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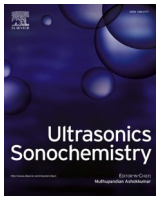

High-power ultrasound pretreatment for enhanced protein extraction from lupin flour: Impact on yield, anti-technological and anti-nutritional factors, and techno-functional properties

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ABSTRACT

Quality of lupin protein is considerably compromised by the presence of anti-nutritional (ANF), such as polyphenols, alkaloids and saponins and anti-technological factors (ATF), like polyphenols and fat. This work explores how high-power ultrasound-assisted (HPU) pretreatment of lupin flour (LF) before protein extraction could affect ANF and ATF content, as well as protein yield and techno-functional properties. LF pretreatments for 3 and 9 min, using water and ethanol–water (1:4 v/v), were carried using conventional mechanical stirring (952 rpm) and HPU (sonotrode, 24 kHz) at 30 and 60 °C. Ultrasonic field was characterized by computing acoustic pressure from frequency spectra analysis.

In general terms, pretreatment reduced noticeably ANF and ATF in both LF and protein isolate (LPI), while also producing a protein concentrate (LPC, avg. 65 g protein/100 g) with low ANF and ATF content. Total protein yield, adding LPC and LPI, was increased by 15 % due to the pretreatment, which also enhanced the techno-functional properties of LPI, such as water absorption index (avg. 26 %) and foaming (avg. 8 %) and emulsifying properties (avg. 14 %). But, the efficiency of the pretreatment was largely affected by process variables. In particular, HPU reduced fat (avg. 27 %), saponin (avg. 21 %) and phenolic content (avg. 17 %), as well as antioxidant activity (avg. 11 %). In addition, the highest protein yield was achieved by sonication using water at 30 °C. This may be explained by ultrasonic field measurements, which revealed higher acoustic pressure levels under these conditions.

1. Introduction

The global population is growing rapidly, driving an increased demand for novel protein sources. In this context, legume proteins have emerged as a promising alternative that offers greater sustainability than traditional animal-based proteins [1]. *Lupinus luteus*, native to the Mediterranean region, stands out for its high protein content and exceptional adaptability, growing in soils and climates where soybean cultivation is unfeasible. Despite its numerous advantages, the main handicap of this legume is the presence of anti-nutritional factors (ANF), such as polyphenols, saponins and alkaloids. These compounds can potentially hinder the absorption and use of essential minerals, vitamins and proteins, thereby reducing their bioavailability and digestibility. They could also affect the sensory attributes of lupin protein isolates, contributing to bitterness and astringency. In addition, the presence of alkaloids can also lead to serious health risks due to their inherent toxicity [2,3,4,5,6]. Moreover, lupin seeds also contain anti-technological factors (ATF), such as fat, which reduce both yield and purity of protein isolates. Similarly, polyphenols are also classified as ATF as they induce color changes in protein isolates and decrease extraction yield and purity by phenolic-protein binding [2,7]. Consequently, optimizing extraction processes is essential for obtaining high yields and purity, as well as high-quality lupin protein isolates (LPI), facilitating their subsequent incorporation into food formulations. The extraction of protein isolates typically involves (i) solubilizing proteins at alkaline pH and (ii) precipitating them at their isoelectric point [7]. Although ANF and ATF are reduced during the protein extraction process, some undesirable compounds may still remain in the final isolates.

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Therefore, pretreating lupin flour (LF) before protein solubilization could present an effective strategy to eliminate these compounds and enhance protein extraction efficiency. Emerging technologies like high-power ultrasound (HPU), moderate and pulsed electric fields (MEF and PEF, respectively) or supercritical fluids (SF) have shown potential for effective ANF and ATF removal, while reducing energy consumption, shortening extraction time and minimizing the use of solvents [7,8]. HPU-assisted extraction is widely employed to extract ANF and ATF from different plant matrices such as saponins from lentils, fenugreek and lupin [6], alkaloids from lupin [9], polyphenols from red beans [10], antioxidant compounds from chickpeas [11]or fat from rapeseed flakes [12], among others. Moreover, these pretreatments may induce structural changes of proteins altering the isolate techno-functional properties like foaming capacity and stability [13], emulsifying capacity and stability [14]or water and fat absorption indexes [15], which are critical factors for the development of food products. Additionally, it is necessary to consider that the effectiveness of HPU-assisted extraction is influenced by process variables, such as the ultrasonic power, treatment duration, temperature and solvent choice [16]. However, there is currently limited research investigating the effect of these HPU variables in the context of lupin extraction. Therefore, this study aimed to assess the impact of HPU pretreatment on lupin flours and protein isolates regarding yields, content of ANF and ATF and techno-functional properties.

2. Materials and methods

2.1. Sample preparation

Seed samples of yellow lupin (*Lupinus luteus* L. var. *tremosilla*) were purchased from Semillas Batlle S.A (Barcelona, Spain). Seeds were milled by an industrial vertical hammer mill (Sitem-gran Ibérica S.L., 22 kW and 3 mm screen size) to finally obtain a flour with a particle size range from 200 to 1000 μm.

2.2. Conventional and high-power ultrasound pretreatments of flour

The experimental setup for LF pretreatments is shown in Fig. 1. Conventional extraction was conducted at 952 rpm using a mechanical stirrer (RZR 2021, Heidolph, Germany). On the other hand, an ultrasonicator (UP400St, Heilscher Ultrasound Technology, Germany) with a 2 cm diameter tip was employed in HPU pretreatments. Both pretreatments were conducted with a flour-to-solvent extraction ratio of 1:6 w/v, using water or an ethanol–water mixture (1:4 v/v) as solvents, at different times (3 and 9 min) and temperatures (30 and 60 °C) and trials were performed in triplicate. For temperature control, extractions were carried out in a closed 200 mL jacketed vessel equipped with a chiller-heating unit (Model 89202–974, VWR, Pennsylvania, United States). It is important to note that the vessel had a 4 cm headspace, which could lead to volatile accumulation due to ethanol evaporation when pretreating at 60 °C. Treated flours were centrifuged at 5000 rpm for 10 min, freeze-dried for 48 h to reach a final moisture content close to 7 % wet basis and stored at −26 °C until further chemical analysis. The yield of extraction was calculated with Eq. (1).

$$PLF\ Extraction\ yield\left(\frac{g\ PLF}{100\ g\ LF}\right) = \frac{m_{PLF}}{m_{LF}} \times 100 \quad (1)$$

where, m_{PLF} is the mass of the resulting pretreated lupin flour (PLF) and m_{LF} is the initial mass of dried lupin flour (LF).

After pretreatments, a significant fraction of the LF was solubilized in the medium. Proteins of this fraction were recovered by adjusting the pH to the isoelectric point (pI) of lupin proteins (4.7) [17], using 4 N HCl to facilitate precipitation. Then, the resulting precipitates were centrifuged at 5000 rpm for 10 min, freeze-dried for 48 h to reach a final moisture content close to 7 % wet basis and stored at −26 °C. This dried precipitate, known as lupin protein concentrate (LPC), was subjected to chemical analysis and techno-functional characterization. The yield of extraction and protein yield was calculated with Eqs. (2) and (3), respectively.

$$LPC\ Extraction\ yield\left(\frac{g\ LPC}{100\ g\ LF}\right) = \frac{m_{LPC}}{m_{LF}} \times 100 \quad (2)$$

$$LPC\ Protein\ yield\ \left(\frac{g}{100\ g\ LF}\right) = PC_{LPC} \times \frac{m_{LPC}}{m_{LF}} \quad (3)$$

where, m_{LPC} is the mass of lupin protein concentrate (LPC), m_{LF} is the initial mass of dried lupin flour (LF) and PC_{LPC} is the protein content of the LPC determined as explained in Section 2.5.

