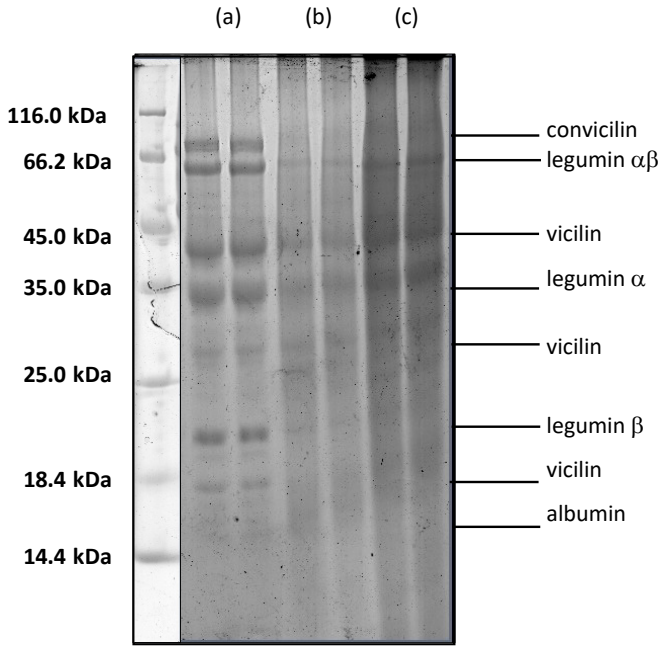


Figure 6.4. Electrophoretic profiles and molecular weight distribution of glutenin extracts from pea commercial flour (a), dry pea by-product (b) and pea protein concentrate (c). The first lane on the left displays the weight markers.

6.3.5 Microbiological analysis of protein concentrate

Microbiological counts on the protein concentrate are reported in Table 6.2. In the absence of specific microbiological criteria concerning protein isolates, a comparison may be attempted with reasonably similar products (i.e., dried vegetables and cereal flours). Micro-organisms commonly isolated from dried vegetables include lactic acid bacteria, *Enterococcus faecalis*, staphylococci, *Bacillus* spp. spores, yeasts, and moulds (*Penicillium* and *Aspergillus* spp.; ICMSF, 2005). The total mesophile bacterial count of the concentrate (2.9×10^5 CFU/g) was lower than the reference values proposed by the Fédération du Commerce et de la Distribution (FCD, 2023) for wholemeal flours (5×10^5 CFU/g) and by Gilbert et al. (2000) in various ready-to-eat foods including dried vegetables (10^6 CFU/g), whereas 10^4 CFU/g has been reported as the guide value for commercial vegetable protein ingredients (D'Agostina et al., 2005). Previously, TBC ranging from 10^1 to 1.5×10^4 CFU/g were reported among 35 different commercial protein isolates (26 from pea, 7 from faba bean, 1 from mung bean and 1 from chickpea; Kyrylenko et al., 2023), whereas 1.9×10^7 CFU/g count was determined in lupin protein isolate (Melde et al., 2016). In the protein concentrate, the

greatest contribution to TBC came from presumptive lactic acid bacteria (2.4×10^5 CFU/g). The Enterobacteriaceae (3.5×10^2 CFU/g) did not exceed the safety criteria for dried vegetables (10^4 CFU/g) reported by Gilbert et al. (2000) or the hygienic criteria in the processing of cereals (10^2–10^3 CFU/g) stated by ICMSF (2011). The sum of yeasts and moulds (2.1×10^3 CFU/g) was lower than the moulds limit specified by the FCD (2023). *Salmonella* was absent, as it was in the by-product. Given that the native by-product had an average TBC of 2.4×10^7 CFU/g, the extraction significantly reduced microbiological contamination. In fact, sonication has a sanitizing effect because of the mechanical damage on cells (Bhargava et al., 2021); additionally, the first centrifugation precipitated cells and non-protein pellets, further reducing the number of bacteria in the solubilized protein extract. Finally, as previously discussed for other parameters, the microbiological quality of the extract could be dramatically improved by in-line processing of the by-product or by the adoption of a hygienic design at the collection point to minimize cross-contamination from pre-fermented residues.

6.4 Conclusion

The ultrasonication increased three-fold the extraction recovery of protein (from 21.5% to 66.6%) and reduced the treatment time from 4 to 1 h compared to the traditional method. The protein concentrate obtained under the optimal conditions presented a 74.9 g/100 g DM protein content and a gelling capacity equal to that of pea flour. Protein degradation, probably due to fermentation and enzymatic hydrolysis occurred before sampling, was observed in the by-product and concentrate. Total bacterial count, Enterobacteriaceae and moulds in the concentrate were always below the guideline values.

The pea canning by-product stands out as a valuable raw material for protein recovery. Due to logistical and construction characteristics of the processing plant, the by-product had a high moisture content at the collection point. Therefore, for a scale-up of the extraction process a direct treatment of the by-product, avoiding the drying costs, would be advantageous. Furthermore, a direct and rapid processing would limit or prevent the fermentation phenomena which seem to partially compromise quality of the concentrate and protein recovery.

References

AOAC. (2000). *Official Methods of Analysis of AOAC International, 17th ed.* Association of Official Analytical Chemists International.

Bai, L. T., Song, Y., Li, Q. M., Pan, L. H., Zha, X. Q., & Luo, J. P. (2019). Emulsifying and physicochemical properties of lotus root amylopectin-whey protein isolate conjugates. *LWT - Food Sci Technol*, *111*, 345–354. https://doi.org/10.1016/J.LWT.2019.05.052

Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Zilic, S., Macej, O., & Ristic, N. (2010). Profile and functional properties of seed proteins from six pea (*Pisum sativum*) genotypes. *Int J Mol Sci*, *11*, 4973–4990. https://doi.org/10.3390/IJMS11124973

Belghith Fendri, L., Chaari, F., Kallel, F., Koubaa, M., Zouari-Ellouzi, S., Kacem, I., Ellouz-Chaabouni, S., & Ghribi-Aydi, D. (2022). Antioxidant and antimicrobial activities of polyphenols extracted from pea and broad bean pods wastes. *J Food Meas Charact*, *16*, 4822–4832. https://doi.org/10.1007/S11694-022-01547-3

Belghith Fendri, L., Chaari, F., Kallel, F., Zouari-Ellouzi, S., Ghorbel, R., Besbes, S., Ellouz-Chaabouni, S., & Ghribi-Aydi, D. (2016). Pea and broad bean pods as a natural source of dietary fiber: The impact on texture and sensory properties of cake. *J Food Sci*, *81*, C2360–C2366. https://doi.org/10.1111/1750-3841.13448

Belghith-Fendri, L., Chaari, F., Jeddou, K. B., Kallel, F., Bouaziz, F., Helbert, C. B., Abdelkefi-Mesrati, L., Ellouz-Chaabouni, S., & Ghribi-Aydi, D. (2018). Identification of polysaccharides extracted from pea pod by-products and evaluation of their biological and functional properties. *Int J Biol Macromol*, *116*, 947–954. https://doi.org/10.1016/J.IJBIOMAC.2018.05.095

Beuchat, L. R. (1977). Functional and electrophoretic characteristics of succinylated peanut flour protein. *J Agr Food Chem*, *25*, 258–261. https://doi.org/10.1021/JF60210A044

Bhargava, N., Mor, R. S., Kumar, K., & Sharanagat, V. S. (2021). Advances in application of ultrasound in food processing: A review. *Ultrason Sonochem*, *70*, 105293. https://doi.org/10.1016/j.ultsonch.2020.105293

Boukid, F., Rosell, C. M., & Castellari, M. (2021). Pea protein ingredients: A mainstream ingredient to (re)formulate innovative foods and beverages. *Trends Food Sci Tech*, *110*, 729–742. https://doi.org/10.1016/J.TIFS.2021.02.040

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Res Int*, *43*, 414–431. https://doi.org/10.1016/J.FOODRES.2009.09.003

Chang, L., Lan, Y., Bandillo, N., Bom Ohm, J., Chen, B., & Rao, J. (2022). Plant proteins from green pea and chickpea: Extraction, fractionation, structural characterization and functional properties. *Food Hydrocoll*, *123*, 107165. https://doi.org/10.1016/J.FOODHYD.2021.107165

D'Agostina, A., Antonioni, C., Resta, D., Arnoldi, A., Bez, J., Knauf, U., & Wäsche, A. (2005). Optimization of a pilot-scale process for producing lupin protein isolates with valuable technological properties and minimum thermal damage. *J Agr Food Chem*, *54*, 92–98. https://doi.org/10.1021/JF0518094

Dueñas, M., Estrella, I., & Hernández, T. (2004). Occurrence of phenolic compounds in the seed coat and the cotyledon of peas (*Pisum sativum* L.) *Eur Food Res Technol*, *219*, 116–123. https://doi.org/10.1007/S00217-004-0938-X

Estivi, L., Brandolini, A., Condezo-Hoyos, L., & Hidalgo, A. (2022). Impact of low-frequency ultrasound technology on physical, chemical and technological properties of cereals and pseudocereals. *Ultrason Sonochem*, *86*, 106044. https://doi.org/10.1016/j.ultsonch.2022.106044

FCD. (2023). Critères microbiologiques applicables à partir de 2024 aux marques de distributeurs, marques premiers prix et matières premières dans leur conditionnement initial industriel. *Fédération du Commerce et de la Distribution*.

Fernando, S. (2021). Production of protein-rich pulse ingredients through dry fractionation: A review. *LWT - Food Sci Technol*, *141*, 110961. https://doi.org/10.1016/J.LWT.2021.110961

Gao, Z., Shen, P., Lan, Y., Cui, L., Bom Ohm, J., Chen, B., & Rao, J. (2020). Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Res Int*, *131*, 109045. https://doi.org/10.1016/J.FOODRES.2020.109045

Gilbert, R. J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C. D., Richards, J., Roberts, D., & Bolton, F. J. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. PHLS Advisory Committee for Food and Dairy Products. *Communicable disease and public health*, *3*, 163–167.

Hall, C., Hillen, C., & Garden Robinson, J. (2017). Composition, nutritional value, and health benefits of pulses. *Cereal Chem*, *94*, 11–31. https://doi.org/10.1094/CCHEM-03-16-0069-FI

He, C. A., Qi, J. R., Liao, J. S., Song, Y. T., & Wu, C. L. (2023). Excellent hydration properties and oil holding capacity of citrus fiber: Effects of component variation and microstructure. *Food Hydrocoll*, *144*, 108988. https://doi.org/10.1016/J.FOODHYD.2023.108988

ICMSF. (2005). Vegetables and vegetable products. In T. A. Roberts, J.-L. Cordier, L. Gram, R. B. Tompkin, J. I. Pitt, L. G. M. Gorris, & K. M. J. Swanson (Eds.), *Micro-organisms in foods 6. Microbial ecology of food commodities* (pp. 277–325). Springer US. https://doi.org/10.1007/0-387-28801-5_5

ICMSF. (2011). Cereals and cereal products. In K. M. J. Swanson (Ed.), *Microorganisms in foods 8. Use of data for assessing process control*

and product acceptance (pp. 209–225). Springer US. https://doi.org/10.1007/978-1-4419-9374-8_15

ISO. (1997). Native starch—Determination of starch content. Ewers polarimetric method (ISO standard 10520:1997).

ISO. (1998). Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of mesophilic lactic acid bacteria: Colony-count technique at 30 °C (ISO standard 15214:1998).

ISO. (2008). Microbiology of food and animal feeding stuffs—Horizontal method for the enumeration of yeasts and moulds Part 2: Colony count technique in products with water activity less than or equal to 0.95 (ISO standard 21527-2:2008).

