Use of supercritical CO_2 to improve the quality of lupin protein isolate

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Abstract

Lupins are an excellent source of protein which can be used to obtain protein isolates with potential use in the food industry. Some studies use supercritical CO_2 (SC-CO_2) to defat legume flours, but no study analyzes the effect of applying this technology directly to the protein isolate. This article has proposed the use of SC-CO_2 to improve lupin protein isolate (LPI) quality. SC-CO_2 increased the LPI purity while reducing oil and other antitechnological factors (saponins and polyphenols). The treatment significantly improved the LPI color due to the elimination of the lipid fraction and lipophilic pigments (carotenoids). No changes in amino acid contents or chemical score were observed due to the SC-CO_2. Finally, the treatment improved or did not affect the main LPI technofunctional properties. Therefore, SC-CO_2 is a promising technique to enhance the quality of protein isolates, without affecting or improving their functional properties.

Keywords: *Lupinus luteus*; functional properties; chemical composition; amino acids; vegetable protein

1- Introduction

Nowadays, deficiency of protein is a serious global problem due to the fast increase of the world population and present production abilities cannot meet the growing demands for proteins (Li, Shi, Scanlon, Xue, & Lu, 2021). Thus, alternative protein sources are required. In this sense, in recent years, many works have focused on obtaining proteins from legumes (Czubinski, Grygier, & Siger, 2021). Of the many types of legumes that exist, lupin perhaps presents important benefits over other legumes, since it has a high protein content, an excellent amino acid profile, and low to no starch (Boukid & Pasqualone, 2022; Czubinski et al., 2021; Devkota, Kyriakopoulou, Bergia, & Dhital, 2023). Despite these potential properties, lupin is still little used for human consumption (Chukwuejim, Utioh, Choi, & Aluko, 2023), mainly linked to the presence of alkaloids that are toxic to humans, so studies related to its characterization, protein extraction, purification, and functionalization are necessary to clarify and enhance its use as an ingredient in the human diet.

The production of protein isolates and concentrates has emerged as a solution for the food industry, both for food fortification due to their bioactive peptide sequences (Chukwuejim et al., 2023) and for their application in multiple industrial processes due to their technofunctional characteristics (emulsifying, foaming, and gelling capacity, water retention capacity, etc.) (Bou et al., 2022; Boukid & Pasqualone, 2022). The most important technique for obtaining protein isolates is focused on performing an alkaline extraction followed by precipitation of the proteins at the isoelectric point (Domínguez, Bermúdez, Pateiro, Lucas-González, & Lorenzo, 2023) since it is simple and easily scalable to the industry while producing high-purity protein isolates. However, the oil present in legume seeds is generally considered an antitechnological agent, since it is not wanted in the protein isolates. Although the oil may be nutritionally positive for human nutrition, its presence in protein isolates has a negative impact on the purity of the isolate, while affecting technological properties, including lipophilic pigments that alter color, and can even promote oxidative processes that worsen the quality of the protein isolate. Thus, during protein

extraction procedures, defatting is carried out in legume flour before further processing (Boukid & Pasqualone, 2022; Domínguez et al., 2023). In lupins, during this step, oil and carotenoids which are present in the flour are removed to increase protein extraction yield and isolate purity, improve color, de-flavoring flour, reduce oxidative degradation, and improve technofunctional properties (Bou et al., 2022; Vatansever, Ohm, Simsek, & Hall, 2022).

The general defatting procedure consists of the use of organic solvents that eliminate unwanted nonpolar compounds, followed by a solvent removal phase at relatively high temperatures (Delgado-García, Luna-Suárez, López-Malo, & Morales-Camacho, 2022). The most used solvent is hexane (Delgado-García et al., 2022; Devkota et al., 2023; Nahimana et al., 2023; Schlegel et al., 2019) due to its high oil extraction efficiency and its availability, but it is flammable and toxic. Thus, due to its harm to human health and the environment, there is a tendency to limit its use and increase interest in alternative techniques. Furthermore, the use of solvents and moderate temperatures promotes protein denaturation, which reduces protein solubility and recovery, negatively affects the technofunctional properties of protein isolates (Chukwuejim et al., 2023; Shrestha, Hag, Haritos, & Dhital, 2021), and produces undesirable off-flavors (Kang et al., 2018).

Due to these limitations, the use of supercritical CO_2 (SC-CO_2) oil extraction has gained attention in the last decade because it is an efficient and alternative process to the use of solvent-based extraction methods (Aussanasuwannakul, Boonbumrung, & Pantoa, 2023). Moreover, innovative SC-CO_2 extraction has attracted interest in the food industry due to reducing energy, time, and solvent consumption (Bou et al., 2022) and high reproducibility (Buszewski et al., 2019). The use of this technique has recognized benefits since it reduces environmental impacts and produces solvent-free products, and minimal post-extraction manipulation is required. The use of low or moderate temperatures allows minimal degradation of thermolabile compounds as well as limiting denaturation of proteins (Yu, Kniepkamp, Thie, Witkamp, & van Haren, 2023). Additionally, the SC-CO_2 technique is cost-efficient, 100% food grade, and produces minimum damage to raw material, while carbon dioxide is recognized as GRAS, non-toxic, non-flammable,

chemically inert, cheap, easily available, highly selective to nonpolar molecules, and non-corrosive (Amaral et al., 2017; Delgado-García et al., 2022; Sheikh, Saini, & Sharma, 2023). Thus SC-CO_2 can be applied to increase the removal of unwanted compounds of lupin protein isolate (LPI) (Bou et al., 2022) and improve both, purity and nutritional LPI quality. However, it is important to highlight that SC-CO_2 can modify protein structure and conformation, which affects the technofunctional properties of LPI. In fact, various studies reported protein changes (structural and conformational modifications) after supercritical treatment (Amaral et al., 2017; Sheikh et al., 2023) and also after pressure treatment (Chao, Jung, & Aluko, 2018). However, the protein changes derived from SC-CO_2 treatment can enhance and improve protein isolate technofunctional properties (Sheikh et al., 2023).

In a recent study, our research group developed a procedure to obtain a high-purity lupin protein isolate through a simple, fast, and effective procedure (Domínguez et al., 2023) using an alkaline extraction and protein precipitation procedure, where in about 4 hours it can be obtained the protein isolate (without lyophilizing). In the previous study, full-fat lupin flour was used. On the contrary, in the present study, the defatting phase (using SC-CO_2) could have positive effects on the protein isolate quality (purity), which is why this research was proposed. However, the defatting phase has a clear impact on the conformation and denaturation of proteins (Kang et al., 2017; Lee et al., 2019; Nahimana et al., 2023), which directly affects (positively or negatively) the technofunctional properties of protein isolates. Consequently, clarifying the impacts of SC-CO_2 treatment in lupin protein isolate composition and functionality is imperative for its use in the food industry. Moreover, there is a need to enhance the functionality of LPI and the use of SC-CO_2 is a promising technique.

To our best knowledge, there is currently no information available about the use of SC-CO_2 directly to the LPI, and its impact on the LPI composition and properties. In a preliminary study, we verified that the application of SC-CO_2 in lupin flour produced a slight reduction in the LPI oil content. However, it did not lead to an increase in protein extraction or important changes in

the color characteristics and technofunctional properties of the LPI obtained from treated flour in comparison with the control isolate. On the contrary, significant changes were observed when applying the SC-CO_2 treatment to the LPI. In addition, it is also worth noting that performing the SC-CO_2 in the LPI allows treating approximately 4 times more sample volume in comparison to the application of SC-CO_2 in lupin flour (due to subsequent LPI extraction yield), with the same consumption (energy consumption, water, etc.), which represents a clear advantage for the food industry. Taking into account the advantages implied by the direct application of SC-CO_2 on the isolate instead of on the flour, the hypothesis of improving the purity, nutritional quality, color, as well as the main technofunctional properties of the LPI through the application of SC-CO_2 is proposed, which is essential for future food applications.

Therefore, the main objective of the present research was to study the main effects of SC-CO_2 treatment on chemical composition, color characteristics, amino acids, and technofuntional properties of lupin protein isolate. This research also provides new and innovative information on the use of SC-CO_2 (in LPI and not on flour) to obtain valuable and functional ingredients for the food industry, which helps food processors to better design food processing systems that involve lupin protein isolates.