2.3. Protein isolation procedure

After pretreatments, protein isolates (LPI) were obtained from PLF following the experimental procedure exhaustively detailed by Navarro-

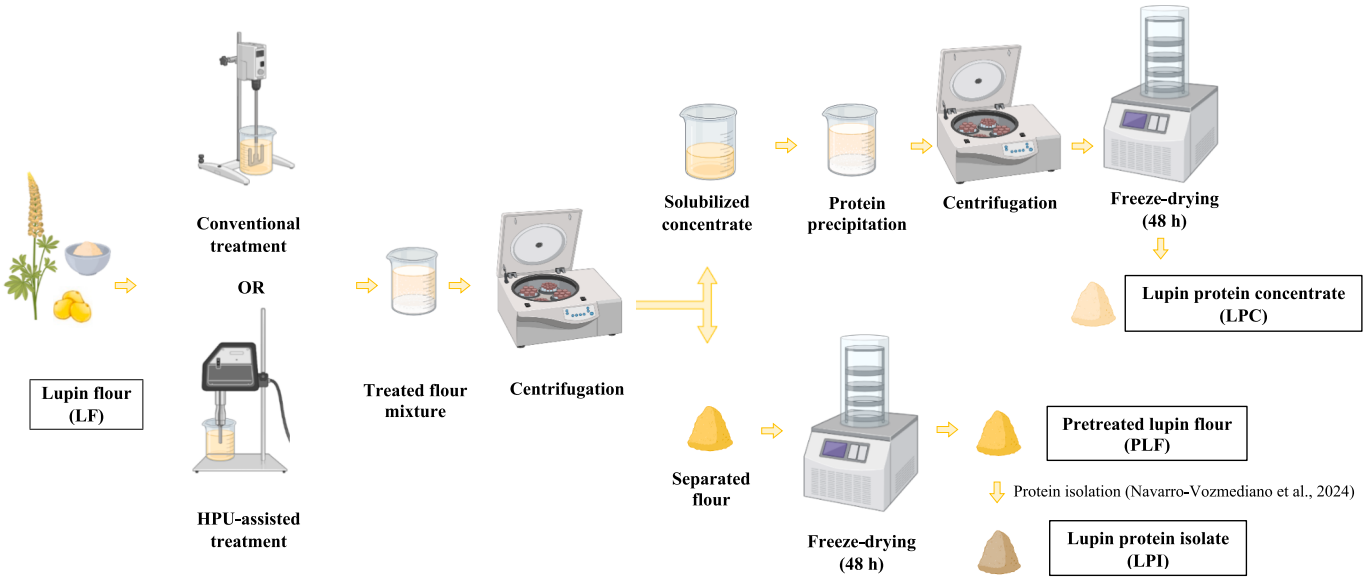


Fig. 1. Scheme of flour pretreatments.

Vozmediano et al. [5]. 40 g of PLF were homogenized with 320 mL of distilled water using an Ultra-Turrax DI 25 Basic (IKA, Staufen, Germany) at 12000 rpm for 5 min and the pH was adjusted to 10.3 with 2 M NaOH. Suspension was stirred for 90 min (RZR 2021, Heidolph, Germany) and then centrifuged for 10 min at 5000 rpm (Digicen 21 R, Orto Alresa, Madrid, Spain). The supernatant pH was then readjusted to the pI of proteins (4.7) using 4 N HCl to induce precipitation. Finally, after centrifugation at 5000 rp m for 10 min (Digicen 21 R, Orto Alresa, Madrid, Spain), protein isolates were freeze-dried and stored at −26 °C until further chemical analysis and techno-functional characterization. Extraction and protein yields of LPI were calculated using equivalent expressions to the one shown in Eqs. (2) to (3) for the LPC.

2.4. Characterization of ultrasonic field

Two different approaches were used to characterize the ultrasonic field generated in HPU pretreatments. On the one hand, the widely used calorimetric method [16]was employed, where the specific heat of the lupin seeds was assumed to be similar to that of soybeans (1.84 J/g°C) [18]. On the other hand, the acoustic pressure was measured according to the recently published standard by the International Electrotechnical Commission (IEC TS 63001: 2019). This methodology, to our knowledge never used before in ultrasonic applications in foods, is based on the spectral analysis of the ultrasonic signal, which allows to split the acoustic pressure in three components: (i) field pressure (P_0), (ii) stable cavitation pressure (P_s) and (iii) transient cavitation pressure (P_t) while the total acoustic pressure (P_T) may be estimated from Eq. (4).

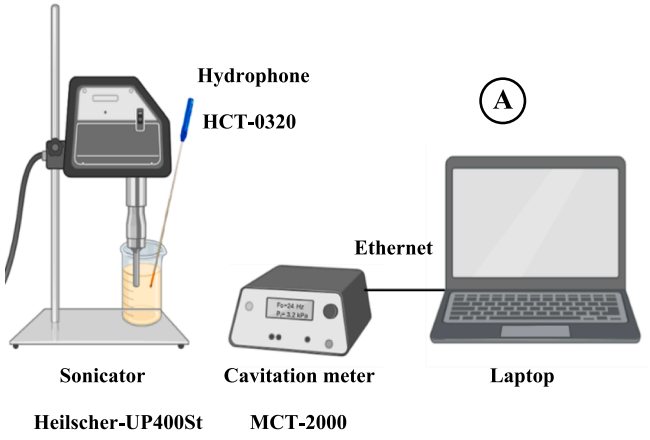
$$P_T = \sqrt{P_0^2 + P_s^2 + P_t^2} \quad (4)$$

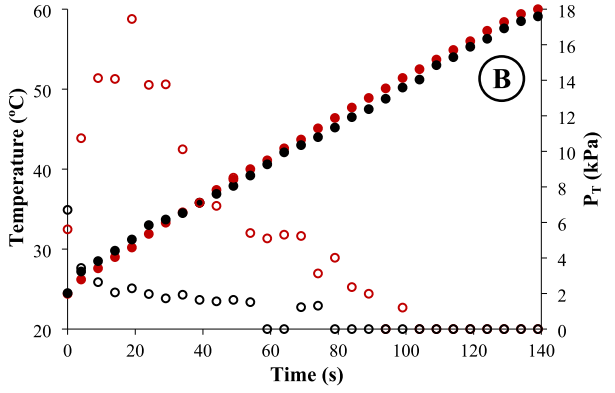
Fig. 2A illustrates the experimental set-up used for the measurement of the acoustic pressure in which the hydrophone (HCT-0320, Ondasonics, California, United States) was positioned at a 5° angle and displaced 2 cm from the sonicator tip. The hydrophone was connected to a cavitation meter (MCT-2000, Ondasonics, California, United States), which performs the spectral analysis and converted the signals into pressure (kPa). Measurements of the ultrasonic field were performed at 100 % amplitude using water and ethanol–water solvents and flour dispersions (1:6 ratio w/v) and were extended for 140 s during calorimetry test.

2.5. Chemical analyses

2.5.1. Protein content

The protein content was determined using the Kjeldahl method 2.062 [19]considering a protein factor of N × 6.25. Results were expressed as grams of protein per 100 g of dry matter (g/100 g dm).





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	P_o (kPa)	P_s (kPa)	P_t (kPa)	P_T (kPa)	P (W)
Water	12.8±1.7^b	12.6±3.0^a	11.4±0.8^a	21.4±2.2^b	105±12^a
Ethanol-water	16.9±4.7^a	13.5±3.2^a	12.4±2.8^a	25.1±5.5^a	88±7^a
Water+LF	9.4±3.1^c	4.2±2.2^b	3.9±1.6^b	11.1±3.8^c	111±13^a
Ethanol-water+LF	2.1±1.4^d	1.2±0.7^c	0.8±0.3^c	2.5±1.5^d	103±8^a

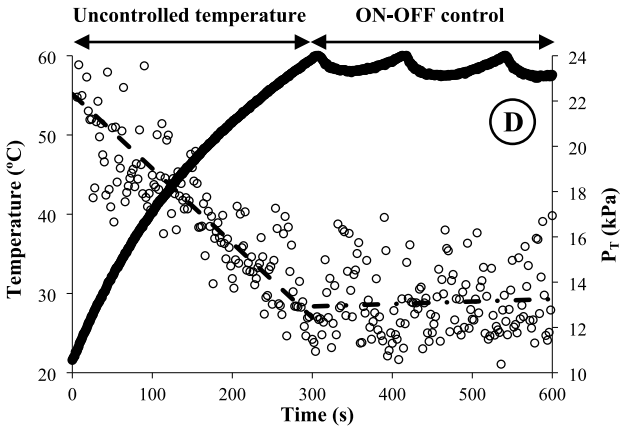

Fig. 2. Acoustic field characterization set-up, A. Experimental set-up, B. Evolution of temperature (filled dots) and total acoustic pressure (P_T) (unfilled dots) during calorimetry tests of lupin flour dispersions in water (red) and ethanol–water solvents (black), C. Average and standard deviation (from 0 to 60 s) of the direct field (P_0), stable cavitation (P_s), transient cavitation (P_t) and total acoustic pressure (P_T) and acoustic power measured (P) during calorimetry tests. Different lowercase letters in columns indicate significant ($p < 0.05$) differences, D. Evolution of temperature (filled dots) and total acoustic pressure (P_T) (unfilled dots) during sonication of water solvent with (ON-OFF control) and without temperature control (dashed line shows the linear fit in both periods). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5.2. Fat content

The Soxhlet extraction method 991.36 [20]was employed to measure the fat content. Results were expressed as grams of fat per 100 g of dry matter (g/100 g dm).