ISO. (2013). Microbiology of the food chain—Horizontal method for the enumeration of microorganisms. Part 1: Colony count at 30 °C by the pour plate technique (ISO standard 4833-1:2013).

ISO. (2017a). Microbiology of the food chain—Horizontal method for the detection and enumeration of Enterobacteriaceae Part 2: Colony-count technique (ISO standard 21528-2:2017).

ISO. (2017b). Microbiology of the food chain—Horizontal method for the detection, enumeration and serotyping of *Salmonella* Part 1: Detection of *Salmonella* spp. (ISO standard ISO 6579-1:2017).

Jahan, K., Ashfaq, A., Younis, K., Yousuf, O., & Ul Islam, R. (2022). A review of the effects of ultrasound-assisted extraction factors on plant protein yield and functional properties. *J Food Meas Charact*, *16*, 2875–2883. https://doi.org/10.1007/S11694-022-01390-6

Kamani, M. H., Neji, C., Fitzsimons, S. M., Fenelon, M. A., & Murphy, E. G. (2023). Unlocking the nutritional and functional potential of legume waste to produce protein ingredients. *Crit Rev Food Sci*. https://doi.org/10.1080/10408398.2023.2184322

Karabulut, G., Yildiz, S., Can Karaca, A., & Yemiş, O. (2023). Ultrasound and enzyme-pretreated extraction for the valorization of pea pod proteins. *J Food Process Eng*. https://doi.org/10.1111/JFPE.14452

Kornet, R., Yang, J., Venema, P., van der Linden, E., & Sagis, L. M. C. (2022). Optimizing pea protein fractionation to yield protein fractions with a high foaming and emulsifying capacity. *Food Hydrocoll*, *126*, 107456. https://doi.org/10.1016/J.FOODHYD.2021.107456

Kumar, P. K., Sivabalan, S., Parhi, A., & Sablani, S. S. (2022). Modification of pea protein isolate functionality by freeze–thaw cycling. *J Food Meas Charact*, *16*, 162–170. https://doi.org/10.1007/S11694-021-01151-X

Kyrylenko, A., Eijlander, R. T., Alliney, G., van de Bos, E. L., & Wells-Bennik, M. H. J. (2023). Levels and types of microbial contaminants in different plant-based ingredients used in dairy alternatives. *Int J Food Microbiol*, *407*, 110392. https://doi.org/10.1016/J.IJFOODMICRO.2023.110392

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: Structure, extraction, and functionality. *Food Rev Int*, *34*, 126–147. https://doi.org/10.1080/87559129.2016.1242135

Lee, M. K., Kim, J. K., & Lee, S. Y. (2018). Effects of fermentation on SDS-PAGE patterns, total peptide, isoflavone contents and antioxidant activity of freeze-thawed tofu fermented with *Bacillus subtilis*. *Food Chem*, *249*, 60–65. https://doi.org/10.1016/J.FOODCHEM.2017.12.045

Li, K., Ma, H., Li, S., Zhang, C., & Dai, C. (2017). Effect of ultrasound on alkali extraction protein from rice dreg flour. *J Food Process Eng*, *40*, e12377. https://doi.org/10.1111/JFPE.12377

Lund, D., & Lorenz, K. J. (1984). Influence of time, temperature, moisture, ingredients, and processing conditions on starch gelatinization. *Crit Rev Food Sci*, *20*, 249–273. https://doi.org/10.1080/10408398409527391

Ma, X., Hou, F., Zhao, H., Wang, D., Chen, W., Miao, S., & Liu, D. (2020). Conjugation of soy protein isolate (SPI) with pectin by ultrasound treatment. *Food Hydrocoll*, *108*, 106056. https://doi.org/10.1016/J.FOODHYD.2020.106056

Mateos-Aparicio, I., Redondo-Cuenca, A., Villanueva-Suárez, M. J., Zapata-Revilla, M. A., & Tenorio-Sanz, M. D. (2010). Pea pod, broad bean pod and okara, potential sources of functional compounds. *LWT - Food Sci Technol*, *43*, 1467–1470. https://doi.org/10.1016/J.LWT.2010.05.008

Melde, D., Wiacek, C., & Braun, P. G. (2016). Physical decontamination of lupin (*Lupinus angustifolius*) protein isolates, seeds and flakes: Effects on microbiological status and micellar protein yield. *LWT - Food Sci Technol*, *66*, 651–656. https://doi.org/10.1016/J.LWT.2015.11.030

Miller, R., & Groninger, H. S. (1976). Functional properties of enzyme-modified acylated fish protein derivatives. *J Food Sci*, *41*, 268–272. https://doi.org/10.1111/J.1365-2621.1976.TB00598.X

Montgomery, D. C. (2019). *Design and analysis of experiments 10th ed.* John Wiley & Sons.

Morel, M. H. (1994). Acid-polyacrylamide gel electrophoresis of wheat gluten-ins: A new tool for the separation of high and low molecular weight subunits. *Cereal Chem*, *71*, 238–242.

Pielichowski, K., Tomasik, P., & Sikora, M. (1998). Kinetics of gelatinization of potato starch studied by non-isothermal DSC. *Carbohydr Polym*, *35*, 49–54. https://doi.org/10.1016/S0144-8617(97)00236-1

Prestes Fallavena, L., Poerner Rodrigues, N., Ferreira Marczak, L. D., & Domeneghini Mercali, G. (2022). Formation of advanced glycation end products by novel food processing technologies: A review. *Food Chem*, *393*, 133338. https://doi.org/10.1016/J.FOODCHEM.2022.133338

Quintero-Quiroz, J., Celis-Torres, A., Ciro-Gómez, G., Torres, J., Corrales-García, L., & Rojas, J. (2022). Physicochemical properties and functional characteristics of ultrasound-assisted legume-protein isolates: A comparative study. *J Food Sci Tech*, *59*, 1665–1676. https://doi.org/10.1007/S13197-021-05126-0

Sablani, S. S., Kasapis, S., Al-Tarqe, Z. H., Al-Marhubi, I., Al-Khuseibi, M., & Al-Khabori, T. (2007). Isobaric and isothermal kinetics of gelatinization of waxy maize starch. *J Food Eng*, *82*, 443–449. https://doi.org/10.1016/J.JFOODENG.2007.02.056

Sareen, J., Shi, D., Stone, A. K., Xu, C., Polley, B., House, J. D., Bhowmik, P., Rajagopalan, N., Tanaka, T., & Nickerson, M. T. (2023). Effect of enzyme hydrolysis on the physicochemical, functional, and nutritional properties of pea and faba bean protein isolates. *Eur Food Res Technol*, *249*, 3175–3190. https://doi.org/10.1007/S00217-023-04358-Y

Sathe, S. K., Deshpande, S. S., & Salunkhe, D. K. (1982). Functional properties of lupin seed (*Lupinus mutabilis*) proteins and protein concentrates. *J Food Sci*, *47*, 491–497. https://doi.org/10.1111/J.1365-2621.1982.TB10110.X

Schlegel, K., Lidzba, N., Ueberham, E., Eisner, P., & Schweiggert-Weisz, U. (2021). Fermentation of lupin protein hydrolysates—Effects on their functional properties, sensory profile and the allergenic potential of the major lupin allergen Lup an 1. *Foods*, *10*, 281. https://doi.org/10.3390/FOODS10020281

Shanthakumar, P., Klepacka, J., Bains, A., Chawla, P., Dhull, S. B., & Najda, A. (2022). The current situation of pea protein and its application in the food industry. *Molecules*, *27*, 5354. https://doi.org/10.3390/MOLECULES27165354

Shi, L., Arntfield, S. D., & Nickerson, M. (2018). Changes in levels of phytic acid, lectins and oxalates during soaking and cooking of Canadian pulses. *Food Res Int*, *107*, 660–668. https://doi.org/10.1016/J.FOODRES.2018.02.056

Singh, N. K., Shepherd, K. W., & Cornish, G. B. (1991). A simplified SDS—PAGE procedure for separating LMW subunits of glutenin. *J Cereal Sci*, *14*, 203–208. https://doi.org/10.1016/S0733-5210(09)80039-8

Stamatie, G. D., Duță, D. E., Belc, N., Zoani, C., & Israel-Roming, F. (2021). Nutritional and functional properties of some protein sources. *AgroLife Sci J*, *10*, 214–220. https://doi.org/10.17930/AGL2021124

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2015). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Res Int*, *76*, 31–38. https://doi.org/10.1016/J.FOODRES.2014.11.017

Sun, Y., Wang, M., Ma, S., & Wang, H. (2020). Physicochemical characterization of rice, potato, and pea starches, each with different crystalline pattern, when incorporated with Konjac glucomannan. *Food Hydrocoll*, *101*, 105499. https://doi.org/10.1016/J.FOODHYD.2019.105499

Tamnak, S., Mirhosseini, H., Tan, C. P., Ghazali, H. M., & Muhammad, K. (2016). Physicochemical properties, rheological behavior and morphology of pectin-pea protein isolate mixtures and conjugates in aqueous system and oil in water emulsion. *Food Hydrocoll*, *56*, 405–416. https://doi.org/10.1016/J.FOODHYD.2015.12.033

Tassoni, A., Tedeschi, T., Zurlini, C., Cigognini, I. M., Petrusan, J.-I., Rodríguez, Ó., Neri, S., Celli, A., Sisti, L., Cinelli, P., Signori, F., Tsatsos, G., Bondi, M., Verstringe, S., Bruggerman, G., & Corvini, P. F. X. (2020). State-of-the-art production chains for peas, beans and chickpeas—Valorization of agro-industrial residues and applications of derived extracts. *Molecules*, *25*, 1383. https://doi.org/10.3390/molecules25061383

Thirunavookarasu, N., Kumar, S., & Rawson, A. (2022). Effect of ultrasonication on the protein–polysaccharide complexes: A review. *J Food Meas Charact*, *16*, 4860–4879. https://doi.org/10.1007/S11694-022-01567-Z

Vogelsang-O'Dwyer, M., Petersen, I. L., Joehnke, M. S., Sørensen, J. C., Bez, J., Detzel, A., Busch, M., Krueger, M., O'Mahony, J. A., Arendt, E. K., & Zannini, E. (2020). Comparison of faba bean protein ingredients produced using dry fractionation and isoelectric precipitation: Techno-functional, nutritional and environmental performance. *Foods*, *9*, 322. https://doi.org/10.3390/FOODS9030322

Wang, F., Zhang, Y., Xu, L., & Ma, H. (2020). An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. *LWT - Food Sci Technol*, *127*, 109348. https://doi.org/10.1016/j.lwt.2020.109348

Wang, S., & Copeland, L. (2012). Effect of alkali treatment on structure and function of pea starch granules. *Food Chem*, *135*, 1635–1642. https://doi.org/10.1016/J.FOODCHEM.2012.06.003

Wu, D.-T., Li, W.-X., Wan, J.-J., Hu, Y.-C., Gan, R.-Y., Zou, L. A., Wu, D.-T., Li, W.-X., Wan, J.-J., Hu, Y.-C., Gan, R.-Y., & Zou, L. (2023). A comprehensive review of pea (*Pisum sativum* L.): Chemical composition, processing, health benefits, and food applications. *Foods*, *12*, 2527. https://doi.org/10.3390/FOODS12132527

Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Res Int*, *109*, 260–267. https://doi.org/10.1016/J.FOODRES.2018.04.044

Zielinska, M., Zapotoczny, P., Alves-Filho, O., Eikevik, T. M., & Blaszczak, W. (2013). A multi-stage combined heat pump and microwave vacuum

drying of green peas. *J Food Eng*, *115*, 347–356. https://doi.org/10.1016/J.JFOODENG.2012.10.047

7

Fermented pea by-product to fortify bread in vitamin B12

The contents of this chapter are under review as:

Estivi, L., Chamlagain, B., Edelmann, M., Varmanen, P., Hidalgo, A., & Piironen, V. (n.d.). By-product of canned green peas as a substrate for *Propionibacterium freudenreichii* fermentation to fortify bread with vitamin B12.