2. Material and Methods

2.1. Sample preparation

Lupin seed flour (*Lupinus luteus* L. [Tremosilla]) was used in this study to obtain LPI. The lupin seeds were purchased from Semillas Batlle S.A. (Barcelona, Spain) and subsequently ground at the Universitat Politècnica de València. Different LPIs were obtained from lupin flour using basic solubilization and isoelectric precipitation according to the procedure optimized in a previous study (Domínguez et al., 2023). Briefly, the lupin flour was mixed with distilled water (ratio 1:8), and after an initial homogenization, the proteins were extracted at pH 10.3 (alkaline extraction) for 90 min (room temperature and with magnetic stirring). The samples were centrifuged to separate the insoluble fraction, and the supernatant with the solubilized proteins was filtered. To

recover the proteins, they were precipitated at the isoelectric point (pH 4.7) and kept for 90 min in an ice bath to promote precipitation. The protein isolate was recovered by centrifugation and freeze-dried. Three independent LPIs (replicas of different extractions following the same procedure) were used for this experiment. From each LPI, 100 g were used as "control" (LPI-Control), while the other 100 g were treated with supercritical CO_2 (LPI-SF).

2.2. Defatting lupin protein isolates by supercritical CO_2

A supercritical CO_2 system (Sitec, model 101-300-AF, Zurich, Switzerland) was used for defatting LPI, and CO_2 (99.99%) was used as solvent. For each replicate (x3), 100 g of LPI was placed in the insert basket (600 mL) equipped with two stainless steel filters (10 µm filter porosity) and introduced into the extraction vessel (1 L). Oil extraction was conducted by pumping CO_2 into the extraction vessel at a constant temperature of 40 ºC. The procedure involves dynamic extraction under the pressure of 400 Bar for 102 min with a CO_2 pump flow rate of 4 kg/h (CO_2 density: 988±6.11 kg/m^3). The SF-CO_2 extraction conditions were optimized in a preliminary study (unpublished results). Using a Box-Behnken experimental design, the effect of CO_2 flow, time, and pressure applied during SC-CO_2 treatment on the oil and antitechnological factors extraction were analyzed, and the treatment conditions were optimized using the RSM procedure. After the process, the extraction vessel was progressively depressurized and the treated LPI (LPI-SF) was obtained. Additionally, the extracted oil was collected in the "collection vessel", and recovered for the determination of the extracted oil percentage. Both LPI-Control and LPI-SF were stored at -20 ºC until their analysis. All experiments were performed in triplicate.

2.3. Chemical composition and color determination

The chemical composition of LPI was determined according to ISO procedures for protein (ISO 937, 1978) (N x 6.25 and N x 5.7) and ash (ISO 936, 1998), while lipid content was determined according to the procedure described by Bligh & Dyer (1959) with the modifications proposed by Barros et al. (2020). Briefly, to determine the total oil content, 10 g of LPI were homogenized with 10 mL of chloroform and 20 mL of methanol for 30 seconds (Ultraturrax; 12,000 rpm). After,

168 the mixture was added of 10 mL of chloroform and 10 mL of NaCl (1% in distilled water) and
169 homogenized for 30 seconds. Then, the chloroform layer (with the lipid fraction) was separated
170 from the residues and aqueous layer by centrifugation (3.100 g for 10 min). Finally, 5 mL of
171 chloroform fraction was evaporated using N_2 gas (Turbovap; 56 ºC, 1.2 Bar N_2 pressure), and the
172 oil was weighed. Total oil content was calculated gravimetrically, according to the next equation.

173 $$Total\ oil\ \ (g/100\ g) = \frac{Oil\ weight\ (g) \times 4}{Sample\ weight\ (g)} \times 100$$

174 For the extraction of the alkaloids, 1 g of LPI was mixed with 32 mL of HCl (0.5N) and sonicated
175 for 30 min in a bath. This step was repeated 2 times, and the supernatants were pooled and
176 centrifuged (7,140 g) for 10 min. The pH was adjusted to 10 with NaOH (4N), and 50 mL of
177 dichloromethane was added. The mixture was mixed and centrifuged again (7,140 g/15 min), and
178 the organic phase was recovered. The extraction was repeated with additional 25 mL of
179 dichloromethane. The solvent was evaporated on a rotary evaporator, and the residue was
180 redissolved with 1 mL of methanol. The quantification of total alkaloids was carried out by
181 volumetric titration, using 0.1% ethyl tetrabromophenolphthaleinate in ethanol as an indicator,
182 and lupanine as a standard. Alkaloid results are shown in mg/kg.

183 For the extraction of saponins, 10 mL of ethanol was added to 1 g of LPI and sonicated for 15
184 min in a bath. After this step, it was left with magnetic stirring for additional 15 min. The mixture
185 was centrifuged (3,400 g/10 min). The solvent from the supernatant was removed in a rotary
186 evaporator and the residue was redissolved with methanol, leading to a final volume of 10 mL.
187 For the saponins determination, 125 µL of methanolic extract (or standard) was mixed with 1.25
188 mL of sulfuric acid (72%) and 125 µL of anisaldehyde (10%, v/v in methanol). The samples were
189 heated at 60 ºC for 1 hour and subsequently cooled to room temperature (protecting the samples
190 from light). To extract the saponins, 1 mL of dichloromethane was added, and the mixture was
191 vortexed and waited for the phases to separate. The organic phase was collected and the extraction
192 was repeated with 1 mL additional of dichloromethane. The two recovered organic phases were
193 mixed, and measured in a spectrophotometer at 535 nm. Quantification was carried out by

194 external standard method, using oleanolic acid as a standard. The results were expressed as g
195 oleanolic acid/100 g.

196 Total polyphenol content (TPC) was calculated according to the procedure described by
197 Singleton, Orthofer, & Lamuela-Raventos (1999), using acidified methanol (0.2 M HCl in
198 methanol) as extracting solvent.

199 Color parameters were measured in the CIELAB space using a portable CR-400 colorimeter
200 (Konica Minolta Sensing Inc., Osaka, Japan), at 10º viewing angle geometry and 8 mm aperture
201 size equipped with pulsed xenon arc lamp filtered to illuminant D65 lighting. Before analysis, the
202 colorimeter was calibrated with a white ceramic tile according to manufacturer recommendations.
203 The LPI samples were placed in a petri dish (sample thickness: 5 mm) and the color was measured
204 at three different points on the LPI surface. The color parameters lightness (L*), redness (a*), and
205 yellowness (b*) were recorded.

206 *2.4. Amino acid analysis and chemical score*

207 The amino acid content of LPI was determined using liquid chromatography following the AccQ-
208 Tag method, with the modifications described by Munekata et al. (2020). Briefly, the LPI samples
209 (0.1 g) were hydrolyzed with HCl (6N) for 24h at 110ºC. Then, the extracts were derivatized using
210 the AccQ-Tag reagent (Waters, Milford, MA, USA), and the separation, identification, and
211 quantification were performed in a high-performance liquid chromatography (Alliance 2695
212 model, Waters, Milford, MA, USA) using a scanning fluorescence detector (model 2475, Waters).
213 All results were expressed as mg/g protein.

214 The amino acid composition of LPI was used for the determination of chemical score, considering
215 the values of essential amino acids of the sample (EAAs) and the pattern concentration (EAAp)
216 according to FAO/WHO/UNU (2007) for adults:

217 $$Chemical\ Score\ (\%) = \frac{Essential\ amino\ acid\ in\ sample\ [\frac{mg}{g\ protein}]}{Essential\ amino\ acid\ pattern\ concentration\ [\frac{mg}{g\ protein}]}\ x\ 100 \quad \text{(Eq. 1)}$$

218 *2.5. Analysis of LPI technofunctional properties*

219 2.5.1. Water and oil absorption capacity

220 For the water and oil absorption capacity measurement, 0.5 g of LPI was weighed and mixed with
221 5 mL of water or soybean oil. The mixture was vortexed for 1 min and left to settle for 30 min at
222 room temperature. Then, samples were centrifuged (1600 g for 25 min; Beckman Coulter, model
223 Allegra X-22R, rotor SX4250), and the supernatant was discarded. The sample was then weighted
224 again, and the water or oil absorbed was expressed as g oil or water/g LPI.

225 2.5.2. Foam properties

226 Foam properties were evaluated according to the procedure described by Domínguez et al. (2023).
227 A LPI solution (1% w/v) adjusted to pH 7 was used. The solution (V_l = 100 mL) was placed in a
228 250 mL graduated cylinder and homogenized using an UltraTurrax disperser (17,500 rpm for 2
229 min). The foam volume was recorded after homogenization (V_0) and after 30 min (V_{30}). The foam
230 capacity properties were calculated using the following equations.