2.5.3. Total polyphenol content and antioxidant activity

Methanolic extracts for total polyphenol content and antioxidant activity quantification were obtained by combining 0.5 g of sample with 5 mL of 0.2 M HCl in CH_3OH and mixed for 1 min at 9500 rpm using an Ultra-Turrax (DI 25 Basic, IKA, Staufen, Germany). Mixtures were ultrasonicated in a water bath for 15 min (USC500T, VWR, United States) and stirred for 15 min on an orbital shaker at 125 rpm (Rotabit, PSelecta). Then, mixtures were centrifuged at 10000 rpm for 10 min (Medifriger BL-S, P-Selecta, Barcelona, Spain) and filtered through a 0.45 μm nylon syringe filter (Filter-Lab, Barcelona, Spain). Samples were stored in the dark at −28 °C until analysis.

Determination of total polyphenol content.

Quantitative analysis of total polyphenol content was carried out following the Folin-Ciocalteu method [21]with slight modifications. The procedure involves mixing 10 μL of extracts with 50 μL of Folin − Ciocalteu reagent, 100 μL of 20 % Na_2CO_3 and 840 μL of distilled water. After mixing, the samples were allowed to rest in darkness for 30 min at room temperature before measuring their absorbance at 700 nm using a Biochrom EZ Read 2000 spectrophotometer (Biochrom, Cambridge, United Kingdom). A calibration curve was established using known concentrations of gallic acid in CH_3OH. The experiments were conducted in triplicate and the results were reported as milligrams of gallic acid per 100 g of dry matter (mg GAE/100 g dm).

Determination of antioxidant activity.

Antioxidant activity (AA) was determined by the ferric reducing antioxidant power assay (FRAP) [22]with slight modifications to adapt to microplates. Briefly, 10 μL of extract were mixed with 180 μL of FRAP reagent (composed by 0.01 M TPTZ in 0.04 M HCl, 0.02 M $FeCl_3{\cdot}6H_2O$ and acetate buffer at pH 3.6 in the ratio 1:1:1 (v/v/v)). The mixtures were then incubated in darkness at 37 °C for 30 min. Absorbance was measured at 595 nm using a Biochrom EZ Read 2000 spectrophotometer (Biochrom, Cambridge, United Kingdom). A calibration curve was established with known concentrations of Trolox in CH_3OH. The tests were performed in triplicate and results were reported as micromoles of Trolox per 100 g of dry matter (μM Trolox/100 g dm).

2.5.4. Alkaloid content

Alkaloid extracts were obtained following the guidelines set by [23]. Alkaloid content was quantified using a modified version of Ruiz [24] titration method. Briefly, 1 g of samples was mixed with 32 mL 0.5 N HCl, followed by sonication for 30 min (Ultrasonic bath, ATM 12L, ATU, Paterna, Valencia) and centrifugation at 5000 rpm for 10 min (Digicen 21 R, Orto Alresa, Madrid, Spain). The pH of the resulting extracts was adjusted to 10 with 4 N NaOH. Subsequent steps involved two CH_2Cl_2 extractions, after which the organic phases were rotaevaporated and resuspended in 1 mL of CH_3OH. Titration was performed using 0.01 N $CH_3C_6H_4SO_3H$ in CH_3OH and 0.1 % $C_{22}H_{14}Br_4O_4$ in CH_3OH as an indicator. Measurements were repeated at least in triplicate, and results were expressed as grams of lupinine per 100 g of dry matter (g lupinine/100 g dm).

2.5.5. Total saponin content

Total Saponin Content (TSC) was determined using a spectrophotometric method outlined by Navarro del Hierro et al. [25]. Initially, 1 g of samples was mixed with 10 mL of CH_3OH, followed by 15 min of sonication (Ultrasonic bath, ATM 12L, ATU, Paterna, Valencia) and 10 min centrifugation at 5000 rpm (Digicen 21 R, Orto Alresa, Madrid, Spain). Subsequently, 25 μL of the extracts were combined with 100 μL of $C_8H_8O_3$ in C_2H_6O (1:10 w/v) and 1mL of 72 % H_2SO_4. Mixtures were then heated at 60 °C for 10 min and quickly cooled in an ice bath for 5 min. Absorbance was measured at 540 nm using a spectrophotometer (Helios gamma 9423 UVG 1702E, Thermo, England). A calibration curve was established with known concentrations of oleanolic acid solutions. The tests were repeated in triplicate and the results were expressed as grams of oleanolic acid per 100 g of dry matter (g oleanolic acid/100 g dm).

2.5.6. Fat, saponin and polyphenol content retention

Additionally, the percentages of fat content (FC), total saponin content (TSC) and total polyphenol content (TPC) retention in the pretreated flour (PLF), concentrates (LPC) and isolates (LPI), relative to unpretreated flour (LF), were calculated with Eq. (5).

$$Retention\ (\%) = \frac{C_x \times Ey_x}{C_{LF}} \tag{5}$$

where, Cx is the content of the compound in the PLF, LPC or LPI, Eyx is the extraction yield of PLF, LPC or LPI and CLF is the content of the compound in the LF, determined as described in Sections 2.2, 2.3., 2.5.2., 2.5.3. and 2.5.5.

2.6. Techno-functional properties of LPI and LPC

2.6.1. Water and fat absorption capacity

Water and fat absorption indexes (WAI and FAI) were evaluated following the method proposed by Lian et al. [26] with minor modifications. 0.5 g of LPI and LPC were homogenized with 5 mL of distilled water or sunflower oil. After standing for 30 min at room temperature, mixtures were centrifuged at 4000 rpm for 25 min (Medifriger BL-S, P-Selecta, Barcelona, Spain). Supernatants were discarded and absorption capacities were expressed as grams of water or oil absorbed per 100 g of dry matter (g water/100 g dm and g oil/100 g dm).

2.6.2. Foaming and emulsifying properties

Foaming and emulsifying properties were determined at pH 7, according to Wang et al. [27] and Akasha et al. [28], respectively.

For foaming capacity and stability, 20 mL of LPI and LPC solution at 1.5 % were stirred at 12000 rpm in a 2.8 cm diameter tube for 1 min using an Ultra-turrax (DI 25 Basic, IKA, Staufen, Germany). Foam height was measured immediately to calculate foaming capacity as a percentage of increased volume. After 1 and 2 h, remaining foam height was also measured to calculate foam stability. Finally, foaming capacity (FCA) and foam stability (FS) after 1 and 2 h were calculated by Eqs. (6) and (7), respectively.

$$FCA(\%) = \frac{Vf_0}{V} \times 100 \tag{6}$$

$$FS(\%) = \frac{Vf_t}{Vf_0} \times 100 \tag{7}$$

where, Vf_0 is the volume of foam at 0 min, V is the volume of sample (20 mL) and Vf_t is the volume of foam after 1 or 2 h.