Abstract

This study focused on upcycling the industrial by-product of canned green peas for the synthesis of vitamin B12 by *in-situ* fermentation and subsequent bread fortification. The by-product, rich in protein (24.3 g/100 g dry matter, DM) and dietary fibre (33.2 g/100 g DM), was fermented with *Propionibacterium freudenreichii*, resulting in significant vitamin B12 production (1374–1535 ng/g DM). The fermented material was then incorporated into wheat bread with two percentages of enrichment (15% and 20%), aiming to address the deficiency of vitamin B12 in strictly plant-based diets. From 40 to 70 g of fortified bread provided the recommended daily intake of vitamin B12 (2.4 µg/g), along with minimal losses in volume development and no significant differences in texture when compared to the controls. The study underscores the potential of using food-grade by-products for enhancing the nutritional value of plant-based products, while also contributing to food-waste reduction.

7.1 Introduction

In 2011, approximately one-third (about 1.3 billion tonnes) of global food production was wasted worldwide annually (FAO, 2011). FAO (FAO, 2019) defines food loss and waste (FLW) as "the decrease in quantity or quality of food along the food supply chain", resulting from decisions and actions by suppliers (food loss) and by consumers, retailers, or food service providers (food waste). On average, about 14% of current food production is lost, with the greatest contribution from roots, tubers and oil-bearing crops (25.5% loss), followed by fruit and vegetables (21.6%), meat and animal products (12%), and cereals and pulses (8.7%; FAO, 2019). Approximately 17% of food is wasted by households (67%), food services (26%) and retailers (13%), corresponding to 931 million metric tonnes in 2019 (UNEP, 2021). Food diverted to feed, seeds or industrial purposes is considered lost or wasted only if originally destined for human consumption. Inedible parts, though not considered food, cannot be deducted from "food loss" calculations (FAO, 2019). However, some inedible residues (e.g., fruit and vegetable pomace, seeds, or peels) may be considered as non-food side-streams (i.e., waste), when disposed of, or source of food, when utilized. In fact, the Directive 2008/98/EC (2008) distinguishes "waste", intentionally discarded substances/objects (art. 3), from "by-products", resulting from a production process without being its primary aim and certainly employed in a subsequent stage (art. 5). Upcycling of food-grade by-products has emerged as a major strategy to dispose of effluents and take advantage of valuable macro- (i.e., dietary fibre and protein above all) or micro-nutrients (especially antioxidants and bioactive compounds; Plazzotta & Manzocco, 2019). However, re-use of by-products as a food implies appropriate segregation, storage and/or stabilization to prevent microbial proliferation, an often-overlooked aspect in current studies, as well as investigation of possible contaminants (e.g., heavy metals, pesticides, dioxin, PCBs, pathogens and their toxins; Socas-Rodríguez et al., 2021). In the European Union, by-products employable as ingredients must have a solid history of safe use; otherwise, they come under the novel food regulation, being authorized only after safety assessment (Reg. 2015/2283/EU, 2015).

Legumes processing generates high volumes of wastes, encouraging the food industry to adopt novel strategies for their exploitation, although currently they are merely incorporated into traditional formulations or, more rarely, used for protein extraction (Tassoni et al., 2020). Various legume dried by-products (pods, hulls, broken seeds), or their fractions, were successfully experimented for protein or dietary fibre enrichment of bakery products, such as bread (Belghith Fendri, Chaari, Maaloul, et al., 2016; Ni et al., 2020; Niño-Medina et al., 2019), cakes (Belghith Fendri, Chaari, Kallel, et al., 2016), crackers (Mousa et al., 2021) or biscuits (Tiwari et al., 2011).

In recent years, alongside the increasing demand for vegan and vegetarian products, there has been a gradual rise in the demand for functional foods

enriched with bioactive compounds of plant origin, driven by consumers awareness of the close relationship between nutrition and health (Kumar et al., 2017; Willett et al., 2019). However, the deficiency of vitamin B12 intake poses a major risk in strictly vegetable-based diets, naturally devoid of it (Ball, 2006). Moreover, the vast variety of commercial B12 supplements from algae, often containing the inactive pseudo vitamin, contributes to misleading consumers (van den Oever & Mayer, 2022). *In-situ* fermentation by *Propionibacterium freudenreichii* has been recognized as a feasible strategy for vitamin B12 fortification of cereal and legume ingredients (Xie et al., 2021). Although previously enrichment in B12 was tested fermenting cereal side-products (i.e., wheat and rice bran; Xie et al., 2019, 2021), no attempts were made using industrially-generated legume by-products.

Therefore, the aim of this study was to test the industrial by-product of canned green peas as a potential substrate for the synthesis of vitamin B12 by *in-situ* fermentation. The fermented material was subsequently used to fortify bread in vitamin B12.

7.2 Materials and methods

7.2.1 Materials

The by-product of a canned peas production line was sampled on three different days at the Casalasco Società Agricola SPA plant (Gariga di Podenzano, Italy), dried at 55 °C for 24 h in a Venticell 55 ventilated oven (Medcenter Einrichtungen GmbH, Planegg, Germany), milled at 8000 rpm in a ZM200 ultra centrifugal mill (Retsch, Haan, Germany) equipped with a 0.5 mm sieve, and stored at −20 °C until use. An equal mix of three by-product lots was used in the trials.

7.2.2 Fermentation of pea by-product

The procedure to produce the fermented B12 vitamin-rich materials was fine-tuned in preliminary trials with incremental adjustments. A 20% (w/w) ratio of by-product and water was used in the first two trials. The batter was transferred in three 50 mL falcon tubes and inoculated as detailed below. In the second trial, the pH value was also adjusted to 6.0 prior to inoculation. The third preliminary trial, dedicated to establishing the pasteurization conditions, was conducted at 70 °C for 20 min with 15% (w/w) ratio and native pH.

For baking trials, 680 g of a 15% (w/w) batter were prepared mixing the powdered by-product with deionized water. The batter was then neutralized to pH 6.0 with 10 M NaOH, transferred into a 1 L-sized polyethylene bottle, pasteurized at 70 °C for 20 min in a water bath, and inoculated (10%) with *P. freudenreichii* DSM 20271 (Chamlagain et al., 2016; Xie et al., 2019) to achieve an initial cell count of approximately 8.5 log CFU/g. After cells

dispersion by mixing, six 30 g portions of batter were aseptically transferred into 50 mL falcon tubes, leaving 500 g batter in the bottle. The fermentation took place in an orbital shaker (Certomat H, Sartorious, Aubagne, France) set at 200 rpm and 30 °C for 72 h, positioning three tubes horizontally, while the remaining three and the bottle were kept vertically. Two independent batches were fermented as explained above.

7.2.3 Bread making

Refined wheat flour, rapeseed oil, fresh yeast, salt and sugar used in bread-making were acquired from a local supermarket (Helsinki, Finland). The batters fermented in the 1 L bottle were used for baking. Six lots of bread were baked with the straight dough method, following the recipe of Edelmann et al. (2016) with some modifications, as detailed in the Supplementary Table S.16: white bread (Control), a second control with the addition of 20% of non-fermented by-product batter (Control-NF), two lots with 15% enrichment (1a and 2a), one from each of the two independent batches of fermented material, two lots with 20% enrichment (1b and 2b). All the ingredients were mixed in a BE10 mixer (Metos, Instrumentarium Ltd., Kerava, Finland) for 1 min at the lowest speed and then for 4 min at the middle speed. Three 150 g pieces of dough were put in aluminium moulds and proofed for 45 min at 35 °C and 85% relative humidity in a proofing cabinet (Lillnord TopLine, Odder, Denmark). A dough sample was taken after proofing and immediately stored at −20 °C. The breads were baked in a convection oven (Sveba Dahlen, Fristad, Sweden) at 200 °C for 15 min with an initial 15 s-long steam injection. After cooling for 1 h, the breads were weighted. Three slices (one from each bread) were cut from the central part. The slices (25 mm-thick, approximately 30 g) were crushed in a GM200 cutter mill (Retsch, Haan, Germany) at 7000 rpm for 30 s. The crushed breads were packaged in plastic bags and stored at −20 °C until further analyses.

7.2.4 Proximate composition

The moisture content of the by-product, breads and doughs was determined gravimetrically according to the method 925.10 (AOAC, 2000). Proximate composition of by-product was determined according to AOAC (2000): protein by Kjeldahl ($N \times 6.25$) method 920.87; fat by Soxhlet method 920.39C; raw fibre by method 962.09; ash by method 923.03. Results are expressed as g/100 g dry matter (DM).

7.2.5 Microbial counts

Presumptive lactic acid bacteria (LAB), propionic acid bacteria (PAB) and Enterobacteriaceae were enumerated in the pea by-product batter immediately after inoculation (0 h) and post-fermentation (72 h). Ten grams of

batter were weighed, homogenized with 90 mL of sterile 0.9% NaCl solution for 60 s in a stomacher bag using a BagMixer 400 (Interscience, Puycapel, France), and then serially diluted. LAB counts were determined from appropriately diluted samples using Lactobacilli MRS Agar (Neogen, Auchincruive, UK) and the pour-plate method, followed by incubation of plates at 30 °C under microaerobic conditions. For Enterobacteriaceae, Violet Red Bile Glucose Agar (VRBGA, Neogen) was utilized and the plates were incubated at 37 °C for 24–48 h. PAB counts were performed on yeast extract lactate (YEL) agar, employing incubation in anaerobic jars with an oxygen absorbent (Oxoid, Basingstoke, UK) at 30 °C for 5 days. Results are expressed as log CFU/g FM.

7.2.6 pH and organic acids

The pH was measured by a Mettler Toledo (Greifensee, Switzerland) pH meter. To determine the organic acids, 1 g of fermented batter was diluted with 9 mL of MilliQ water (MilliQ Plus system, Millipore Corporation, Bedford, MA, USA) and after centrifugation (6900 *g*) the supernatant was filtered through 0.45 µm PTFE membrane (Millipore, Carrigtwohill Co., Cork, Ireland) into HPLC vials as previously explained (Chamlagain et al., 2018). Succinic, lactic, acetic, propionic and butyric acids were quantified by injecting 60 µL of the extract in an HPLC system equipped with an Hi-Plex H column (300 × 6.5 mm; Agilent, Santa Clara, CA, USA) and a guard column (Hi-Plex H 50 × 7.7 mm; Agilent), thermostated at 40 °C, and coupled to a 996 photodiode array detector (Waters, Milford, MA, USA) and a 2414 refractive index detector (Waters). The mobile phase consisted of 10 mM sulphuric acid in water with a flow rate of 0.5 mL/min. The identification was based on RI and UV detection, while for quantification RI was used with external standard curves of 0.12–40 µg per injection of each analyte. The results are expressed as mg/g fresh matter (FM).