231 $$Foaming\ capacity\ (\%) = \frac{V_0}{V_l} \times 100$$
232
233 $$Foaming\ stability\ (\%) = \frac{V_{30}}{V_0} \times 100$$
234

235 2.5.3. Emulsifying properties

236 Emulsifying properties were assessed using the protocol described by Domínguez et al. (2023).
237 15 mL of LPI solution (1%) at pH 7 was placed in a 50 mL volume falcon tube, and homogenized
238 for 15 seconds (UltraTurrax, 12000 rpm). Then, 15 ml of soybean oil were added and
239 homogenized again for 1 min. The emulsion was centrifuged at 1300 g for 5 min (Beckman
240 Coulter, model Allegra X-22R, rotor SX4250) and at room temperature. The emulsifying capacity
241 was calculated according to the next equation.

242
243 $$Emulsifying\ capacity\ (\%) = \frac{Volume\ of\ the\ emulsified\ layer\ after\ centrifugation}{Volume\ of\ emulsion\ before\ centrifugation} \times 100$$

For the determination of emulsifying stability, the emulsion obtained after homogenization was heated for 30 min at 80 ºC. Then, they were cooled at room temperature and centrifuged (1300 g for 5 min; Beckman Coulter, model Allegra X-22R, rotor SX4250), and the emulsifying stability was calculated according to the next equation.

$$Emulsifying\ stability\ (\%) = \frac{Volume\ of\ the\ emulsified\ layer\ after\ heating}{Volume\ of\ emulsion\ before\ centrifugation} \times 100$$

2.5.4. Protein solubility

The LPI solubility (%) was determined over the pH range of 3-9 following the procedure described by Domínguez et al. (2023). 0.75 g of LPI was mixed with 25 mL of distilled water, and pH of each solution was adjusted using 1M NaOH or 1M HCl. Then, the suspension was magnetically stirred at room temperature for 1 hour. Finally, the suspensions were centrifuged (10,000 g for 15 min), and 5 mL of the supernatant was used for the nitrogen determination using the Kjeldahl method (ISO 937, 1978). The protein solubility was calculated according to the following equation.

$$Protein\ solubility\ (\%) = \frac{Volume\ [mL]\ \times\ protein\ content\ [\frac{g}{mL}]}{Sample\ weight\ [g]\ \times\ purity\ [\frac{g\ protein}{g\ LPI}]} \times 100$$

2.6. Statistical analysis

After checking the normal distribution and variance homogeneity were tested (Shapiro–Wilk), SPSS software (version 25.0, SPSS Inc., Chicago, USA) was used to analyze the data from lupin protein isolate characterization using one-way analysis of variance (one-way ANOVA), and the results were presented as mean and standard deviation.

3. Results and Discussion

3.1. Effect of SC-CO_2 treatment on chemical composition

The total yield with SC-CO_2 treatment was 90.5 g/100 g LPI, removing 9.63 g oil/100 g LPI (Table 1). Therefore, the treatment has extracted 62% of the oil present in the initial sample (LPI-Control). The protein-lipid interaction can explain the remaining oil content after SC-CO_2 treatment since these complexes resist the defatting treatment. This percentage was similar to those reported by Spina et al. (2022) (60-62%) in *L. luteus* and slightly lower than that of another study, where oil removal from Andean lupin was more than 80% (Yu et al., 2023). It is well known that CO_2 density is an important factor for the extraction of nonpolar compounds, but also a balance between mass transfer and solvation power should be maintained to improve the extractability efficiency (Santana & Meireles, 2024). The discrepancies among different researches are expected since oil extraction is also influenced by multiple factors such as particle size, original material, oil and water contents, use of co-solvents, and flow-pressure-temperature combinations during SC-CO_2 treatment (Aussanasuwannakul et al., 2023). Therefore, variations in processing conditions could justify these differences between studies.

The chemical composition values of the control and treated LPI are shown in Table 1. The moisture content was zero in both cases, which is logical when considering that the final step in obtaining the LPI is freeze-drying, which eliminates moisture from the samples. The oil content in LPI-Control was 15.6 g/100 g, which was higher than those obtained in a previous study (8.87 g/100 g) conducted with the same lupin flour and using the same protein isolate procedure (Domínguez et al., 2023). These variations were due to the different procedure analyses. In the previous study, the AOCS protocol was employed (AOCS, 2005) for the determination of total lipids, however, after carrying out several extractions it has been proven that, even with acid hydrolysis, the oil content determined with this procedure is underestimated. The difference between the procedures is that AOCS uses petroleum ether to extract the lipid fraction, while the methodology described by Bligh & Dyer (1959) uses a mixture of methanol and chloroform, solvents that have a higher polarity than ether. In this case, it seems that this increase in polarity improves the extraction and is a more suitable procedure for the determination of oil in protein isolates, since the AOCS method is not capable of quantifying the oil content consistently and

reliably. For this reason, the determination of the oil content with the Bligh & Dyer (1959) procedure has been chosen, with much more appropriate and consistent results. The oil values obtained by other authors in LPI vary from low values (0.5-2%) (D'Agostina et al., 2006; Piornos et al., 2015) to high values (10-17%) (Devkota et al., 2023). These differences between studies are related not only to extraction procedures but also to the initial oil content in the lupin flour.

During the alkaline extraction, the use of NaOH to adjust the pH has been a determining factor in saponifying and emulsifying the lipid fraction (Chukwuejim et al., 2023), which makes the oil more "soluble" in the aqueous phase. The oil is incorporated into the protein matrix, and extracted with protein thereby increasing the total oil content of the protein isolate (Chukwuejim et al., 2023). Thus, the oil content in LPI (15.6 g/100 g) is higher than in the lupin flour (6.06 g/100 g) (Domínguez et al., 2023). This fact has been reported by multiple studies (Berghout, Boom, & Van Der Goot, 2014; Devkota et al., 2023; Domínguez et al., 2023), and is undesirable since it negatively affects the purity and quality of the final isolate obtained. In this study, it is observed that applying SC-CO_2 treatment on this isolate eliminates a large part of the oil from the LPI-Control since the LPI-SF had an oil content of 5.27 g/100 g. This represents a reduction of 66.2% with respect to the control, which agrees with the extraction percentage obtained during SC-CO_2 extraction. Therefore, the application of SC-CO_2 directly on the protein isolate produces an effective elimination of the lipid fraction. This result was expected due to the nonpolar character of CO_2, which promotes the extraction of the lipophilic compounds contained in the sample (Bou et al., 2022). This fact coincides with the results reported by multiple authors on legume flours (Kang et al., 2018, 2017; Lee et al., 2019; Rosas-Quina & Mejía-Nova, 2021; Shin, Kim, & Kim, 2014). Aussanasuwannakul et al. (2023) also observed a significant decrease in fat (2.88% vs. 0.24%) content in soybean okara after the application of SC-CO_2.

Removal of oil not only improves the composition of the protein isolate but also plays a vital role in the stability of the LPI. It is well known that unsaturated fatty acids, which are the majority of LPI (~80%; 40% MUFA and 39% PUFA) (Domínguez et al., 2023), are highly susceptible to

oxidation degradation processes. Furthermore, the radicals and oxidized forms derived from lipid oxidation promote protein oxidation, and it is known that lipidic and proteinic oxidation, are interrelated and one enhances the other (Domínguez et al., 2019). Protein oxidation, on the other hand, can have very negative effects on the protein conformation and properties (Domínguez et al., 2022), which can modify the composition and technofunctional properties of LPI (Peng et al., 2021). Furthermore, the decrease in the lipoxygenase activity after SC-CO_2 was also another factor that limited oxidative degradation (Russin, Boye, Arcand, & Rajamohamed, 2011). Therefore, it is clear that removing oil and/or inactive pro-oxidative enzymes limits oxidation, and increases the oxidative stability of LPI during storage (Devkota et al., 2023; Lee et al., 2019).

It is well known that both, oil and protein content are the two key factors to determine the protein isolate quality. In this study, the protein content in LPI-Control was 86.3 g/100 g (N x 6.25) or 78.7 g/100 g (N x 5.7), while after SC-CO_2 treatment, this value increased dramatically to 96 g/100 g (N x 6.25) or 87.6 g/100 g (N x 5.7). Although most studies calculate the purity of protein isolates from legumes using the nitrogen conversion factor "6.25", for legumes the factor “5.7” should be used (Albe-Slabi et al., 2022; Rodriguez & Beyrer, 2023). In fact, in the present study, using the factor 6.25 there is an overestimation of the protein content, while with the factor 5.7, the sum of protein, fat, and ash presents a content close to 100%. Therefore, to compare our results with those of other studies, both conversion factors were calculated and shown in Table 1. In any case, the application of SC-CO_2 treatment improves the purity of protein isolates. Obviously, the extraction of oil from LPI after SC-CO_2 treatment determines the increase of its protein purity. Our purity was higher than those observed by Albe-Slabi et al. (2022) (66-75%) and similar to those reported for *L. albus* (83.96-94.4%) (D’Agostina et al., 2006; Kebede & Teferra, 2023; Vogelsang-O’Dwyer et al., 2020), defatted *L. campestris* (93.2%) (Rodríguez-Ambriz, Martínez-Ayala, Millán, & Dávila-Ortíz, 2005), or *L. angustifolius* (81.2-92.6%) (Kebede & Teferra, 2023; Vogelsang-O’Dwyer et al., 2020). Moreover, the same values of protein content in lupin protein isolates from *L. luteus* (87.7%) were obtained by us in a previous study (Domínguez et al., 2023), and from *L. albus* and *L angustifolius* (87-94%) (Devkota et al., 2023). Additionally, Devkota et

al. (2023) observed that LPI obtained from full-fat flour presented significantly lower purity than those obtained from defatted flours, which agrees with our findings. Similarly, the LPI obtained from defatted (SC-CO_2) lupin flour had protein content ranging between 92 and 94 g/100 g (Vogelsang-O'Dwyer et al., 2020). The treatment of soybean okara with SC-CO_2 also increased its protein content from 35.5% in full-fat samples to 44% in defatted samples (Aussanasuwannakul et al., 2023).