For emulsifying activity and stability, 6 mL of LPI and LPC solution at 1 % in distilled water were stirred with 2 mL of sunflower oil (Aceites Abensa, Jobellan S.L, Alicante, Spain) for 1 min at 12000 rpm (DI 25 Basic, IKA, Staufen, Germany). Aliquots (20 μL) of emulsion and 1.5 mL of 1 % (w/v) SDS solution were mixed and absorbance at 500 nm was measured immediately and after 10 min. Finally, emulsifying capacity (as emulsifying activity index, EAI) and emulsion stability (as emulsion stability index, ESI) were calculated by following Eqs. (8) and (9), respectively.

$$EAI\left(\frac{m^2}{g}\right) = \frac{2 \times T \times A_0 \times F}{\varphi \times C \times L} \tag{8}$$

$$ESI\ (\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10 \tag{9}$$

where, T is 2.303, A_0 is the absorbance of emulsion at 0 min, F is the dilution factor (76), φ is the oil volumetric ratio, C is protein concentration (g/m^3), L is the length of the light path (0.007 m) and A_{10} is the absorbance of the emulsion after 10 min.

2.6.3. Instrumental color

The color of the LPI and LPC was assessed using a colorimeter (Konica Minolta CM-2600D, Tokyo, Japan). The instrument was previously calibrated with illuminant D65 and a 10° visual angle. CIELab system parameters L* (lightness), a* (green–red), and b* (blue-yellow) were recorded and the h* (hue angle), C* (chroma), and ΔE (total color differences between LPI and LPC) were calculated [29].

2.7. Statistical analysis

Experimental results were reported as mean value ± SD and were analyzed by one-way variance analysis (ANOVA, $p < 0.05$) to determine significant differences between samples. Additionally, the effects of high-power ultrasound application, time, temperature and type of solvent on chemical composition of PLF, LPC and LPI and techno-functional properties of LPC and LPI were examined by multifactorial analysis of variance (ANOVA, $p < 0.05$) considering interactions of level 2 between the factors. LSD (Least Significant Difference) intervals were used to determine significant differences between averages. Statistical tests were carried out using the Statgraphics Centurion XVIII software (Statpoint Technologies, Virginia, United States).

3. Results and discussion

3.1. Ultrasonic field characterization

Fig. 2B reports an example of calorimetry test and the measurement of the acoustic pressure for the pretreatment of LF in water and ethanol–water solvents and average values (from 0 to 60 s) are shown in Fig. 2C. The calorimetry and acoustic pressure measurements carried out in the solvents were very similar (Fig. 2C), which denotes that water and ethanol do not present noticeable differences in the acoustic wave propagation. However, when the acoustic pressure measurements were carried out in the flour dispersions significant ($p < 0.05$) differences were found between both solvents (Fig. 2B and 2C). Thereby, all the terms of the acoustic pressure, P_s, P_t and P_0, were much higher in water + LF than ethanol–water + LF dispersions (Fig. 2C). This fact provides evidence that the solvent noticeable affects the elastic properties of the medium, probably linked to modifications of its viscosity caused by different solute solubility in water than ethanol of the LF, resulting in modifications of the acoustic pressure levels. Despite the differences observed in the acoustic pressure, differences in the calorimetry tests were minimal, as observed in Fig. 2B, as well as the average acoustic power (P) computed (Fig. 2C). Therefore, heat release to the medium seems to be independent of the physical properties since (i) high attenuating media generate heat due to friction forces and (ii) low attenuating media generate cavitation voids whose implosion also generate heat. A thorough analysis of these aspects is a critical area of research and must be addressed in future studies to optimize liquid-borne ultrasonic applications and fully understand the associated phenomena. The addition of the LF to the solvent brought about a significant ($p < 0.05$) reduction of the acoustic pressure, which was noticeable for the 3 acoustic pressure components (Fig. 2C). Thus, in the case of the water solvent, P_0, P_s, P_t were reduced from 12.8, 12.6, 11.4 to 9.4, 4.2, 3.9 kPa, respectively. Similar reductions were found for the ethanol–water solvent. However, differences in the acoustic power supplied to the medium were much smaller and only affected by specific heat of the flour.

Fig. 2B illustrates how the acoustic pressure is reduced as the temperature increased during the calorimetry test. The negative effect of the temperature on the acoustic pressure has been extensively reported in the literature. However, the observed temperature reduction could also be related to the dissipation of acoustic energy. To clarify this aspect, acoustic pressure was monitored during the ultrasonic treatment of water solvent from room temperature up to 60 °C, and the temperature was then controlled as described in Section 2.2 using an ON-OFF control loop (Fig. 2D). The experimental results again illustrated the reduction in acoustic pressure as the temperature increased and how it remained fairly constant once the temperature was controlled. In LF dispersions, the increase in temperature involves a reduction of the acoustic pressure to values close to 0 at temperatures above 45 °C for the ethanol–water + LF dispersion and above 50 °C for the water + LF dispersion (Fig. 2B). Although, it has to be considered that the acoustic field brought about by the sonicator is not homogenous and the acoustic pressure figures below the tip will be much higher than the ones measured (2 cm apart from the tip). The measurement of the acoustic pressure below the tip is strongly discouraged by the manufacturer to avoid damage on the microphone due to cavitation.

3.2. Effect of pretreatment process parameters on the yield and protein content

Extraction yields of PLF ranged from 57.9 to 73.2 g/100 g LF (avg. 65.2 g/100 g LF) and protein content ranged from 31.5 to 42.0 g protein/100 g PLF (avg. 36.6 g protein/100 g PLF) (Table 1). The experimental results revealed a significant ($p < 0.05$) effect of pretreatment conditions on both PLF yield and protein content. Thereby, sonication and longer extraction times led to a significant ($p < 0.05$) decrease of extraction yield (6 and 7 %, respectively, Table 1). In addition, sonication also led to a significant ($p < 0.05$) protein content reduction of PLF (10 %), while temperature rise from 30 to 60 °C or ethanol–water solvent significantly ($p < 0.05$) increased protein content by 5 and 9 %, respectively (Table 1).

It is important to highlight that the observed mass loss of flour is due to the solubilization of a soluble fraction during the pretreatment, which results in the LPC. Thus, LPC extraction yield ranged from 9.6 to 27.0 g/100 g LF (avg. 18.8 g/100 g LF), protein content from 58.9 to 72.6 g protein/100 g LPC (avg. 64.8 g protein/100 g LPC) and protein yield ranged from 6.2 to 15.8 g/100 g LF (avg. 12.4 g/100 g LF) (Table 1). The high yield and protein content qualify LPC as a valuable and sustainable source of plant-based protein for food industry, comparable to concentrates from other legumes such as cowpea (18 g concentrate/100 g and 79 g protein/100 g) or horsegram (13.5 g concentrate/100 g and 79 g protein/100 g) [30]. As expected, the effect of pretreatment conditions on LPC was the opposite of that observed for PLF. Thereby, sonication and longer pretreatments resulted in a significant ($p < 0.05$) increase in LPC extraction yield (32 and 37 %, respectively) and protein yield (31 and 39 %, respectively) (Table 1). While, statistical analysis revealed that the influence of temperature and solvent was not significant ($p > 0.05$) on LPC extraction and protein yields and the effect on protein content was very moderate (Table 1). Experimental results agree with the previous literature about the HPU capability of improving protein solubility [31,32]. HPU technology induces cavitation and micro-agitation, generating increased turbulence and disruption of the boundary layer. Moreover, these phenomena can also disrupt cell structures, facilitating the interaction between solvent and plant matrix by improving solvent penetration and solute diffusion, which in turn improves extraction efficiency [31,33,32].

As for the LPI, extraction ranged from 7.7 to 17.1 g/100 g LF (avg. 12.2 g/100 g LF), protein content from 69.9 to 88.5 g protein/100 g LPI (avg. 82.7 g protein/100 g LPI) and protein yield from 6.4 to 14.0 g/100 g LF (avg. 10.6 g/100 g LF) (Table 1). HPU application and increasing temperature from 30 to 60 °C reduced ($p > 0.05$, Table 1) by 25 and 10 % LPI extraction yield, respectively. While as for protein content, the effect of pretreatment variables was minimal (Table 1). In overall terms, protein yield from LPC was higher than the one from LPI for the different pretreatments. In addition, total protein yield, adding LPC and LPI, was

Table 1
Extraction yield, protein content and protein yield of pretreated lupin flours (PLF) and their concentrates (LPC) and isolates (LPI).