7.2.7 Vitamin B12

The vitamin B12 content was determined in breads, doughs and fermented batters by ultra-high performance liquid chromatography (UHPLC), as previously described (Chamlagain et al., 2015; Wang et al., 2022). Briefly, ca. 2 g of each sample were mixed with 15 mL of extraction buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid, pH 4.5) and 100 µL of sodium cyanide 1% (w/v) in water, boiled for 30 min with occasional shaking and then cooled on ice. Only in the case of dough or bread samples, after rapid cooling, 750 µL of α-amylase solution (20 mg/mL) were added to digest the starch, incubating the samples at 37 °C for 30 min. The supernatant was recovered after centrifugation (6900 *g* 10 min). The pellets were washed with 5 mL of extraction buffer and, after a second centrifugation, the supernatants were combined and the final volume was adjusted to 25 mL with the extraction

buffer. An exact volume of the extract was purified through an Easi-Extract immunoaffinity column (R-Biopharma, Glasgow, UK), recovered with 300 µL of MilliQ water and filtered via PTFE 0.2 µm (Pall, USA) into UHPLC vials. Vitamin B12 was determined employing a Waters UHPLC system (Milford, MA, USA) equipped with a photodiode array detector (set at 361 nm) and an Acquity HSS T3 C18 column (100 × 2.1 mm, 1.8 µm). The mobile phase consisted of a 0.32 mL/min gradient flow of Milli-Q water and acetonitrile, both with the addition of 0.025% trifluoroacetic acid. A multilevel standard curve of cyanocobalamin was used to quantitate B12 (0.4–8 ng/injection). Pseudo vitamin B12 was identified based on its absorption spectrum and retention time (Chamlagain et al., 2015), and quantified as cyanocobalamin. Results are expressed in ng/g FM.

7.2.8 Bread volume and texture analysis

The bread volume profile was acquired using a Volscan Profiler 300 (Stable Micro Systems, Godalming, UK). Crumb texture analysis was performed after one day with a TA.XT2i texture analyser (Stable Micro Systems) according to Sammalisto et al. (2021). 25 mm-thick slices were taken from the centre of breads, removing crusts to obtain 40 × 40 × 25 mm crumb parallelepipeds. The crumb was compressed two times to a 40% deformation at 5 mm/s speed under a 36 mm diameter cylindrical probe using a 10 kg load cell.

7.2.9 Statistical analysis

All analyses were performed in triplicate. Average values and standard deviations were computed using Excel (Microsoft, Redmond, WA, USA). After verifying the normal distribution of data, one-way analysis of variance (ANOVA) was performed with the Statgraphics Centurion 18 statistical software (Statpoint Technologies Inc., Warrenton VA, USA). When significant differences were found ($p \leq 0.05$), Fisher's lowest significant difference (LSD) at 95% significance was computed.

7.3 Results and discussion

7.3.1 By-product characterization

The pea by-product, consisting of pods, leaves, hulls and faulty, broken or spotted seeds, had a moisture content of 77.9 ± 1.0 g/100 g, while protein (24.3 ± 2.2 g/100 g DM), fat (3.9 ± 0.2 g/100 g DM), raw fibre (33.2 ± 1.0 g/100 g DM), ash (3.4 ± 0.5 g/100 g DM) and residual carbohydrates (35.2 ± 2.0 g/100 g DM) constituted the dry matter. These values were consistent considering the average contents in by-product components. In fact, pea pods are constituted by protein (10.8–13.4 g/100 g DM),

fat (1.1–1.3 g/100 g DM), soluble carbohydrates (24.3–26.4 g/100 g DM; mainly glucose and sucrose), starch (3.7–6.8 g/100 g DM), insoluble (35.6–54.5 g/100 g DM) and soluble (4.2–8.3 g/100 g DM) dietary fibre, and ash (6.6–8.1 g/100 g DM; Belghith Fendri, Chaari, Kallel, et al., 2016; Mateos-Aparicio et al., 2010). Instead, pea cotyledons contain 14–31 g/100 g DM of protein, 1–4 g/100 g DM fat, 5–12 g/100 g DM sugars, 30–49 g/100 g DM starch, 10–20 g/100 g insoluble fibre DM, 2–9 g/100 g DM soluble fibre and 2.3–3.7 g/100 g ash (Hall et al., 2017).

7.3.2 Preliminary fermentation trials

The preliminary trials are summarized in Table 7.1. In the first trial, the pH decreased from 5.1 to 3.8 during the fermentation, primarily due to lactic acid abundance (17.15 mg/g) produced by resident lactic acid bacteria pre- and post-inoculum. PAB exhibited no growth, decreasing from the theoretical initial cell concentration of 8.5 log CFU/g to 8.1 after 72 h. This explains the absence of propionic acid and the modest vitamin B12 production (12.5 ng/g). The already low pH of the matrix, a critical factor limiting *Propionibacterium* growth (Xie et al., 2019), prompted pH adjustment to 6, making the lactic acid available in its neutral form—the preferred carbon source of *Propionibacterium*. The beneficial effect of pH control on vitamin synthesis was previously confirmed by Xie et al. (2019) following co-fermentation of non-sterile wheat bran containing resident or inoculated LAB. The second trial resulted in the production of 42.9 ng/g of vitamin B12, but also 76.3 ng/g of pseudo vitamin was observed, likely due to insufficient synthesis of the lower ligand of the active form (i.e., 5,6-dimethylbenzimidazole) attributable to oxygen scarcity (Chamlagain et al., 2018). To address this, the solids ratio was reduced to 15% (w/w) to get a less viscous batter, thereby improving aeration during shaking in the subsequent trials. Heavy presence of contaminants (Enterobacteriaceae and LAB) led to the adoption of a pasteurization stage to ensure safety and improve competitiveness of PAB. In the third preliminary trial, a 20-min pasteurization at 70 °C proved effective in eradicating Enterobacteriaceae, from an initial count of 3.18 ± 1.01 log CFU/g, and further reducing LAB from 5.75 to 3.05 log CFU/g.

7.3.3 Characterization of fermented batters

Table 7.2 and Table 7.3 summarize the results for two independent lots of fermented batter. PAB exhibited nearly a 20-fold growth in horizontally-positioned tubes and in the half-filled 1 L bottle, where shaking was most effective. This improved growth aligns with the observed vitamin B12 contents, ranging from 189 to 211 ng/g FM, equivalent to 1374–1535 ng/g DM. Notably, these B12 levels surpass those reported by Xie et al. (2021) in various matrices from cereals and legumes (51–742 ng/g DM; average: 301). Propionic (4.05–4.52 mg/g FM or 29.5–32.7 mg/g DM) and acetic acid (2.81 mg/g FM or

Table 7.1. Preliminary trials: characterization of fermented batters (72 h) containing 20% of dried pea by-product (Trials 1 and 2) and effect of pasteurization on the microbial count of a batter with 15% by-product (Trial 3).

Trial 1	
pH	3.79 ± 0.01
B12 (ng/g FM)	12.5 ± 1.6
Succinic acid (mg/g FM)	0.32 ± 0.05
Lactic acid (mg/g FM)	17.15 ± 1.35
Acetic acid (mg/g FM)	2.72 ± 0.31
Propionic acid (mg/g FM)	nd
Enterobacteriaceae (log CFU/g)	3.45 ± 0.31
Propionic acid bacteria (log CFU/g)	8.08 ± 0.15
Trial 2 (initial pH adjusted to 6)	
pH	4.1
Pseudo B12 (ng/g FM)	76.3
B12 (ng/g FM)	42.9
Trial 3 (pasteurization)	
Enterobacteriaceae (raw, log CFU/g)	3.18 ± 1.01
Enterobacteriaceae (pasteurized, log CFU/g)	absent
Lactic acid bacteria (raw, log CFU/g)	5.75 ± 0.16
Lactic acid bacteria (pasteurized, log CFU/g)	3.05 ± 0.74

nd, not detected.

20.4 mg/g DM) levels were comparable to those reported by Chamlagain et al. (2018) in fermented barley malt (4.2–4.7 and 3.2–4.0 mg/g FM respectively), and by Xie et al. (2021) in fermented buckwheat bran (27.9–32.8 and 16.2–18.0 mg/g DM). Conversely, vertically shaken tubes exhibited greater variability in vitamin B12 content (87.2–130.8 ng/g FM), with a significant portion in the inactive pseudo form (64.6–88.1 ng/g FM). This underscores the role of sufficient oxygen availability in the synthesis of the lower ligand, DMBI. Furthermore, only in vertically-shaken tubes butyric acid (4.99 mg/g FM) and some extra acetic acid were detected, possibly due to sporulating obligate anaerobes contaminants. In fact, the higher oxygen availability appeared to inhibit them, aligning with the known fermentation patterns of contaminants like *Clostridium butyricum* and *C. tyrobutyricum*, recognized for starch, sugar, and lactate fermentation, producing butyrate and acetate (Ljungdahl et al., 1989).

7.3.4 Characterization of breads and doughs

The vitamin B12 content, presented in Table 7.4, ranged from 32.0–39.7 to 51.1–52.3 ng/g FM in 15% and 20%-enriched breads, respectively, with consistent results in the two batches. The average levels of B12 in the doughs ranged from 51.7 to 87.1 ng/g DM and the analysis of variance did not

Table 7.2. Average values (mean ± standard deviation; $n = 3$) of propionic acid bacteria (PAB), lactic acid bacteria (LAB) and vitamin B12 amount in pea by-product batters before (0 h) and after (72 h) fermentation using different types of shaking.

Batch	Time (h)	Shaking	PAB	LAB	pseudo B12	B12
			(log CFU/g)		(ng/g FM)	
1	0		8.64 ± 0.09	3.13 ± 0.12		
	72	Horizontal (tubes)	9.87 ± 0.11^{a}	4.38 ± 0.35^{b}	nd	189.4 ± 17.2^{a}
	72	Vertical (bottle)	9.93 ± 0.04^{a}	4.86 ± 0.20^{ab}	2.8 ± 0.3^{c}	211.3 ± 22.9^{a}
	72	Vertical (tubes)	9.57 ± 0.16^{b}	3.09 ± 0.35^{c}	88.1 ± 4.1^{a}	130.8 ± 6.5^{b}
2	0		8.63 ± 0.04	3.76 ± 0.07		
	72	Horizontal (tubes)	9.94 ± 0.05^{a}	5.46 ± 0.29^{a}	nd	192.8 ± 25.0^{a}
	72	Vertical (bottle)	9.98 ± 0.01^{a}	5.31 ± 0.51^{a}	6.1 ± 0.8^{c}	206.5 ± 25.8^{a}
	72	Vertical (tubes)	9.67 ± 0.12^{b}	3.12 ± 0.16^{c}	64.6 ± 4.3^{b}	87.2 ± 4.6^{c}

nd, not detected. Enterobacteriaceae were absent in all samples. Different letters in the same column indicate significant differences between fermented samples according to the LSD test ($p < 0.05$).

Table 7.3. Average values (mean ± standard deviation; $n = 3$) of pH values and organic acids content (mg/g FM) in fermented (72 h) pea by-product batters.