It is important to highlight that protein and oil content are vital factors for the technofunctional properties of LPI. Removing oil from LPI with SC-CO_2 concentrates its protein content. The higher protein content in LPI increases the polar and nonpolar groups disponible for both, oil and water interactions. The high amount of protein also produces higher stabilization of air bubbles and thus, improves foaming properties (Vatansever et al., 2022; Vogelsang-O'Dwyer et al., 2020). Similarly, the oil removal eliminates the "oil film" and bound lipids and enhances hydrophilic properties, hydration, and protein-water interactions (Delgado-García et al., 2022), which improves protein isolate solubility and water absorption capacity. Moreover, the elimination of oil also increased the hydrophobicity of proteins resulting in the higher foaming ability of defatted LPI than the control.

Additionally, although a large part of the oil (~66%) is removed during the SC-CO_2 treatment, it should be noted that approximately 5% still remains, so this highly unsaturated oil could promote oxidative processes. Thus, it is vital in future studies to determine the stability of the LPI, and to determine the effect that the residual oil has on the stability of the protein isolate.

The ash content was 5.19 g/100 g in LPI-Control and 6.41 g/100 g in LPI-SF. These results agree with those found in protein isolates from *L. albus*, *L. luteus*, and *L. angustifolius* (3.14-5.3%) (D'Agostina et al., 2006; Domínguez et al., 2023; Kebede & Teferra, 2023; Vogelsang-O'Dwyer et al., 2020). In contrast, other authors described lower ash content (1.42%) in *L. luteus* isolates (Piornos et al., 2015). In our study, the ash content was not affected by the SC-CO_2 treatment. It could be due to the treatment did not produce modification of dry matter. Our findings agree with

371 those reported in amaranth flour (Delgado-García et al., 2022) and okara residue
372 (Aussanasuwannakul et al., 2023), where the SC-CO_2 treatment did not produce any influence on
373 ash content.

374 Other important antitechnological factors found in lupins are saponins, polyphenols, and
375 alkaloids. In this sense, the debittering process is a typical and necessary step with removes these
376 compounds (Curti, Curti, Bonini, & Ramón, 2018). In our study, the treatment of LPI with SC-
377 CO_2 reduced saponins (0.99 *vs*. 0.86 g oleanolic acid/100 g) and total polyphenols content (162
378 *vs*. 127 mg GAE/100 g), while did not produce any change in the alkaloid content (~658 mg/kg).
379 The main reason for these results is that the use of SC-CO_2 also removes relatively low-polarity
380 molecules, such as saponins and polyphenols (Al Khawli et al., 2019; Bou et al., 2022; Chemat
381 et al., 2020).

382 Quinolizidine alkaloids are the most important antinutrient in lupins, where lupanine is the main
383 alkaloid. The maximum value of alkaloids was fixed at 200 mg/kg (Boukid & Pasqualone, 2022;
384 Rosas-Quina & Mejía-Nova, 2021), and all values below this critical value are not toxic for
385 humans. It is important to highlight that in the present study, the total alkaloid content exceeds
386 this limit. The very low time (~3 hours) and low water amount (1:8 lupin flour/water ratio) used
387 for the protein extraction could be related to this high alkaloid content in LPI. Boukid &
388 Pasqualone (2022) reported that the most effective methods to decrease alkaloid level below 200
389 mg/kg require successive washes with large water amounts and during long processing times.
390 Additionally, alkaloids are water-soluble compounds (Rodríguez-Ambriz et al., 2005), and the
391 use of pure CO_2 (nonpolar) as solvent, with the low dielectric constant justifies the low
392 extractability of polar molecules (Santana & Meireles, 2024) such as alkaloids, and explain our
393 results. To solve this drawback, some authors proposed the use of ethanol (10%) to increase the
394 polarity of CO_2 and to improve alkaloid extraction from *Lupinus* spp. (Nossack, Vilegas, Von
395 Baer, & Lanças, 2000).

396 Similarly, polyphenols removal is desirable to improve the functional properties of LPI (Lqari,
397 Vioque, Pedroche, & Millán, 2002). As in the alkaloids, the high nonpolar character of CO_2 has
398 also a limited impact on polar polyphenols extraction, and thus it remains in the LPI. Several
399 authors use co-solvents to increase the polarity of CO_2 and enhance polyphenol extraction. For
400 example, in a recent study, the authors optimized the co-solvent percentage (ethanol 10-30%) for
401 the maximum extraction of polyphenols in lupin seeds (Buszewski et al., 2019), and at the lowest
402 co-solvent percentage obtained the lowest recovery and polyphenols extraction yield. This fact
403 justifies our findings, and although a significant polyphenol reduction was observed after SC-CO_2
404 treatment, an important content of polyphenols remains in the LPI.

405 Unfortunately, there is no information about the effect of SC-CO_2 in the LPI instead of lupin flour.
406 Therefore, the present study provides innovative and necessary data for the scientific community
407 and food processors. As a general conclusion, the application of SC-CO_2 increased the LPI purity,
408 while removing the oil (the main antitechnological factor), and produced a slight reduction of
409 saponins and polyphenols, which resulted in a higher nutritional LPI quality.

410 *3.2. Effect of SC-CO_2 treatment on color parameters*

411 Color parameter values of both LPI are shown in Table 1, while the visual aspect is shown in
412 Figure 1. As can be seen in Figure 1, LPI-Control has an intense light-yellow color, while LPI-SF
413 presents a pale cream color. This fact agrees with those found by multiple authors, who reported
414 that LPI presented a yellow tone (Albe-Slabi et al., 2022; Domínguez et al., 2023). The values of
415 L*, a*, and b* are consistent with those described in a previous study (Domínguez et al., 2023).
416 The use of SC-CO_2 treatment produced significant (p<0.001) changes in color parameters. The
417 L* value increased, while a* and b* decreased after treatment. The higher a* and b* coordinates
418 in untreated isolate imply that LPI-Control has a higher yellow intense color than LPI-SF, which
419 agrees with the visual appearance of the LPI obtained in the present study. Other authors found
420 the same trend. In this regard, the application of SC-CO_2 in soy flour produced an increase of L*
421 and a significant reduction of the a* and b* parameters of tofu (Kang et al., 2018; Lee et al., 2019)

422 and also in defatted flour (Shin et al., 2014). The main reason for these color changes after the
423 treatment with SC-CO_2 is the removal of pigments present in the raw material
424 (Aussanasuwannakul et al., 2023). Vegetable tissues have different pigments, and the main
425 lipophilic coloring agents are carotenoids. It is well known that lupins have important amounts of
426 carotenoids (Bou et al., 2022). However, the composition and total content of carotenoids in lupins
427 varies between species. Czubinski et al. (2021) found in *L. mutabilis* that the total carotenoid
428 content was 4.51 mg/kg, and the most important carotenoid was lutein (73.2%) and zeaxanthin
429 (15.7%), while other minor contents of violaxanthin, α- and β-carotene were also identified.
430 Similarly, Siger, Grygier, & Czubinski (2023) quantified total carotenoids in different cultivars of
431 *L. angustifolius* (17.2-62.5 mg/kg), *L. albus* (8.94-12.7 mg/kg) and *L. luteus* (~6.2 mg/kg), and
432 observed that lutein and zeaxanthin are the most important carotenoids, although α- and β-
433 carotene were also identified in most of the species. In contrast, another study found that β-
434 carotene was the most important carotenoid in both, *L. albus* (~98 mg/kg) and *L. termis* (~66
435 mg/kg), with minor contents of lutein and zeaxanthin (Al-Amrousi et al., 2022). Therefore, it
436 seems clear that lupins have a high amount of carotenoids, which have an intense color that varies
437 between yellow and orange and explains the color of the LPI-Control.