	T (°C)		t (min)	Extraction yields (g/100 g LF)			Protein content (g protein/100 g)			Protein yield (g/100 g LF)	
				PLF	LPC	LPI	PLF	LPC	LPI	LPC	LPI
Water	30	no-HPU	3	73.2 ± 2.9^{a}	9.6 ± 1.4^{k}	17.1 ± 0.5^{a}	35.2 ± 0.8^{def}	65.1 ± 0.6^{cde}	82.4 ± 0.6^{cd}	6.2 ± 0.9^{h}	14.1 ± 0.4^{a}
			9	67.3 ± 0.6^{bcd}	13.8 ± 1.5^{ij}	13.9 ± 3.3^{bcd}	36.9 ± 2.4^{cde}	66.5 ± 1.9^{bcd}	81.9 ± 1.3^{d}	9.2 ± 1.0^{g}	11.4 ± 2.7^{bcde}
	60		3	60.9 ± 2.8^{efg}	20.7 ± 0.5^{def}	14.2 ± 1.7^{abcd}	38.1 ± 0.1^{bcd}	64.8 ± 0.6^{de}	82.9 ± 1.2^{bcd}	13.4 ± 0.3^{bc}	11.8 ± 1.4^{abcde}
			9	66.2 ± 1.9^{bcd}	24.3 ± 1.1^{bc}	12.0 ± 1.5^{def}	34.9 ± 2.4^{defg}	68.9 ± 0.5^{b}	84.3 ± 1.1^{bcd}	16.8 ± 1.1^{cd}	10.1 ± 1.3^{defg}
	30	HPU	3	64.7 ± 3.0^{cde}	15.9 ± 1.3^{hi}	10.0 ± 1.1^{efg}	31.5 ± 0.0^{g}	72.6 ± 1.5^{a}	85.7 ± 1.6^{abc}	11.6 ± 0.9^{f}	8.6 ± 1.0^{fgh}
			9	57.9 ± 0.1^{g}	26.3 ± 1.8^{ab}	15.4 ± 0.7^{abc}	32.2 ± 0.4^{fg}	62.7 ± 0.6^{ef}	83.7 ± 1.4^{bcd}	16.5 ± 1.1^{b}	12.9 ± 0.5^{abc}
	60		3	70.6 ± 1.3^{ab}	19.6 ± 0.8^{fg}	9.2 ± 0.6^{fg}	36.6 ± 2.1^{cde}	61.5 ± 1.0^{fg}	86.1 ± 1.0^{ab}	12.0 ± 0.5^{ef}	8.0 ± 0.6^{gh}
			9	63.3 ± 2.5^{def}	22.4 ± 1.6^{cd}	7.7 ± 0.3^{g}	35.2 ± 1.1^{def}	67.3 ± 2.2^{bcd}	82.9 ± 0.7^{bcd}	15.1 ± 1.0^{bc}	6.4 ± 0.2^{h}
Ethanol-water	30	no-HPU	3	72.8 ± 0.7^{a}	11.7 ± 0.3^{jk}	15.4 ± 2.0^{abc}	39.7 ± 1.7^{abc}	60.8 ± 2.2^{fg}	81.0 ± 1.7^{d}	7.1 ± 0.2^{h}	12.5 ± 1.7^{abcd}
			9	59.7 ± 2.6^{fg}	23.6 ± 1.8^{c}	12.9 ± 1.4^{cde}	39.6 ± 0.1^{abc}	67.0 ± 0.5^{bcd}	75.8 ± 3.1^{e}	15.8 ± 1.2^{a}	9.8 ± 1.1^{efg}
	60		3	69.8 ± 2.5^{ab}	11.9 ± 0.9^{jk}	15.8 ± 0.6^{abc}	42.0 ± 0.4^{a}	58.9 ± 0.5^{g}	88.5 ± 1.0^{a}	7.0 ± 0.6^{h}	14.0 ± 0.5^{a}
			9	68.0 ± 1.6^{bc}	14.5 ± 1.3^{i}	16.2 ± 1.4^{ab}	41.4 ± 1.2^{ab}	62.9 ± 0.2^{ef}	81.5 ± 0.5^{d}	9.1 ± 0.8^{g}	13.2 ± 1.1^{ab}
	30	HPU	3	63.0 ± 2.0^{def}	19.8 ± 0.4^{efg}	11.6 ± 0.7^{def}	34.5 ± 0.7^{efg}	67.4 ± 2.1^{bc}	69.9 ± 2.9^{f}	13.3 ± 0.2^{de}	8.1 ± 0.5^{fgh}
			9	59.9 ± 0.2^{fg}	27.0 ± 0.7^{a}	11.9 ± 0.1^{def}	36.5 ± 1.8^{cde}	63.2 ± 0.3^{ef}	88.5 ± 0.9^{a}	17.1 ± 0.5^{bcd}	10.5 ± 0.1^{cdef}
	60		3	66.2 ± 2.8^{bcd}	18.0 ± 0.7^{gh}	11.5 ± 1.8^{def}	37.5 ± 1.5^{cde}	61.5 ± 0.5^{fg}	81.7 ± 3.6^{d}	11.1 ± 0.5^{f}	9.4 ± 1.5^{efg}
			9	59.5 ± 3.4^{fg}	22.2 ± 0.5^{cde}	10.1 ± 0.5^{efg}	34.2 ± 3.9^{efg}	64.9 ± 0.1^{cde}	86.5 ± 1.3^{ab}	14.4 ± 0.3^{cd}	8.7 ± 1.0^{fgh}

EY, extraction yield; PC, protein content; PY, protein yield; HPU, refers to sonicated pretreatments; no-HPU, refers to unsonicated pretreatments.
Values are presented as average ± SD. Different lowercase letters in columns indicate significant differences ($p < 0.05$).

higher (avg. 22.8 g/100 g LF) than the one obtained from LF (19.8 g/100 g LF) regardless of the pretreatment conditions. Thereby, pretreatment allowed achieving an average extraction efficiency of 54 % of the total protein available in the LF (42.4 g/100 g dm). It was obvious that longer extraction times significantly ($p < 0.05$) increased LPC + LPI protein yield. The highest protein yield was observed when sonication was performed at 30 °C using water as the solvent. This can be explained by the findings presented in Section 3.1, which demonstrated that acoustic pressure was higher in water compared to ethanol–water dispersions and it decreased as the temperature increased.

3.3. Effect of pretreatment process parameters on the ANF and ATF content of PLF, LPC and LPI

3.3.1. Fat content

Fat content (FC) of PLF ranged from 2.11 to 4.77 g/100 g PLF, which illustrates that pretreatment may reduce FC up to 44 % compared to LF (3.79 g/100 g LF) (Table 2). However, pretreatment may also increase FC depending on experimental conditions tested. Thus, HPU or the use of ethanol–water as solvent, significantly ($p < 0.05$) decreased the FC of PLF by 27 and 14 %, respectively (Table 2). Similar findings were reported by Perrier et al. [12] and Krishnan et al. [34], where HPU-assisted treatments decreased FC from rapeseed flakes (39 %) and rice bran (78 %), respectively, compared to control samples. Due to the nonpolar nature of fat and the relatively lower polarity of ethanol compared to water, ethanol proves to be a more effective solvent for fat extraction [35,12]. This enhanced extraction capability may also elucidate the slight increase in PLF protein content observed in Section 3.2. when using an ethanol–water solvent, as the removal of fat potentially leads to a higher concentration of protein. Moreover, statistical analysis showed that the influence of temperature and pretreatment time was not significant ($p > 0.05$).

It is reasonable to expect that conditions promoting fat reduction in flour would have the opposite effect on LPC, as the extracted fat will either remain in the solvent or be retained in the concentrate. This assumption was supported by data, which showed that PLF and LPC retained an average value of 65 and 34 % of the FC present in LF, respectively. The FC of LPC ranged from 3.94 to 9.27 g/100 g LPC (Table 2) and was also affected by pretreatment conditions. Specifically, HPU significantly ($p < 0.05$) increased FC of LPC 26 %, while the temperature rise from 30 to 60 °C resulted in a significant ($p < 0.05$) 21 % reduction of FC (Table 2). For LPC, the effect of the solvent and pretreatment time on FC was not significant ($p > 0.05$).