Batch	Shaking	pH	Organic acids (mg/g FM)				
			Succinic	Acetic	Propionic	Butyric	Ratio C_3:C_2
1	Horizontal (tubes)	4.81 ± 0.02^{b}	1.85 ± 0.25^{b}	2.81 ± 0.03^{b}	4.52 ± 0.04^{a}	nd	1.61 ± 0.03^{a}
	Vertical (bottle)	4.79 ± 0.08^{b}	1.67 ± 0.01^{b}	2.81 ± 0.01^{b}	4.05 ± 0.01^{b}	nd	1.44 ± 0.01^{b}
	Vertical (tubes)	4.55 ± 0.01^{c}	1.83 ± 0.15^{b}	4.69 ± 0.15^{a}	3.75 ± 0.21^{c}	4.97 ± 0.03	0.80 ± 0.07^{c}
2	Horizontal (tubes)	4.83 ± 0.02^{b}	2.44 ± 0.07^{a}	2.80 ± 0.01^{b}	4.50 ± 0.04^{a}	nd	1.60 ± 0.02^{a}
	Vertical (bottle)	4.90 ± 0.01^{a}	2.61 ± 0.01^{a}	2.78 ± 0.01^{b}	4.03 ± 0.01^{b}	nd	1.45 ± 0.01^{b}
	Vertical (tubes)	4.57 ± 0.02^{c}	0.33 ± 0.01^{c}	4.81 ± 0.15^{a}	3.68 ± 0.09^{c}	5.01 ± 0.17	0.77 ± 0.04^{c}

nd, not detected. Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

highlight significant differences with the corresponding breads, confirming that no loss of vitamin occurred due to baking. In fact, B12 displays higher stability when is produced *in-situ* rather than supplemented, and its greater retention is associated with the lower acidity typical of straight-dough breadmaking compared to sourdough (Edelmann et al., 2016). Similar B12 values were previously published for six wheat breads supplemented with rice bran or soybean flour obtained by co-fermentation (29.9–48.7 ng/g FM; Wang et al., 2022). Lower content of vitamin (14 ng/g FM, but starting from 8.9) was instead reported by Zhang et al. (2023) for a whole meal bread obtained by the sourdough method with a co-colture of *L. lactis* and *P. freudenreichii*. From 40 to 70 g of the breads in the present study would provide enough vitamin according to the recommend daily intake (2.4 µg per day; National Academy of Medicine, 1998).

Table 7.4. Average values (mean ± standard deviation; $n = 3$) of vitamin B12 content in enriched doughs (after proofing) and breads.

Sample	Enrichment	B12 (ng/g FM)		B12 (ng/g DM)	
	(%)	Dough	Bread	Dough	Bread
1a	15	32.8 ± 5.4^{b}	39.7 ± 0.5^{b}	59.9 ± 10.0^{b}	63.8 ± 0.7^{b}
2a	15	28.3 ± 1.9^{b}	32.0 ± 4.1^{b}	51.7 ± 3.5^{b}	51.3 ± 6.6^{b}
1b	20	36.6 ± 6.4^{ab}	52.3 ± 6.3^{a}	66.6 ± 11.6^{ab}	83.7 ± 10.0^{a}
2b	20	37.3 ± 9.6^{a}	51.1 ± 3.5^{a}	87.1 ± 15.7^{a}	81.2 ± 5.6^{a}

Different letters indicate significant differences between samples in the same column according to the LSD test ($p \leq 0.05$).

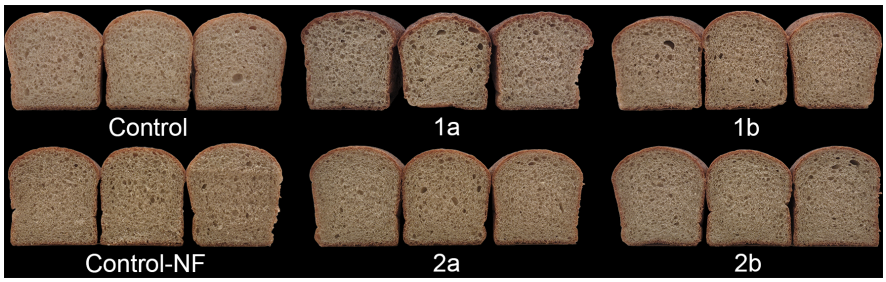


Figure 7.1. Control and enriched breads

The Figure 7.1 shows the control and enriched breads. The addition of fermented materials had minor or negligible impact on bread volume. In fact, as reported in Table 7.5, significant but limited decreases in specific volume were registered, especially with the highest enrichment percentage, also resulting in superior crumb hardness. The addition of non-fermented by-product caused a decrease in hardness, as previously noted by Belghith Fendri,

Chaari, Maaloul, et al. (2016) with very similar values: from 15.2 N in the control to 12.8–13.8 N in breads enriched with 1% of fibre extracted from pea or faba bean pods. The specific volumes (2.69–2.94 mL/g) were lower than those described by Wang et al. (2022; 3.32–4.37 mL/g) and Belghith Fendri, Chaari, Maaloul, et al. (2016; 3.00–3.59 mL/g), but included in the intervals reported by Kasprzak and Rzedzicki (2010) for pea seed coat-enriched breads (2.47–3.75 mL/g). The baking loss ranged from 12.2% to 13.3% but did not show a clear pattern. Based on subjective tasting, the addition of fermented materials did not affect the overall taste of the bread but resulted in a barely perceivable sandy texture.

Table 7.5. Average values (mean ± standard deviation; $n = 3$) for baking loss, specific volume and texture analysis in control and enriched breads.

Sample	Baking loss (%)	Specific volume (mL/g)	Hardness (N)	Cohesiveness	Gumminess (N)
Control	13.3 ± 0.5^{a}	2.94 ± 0.03^{a}	14.1 ± 1.1^{bc}	0.76 ± 0.01^{a}	10.6 ± 0.6^{bc}
Control-NF	12.2 ± 0.7^{d}	2.89 ± 0.03^{b}	11.9 ± 0.8^{c}	0.72 ± 0.01^{c}	8.5 ± 0.5^{d}
1a	12.3 ± 0.3^{cd}	2.89 ± 0.03^{b}	16.2 ± 1.6^{ab}	0.75 ± 0.01^{a}	12.1 ± 1.3^{ab}
2a	13.0 ± 0.2^{ab}	2.82 ± 0.02^{c}	14.1 ± 1.1^{bc}	0.74 ± 0.01^{ab}	10.4 ± 0.8^{c}
1b	12.5 ± 0.2^{bcd}	2.73 ± 0.03^{d}	17.8 ± 2.0^{a}	0.71 ± 0.02^{c}	12.7 ± 1.2^{a}
2b	13.0 ± 0.2^{abc}	2.69 ± 0.01^{d}	16.3 ± 0.6^{ab}	0.72 ± 0.01^{bc}	11.8 ± 0.6^{abc}

Different letters indicate significant differences between samples in the same column according to the LSD test ($p < 0.05$).

7.4 Conclusions

The by-product of industrial canned green peas processing was successfully utilized to produce a vitamin B12-rich batter (1374–1535 ng/g DM). This high vitamin content led to remarkable values in the fortified breads (32.0–52.3 ng/g FM) with relatively moderate additions, and with minimal impact on volume and texture. From 40 to 70 g of enriched bread supply the recommended dietary intake of vitamin B12, addressing the nutritional deficiencies typical of strictly plant-based diets. This study emphasizes the importance of upcycling by-products, which can contribute to the reduction of food waste while enhancing the nutritional profile of foods. The high level of microbial contaminants, especially lactic acid bacteria, imposed a pasteurization stage, stressing the necessity to segregate the by-products when they have to be treated as food-grade material. These findings encourage further exploitation of legumes by-products, because as the demand for sustainable and nutritious foods grows, the strategies that create value from disposable leftovers collect interest and favour from industries and consumers.

References

AOAC. (2000). *Official Methods of Analysis of AOAC International, 17th ed.* Association of Official Analytical Chemists International.

Ball, G. F. M. (2006). *Vitamins in foods: Analysis, bioavailability, and stability.* Taylor & Francis.

Belghith Fendri, L., Chaari, F., Kallel, F., Zouari-Ellouzi, S., Ghorbel, R., Besbes, S., Ellouz-Chaabouni, S., & Ghribi-Aydi, D. (2016). Pea and broad bean pods as a natural source of dietary fiber: The impact on texture and sensory properties of cake. *J Food Sci*, *81*, C2360–C2366. https://doi.org/10.1111/1750-3841.13448

Belghith Fendri, L., Chaari, F., Maaloul, M., Kallel, F., Abdelkafi, L., Ellouz-Chaabouni, S., & Ghribi-Aydi, D. (2016). Wheat bread enrichment by pea and broad bean pods fibers: Effect on dough rheology and bread quality. *LWT - Food Sci Technol*, *73*, 584–591. https://doi.org/10.1016/J.LWT.2016.06.070

Chamlagain, B., Deptula, P., Edelmann, M., Kariluoto, S., Grattepanche, F., Lacroix, C., Varmanen, P., & Piironen, V. (2016). Effect of the lower ligand precursors on vitamin B12 production by food-grade Propionibacteria. *LWT - Food Sci Technol*, *72*, 117–124. https://doi.org/10.1016/j.lwt.2016.04.023

Chamlagain, B., Edelmann, M., Kariluoto, S., Ollilainen, V., & Piironen, V. (2015). Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B12 in cells of *Propionibacterium* and fermented cereal matrices. *Food Chem*, *166*, 630–638. https://doi.org/10.1016/J.FOODCHEM.2014.06.068

Chamlagain, B., Sugito, T. A., Deptula, P., Edelmann, M., Kariluoto, S., Varmanen, P., & Piironen, V. (2018). *In situ* production of active vitamin B12 in cereal matrices using *Propionibacterium freudenreichii*. *Food Sci Nutr*, *6*, 67–76. https://doi.org/10.1002/FSN3.528

Directive 2008/98/EC of the European Parliament and of the Council of 19 november 2008 on waste and repealing certain Directives (2008).

Edelmann, M., Chamlagain, B., Santin, M., Kariluoto, S., & Piironen, V. (2016). Stability of added and *in situ*-produced vitamin B12 in breadmaking. *Food Chem*, *204*, 21–28. https://doi.org/10.1016/J.FOODCHEM.2016.02.071

FAO. (2011). *Global food losses and food waste — Extent, causes and prevention*. Rome, IT. https://www.fao.org/3/i2697e/i2697e.pdf

FAO. (2019). *The state of food and agriculture 2019. Moving forward on food loss and waste reduction*. Rome, IT. http://www.fao.org/3/ca6030en/ca6030en.pdf

Hall, C., Hillen, C., & Garden Robinson, J. (2017). Composition, nutritional value, and health benefits of pulses. *Cereal Chem*, *94*, 11–31. https://doi.org/10.1094/CCHEM-03-16-0069-FI

Kasprzak, M., & Rzedzicki, Z. (2010). Effect of pea seed coat admixture on physical properties and chemical composition of bread. *Int Agrophys*, *24*, 149–156.

Kumar, P., Chatli, M. K., Mehta, N., Singh, P., Malav, O. P., & Verma, A. K. (2017). Meat analogues: Health promising sustainable meat substitutes. *Crit Rev Food Sci Nutr*, *57*, 923–932.

Ljungdahl, L. G., Hugenholtz, J., & Wiege, J. (1989). Acetogenic and acid-producing Clostridia. In N. P. Minton & D. J. Clarke (Eds.), *Clostridia* (pp. 145–192). Springer Science Business Media.

Mateos-Aparicio, I., Redondo-Cuenca, A., Villanueva-Suárez, M. J., Zapata-Revilla, M. A., & Tenorio-Sanz, M. D. (2010). Pea pod, broad bean pod and okara, potential sources of functional compounds. *LWT - Food Sci Technol*, *43*, 1467–1470. https://doi.org/10.1016/J.LWT.2010.05.008

Mousa, M. M. H., El-Magd, M. A., Ghamry, H. I., Alshahrani, M. Y., El-Wakeil, N. H. M., Hammad, E. M., & Asker, G. A. H. (2021). Pea peels as a value-added food ingredient for snack crackers and dry soup. *Sci Rep*, *11*, 1–11. https://doi.org/10.1038/s41598-021-02202-5

National Academy of Medicine. (1998). *Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline*. National Academies Press. https://doi.org/10.17226/6015

Ni, Q., Ranawana, V., Hayes, H. E., Hayward, N. J., Stead, D., & Raikos, V. (2020). Addition of broad bean hull to wheat flour for the development of high-fiber bread: Effects on physical and nutritional properties. *Foods*, *9*, 1192. https://doi.org/10.3390/FOODS9091192

Niño-Medina, G., Muy-Rangel, D., De La Garza, A. L., Rubio-Carrasco, W., Pérez-Meza, B., Araujo-Chapa, A. P., Gutiérrez-Álvarez, K. A., & Urías-Orona, V. (2019). Dietary fiber from chickpea (*Cicer arietinum*) and soybean (*Glycine max*) husk byproducts as baking additives: Functional and nutritional properties. *Molecules*, *24*, 991. https://doi.org/10.3390/MOLECULES24050991

Plazzotta, S., & Manzocco, L. (2019). Food waste valorization. In C. M. Galanakis (Ed.), *Saving food: Production, supply chain, food waste and food consumption* (pp. 279–313). Academic Press. https://doi.org/10.1016/B978-0-12-815357-4.00010-9

Reg. 2015/2283/EU of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (2015).