438 Therefore, the color reduction (redness and yellowness) after the use of SF-CO_2 is due to it is a
439 selective and efficient technique to remove nonpolar molecules such as carotenoids from a
440 complex matrix (Santana & Meireles, 2024). This aspect is clear when observing the visual
441 appearance of the oil extracted during the treatment with SC-CO_2 (Figure 1C) since an intense
442 and dark orange-reddish color can be seen, which confirms that together with the oil a large
443 amount of the LPI carotenoids was extracted. Thus, the application of SC-CO_2 removes a high
444 amount of carotenoids from lupin isolates and produces a more neutral and desirable color of LPI
445 for food purposes. The supplementation of food products with lupin protein ingredients can be a
446 powerful approach for improving their nutritional value (Boukid & Pasqualone, 2022) or as a
447 functional ingredient that improves its physicochemical properties, due to the excellent LPI
448 technofunctional properties. However, the fact that LPI has an intense color (intense yellow) may

449 be undesirable for its application in certain foods. In fact, color is one of the most important
450 parameters that determine the purchase intention of food, since consumers relate it to aspects such
451 as freshness or quality. Therefore, the elimination of the pigments and most of the color after the
452 application of SC-CO_2 allows it to be applied to multiple foods, without modifying its color, which
453 is vital for its application in the food industry.

454 *3.3. Effect of SC-CO_2 treatment on amino acids content and chemical score*

455 Lupins, characterized by having a well-balanced amino acid composition could mitigate the
456 global high-quality protein deficiency problem (Czubinski et al., 2021; Domínguez et al., 2023).
457 However, in order to be able to include them in the human diet, it is vital to carry out a complete
458 characterization of the amino acid composition of the protein isolates, as well as their quality
459 (chemical score) to ensure that these proteins provide the nutritional needs of humans.

460 Table 2 shows the effect of SC-CO_2 treatment on the amino acid content of LPI. In both cases, the
461 major amino acid was glutamic acid (~216 mg/g protein), followed by arginine (113 mg/g protein)
462 and aspartic acid (~96 mg/g protein). In contrast, the lowest values were observed for methionine
463 (6 mg/g protein), cysteine (17.9 mg/g protein), and histidine (29 mg/g protein). This profile was
464 also reported in our previous study with *L. luteus* protein isolate, in which the sum of glutamic
465 acid, arginine, aspartic acid and leucine represent more than 50% of total amino acids (Domínguez
466 et al., 2023). Similarly, in LPI from other lupin species, the same contents of amino acids were
467 described. In *L. albus* and *L. angustifolius* LPI (Devkota et al., 2023; Lqari et al., 2002; Muranyi,
468 Volke, et al., 2016; Vogelsang-O'Dwyer et al., 2020), glutamic acid content ranging about 23-
469 27%, while aspartic acid, arginine, and leucine represent each about 7-13%. In our study, low
470 amounts of the main sulfur-containing amino acids, methionine, and cysteine were found
471 (representing <2.2%). In contrast, important amounts of aromatic amino acids (phenylalanine and
472 tyrosine) were observed, reaching in LPI-Control 8.35% and LPI-SF 8.61% of total amino acids.
473 It is well known that legumes are deficient in sulfur-containing amino acids, which explains the

474 values obtained in this study and agrees with other lupin protein isolates (Vogelsang-O'Dwyer et
475 al., 2020).

476 In our study, the total essential amino acids reached 34.3% in LPI-Control and 34.9% in LPI-SF,
477 similar to the FAO/WHO/UNU reference value of 36%. Moreover, these contents also agree with
478 values reported by other authors in previous researches carried out in protein isolates from *L.*
479 *luteus* (~32%) (Domínguez et al., 2023) and from *L. albus* (~31%) and *L. angustifolius* (~31%)
480 (Devkota et al., 2023). Additionally, the slightly higher essential amino acids in this study could
481 be related to *L. luteus* presenting a higher content of these amino acids than other lupin species
482 (Boukid & Pasqualone, 2022). Although some minor differences can be observed between
483 different studies' amino acid content, the results proved that the amino acid profile did not vary
484 among the lupin protein isolates, even produced with different procedures and/or lupins species.

485 On the other hand, the use of SC-CO_2 did not produce important differences in the LPI amino acid
486 profile. Only three individual amino acids presented significant differences. The content of
487 isoleucine (47.7 *vs*. 48.7 mg/g protein), leucine (85.8 *vs*. 87.8 mg/g protein), and phenylalanine
488 (47.9 *vs*. 50.0 mg/g protein) increased (p<0.05) after SC-CO_2 treatment. It is important to note
489 that these differences, although significant, were very small, so it is assumed that their influence
490 on the nutritional quality of the isolate is negligible. In any case, the application of the SC-CO_2
491 treatment increased the content of these three essential amino acids, so the treatment had a positive
492 impact. The fact that the application of SC-CO_2 has no effects on amino acids content or profile
493 could be due, as has been commented throughout the manuscript, to the nonpolar character of
494 CO_2, which determines that the amino acids, by not soluble, neither modified nor extracted nor
495 degraded by treatment.

496 Another way to determine the quality of proteins is to examine whether they cover the needs of
497 humans for essential amino acids, for which chemical scores are calculated. The values of the
498 chemical score from both LPI, as proposed by FAO/WHO/UNU (2007) for humans (adults) are
499 shown in Table 3. It is important to highlight that both isolates present similar chemical scores,

500 with only the values for leucine and isoleucine in LPI-SF being significantly higher than the
501 control. This is directly related to the slightly higher content of these amino acids that have been
502 detected after treatment with SC-CO_2. Therefore, it can be concluded that the treatment with SC-
503 CO_2, as occurs in the case of the amino acid content, did not affect the protein quality (chemical
504 score) either.

505 In our study, the chemical score values of both LPI indicated important amounts of cysteine
506 (sulfur-derived amino acid) (298%) and aromatic amino acids (phenylalanine + tyrosine) (223%).
507 Additionally, high amounts (>140%) were described for histidine, isoleucine, leucine, and
508 threonine, and also cover the amino acid supply requirements (>100%) of lysine, sulfur-
509 containing amino acids (methionine + cysteine) and valine. The LPI only were deficient in
510 methionine (~38%), which did not reach the WHO/FAO/UNU requirements. Similarly, in a
511 previous study, the LPI obtained from *L. luteus* showed that valine (95%) and methionine (22%)
512 were the limiting amino acids (Domínguez et al., 2023), although in the present study, valine was
513 no limiting amino acid.

514 In contrast to our results, other authors reported that lupins were deficient in sulfur-containing
515 amino acids (methionine + cysteine) (Chukwuejim et al., 2023; Vogelsang-O'Dwyer et al., 2020),
516 threonine (Boukid & Pasqualone, 2022), valine (Boukid & Pasqualone, 2022; Domínguez et al.,
517 2023; Vogelsang-O'Dwyer et al., 2020) and lysine (Lqari et al., 2002; Vogelsang-O'Dwyer et al.,
518 2020). Additionally, although several authors reported that lupin protein contained low amounts
519 of sulfur-containing amino acids (Shrestha et al., 2021; Vogelsang-O'Dwyer et al., 2020), this fact
520 did not agree with our results, in which both LPI supply 109% of the requirements from these
521 amino acids.

522 Based on the mean requirement estimates for the total indispensable (essential) amino acids,
523 FAO/WHO/UNU (2007) recommended 277 mg/g protein. The total indispensable amino acids
524 content in lupin isolates of the present research was higher (400 mg/g protein) than the
525 recommended value, based on 10 amino acids (histidine, isoleucine, leucine, lysine, methionine,

526 cysteine, phenylalanine, tyrosine, threonine, and valine), which demonstrated the great nutritional
527 quality of both isolates. The higher amount of chemical score obtained in the present study than
528 in others could be related to *L. luteus* showed the highest values of essential amino acids in
529 comparison with other lupins (Boukid & Pasqualone, 2022).

530 *3.4. Effect of SC-CO_2 treatment on LPI technofunctional properties*

531 Technofunctional properties are vital when considering including protein isolates in foods.
532 Among them, properties such as water and oil absorption capacity, emulsifying and foaming
533 capacity, as well as solubility stand out. However, it is well known that several processes and
534 techniques can produce changes in proteins, leading protein isolates with decreased
535 technofuntional functionality. In this sense, SC-CO_2 technology was proposed for its potential use
536 in modifying the morphological, structural, and technofuntional properties of different
537 biopolymers, which include proteins (Sheikh et al., 2023). Therefore, it is necessary to analyze
538 and study the effect that the SC-CO_2 treatment applied to the LPI has on its technofunctional
539 properties.