As for the LPI from PLF, their average FC (6.58 g/100 g LPI) was 36 % lower than that of the LPI from LF (10.33 g/100 g LPI). FC of LPI ranged between 3.19 to 10.19 g/100 g LPI (Table 2). Longer LF pretreatments significantly ($p < 0.05$) reduced by 46 % the FC of LPI, while increasing temperature from 30 to 60 °C led to a 17 % FC increase. Interestingly, most LPI showed a higher FC than PLF and LF (Table 2), which reveals a concentration effect of protein isolation. Nevertheless, LPI from PLF only retained an average of 22 % of the FC originally present in the LF compared to a retention of 39 % in LPI from LF. It is noteworthy that no previous studies have evaluated the effect of HPU, time, temperature and type of solvent on the FC of PLF, LPC and LPI.

3.3.2. Total polyphenol content and antioxidant activity

LF pretreatment resulted in a 43 % reduction in the total polyphenol content (TPC) of PLF compared to LF (2.72 mg gallic acid/100 g LF). Despite the low TPC in LF, avoiding their presence is recommended, as these compounds can adversely impact the nutritional quality of the LPI

Table 2
Anti-nutritional and anti-technological compounds of pretreated lupin flours (PLF) and their concentrates (LPC) and isolates (LPI).

	T (°C)		t (min)	FC (g/100 g dm)			TPC (mg gallic acid/g dm)			AA (μM Trolox/g dm)		
				PLF	LPC	LPI	PLF	LPC	LPI	PLF	LPC	LPI
Water	30	no-HPU	3	4.67 ± 0.71^{ab}	5.51 ± 0.28^{f}	9.30 ± 0.52^{abc}	1.96 ± 0.15^{a}	1.07 ± 0.11^{hi}	1.40 ± 0.11^{ab}	6.36 ± 0.24^{a}	4.92 ± 0.59^{e}	5.62 ± 0.40^{b}
			9	4.59 ± 0.05^{ab}	8.13 ± 0.07^{b}	4.28 ± 0.16^{ef}	1.92 ± 0.17^{ab}	1.37 ± 0.12^{bcd}	1.46 ± 0.13^{a}	5.39 ± 0.48^{bc}	5.27 ± 0.25^{e}	6.27 ± 0.59^{a}
	60		3	4.77 ± 0.63^{a}	4.24 ± 0.75^{g}	7.38 ± 1.11^{d}	1.93 ± 0.13^{b}	1.18 ± 0.07^{gh}	1.43 ± 0.18^{a}	5.04 ± 0.72^{cd}	5.03 ± 0.35^{e}	5.32 ± 0.36^{b}
			9	4.05 ± 0.45^{abc}	3.94 ± 0.28^{g}	6.74 ± 0.92^{d}	1.58 ± 0.09^{ef}	1.34 ± 0.20^{bcdef}	1.43 ± 0.04^{a}	5.22 ± 0.48^{cd}	3.80 ± 0.23^{g}	3.97 ± 0.34^{cd}
	30	HPU	3	3.77 ± 0.05^{abcd}	9.27 ± 0.04^{a}	8.26 ± 0.77^{bcd}	1.78 ± 0.14^{c}	1.44 ± 0.11^{abc}	1.08 ± 0.13^{def}	4.81 ± 0.41^{de}	4.20 ± 0.37^{f}	5.37 ± 0.61^{b}
			9	3.29 ± 0.75^{cde}	7.43 ± 0.55^{c}	3.45 ± 0.66^{ef}	0.92 ± 0.05^{j}	1.47 ± 0.13^{abc}	1.15 ± 0.16^{cde}	3.90 ± 0.29^{f}	4.24 ± 0.25^{f}	3.23 ± 0.32^{g}
	60		3	3.68 ± 0.33^{bcd}	5.70 ± 0.01^{ef}	10.19 ± 0.08^{a}	1.53 ± 0.13^{efg}	1.20 ± 0.03^{fgh}	1.03 ± 0.16^{ef}	5.24 ± 0.49^{cd}	6.11 ± 0.52^{bcd}	3.50 ± 0.21^{defg}
			9	3.53 ± 0.28^{cd}	7.83 ± 0.34^{bc}	7.69 ± 1.67^{cd}	1.73 ± 0.09^{cd}	1.42 ± 0.06^{abc}	1.16 ± 0.09^{cde}	4.38 ± 0.05^{e}	5.77 ± 0.33^{d}	4.30 ± 0.41^{c}
Ethanol-water	30	no-HPU	3	4.26 ± 0.20^{abc}	6.25 ± 0.16^{de}	9.45 ± 0.74^{ab}	1.79 ± 0.19^{bc}	1.22 ± 0.08^{efgh}	1.13 ± 0.07^{de}	4.99 ± 0.26^{cd}	3.99 ± 0.22^{fg}	3.39 ± 0.28^{fg}
			9	4.06 ± 0.10^{abc}	7.73 ± 0.22^{bc}	4.70 ± 1.51^{ef}	1.33 ± 0.14^{hi}	1.23 ± 0.08^{defgh}	1.19 ± 0.15^{cd}	4.48 ± 0.46^{e}	5.19 ± 0.31^{e}	3.41 ± 0.41^{efg}
	60		3	4.24 ± 0.11^{abc}	4.33 ± 0.29^{g}	9.78 ± 0.03^{ab}	1.45 ± 0.14^{gh}	1.36 ± 0.13^{bcde}	1.27 ± 0.12^{bc}	5.71 ± 0.43^{b}	6.39 ± 0.26^{ab}	3.49 ± 0.30^{efg}
			9	4.13 ± 0.33^{abc}	6.49 ± 0.19^{d}	4.92 ± 0.08^{e}	1.57 ± 0.14^{efg}	1.49 ± 0.09^{ab}	1.07 ± 0.07^{def}	4.83 ± 0.23^{de}	6.63 ± 0.25^{a}	5.41 ± 0.34^{b}
	30	HPU	3	3.86 ± 0.47^{abcd}	6.45 ± 0.05^{d}	7.55 ± 0.37^{d}	1.64 ± 0.15^{de}	0.97 ± 0.06^{i}	1.15 ± 0.11^{cde}	5.18 ± 0.58^{cd}	4.06 ± 0.15^{fg}	3.80 ± 0.59^{def}
			9	2.90 ± 0.39^{def}	7.98 ± 0.56^{bc}	3.78 ± 0.28^{ef}	0.87 ± 0.07^{j}	1.33 ± 0.15^{cdefg}	0.98 ± 0.06^{f}	3.90 ± 0.21^{f}	5.80 ± 0.14^{cd}	3.68 ± 0.34^{defg}
	60		3	2.33 ± 0.24^{ef}	8.34 ± 0.18^{b}	9.50 ± 0.08^{ab}	1.20 ± 0.06^{i}	1.33 ± 0.14^{cdefg}	1.12 ± 0.12^{def}	5.44 ± 0.26^{bc}	6.14 ± 0.20^{bc}	3.88 ± 0.46^{cde}
			9	2.11 ± 1.05^{f}	5.70 ± 0.08^{ef}	3.19 ± 0.54^{f}	1.46 ± 0.15^{fg}	1.54 ± 0.07^{a}	1.08 ± 0.12^{def}	4.37 ± 0.30^{e}	6.60 ± 0.31^{a}	3.61 ± 0.43^{defg}