Sammalisto, S., Laitinen, M., & Sontag-Strohm, T. (2021). Baking quality assessment of twenty whole grain oat cultivar samples. *Foods*, *10*, 2461. https://doi.org/10.3390/FOODS10102461

Socas-Rodríguez, B., Álvarez-Rivera, G., Valdés, A., Ibáñez, E., & Cifuentes, A. (2021). Food by-products and food wastes: Are they safe enough for their valorization? *Trends Food Sci Tech*, *114*, 133–147. https://doi.org/10.1016/j.tifs.2021.05.002

Tassoni, A., Tedeschi, T., Zurlini, C., Cigognini, I. M., Petrusan, J.-I., Rodríguez, Ó., Neri, S., Celli, A., Sisti, L., Cinelli, P., Signori, F., Tsatsos, G., Bondi, M., Verstringe, S., Bruggerman, G., & Corvini, P. F. X. (2020). State-of-the-art production chains for peas, beans and chickpeas—Valorization of agro-industrial residues and applications of derived extracts. *Molecules*, *25*, 1383. https://doi.org/10.3390/molecules25061383

Tiwari, B. K., Brennan, C. S., Jaganmohan, R., Surabi, A., & Alagusundaram, K. (2011). Utilisation of pigeon pea (*Cajanus cajan* L) byproducts in biscuit manufacture. *LWT - Food Sci Technol*, *44*, 1533–1537. https://doi.org/10.1016/J.LWT.2011.01.018

UNEP. (2021). *Food waste index. Report 2021*. United Nations Environment Programme. Nairobi, KE. https://wedocs.unep.org/bitstream/handle/20.500.11822/35280/FoodWaste.pdf

van den Oever, S. P., & Mayer, H. K. (2022). Biologically active or just "pseudo"-vitamin B12 as predominant form in algae-based nutritional supplements? *J Food Compos Anal*, *109*, 104464. https://doi.org/10.1016/J.JFCA.2022.104464

Wang, Y., Xie, C., Pulkkinen, M., Edelmann, M., Chamlagain, B., Coda, R., Sandell, M., Piironen, V., Maina, N. E., & Katina, K. (2022). *In situ* production of vitamin B12 and dextran in soya flour and rice bran: A tool to improve flavour and texture of B12-fortified bread. *LWT - Food Sci Technol*, *161*, 113407. https://doi.org/10.1016/J.LWT.2022.113407

Willett, W., Rockström, J., Loken, B., Springmann, M., Lang, T., Vermeulen, S., Garnett, T., Tilman, D., DeClerck, F., Wood, A., Jonell, M., Clark, M., Gordon, L. J., Fanzo, J., Hawkes, C., Zurayk, R., Rivera, J. A., De Vries, W., Majele Sibanda, L., … Murray, C. J. L. (2019). Food in the Anthropocene: The EAT–Lancet Commission on healthy diets from sustainable food systems. *Lancet*, *393*, 447–492. https://doi.org/10.1016/S0140-6736(18)31788-4

Xie, C., Coda, R., Chamlagain, B., Edelmann, M., Varmanen, P., Piironen, V., & Katina, K. (2021). Fermentation of cereal, pseudo-cereal and legume materials with *Propionibacterium freudenreichii* and *Levilactobacillus brevis* for vitamin B12 fortification. *LWT - Food Sci Technol*, *137*, 110431. https://doi.org/10.1016/J.LWT.2020.110431

Xie, C., Coda, R., Chamlagain, B., Varmanen, P., Piironen, V., & Katina, K. (2019). Co-fermentation of *Propionibacterium freudenreichii* and *Lactobacillus brevis* in wheat bran for *in situ* production of vitamin B12. *Front Microbiol*, *10*, 456519. https://doi.org/10.3389/FMICB.2019.01541

Zhang, Y., Momoisea, P., Lin, Q., Liang, J., Burrow, K., & Serventi, L. (2023). Evaluation of sensory and physicochemical characteristics of vitamin B12 enriched whole-meal sourdough bread fermented with *Propionibacterium freudenreichii*. *Sustainability*, *15*, 8157. https://doi.org/10.3390/SU15108157

8

Conclusions and future perspectives

The major objective of this PhD thesis was to develop legume-derived ingredients and foods enriched with bioactive compounds.

Lupin was extensively studied, given its excellent nutritional composition and sustainable crop nature. Andean lupin stood out as exceptionally rich in free phenolic compounds, even after the necessary removal of alkaloids. A relationship between phenolics and FT-NIR spectral bands was established, paving the way for fast, reliable, and non-destructive characterization. An optimized treatment to remove bitter alkaloids, limiting water utilisation, time consumption and loss of water-soluble antioxidants, was proposed. Additionally, the gap of knowledge about conjugated and bound phenolic fractions was partially filled. Germination produced a considerable increase in carotenoid and vitamin E contents, representing a simple and effective treatment to improve lupin nutritional value. Overall, the results support boosting lupin diffusion and consumption, especially among vulnerable populations practicing subsistence agriculture with inadequate essential nutrients intake.

Two strategies for recycling an industrially-generated by-product from the manufacturing of canned green peas were proposed. The first optimized the conditions of ultrasound-assisted protein extraction, increasing three-fold the recovery and reducing four-fold the treatment duration. Secondly, the by-product was fermented, achieving an outstanding amount of vitamin B12 and resulting in remarkably high B12 contents in the fortified bread. It was emphasized that prompt use or stabilization of the by-product is crucial to avoid bacterial growth and acidification. These results stress the importance and feasibility of by-product recycling, supporting food industries headed towards sustainable production, waste reduction, and complete exploitation of raw materials.

Future studies should prioritize the integration of the proposed ingredients into food preparations, investigating the bioaccessibility of nutrients by *in*

vitro digestion, and their potential impact on health and nutritional status by cell culture. Specifically, combining germination and the proposed debittering method could ease the production of antioxidant-rich lupin flour, to be integrated into the preparation of baking goods. Furthermore, the consumers acceptability of the mentioned foods and ingredients should be appraised.

Supplementary materials

Supplementary Table S.1. *Lupinus mutabilis* ecotypes and other *Lupinus* species tested, and their area of origin.

	Code	Department	Area
Lupinus mutabilis			
Cajamarca	CAJ	Cajamarca	A
Congona	CON	Cajamarca	A
Suchubamba	SUC	Cajamarca	A
Altagracia	ALG	La Libertad	B
Paton grande	PAG	La Libertad	B
Andenes 80	AND80	Ancash	C
Chacas	CHA	Ancash	C
Cholo fuerte	CHF	Ancash	C
Huallanca	HUAL	Ancash	C
Pallasca	PAL	Ancash	C
Tauribamba Sihuas 1 BN	TS1BN	Ancash	C
Tauribamba Sihuas 3 N	TS3N	Ancash	C
Vicos	VIC	Ancash	C
Yana Tarwi	YAT	Ancash	C
Churibamba	CHU	Huánuco	D
Huanuco 1 BN	HU1BN	Huánuco	D
Huanuco 1	HU1	Huánuco	D
Huanuco 2	HU2	Huánuco	D
CD Junin 7-2	CDJ7-2	Junín	E
CD Junin 10-2	CDJ10-2	Junín	E
H6 INIA BN	H6BN	Junín	E
H6 INIA BP	H6BP	Junín	E
Moteado beige	MOB	Junín	E
Yanamuclo 008-1	YA1	Junín	E
Yanamuclo 008-3	YA3	Junín	E
Yanamuclo PLGO	YAPL	Junín	E
Lircay	LIR	Huancavelica	F
Andenes INIA	ANDIN	Cusco	G
Comp. blanco semiprecoz	CBSP	Cusco	G
SGC 22	SGC22	Cusco	G
Cheje Copani N	CHC	Puno	H
Puno 2 Blanquita	PU2B	Puno	H
Yunguyo	YUN	Puno	H
Controls			
L. albus - Dulce 7	albus D	Junín	
L. albus - Ares	albus A	Italy	
L. albus - Multitalia	albus M	Italy	
L. angustifolius - Boregine	angust	Italy	
L. luteus- Percoz	luteus	Italy	

Supplementary Table S.2. Free phenolics content (mean ± standard deviation; mg/kg DM) in debittered seeds of *Lupinus* ecotypes.