540 3.4.1. Water and oil absorption capacity

541 Water and oil absorption capacity has a direct impact on some important organoleptic and
542 physicochemical characteristics of foods, such as texture, flavor, and mouthfeel (Chukwuejim et
543 al., 2023). The water absorption capacity is related to the protein-water interactions, while the oil
544 absorption capacity depends on the number of the protein hydrophobic groups exposed
545 (Domínguez et al., 2023; Muranyi, Otto, et al., 2016).

546 In the present study, both attributes were improved with the SC-CO_2 treatment (Table 4). The
547 water capacity was 1.25 g/g LPI for LPI-Control, while this attribute increased to 1.45 g/g after
548 SC-CO_2 treatment. Similarly, oil absorption capacity increased from 1.67 g/g to 1.84 g/g when
549 LPI was defatted with SC-CO_2. The values obtained by us in LPI-Control agree with those
550 reported by other authors in LPI obtained from *L. campestris* (Rodríguez-Ambriz et al., 2005) and
551 *L. luteus* (Domínguez et al., 2023; Piornos et al., 2015). In contrast, other researchers found higher

552 values of water absorption capacity (2.7-4.46 g/g) and oil absorption capacity (1.95-2.5 g/g) in
553 isolates from *L. angustifolius* (Kebede & Teferra, 2023; Lqari et al., 2002), while others reported
554 lower values in *L. albus* (D'Agostina et al., 2006) and *L. angustifolius* (0.85 mL/g) (Muranyi,
555 Otto, et al., 2016) protein isolates.

556 Concerning to the improvement of both parameters after the SC-CO_2 treatment could be related
557 to the fact that supercritical CO_2 penetrates into the protein structure, and disrupts the non-
558 covalent bonds, which result in higher exposition of hydrophobic moieties and cross-linking
559 between proteins (Sheikh et al., 2023). Similarly, other authors found that the defatting process
560 favored a greater exposure of hydrophobic groups (Nahimana et al., 2023). Additionally, the water
561 absorption capacity depends on the polar amino acids availability on the primary sites for
562 protein/water interactions (Rodríguez-Ambriz et al., 2005). Therefore, the conformational
563 changes in proteins, and the higher exposition of both, polar and nonpolar groups in proteins after
564 SC-CO_2 treatment explain the higher water and oil absorption capacities in LPI-SF than in LPI-
565 Control. Another possible reason for the enhancement of water and oil absorption after SC-CO_2
566 is that the LPI-SF had lower fat and higher protein content than LPI-Control, and both attributes
567 were positively related to the protein content (Delgado-García et al., 2022; Kang et al., 2017).
568 The higher protein amount also increased the polar and nonpolar groups disponible for oil and
569 water interactions. Similarly, the use of SC-CO_2 to defat amaranth flours resulted in the
570 elimination of oil film, which allowed better hydration of polar molecules and promotes the
571 trapping of water molecules between the particles (Delgado-García et al., 2022).

572 3.4.2. Protein solubility

573 The protein solubility is the most important attribute of LPI since it influences the other
574 technofunctional properties (Chukwuejim et al., 2023; Shrestha et al., 2021). The effect of SC-
575 CO_2 on the protein solubility is shown in Figure 2. As expected, solubility presented the typical
576 curve with minimal protein solubility near the isoelectric point (pH 4-5) and significantly
577 increased as pH was above and below these pH values. At pH 4, the solubility was <5%, while at

pH 5 this value decreased below 2%. In contrast, at pH 3 (60%) and pH 6 (50%) solubility increased dramatically, being >83% at pH 7 and >92% at pH 8 and 9. The same results were reported in previous studies with LPI from *L. angustifolius* (Muranyi, Otto, et al., 2016; Vogelsang-O'Dwyer et al., 2020), *L. campestris* (Rodríguez-Ambriz et al., 2005), *L. albus* (Vogelsang-O'Dwyer et al., 2020) and *L. luteus* (Domínguez et al., 2023; Piornos et al., 2015). Solubility depends on the protein charge, and whether they are positively charged (acidic pH) or negatively charged (basic pH) they will favor repulsive forces, which favor solubilization (Domínguez et al., 2023; Shrestha et al., 2021). On the contrary, at the isoelectric point, the net charge is zero, which limits the protein-protein interaction, produces aggregation and precipitation, and reduces solubility (Lo, Kasapis, & Farahnaky, 2021). Also, the protein solubility was related to the hydrophilicity/hydrophobicity balance (Rodríguez-Ambriz et al., 2005).

In the present study, the SC-CO_2 treatment did not affect protein solubility at any of the pHs studied. These demonstrated that there was low to no protein denaturation during treatment since the procedures that denature protein result in LPI with lower protein solubility (Domínguez et al., 2023; Lo et al., 2021). Similarly, another study found that the pressurization (200-600 MPa) of pea protein isolate using phosphate buffer did not influence protein solubility (Chao et al., 2018). Therefore, although these authors used a different medium (phosphate buffer solution instead of supercritical CO_2), the results suggest that the pressure during SC-CO_2 treatment had a low effect on the protein aggregates and thus, protein solubility.

In contrast to our findings, the solubility and dispersibility were improved after the SC-CO_2 treatment of protein isolates from the plum kernel (Sheikh et al., 2023) and pea protein isolates (Vatansever et al., 2022). These authors concluded that SC-CO_2 enhanced surface charge and decreased hydrophobicity due to the physical modification of proteins, and thus, increased their solubility (Vatansever et al., 2022). Furthermore, the removal of bound lipids after the treatment also reduced hydrophobicity, which enhanced hydrophilic characteristics and improves solubility.

3.4.3. Foam capacity and stability

The ability to create stable air bubbles is foaming capacity, an important technofunctional property with several applications in food products (Lo et al., 2021). In this case, it is important to measure both, foaming capacity and foaming stability. The results revealed that the foaming capacity of LPI-Control was 55%, while in LPI-SF increased to 73% (Table 4). These values were in agreement with those reported in previous studies with LPI obtained from *L. albus* (31.86-60%), *L. angustifolius* (49.28-60%) (Kebede & Teferra, 2023; Vogelsang-O'Dwyer et al., 2020), and *L. luteus* (89.29%) (Piornos et al., 2015). In contrast, higher foaming capacity ranging between 112% and 242% was observed by other authors in multiple lupins (Albe-Slabi et al., 2022; Domínguez et al., 2023; Lqari et al., 2002) and soy (Zhao, Zhang, Liu, Zhang, & Ao, 2018) protein isolates. However, it is important to highlight that different procedures to measure foaming (different pH, protein content, solvent, etc.) could justify these discrepancies among the studies.

On the other hand, the determination of foam stability is important to measure the foam quality. Foam stability involves the formation of a thick, resistant, cohesive, and viscoelastic membrane around the bubble (Chao et al., 2018; Rodríguez-Ambriz et al., 2005). The value of foam stability of LPI-Control was 83.5%, and in LPI-SF was 87.7%. These outcomes agree with the results reported in LPI from *L. luteus* (76.9%) (Domínguez et al., 2023), and *L. albus* and *L. angustifolius* (~90%) (Vogelsang-O'Dwyer et al., 2020), and were higher than LPI from *L. angustifolius* (66.7%) (Lqari et al., 2002) and *L. albus* (40-70%) (Albe-Slabi et al., 2022).

Our findings showed that SC-CO_2 treatment produced a significant increase in LPI foaming capacity and stability. This fact was also observed in pea protein treated with supercritical CO_2 (Vatansever et al., 2022). These authors theorized that high pressure during the SC-CO_2 treatment promoted protein unfolding which enhanced the air encapsulation and foaming capacity and stability (Vatansever et al., 2022). This may be related to the disruption of disulfide bonds, which increase the exposure of sulfhydryl groups (Sheikh et al., 2023). The same conclusion was observed in pea protein isolate since pressure-induced protein unfolding promotes the foaming capacity (Chao et al., 2018). Not only pressure, but the formation of amide bonds by the

630 interaction of CO_2 and amine groups of specific amino acids alters the secondary structure of the
631 proteins. The changes in protein structure due to CO_2-protein interaction increased exposure of
632 embedded hydrophobic and free-SH groups (Sheikh et al., 2023), and it is well-known that the
633 presence of multiple hydrophobic sites facilitates protein interactions in the air-water interface
634 (Bou et al., 2022). Moreover, the higher protein amount with surface-active groups in LPI-SF is
635 positively related to the foaming capacity and stability since it is well known that foaming
636 increases with increased protein content (Vatansever et al., 2022; Vogelsang-O'Dwyer et al.,
637 2020). Finally, the removal of fat and the improvement of fat dispersion after SC-CO_2 treatment
638 increased the surface hydrophobicity of proteins, which can also explain the higher foaming
639 ability of LPI-SF than LPI-Control.