	T (°C)		t (min)	TSC (g oleanolic acid/100 g dm)			AC (g lupinine/100 g dm)		
				PLF	LPC	LPI	PLF	LPC	LPI
Water	30	no-HPU	3	1.67 ± 0.08^{ab}	1.50 ± 0.15^{g}	2.02 ± 0.14^{a}	0.33 ± 0.03^{a}	0.017 ± 0.004^{bc}	nd
			9	0.77 ± 0.01^{fgh}	2.00 ± 0.12^{f}	1.78 ± 0.09^{abc}	0.29 ± 0.02^{ab}	0.013 ± 0.000^{de}	nd
	60		3	1.58 ± 0.16^{b}	2.14 ± 0.14^{def}	2.03 ± 0.03^{a}	0.23 ± 0.02^{cd}	0.016 ± 0.002^{bcd}	nd
			9	1.78 ± 0.07^{a}	1.46 ± 0.07^{gh}	1.83 ± 0.25^{abc}	0.12 ± 0.02^{e}	0.009 ± 0.001^{fg}	nd
	30	HPU	3	1.48 ± 0.09^{b}	3.01 ± 0.06^{a}	2.04 ± 0.20^{a}	0.25 ± 0.02^{bcd}	0.007 ± 0.000^{f}	nd
			9	1.15 ± 0.17^{cd}	2.95 ± 0.08^{a}	1.76 ± 0.08^{bc}	0.25 ± 0.03^{bcd}	0.013 ± 0.001^{de}	nd
	60		3	0.78 ± 0.08^{fgh}	2.63 ± 0.13^{b}	2.02 ± 0.09^{ab}	0.24 ± 0.05^{cd}	0.017 ± 0.001^{bc}	nd
			9	0.83 ± 0.01^{efgh}	2.48 ± 0.08^{c}	1.78 ± 0.19^{abc}	0.20 ± 0.04^{d}	0.008 ± 0.001^{f}	nd
Ethanol-water	30	no-HPU	3	0.63 ± 0.05^{h}	1.36 ± 0.06^{h}	1.16 ± 0.15^{d}	0.27 ± 0.03^{bc}	0.013 ± 0.001^{de}	nd
			9	0.91 ± 0.13^{ef}	1.60 ± 0.12^{g}	1.22 ± 0.24^{d}	0.27 ± 0.01^{bc}	0.012 ± 0.001^{ef}	nd
	60		3	0.98 ± 0.07^{de}	1.49 ± 0.08^{gh}	1.84 ± 0.29^{abc}	0.24 ± 0.01^{cd}	0.023 ± 0.000^{a}	nd
			9	1.27 ± 0.06^{c}	1.53 ± 0.04^{g}	1.69 ± 0.19^{c}	0.20 ± 0.02^{d}	0.018 ± 0.001^{b}	nd
	30	HPU	3	0.81 ± 0.12^{efgh}	2.07 ± 0.08^{ef}	1.33 ± 0.19^{d}	0.30 ± 0.00^{ab}	0.015 ± 0.003^{bcde}	nd
			9	0.86 ± 0.06^{efg}	2.18 ± 0.14^{de}	1.71 ± 0.33^{c}	0.23 ± 0.01^{cd}	0.015 ± 0.001^{cde}	nd
	60		3	0.68 ± 0.11^{gh}	2.26 ± 0.12^{d}	2.01 ± 0.18^{ab}	0.25 ± 0.00^{bcd}	0.014 ± 0.001^{de}	nd
			9	0.96 ± 0.08^{def}	2.89 ± 0.10^{a}	1.34 ± 0.03^{d}	0.21 ± 0.00^{d}	0.018 ± 0.000^{b}	nd

FC, at content; TPC, total polyphenol content; AA, antioxidant activity; TSC, total saponin content; AC, alkaloid content; HPU, refers to sonicated pretreatments; no-HPU, refers to unsonicated pretreatments.
Values are presented as average ± SD; nd: non-detectable. Different lowercase letters in columns indicate significant differences ($p < 0.05$).

by reducing its bioavailability and digestibility through interactions with proteins [2,3,5]. Thus, TPC of PLF ranged between 0.87 to 1.96 mg gallic acid/100 g PLF (Table 2) and was affected by pretreatment conditions. In particular, HPU exhibited a significant ($p < 0.05$) impact, reducing the TPC by 17 %. Similarly, longer pretreatment times and the use of ethanol–water as solvent involved a significant ($p < 0.05$) decrease of TPC content of PLF (14 and 15 %, respectively, Table 2). On the other hand, PLF exhibited an average value of the antioxidant activity (AA) of 4.95 μM Trolox/100 g PLF, which resulted in a 14 % reduction compared to LF (5.76 μM Trolox/100 g LF). Thus, AA of PLF ranged from 3.90 to 6.36 μM Trolox/100 g PLF (Table 2) and was significantly ($p < 0.05$) diminished by long pretreatments (15 %) or

sonication (11 %). These findings are consistent with previous studies, which reported that HPU-assisted treatments reduced the TPC and AA in different food matrices, including sunflower seeds [36], green lentil hulls [37], jackfruit [38], soybeans [39]and wheat bran [40]. As previously mentioned in Section 3.2., the improved polyphenolic extraction with HPU could be attributed to the enhanced mass transfer due to mechanisms of cavitation and microagitation, but also to the degradation and instability of polyphenols when exposed to ultrasonic energy [33,40,41]. Additionally, the choice of solvent has been shown to play a crucial role in this process. Ethanol, with its lower polarity compared to water, has a higher affinity with polyphenols, thereby enhancing their extraction [41,36]. Despite previous research demonstrated that temperatures exceeding 45 °C enhance the reduction of TPC and AA [42,43], statistical analysis showed that the influence of temperature was not significant ($p > 0.05$) on the present work.

TPC and AA values of resulting LPC ranged from 0.97 to 1.54 mg gallic acid/100 g LPC and from 3.80 to 6.63 μM Trolox/100 g LPC, respectively (Table 2). As expected, most pretreatment factors that facilitated phenolic removal on PLF involved the opposite effect on LPC. Therefore, longer pretreatments led to LPC with increased TPC (15 %). Additionally, the AA of LPC was significantly ($p < 0.05$) increased by sonication (4 %), long pretreatments (6 %) or the use of ethanol–water solvent (14 %) (Table 2). Interestingly, increasing temperature from 30 to 60 °C during LF pretreatment significantly ($p < 0.05$) increased the TPC and AA in LPC (8 and 23 %, respectively) despite the negligible temperature effect ($p > 0.05$) on PLF (Table 2). It is noteworthy that only an average of 46 % of TPC from LF was retained after LF pretreatments (avg. 37 % in PLF and avg. 9 % in LPC). In contrast, 73 % of the AA was preserved (avg. 56 % in PLF and avg. 17 % in LPC). These findings suggest that, despite more than 53 % of the polyphenols were removed, the remaining polyphenols or new compounds produced during pretreatment, potentially through polymerization reactions, in PLF and LPC still exhibited noticeable antioxidant capacity [44]; J. [45].

LPI from PLF exhibited 23 and 29 % lower values of TPC and AA, respectively, compared to LPI from LF (1.55 mg gallic acid/100 g LPI and 5.99 μM Trolox/100 g LPI). TPC of LPI from PLF ranged from 0.98 to 1.46 mg gallic acid/100 g LPI, while AA ranged from 3.23 to 6.27 μM Trolox/100 g LPI (Table 2). Therefore, the LPI only exhibited an average retention of 6 and 9 % for the TPC and AA present in LF, respectively, while LPI from LF showed higher retention rates of 13 % for TPC and 23 % for AA. As for pretreatments conditions, significant ($p < 0.05$) reduction of 15 % in both TPC and AA was caused by sonication (Table 2). The results also highlighted that using ethanol–water solvent during pretreatments led to significantly ($p < 0.05$) lower TPC and AA in LPI (11 and 18 %, respectively, Table 2). It is important to note that no previous study has examined the influence of HPU pretreatment on the TPC and AA of PLF, LPC and LPI.

3.3.3. Total saponin content

As illustrated in Table 2, LF pretreatment reduced, on average, the total saponin content (TSC) of PLF by 15 % compared to LF (1.26 g oleanolic acid/100 g LF). Like in TPC, pretreatment conditions also affected the final TSC achieved, which ranged from 0.63 to 1.78 g oleanolic acid/100 g PLF. Thus, sonication and ethanol–water solvent significantly ($p < 0.05$) decreased the TSC of PLF (21 and 29 %, respectively, Table 2). Similar conclusions were drawn by Navarro del Hierro et al. [6], who demonstrated that HPU-assisted extraction was an effective method to reduce TSC from lentils, fenugreek and lupin seeds. Moreover, Navarro del Hierro et al. [6] also reported that using ethanol–water as solvent, instead of water, supposed a 146 % increase in lupin saponin extraction. Therefore, the results suggest that saponins may display a higher affinity for solvents with lower polarity than water, which could enhance their extraction from flour. Furthermore, as previously mentioned in Section 3.2., the cavitation and microagitation due to HPU may facilitate solvent penetration and enhance mass transfer, but also potentially affect the solubility and stability of saponins [6].