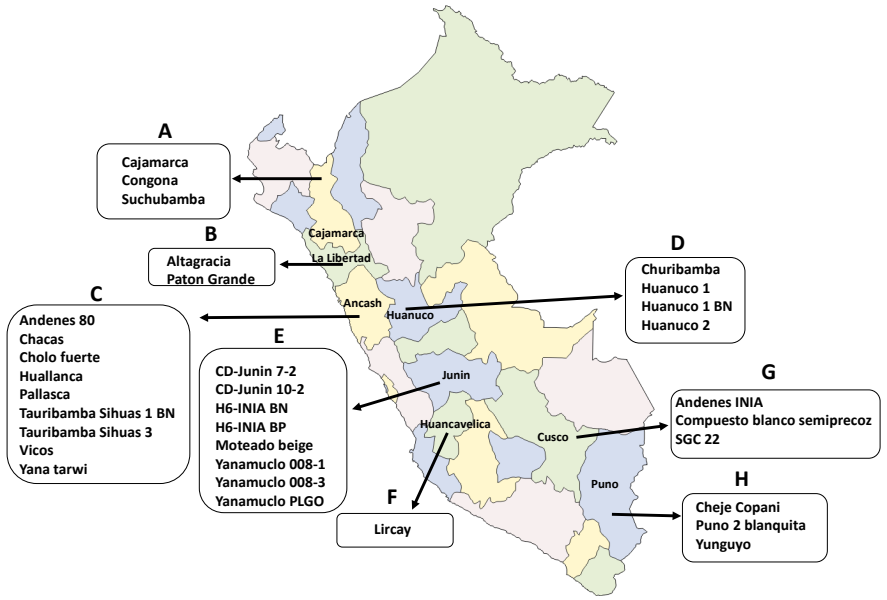
Code	Genistein	Genistein der	Apigenin der	Catechin der	Diosmin der	Naringenin der	Cinnamic acid der
L. mutabilis							
CAJ	135.0 ± 8.3	497.8 ± 36.6	20.1 ± 3.1	4.5 ± 0.9	84.4 ± 13.3	2.8 ± 0.2	1.7 ± 0.1
CON	56.2 ± 3.6	279.2 ± 5.3	3.9 ± 0.1	nd	43.0 ± 1.7	2.6 ± 0.1	5.8 ± 0.1
SUC	72.3 ± 0.7	274.2 ± 9.9	9.2 ± 0.2	nd	138.2 ± 5.9	6.9 ± 0.1	5.0 ± 0.1
ALG	166.8 ± 2.9	456.2 ± 4.7	21.6 ± 0.7	nd	69.5 ± 9.2	7.2 ± 0.1	1.0 ± 0.1
PAG	101.5 ± 1.6	430.7 ± 8.1	15.1 ± 0.1	14.7 ± 0.9	44.0 ± 0.8	4.1 ± 0.3	nd
AND80	203.0 ± 33.1	527.0 ± 97.2	28.0 ± 3.3	nd	71.9 ± 7.0	11.2 ± 0.2	2.6 ± 0.1
CHA	314.4 ± 6.7	946.8 ± 13.3	3.3 ± 0.1	nd	7.2 ± 0.3	8.0 ± 0.4	10.9 ± 0.1
CHF	93.3 ± 3.6	316.1 ± 4.5	11.9 ± 1.1	6.5 ± 0.6	70.3 ± 6.1	7.6 ± 0.2	1.3 ± 0.1
HUAL	118.7 ± 11.5	409.7 ± 35.5	17.4 ± 0.3	7.1 ± 0.4	95.0 ± 5.8	3.0 ± 0.4	2.5 ± 0.1
PAL	50.6 ± 2.1	356.9 ± 0.4	5.0 ± 0.1	nd	42.5 ± 0.8	9.5 ± 0.1	6.6 ± 0.2
TS1BN	167.4 ± 6.5	401.7 ± 5.5	2.7 ± 0.1	nd	25.0 ± 0.1	7.4 ± 0.2	7.9 ± 0.1
TS3N	160.9 ± 0.3	502.0 ± 19.7	3.0 ± 0.1	nd	16.6 ± 0.5	5.2 ± 0.1	5.4 ± 0.1
VIC	86.9 ± 1.8	296.2 ± 11.6	4.4 ± 0.1	nd	8.8 ± 0.5	11.6 ± 0.3	11.1 ± 0.1
YAT	68.1 ± 1.8	376.8 ± 12.5	5.9 ± 0.1	nd	62.3 ± 4.5	5.0 ± 0.1	10.3 ± 0.1
CHU	39.6 ± 0.9	219.6 ± 3.8	6.5 ± 0.3	nd	25.4 ± 0.7	3.8 ± 0.2	nd
HU1BN	352.1 ± 4.0	430.8 ± 5.0	23.9 ± 0.6	5.5 ± 0.2	168.2 ± 17.6	8.2 ± 0.1	1.7 ± 0.3
HU1	96.2 ± 0.9	383.5 ± 7.7	7.0 ± 0.2	nd	130.3 ± 1.2	3.1 ± 0.1	nd
HU2	334.6 ± 14.3	762.3 ± 32.0	17.2 ± 2.1	8.8 ± 1.6	99.5 ± 10.2	4.0 ± 0.3	nd
CDJ7-2	38.0 ± 2.1	257.6 ± 4.0	3.2 ± 0.1	nd	68.4 ± 0.5	3.7 ± 0.1	11.6 ± 0.1
CDJ10-2	57.3 ± 5.0	557.3 ± 0.1	7.3 ± 0.3	nd	30.2 ± 0.8	3.4 ± 0.2	nd
H6BN	110.0 ± 11.3	483.0 ± 37.6	15.2 ± 0.6	11.2 ± 0.2	55.8 ± 5.2	5.2 ± 0.4	2.1 ± 0.1
H6BP	196.4 ± 0.5	1062.8 ± 24.9	22.9 ± 0.2	11.8 ± 2.0	40.6 ± 1.5	5.5 ± 1.2	2.9 ± 0.6

der, derivative; nd, not detected.

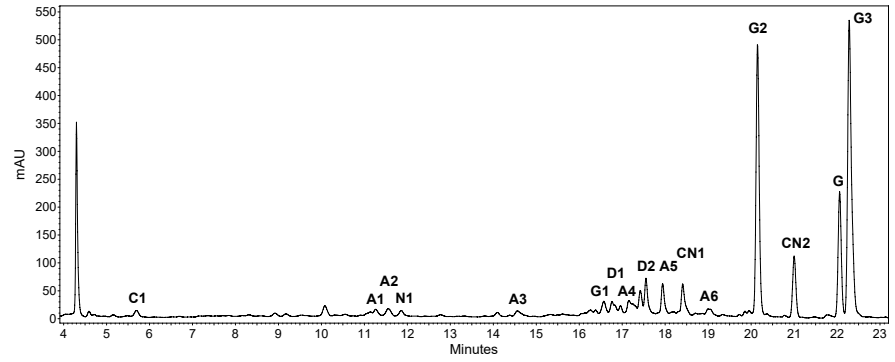
continued on the next page

Code	Genistein	Genistein der	Apigenin der	Catechin der	Diosmin der	Naringenin der	Cinnamic acid der
L. mutabilis							
MOB	225.1 ± 12.0	857.6 ± 40.6	24.9 ± 2.3	19.2 ± 3.3	79.3 ± 6.5	2.5 ± 0.1	nd
YA1	142.4 ± 5.7	438.4 ± 23.7	10.9 ± 2.4	17.1 ± 2.6	67.2 ± 6.6	5.1 ± 0.1	2.5 ± 0.1
YA3	110.5 ± 4.0	354.8 ± 6.1	13.9 ± 0.1	16.2 ± 0.4	71.2 ± 2.0	5.1 ± 0.1	nd
YAPL	61.2 ± 1.7	314.4 ± 13.0	14.7 ± 0.1	16.5 ± 0.4	78.5 ± 6.9	5.2 ± 0.3	3.7 ± 0.6
LIR	293.2 ± 14.2	780.6 ± 31.3	26.5 ± 2.9	nd	64.7 ± 3.3	3.9 ± 0.1	1.7 ± 0.1
ANDIN	132.2 ± 2.7	480.8 ± 5.7	23.2 ± 0.3	nd	72.8 ± 2.6	5.5 ± 0.1	nd
CBSP	159.3 ± 21.0	616.6 ± 83.8	23.8 ± 1.6	8.2 ± 0.2	58.9 ± 4.4	5.2 ± 0.2	1.9 ± 0.1
SGC22	91.0 ± 1.1	369.4 ± 2.6	3.0 ± 0.1	nd	47.0 ± 1.1	8.1 ± 0.1	11.9 ± 0.1
CHC	72.4 ± 2.7	282.9 ± 5.4	3.4 ± 0.1	nd	89.1 ± 2.9	nd	nd
PU2B	97.8 ± 6.9	285.8 ± 10.0	17.6 ± 1.8	36.5 ± 2.0	64.4 ± 4.8	4.4 ± 0.3	1.6 ± 0.1
YUN	76.2 ± 3.1	325.2 ± 2.2	13.1 ± 0.2	17.9 ± 0.4	44.6 ± 1.9	3.1 ± 0.1	1.5 ± 0.1
Controls							
albus D	13.3 ± 3.1	4.9 ± 0.8	13.1 ± 0.2	nd	nd	nd	nd
albus D	nd	nd	6.8 ± 0.3	nd	nd	nd	nd
albus M	nd	nd	17.3 ± 0.8	nd	nd	nd	nd
angust	nd	nd	14.8 ± 2.1	nd	nd	nd	nd
luteus	40.4 ± 5.5	230.0 ± 19.3	27.8 ± 0.6	nd	98.4 ± 7.7	2.0 ± 0.1	nd

der, derivative; nd, not detected.



Supplementary Figure S.1. Areas of origin of the 33 *Lupinus mutabilis* ecotypes analysed.



Supplementary Figure S.2. HPLC chromatogram at 280 nm of the free phenolics in H6 INIA BP ecotype of *Lupinus mutabilis*. A1–A6, apigenin derivatives; C1, catechin derivative; CN1–CN2, cinammic acid derivatives; D1–D2, diosmin derivatives; G, genistein; G1–G3, genistein derivatives; N1, naringenin derivative. Apigenin derivatives are read at 320 nm while diosmin derivatives at 360 nm.

Supplementary Table S.3. Calibration curves equations, coefficients of determination (R^2), ranges, limits of detection (LOD) and limit of quantification (LOQ) for carotenoids and tocopherols.

Compound	Calibration curve	R^2	Range (mg/L)	LOD (mg/L)	LOQ (mg/L)
β-carotene	$y = 357229.0x + 1008.8$	0.9991	0.15–1.50	0.049	0.164
β-cryptoxanthin	$y = 1339215.6x - 1085.7$	0.9994	0.02–0.13	0.003	0.011
Lutein	$y = 775488.9x + 28912.7$	0.9997	0.05–3.00	0.057	0.190
Zeaxanthin	$y = 871271.0x + 885.9$	0.9999	0.05–1.03	0.012	0.041
α-tocopherol	$y = 50933.3x - 2635.4$	1.0000	0.44–109.73	0.377	1.257
β-tocopherol	$y = 23704.4x - 2080.2$	0.9999	0.38–72.20	0.772	2.573
γ-tocopherol	$y = 33671.0x - 5710.1$	0.9998	0.20–19.34	0.236	0.788
δ-tocopherol	$y = 33218.0x - 2243.4$	0.9992	0.05–9.35	0.304	1.012

Supplementary Table S.4. ANOVA and LSD test (mean ± standard error) of alkaloid content (g/kg DM) in *Lupinus albus* seeds after debittering applying different treatments (without and with ultrasound), solvents and soaking times.

ANOVA		
Factor	df	Mean square
Treatment (T)	1	0.006
Solvent (S)	2	57.266***
Time (t)	1	5.980***
T×S	2	0.003
T×t	1	0.004
S×t	2	0.729***
Error	26	0.11
Test LSD		
Treatment		
Without ultrasound	2.65 ± 2.35	
With ultrasound	2.68 ± 2.37	
Solvent		
Water	5.73 ± 0.90^{a}	
NaCl 1%	1.45 ± 0.51^{b}	
Citric acid 1%	0.80 ± 0.24^{c}	
Time		
28.5 h	3.16 ± 2.53^{a}	
45.0 h	2.16 ± 2.04^{b}	

df, degrees of freedom; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; different letters indicate significant differences ($p \leq 0.05$).

Supplementary Table S.5. Analysis of variance (mean square) and LSD test of the alkaloid content (g/kg DM; mean ± standard error) of *Lupinus albus* seeds after debittering without ultrasound, and with different solvents.

ANOVA		
Factor	df	Mean square
Time (t)	2	0.750***
Solvent (S)	4	14.260***
t×S	8	0.092***
Error	30	0.010
Test LSD		
Time		
45 h	1.81 ± 0.48^{a}	
57 h	1.50 ± 0.50^{b}	
69 h	1.26 ± 0.40^{c}	
Solvent		
Water	4.23 ± 0.20^{a}	
NaCl 0.5%	1.29 ± 0.15^{b}	
NaCl 1%	0.85 ± 0.07^{c}	
Citric acid 0.5%	0.84 ± 0.09^{c}	
Citric acid 1%	0.41 ± 0.05^{d}	

df, degrees of freedom; ***, $p \leq 0.001$; different letters indicate significant differences ($p \leq 0.05$).

Supplementary Table S.6. Analysis of variance (mean square) and LSD test of carotenoids and tocopherols content (mg/kg DM; mean ± standard error) of *Lupinus albus* seeds (Lot 1) after debittering without and with ultrasound (US), and employing different solvents.