640 3.4.4. Emulsion capacity and stability

641 Proteins are excellent emulsifiers due to their amphiphilic character (Chukwuejim et al., 2023).
642 In the present study, the emulsion capacity of LPI-Control was 55.8% and LPI-SF was 56.1%
643 (Table 4), while emulsion stability results were 59.8% for LPI-Control and 56.1% for LPI-SF.
644 Similar results were observed in other LPI from *L. albus* (69% capacity and 66.7% stability)
645 (Lqari et al., 2002), *L. albus* and *L. angustifolius* (~49%) (Kebede & Teferra, 2023) and soy isolate
646 (50.94% capacity and 51.22 stability) (Zhao et al., 2018).

647 In our case, the SC-CO_2 did not produce changes in emulsion capacity, while slightly decreasing
648 emulsion stability. Similar findings were reported in canola seed protein isolate (Li et al., 2021)
649 and sesame seed protein concentrate (Abirached et al., 2020), where the treatment with SC-CO_2
650 had no effect on emulsifying properties. The emulsifying capacities are related to protein surface
651 charge, solubility, and hydrophobicity that favor the interaction between protein and water/oil
652 interface and produce a rigid membrane to stabilize the emulsion by spatial repulsion (Li et al.,
653 2021). In contrast to our findings, the treatment with SC-CO_2 improved the emulsion
654 characteristics of pea protein (Vatansever et al., 2022). These authors concluded that the treatment
655 enhanced the charge of protein isolate, which promoted emulsifying properties. However, it is

656 also important to note that they relate this increase in emulsifying capacity and stability to an
657 increase in protein solubility, while we have not observed changes in the solubility of LPI, which
658 could explain the differences in the emulsifying characteristics between our study and the study
659 of Vatansever et al. (2022). In fact, a strong correlation between emulsifying properties and
660 protein solubility was reported by other authors, who concluded that there was more protein in
661 the oil/water interface when protein was highly soluble (Chukwuejim et al., 2023). Additionally,
662 Kang et al. (2017) also noted that defatting soy flour with SC-CO_2 increased the emulsifying
663 properties of soy protein isolate, which may be caused by the higher protein content of this protein
664 isolate.

665 As a general conclusion, in this study, the technofuntional properties were improved or not
666 affected by the SC-CO_2 treatment. This fact was related to the high protein content, the better-
667 structured protein, disruption of non-covalent bonds due to CO_2-protein interactions, and the
668 changes in protein charges, hydrophobicity/hydrophilic rate, and protein´s secondary structure
669 after CO_2 depletion and/or high pressure used during the treatment.

670 **4. Conclusions**

671 The results presented in this manuscript demonstrated that LPI have great potential in the food
672 industry. For this purpose, the goal of the processes for obtaining protein isolates is to achieve
673 maximum purity without affecting their technofunctional properties. With the present research, it
674 has been shown that the application of SC-CO_2 dramatically improved the purity of the LPI,
675 eliminating much of the color (making it neutral and desirable for the food industry) and
676 antitechnological factors such oil, without affecting its nutritional quality (same amino acid
677 content). Furthermore, it improved or did not affect the main technofunctional properties of the
678 LPI.

679 In addition to the improved protein isolate, the extracted oil can be a non-traditional noble source
680 of bioactive compounds (carotenes, essential fatty acids, vitamins, tocopherols, etc.) that can be

used as active ingredients in the food industry, which adds value to the overall process and contributes to a circular economy.

The main restrictions include that currently, the application of this technology is limited, as a general rule, to products with high prices since this technology uses very expensive equipment. Furthermore, the potential for industrial scale-up must be verified. However, the advancement of SC-CO_2 in recent years has led to lower prices, as well as the possibility of treating protein isolates in large quantities, with equipment capable of treating hundreds of L per cycle already on the market. Thus, the need for new protein concentrates and isolates in the food industry, together with the advancement of supercritical fluid extraction technology, presents a potential for joint application to obtain improved protein isolates.

In general conclusion, the SC-CO_2 process produces a significant improvement in the purity, color, and technofunctional properties of LPI, while reducing solvent requirement, processing time, waste production, and energy consumption. Additionally, treated LPI (LPI-SF) presents excellent nutritional quality, extremely high purity, and low content of undesirable substances such as lipids, which suggest its potential application as a new food ingredient (new product formulation and fortification), but further research is needed on their use and functionalization. Thus, although this manuscript provides innovative information for lupin valuation, additional knowledge about the extraction and purification of proteins, their stability, and how protein isolates can be exploited in different foods is required.

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904

905 **Table 1**. Effect of supercritical CO_2 treatment on lupin protein isolate yields, chemical
906 composition, total polyphenol content, alkaloids, saponins, and color parameters

	LPI type		***SEM***	***Sig.***
	LPI-Control	***LPI-SF***		
Total Yield (g treated LPI/100 g of initial LPI)	100	90.5±0.22	1.438	***
Extracted oil (%)	-	9.63±0.44	1.453	-
Chemical composition (g/100 g)				
Moisture	0.00±0.00	0.00±0.00	0.000	ns
Oil	15.6±0.35	5.27±0.14	1.685	***
Protein (N^x6.25)	86.3±0.25	96.0±0.59	1.477	***
Protein (N^x5.7)	78.7±0.23	87.6±0.54	1.347	***
Ash	5.19±1.20	6.41±0.49	0.129	ns
Saponin (g oleanolic acid/100 g)	0.99±0.09	0.86±0.04	0.027	**
Alkaloids (mg/kg)	657±214	659±187	87.93	ns
Total polyphenol content (mg GAE/100 g)	162±21.9	127±6.97	6.902	**
Color parameters				
L*	77.4±0.24	84.6±0.64	1.090	***
a*	4.68±0.08	1.24±0.21	0.521	***
b*	45.8±0.55	22.9±0.27	3.457	***

907 SEM: Standard error of the mean; Sig: significance; ns: not significant; *: $p<0.05$; **: $p<0.01$; ***:
908 $p<0.001$; ns: not significant

909

910 **Table 2**. Effect of supercritical CO_2 treatment on amino acid composition (mg/g protein) of
911 lupin protein isolate

	LPI type		***SEM***	***Sig.***
Amino Acids	***LPI-Control***	***LPI-SF***		
Aspartic acid	97.7±3.36	94.4±1.78	0.894	ns
Serine	52.3±1.40	51.7±0.81	0.330	ns
Glutamic acid	218±8.44	214±2.74	1.877	ns
Glycine	42.0±1.27	42.8±0.62	0.301	ns
Arginine	113±1.77	114±1.54	0.491	ns
Alanine	33.9±1.51	33.7±0.89	0.341	ns
Proline	45.7±1.65	46.4±0.60	0.359	ns
Cysteine	17.9±1.22	17.9±1.56	0.386	ns
Tyrosine	35.6±2.91	36.1±0.53	0.581	ns
Non-Essential Aas	*657±4.54*	*651±1.74*	*1.283*	*ns*
Histidine	29.0±0.81	29.9±0.78	0.260	ns
Threonine	34.5±0.73	34.9±0.74	0.210	ns
Valine	42.9±0.77	43.7±0.43	0.204	ns
Methionine	6.09±0.27	6.04±0.82	0.168	ns
Lysine	49.2±1.23	47.9±0.74	0.342	ns
Isoleucine	47.7±0.93	48.7±0.54	0.259	*
Leucine	85.8±1.84	87.8±1.06	0.512	*
Phenylalanine	47.9±1.48	50.0±0.55	0.436	*
Essential Aas	*343±4.54*	*349±1.74*	*1.283*	*ns*
E/NE	*0.52±0.01*	*0.54±0.00*	*0.003*	*

912 SEM: Standard error of the mean; Sig: significance; ns: not significant; *: $p<0.05$; ns: not significant

913

914 **Table 3**. Effect of supercritical CO_2 treatment on chemical score (%) and total indispensable
915 amino acids (mg/g protein) of lupin protein isolate

		LPI type		*SEM*	*Sig.*
	FAO/WHO/UNU (2007)	***LPI-Control***	***LPI-SF***		
Histidine	10	193±5.43	199±5.21	1.733	ns
Isoleucine	30	159±3.10	162±1.81	0.862	*
Leucine	59	145±3.12	149±1.80	0.867	*
Lysine	45	109±2.73	106±1.63	0.759	ns
Met+Cys	22	109±5.41	109±7.84	1.854	ns
Methionine	16	38.0±1.72	37.8±5.10	1.049	ns
Cysteine	6	298±20.3	298±26.1	6.433	ns
Phe+Tyr	38	220±11.3	226±2.07	2.456	ns
Threonine	23	150±3.15	152±3.20	0.911	ns
Valine	39	110±1.97	112±1.11	0.522	ns
Total indispensable amino acids (mg/g protein)	277	397±6.24	403±2.19	1.598	*