	ANOVA				LSD Treatment		LSD Solvent	
	Treatment (T)	Solvent (S)	T × S	Error	Without US	With US	NaCl 1%	Citric acid 1%
df	1	1	1	4				
Carotenoids								
(α+β)-carotene	0.13**	0.054*	0.097**	0.004	2.46 ± 0.04^{a}	2.21 ± 0.11^{b}	2.42 ± 0.03^{a}	2.26 ± 0.14^{b}
β-cryptoxanthin	0.0028*	0.26***	0.0036*	0.00021	0.26 ± 0.09^{b}	0.30 ± 0.12^{a}	0.11 ± 0.01^{b}	0.46 ± 0.02^{a}
Lutein	1.30*	2.4**	0.34	0.073	8.39 ± 0.25^{a}	7.59 ± 0.44^{b}	8.54 ± 0.18^{a}	7.44 ± 0.36^{b}
Zeaxanthin	0.030*	0.21**	0.025*	0.0032	1.84 ± 0.07^{a}	1.72 ± 0.13^{b}	1.95 ± 0.03^{a}	1.62 ± 0.07^{b}
Total carotenoids	2.6*	3.1*	0.99	0.15	12.97 ± 0.27^{a}	11.83 ± 0.56^{b}	13.02 ± 0.23^{a}	11.77 ± 0.55^{b}
Carotenoids								
α-tocopherol	0.00080	0.0025	0.011*	0.00083	0.48 ± 0.02	0.50 ± 0.03	0.51 ± 0.03	0.47 ± 0.02
β-tocopherol	0.25*	4.2**	0.080	0.023	1.83 ± 0.47^{a}	1.47 ± 0.37^{b}	0.93 ± 0.10^{b}	2.36 ± 0.17^{a}
γ-tocopherol	359	79	0.50	78	185.83 ± 5.29	172.43 ± 2.19	182.28 ± 6.18	175.99 ± 4.23
δ-tocopherol	0.10	0.15	0.011	0.069	1.67 ± 0.16	1.42 ± 0.11	1.69 ± 0.06	1.40 ± 0.18
Total tocopherols	389	53	1.4	78	189.80 ± 5.16	175.82 ± 1.99	185.40 ± 6.19	180.22 ± 4.51

df, degrees of freedom; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

Supplementary Table S.7. Analysis of variance (mean square) and LSD test of phenolic compounds content (mg/kg DM; mean ± standard error) of *Lupinus albus* seeds (Lot 1) after debittering without and with ultrasound (US), and employing different solvents

	ANOVA				LSD Treatment		LSD Solvent	
	Treatment (T)	Solvent (S)	T × S	Error	Without US	With US	NaCl 1%	Citric acid 1%
df	1	1	1	4				
Soluble-free phenolics								
Apigenin der	753***	5511***	183**	4.3	132.93 ± 17.95^{a}	113.53 ± 12.40^{b}	96.99 ± 2.91^{b}	149.47 ± 8.43^{a}
Diosmin der					3.94 ± 0.03^{a}	3.24 ± 0.01^{b}	nd	3.59 ± 0.20
Genistein der	4.2	5068***	351***	1.3	84.67 ± 10.71	86.12 ± 18.37	60.22 ± 3.42^{b}	110.56 ± 4.28^{a}
Naringenin der					4.11 ± 0.07^{a}	3.58 ± 0.01^{b}	nd	3.85 ± 0.16
Sinapic acid der	711*	989*	0.86	50	96.70 ± 6.57^{a}	77.85 ± 7.49^{b}	98.39 ± 5.27^{a}	76.15 ± 6.94^{b}
Total free	2798***	15495***	12	36	318.32 ± 24.75^{a}	280.91 ± 26.30^{b}	255.60 ± 11.51^{b}	343.62 ± 10.66^{a}
Soluble-conjugated phenolics								
Apigenin der					0.52 ± 0.05	0.54 ± 0.01	0.53 ± 0.03	nd
Catechin der					3.54 ± 0.05^{a}	2.53 ± 0.03^{b}	nd	3.04 ± 0.30
Genistein					1.55 ± 0.03^{a}	0.44 ± 0.03^{b}	nd	1.00 ± 0.32
Genistein der	2.8	461	380	79	97.28 ± 9.03	98.46 ± 3.84	90.28 ± 6.53	105.46 ± 3.93
Naringenin der	0.46	0.11	0.24	0.19	2.02 ± 0.22	1.54 ± 0.21	1.90 ± 0.25	1.67 ± 0.25
Total conjugated	0.26	681*	463	81	102.11 ± 10.38	101.75 ± 3.81	92.71 ± 6.56^{b}	111.16 ± 4.75^{a}
Insoluble-bound phenolics								
Apigenin der	0.00080	52***	0.011	0.19	3.43 ± 1.51	3.41 ± 1.45	0.87 ± 0.07^{b}	5.96 ± 0.24^{a}
Catechin der	2.2	708***	0.96	1.8	18.49 ± 5.65	17.44 ± 5.27	27.37 ± 0.83^{a}	8.56 ± 0.41^{b}
Genistein der	9.4	891**	23	16	41.47 ± 7.28	43.63 ± 5.32	53.10 ± 1.64^{a}	31.99 ± 2.27^{b}
Naringenin der	0.000013	28	0.79	4.2	16.82 ± 1.42	16.82 ± 1.34	18.71 ± 1.05	14.94 ± 0.60
Total bound	2.4	2980**	23	47	80.21 ± 12.56	81.30 ± 10.41	100.05 ± 3.18^{a}	61.45 ± 2.79^{b}
Total phenolics	2690*	9212**	179	163	500.64 ± 22.45^{a}	463.96 ± 18.25^{b}	448.37 ± 9.46^{b}	516.23 ± 14.28^{a}

df, degrees of freedom; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; different letters indicate significant differences between samples according to the LSD test ($p \leq 0.05$).

Supplementary Table S.8. Two-ways ANOVA of colour, carotenoids and tocopherols in Andean lupin seeds of ecotypes Cholo fuerte and Altagracia during germination.

	Germination time (G)	Ecotype (E)	G × E	Error
df	3	1	3	23
Colour				
*L**	53.4***	4.59**	8.91***	0.46
*a**	21.4***	0.08	2.01***	0.05
*b**	35.1***	2.16	0.26	0.66
Carotenoids				
(α+β)-carotene	40.7***	0.36	0.11	0.26
β-cryptoxanthin	0.98***	0.01	0.001	0.01
Lutein	178.6***	0.61	1.23*	0.29
Zeaxanthin	2.22***	0.003	0.06	0.02
Total carotenoids	490.9***	2.28	1.46	0.49
Tocopherols				
α-tocopherol	6891.1***	55.2	46.1	22.3
γ-tocopherol	4631.5***	11877.1***	59.8	238.1
δ-tocopherol	0.85***	0.35*	0.07	0.05
Total tocopherols	211	10567.2***	150.1	315.4

df, degrees of freedom; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Supplementary Table S.9. Conditions and results of Experiment 1: Fractional factorial design 2^{4-1} for protein extraction from commercial pea flour.

Std order	Independent variables				Response
	x_1, Ratio (mL/g)	x_2, pH	x_3, Amplitude (%)	x_4, Time (min)	Recovery (%)
1	10	8	30	5	58.33
2	20	8	30	15	62.51
3	10	11	30	15	58.62
4	20	11	30	5	63.96
5	10	8	50	15	58.75
6	20	8	50	5	61.20
7	10	11	50	5	61.49
8	20	11	50	15	67.13
9	15	9.5	40	10	63.23
10	15	9.5	40	10	63.40
11	15	9.5	40	10	64.50

Supplementary Table S.10. Conditions and results of Experiment 2: Full factorial design 2^2 for protein extraction from commercial pea flour.

Std order	Block	Independent variables		Response
		x_1, Amplitude (%)	x_2, Time (min)	Recovery (%)
1	Block 1	60	10	66.09
2	Block 1	80	10	66.72
3	Block 1	60	20	65.81
4	Block 1	80	20	68.77
5	Block 1	70	15	68.83
6	Block 1	70	15	66.83
7	Block 1	70	15	67.03
8	Block 2	60	10	64.56
9	Block 2	80	10	65.78
10	Block 2	60	20	65.08
11	Block 2	80	20	67.91
12	Block 2	70	15	64.59
13	Block 2	70	15	64.71
14	Block 2	70	15	65.46

Supplementary Table S.11. Conditions and results of Experiment 3: Full factorial design 2^2 for protein extraction from the thawed pea by-product.

Std order	Independent variables		Response
	x_1, Amplitude (%)	x_2, Time (min)	Recovery (%)
1	40	5	32.05
2	80	5	47.06
3	40	15	43.47
4	80	15	57.32
5	60	10	52.84
6	60	10	51.07
7	60	10	48.73

Supplementary Table S.12. Results of Experiment 4 following the steepest ascent path method for protein extraction from the thawed pea by-product.

Independent variables		Response
x_1, Amplitude (%)	x_2, Time (min)	Recovery (%)
80	18.75	63.8[b]
80	22.50	64.0[b]
80	26.25	66.1[a]
80	30.00	66.0[a]

Supplementary Table S.13. Conditions and results of Experiment 5: Central Composite Design for protein extraction from the thawed pea by-product.

Std order	Independent variables		Response
	x_1, Amplitude (%)	x_2, Time (min)	Recovery (%)
1	70	25	64.32
2	90	25	62.43
3	70	35	66.59
4	90	35	66.31
5	65.9	30	60.56
6	94.1	30	68.61
7	80	22.9	64.13
8	80	37.1	65.45
9	80	30	68.21
10	80	30	67.22
11	80	30	64.38
12	80	30	66.43

Supplementary Table S.14. Estimates of the regression coefficients of the models and analysis of variance (mean square, MS, and significance) for protein extraction recovery from commercial pea flour.

Source	Experiment 1				Experiment 2			
	df	Coeff	MS	*p*-value	df	Coeff	MS	*p*-value
Intercept		62.10				66.3		
Block					1		10.27	
Model	2		26.16	0.01	1		7.30	0.015
A-Ratio	1	2.20	38.76	0.007	1	0.95	7.30	0.015
B-pH	1	1.30	13.55	0.067				
Residual	8		3.01		11		0.89	
Lack of Fit	6		3.85		7		0.98	0.401
Pure Error	2		0.48		4		0.72	
R^2			0.68				0.43	
Adj-R^2			0.61				0.38	
Pred-R^2			0.40				0.06	
CV%			2.79				1.42	

df, degrees of freedom; Adj-R^2, R^2 adjusted by df; Pred-R^2, R^2 in prediction.

Supplementary Table S.15. Estimates of the regression coefficients of the models and analysis of variance (mean square, MS and significance) for protein extraction recovery from thawed pea by-product.

Source	Experiment 3				Experiment 5			
	df	Coeff	MS	*p*-value	df	Coeff	MS	*p*-value
Intercept		47.51				65.39		
Model	2		162.9	0.030	2		9.32	0.193
A-Amplitude	1	7.22	208.2	0.025	1	1.15	10.63	0.167
B-Time	1	5.42	117.5	0.059	1	1.00	8.01	0.224
Error	4		17.2		9		4.70	
Lack of fit	2		30.1	0.124	6		5.72	0.281
Pure error	2		4.3		3		2.65	
R^2			0.83				0.31	
Adj-R^2			0.74				0.15	
Pred-R^2			0.34				-0.33	
CV%			8.72				3.31	

df, degrees of freedom; Adj-R^2, R^2 adjusted by df; Pred-R^2, R^2 in prediction.

Supplementary Table S.16. Bread recipes of white bread (Control), white bread with 20% non-fermented by-product batter (Control-NF) and breads with 15% (1a, 2a) or 20% (1b, 2b) fermented by-product batters.

Ingredients (g)	Control	Control-NF	1a, 2a	1b, 2b
Wheat flour	360	340.8	345.6	340.8
Tap water	236	127.2	154.4	127.2
Fresh yeast	16	16	16	16
Sugar	12	12	12	12
Rapeseed oil	12	12	12	12
Salt	4	4	4	4
By-product batter		128		
Fermented by-product batter			96	128