916 SEM: Standard error of the mean; Sig: significance; ns: not significant; *: $p<0.05$; ns: not significant

917

Table 4. Effect of supercritical CO_2 treatment on technofunctional properties of lupin protein isolate

	LPI type		*SEM*	*Sig.*
Technofunctional properties	***LPI-Control***	***LPI-SF***		
Water absorption capacity (g/g)	1.25±0.02	1.45±0.03	0.032	***
Oil absorption capacity (g/g)	1.67±0.04	1.84±0.08	0.030	**
Foam capacity (%)	55.0±3.29	73.0±1.10	2.796	***
Foam stability (%)	83.5±2.98	87.7±1.32	0.896	*
Emulsion capacity (%)	55.8±2.08	56.1±3.28	0.757	ns
Emulsion stability (%)	59.8±1.23	56.1±1.69	0.696	**

SEM: Standard error of the mean; Sig: significance; ns: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ns: not significant

Figure Captions

Figure 1. Visual aspect of the LPI-Control (a), LPI-SF treated with supercritical CO_2 (b), and extracted oil (c)

Figure 2. Protein solubility of LPI-Control and LPI-SF at pH range of pH 3 and pH 9

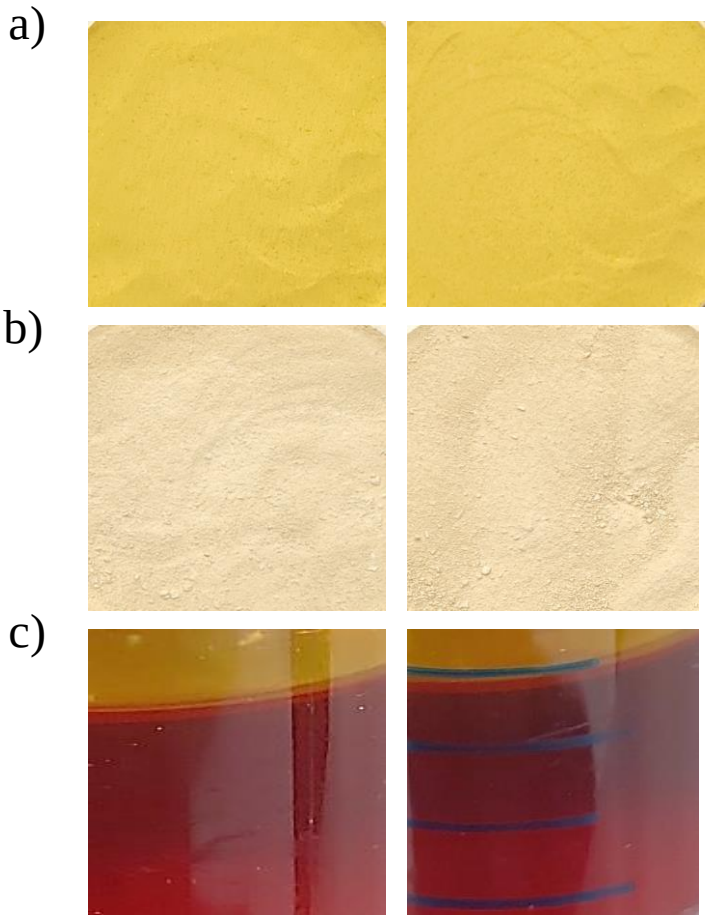
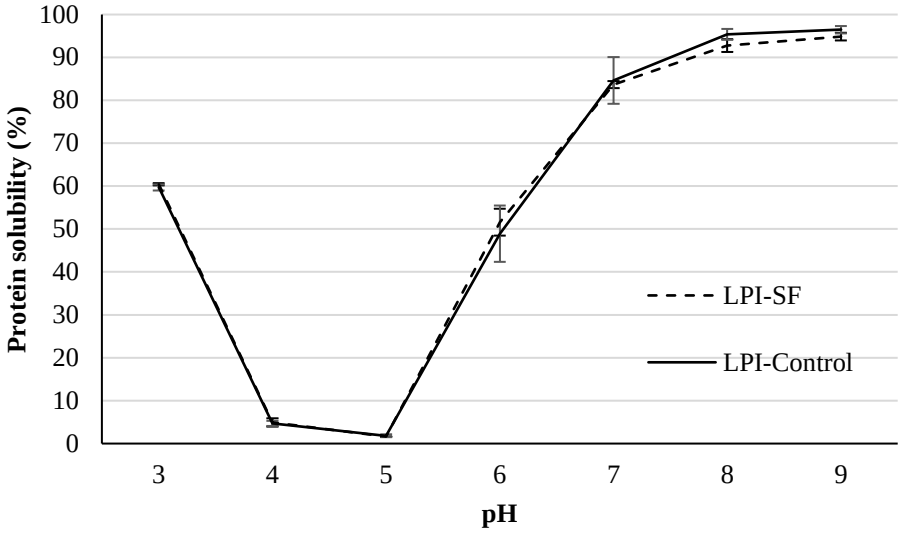


Fig. 1


100
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Protein solubility (%)
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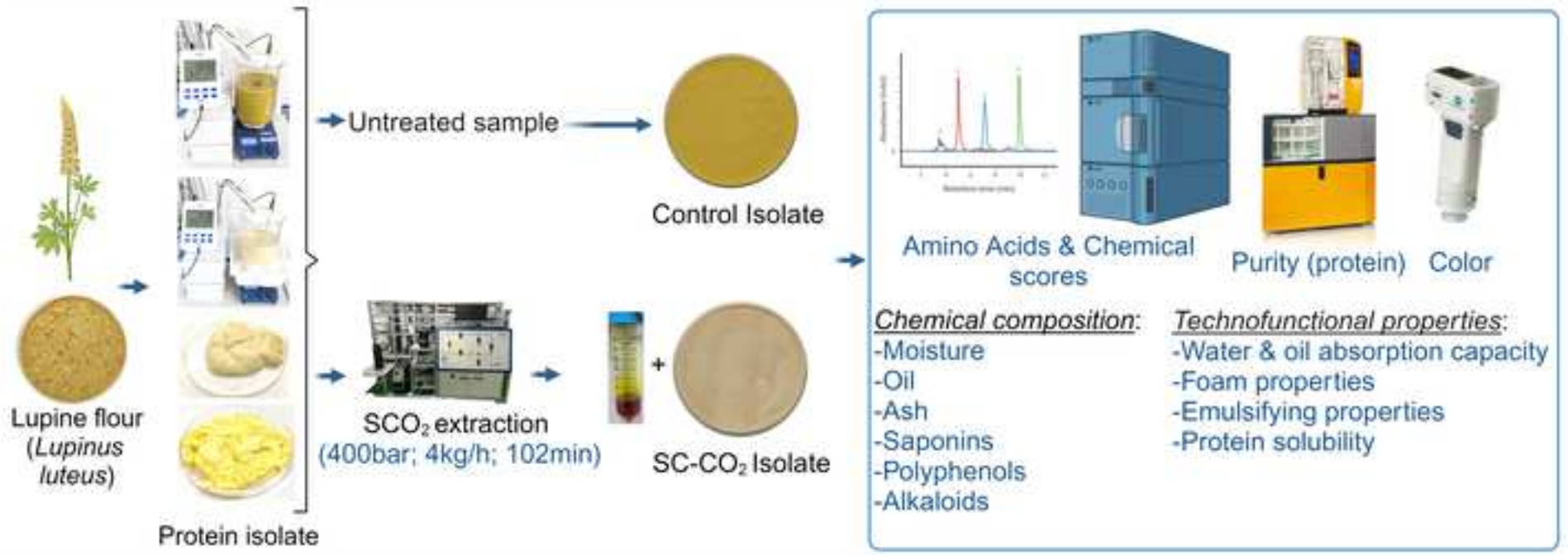
LPI-Control

Fig. 2

Graphical Abstract



Highlights:

- The SC-CO_2 treatment increases the purity of lupin protein isolate (LPI)
- The SC-CO_2 treatment did not modify the LPI amino acids profile & nutritional quality
- The SC-CO_2 improves LPI color by dramatic reduction of a* and b* parameters
- The SC-CO_2 improve or did not affect the technofunctional properties of LPI
- Lupin protein isolate is a valuable resource for functional food ingredients