In the case of LPC, TSC ranged from 1.36 to 3.01 g oleanolic acid/100 g LPC (Table 2). Consistent with the observations in previous sections, it is expected that conditions favoring the removal of saponins from flour had the opposite effect on LPC. Therefore, the TSC of the LPC significantly ($p < 0.05$) increased by 56 % after LF pretreatment with HPU (Table 2). However, using an ethanol–water solvent, instead of water, resulted in a 15 % reduction of TSC (Table 2). Moreover, after pretreatments with ethanol–water solvent, the retention percentages of TSC in both PLF and LPC indicated that approximately 75 % of the saponins originally present in LF were retained (avg. 46 % in PLF and avg. 29 % in LPC), with the remaining 25 % removed. Conversely, when water was used as solvent, nearly 100 % of the saponins were retained when combining both fractions. These results support the earlier observation that saponins possess a higher affinity for low-polar solvents.

On the other hand, LPI from PLF showed an average TSC of 1.72 g oleanolic acid/100 g LPI, resulting in a 13 % reduction compared to LPI from LF (1.99 g oleanolic acid/100 g LPI). In particular, TSC of LPI from PLF ranged from 1.22 to 2.04 g oleanolic acid/100 g LPI (Table 2). It is important to note that protein isolation concentrated TSC, compared to PLF and LF, which could be linked to the pH variations during protein extraction, which cause protein–saponin binding and co-precipitation, thereby elevating the saponin concentration in LPI [46]. However, the TSC of the LPI from PLF only reflected an average retention of 11 to 27 % of the TSC from LF compared to 35 % retention of LPI from LF. The pretreatment variables showed notable effects on TSC. Specifically, using ethanol–water as solvent or longer pretreatment times significantly ($p < 0.05$) decreased the TSC by 19 and 9 %, respectively (Table 2). Conversely, increasing the pretreatment temperature resulted in a significant ($p < 0.05$) rise in TSC by 11 % (Table 2).

3.3.4. Alkaloid content

Regardless of pretreatment conditions, average AC in PLF (0.24 g lupinine/100 g PLF) was 45 % lower than in LF (0.44 g lupinine/100 g LF) (Table 2). AC in PLF ranged from 0.12 to 0.33 g lupinine/100 g PLF and was significantly ($p < 0.05$) affected by pretreatment temperature and time (Table 2). Specifically, PLF at 60 °C showed a 22 % reduction of AC compared at 30 °C and a 16 % reduction if pretreatment for 9 min and 3 min are compared (Table 2). Despite previous research stated that HPU-assisted extraction reduced AC in soursop [47]and lupin seeds [9] compared to conventional method, statistical analysis showed that the influence of HPU was not significant ($p > 0.05$) on the present work.

LPC presented very low alkaloid levels (avg. 0.014 g lupinine/100 g LPC) (Table 2), making them suitable for human consumption, as the AC was below the limit of 0.02 g/100 g [48]. On the other hand, all LPI exhibited non-detectable levels of alkaloids (Table 2). These findings suggested that the water-soluble nature of alkaloids effectively mitigated toxicity concerns by being minimally retained in LPC and completely removed during the protein extraction process of LPI [9,49,50].

In summary, LF pretreatments led to relevant modifications of protein yield and ANF and ATF content and its performance was dependent on process variables. HPU application significantly ($p < 0.05$) intensified the pretreatment, especially at 30 °C, improving the removal of most of undesired compounds. Moreover, the protein yields and ANF and ATF removal reached in HPU experiments using water as solvent were similar to the ones achieved using ethanol–water solvent, which entails a relevant finding for process sustainability and circular economy. In the following section, techno-functional properties of the LPI and LPC with the highest ANF and ATF removal, while preserving great protein yield, were evaluated. Specifically, LPI and LPC from LF pretreated at 30 and 60 °C with HPU using a water solvent, as well as from LF pretreated at 30 and 60 °C with and without HPU using an ethanol–water solvent, were analyzed. The flour pretreatment time was set at 9 min for all treatments.

3.4. Influence of flour pretreatment on the techno-functional properties of LPC and LPI

3.4.1. Water and fat absorption capacities

As seen in Table 3, all LPI from PLF exhibited higher water absorption index (WAI) values, but slightly lower fat absorption index (FAI) values compared to the LPI from LF (1.24 g water/g LPI and 2.63 g oil/g LPI for WAI and FAI, respectively). Thereby, the WAI of LPI from PLF ranged from 1.33 to 1.80 g water/g LPI, while FAI values remained within the 2.03 to 2.33 g oil/g LPI range. These results suggested a stronger affinity of LPI for lipids compared to water. Related to pretreatment conditions, no variable had a statistically significant ($p > 0.05$) effect on WAI. For FAI, the effect of solvent and temperature were not significant ($p > 0.05$). However, the application of HPU during LF pretreatment resulted in LPI with significantly ($p < 0.05$) improved FAI (by 8 %, Table 3). As previously mentioned, the sonication of LF may induce structural changes in proteins, promoting their unfolding and disordering that could ultimately affect the WAI and FAI of the resulting LPC and LPI (Sengar et al., 2022; Yao et al., 2023). Conversely, data presented by Tao et al. [15] showed not significant ($p > 0.05$) differences of WAI and FAI between isolates from untreated and ultrasound pretreated okara flakes. The effects of HPU are highly dependent on the process conditions and the structure of the raw materials used, as extensively discussed in the literature for different applications [51]. This variability contributes to explain the differences observed when compared to previous studies. It must be emphasized that no similar studies have been conducted on lupin or any other legume to date.

As for LPC, both indexes exhibited remarkably high values, ranging from 1.69 to 2.51 g water/g LPC and from 2.15 to 3.28 g oil/g LPC, for WAI and FAI respectively (Table 3). Pretreatment conditions also had a significant ($p < 0.05$) effect on WAI and FAI of LPC. Thus, while increasing temperature or using ethanol–water solvent resulted in 11 and 15 % significant ($p < 0.05$) increase of WAI and FAI, respectively, sonication caused a significant ($p < 0.05$) decrease of 17 % in both indexes (Table 3). Moreover, WAI and FAI of LPC (avg. 2.2 g water/g LPC and 2.8 g oil/g LPC, respectively) were significantly ($p < 0.05$) higher than LPI (avg. 1.6 g water/g LPI and 2.2 g oil/g LPI, respectively), likely due to compositional differences such as the higher FC and potentially greater carbohydrate fraction in LPC.

3.4.2. Foaming properties

The foaming capacity (FCA) of LPI from PLF was, on average, 8 % higher compared to LPI from LF (102.6 %). Concretely, FCA of LPI from PLF ranged from 96.7 to 117.7 % (Table 4) and was influenced by process variables. All measurements were conducted at pH 7. Thus, increasing the temperature to 60 °C significantly ($p < 0.05$) decreased the FCA (9 % compared to 30 °C), while the effect of other pretreatment variables was negligible. Related to foaming stability (FS), the FS_{1h} and FS_{2h} values of LPI from PLF were, on average, 26 and 42 % lower, respectively, compared to LPI from LF (79.1 and 75.5 %, respectively). However, they maintained great stability values, ranging from 44.5 to 74.5 and from 26.8 to 66.5 %, for FS_{1h} and FS_{2h}, respectively (Table 4). In addition, none of the process variables showed a significant ($p < 0.05$) effect on the FS of LPI. In contrast, Tao et al. [15] noted that ultrasound pretreatment of okara flakes diminished FCA of final protein isolates by 21 % but enhanced their FS by more than 100 %, when compared to untreated samples. As previously mentioned in Section 3.4.1, plant characteristics may explain HPU variations, as its effects are influenced by the raw material structure [51].

In the case of LPC, FCA ranged from 40.4 to 110.1 % and FS ranged from 28.8 to 79.0 % and from 9.3 to 68.8 % for 1 and 2 h, respectively (Table 4). Related to LF pretreatment variables, temperatures of 60 °C or using ethanol–water solvent resulted in LPC with significantly ($p < 0.05$) reduced FCA (38 and 28 %, respectively), FS_{1h} (39 and 26 %, respectively) and FS_{2h} (52 and 44 %, respectively) (Table 4). It is important to note that the foaming properties are influenced by the specific protein fractions solubilized at each processing step and could also be related to the fat or carbohydrate content in protein concentrates and isolates [52]; J. C. [53]. In addition, several researchers have demonstrated that the foaming properties of protein isolates are closely linked to their saponin content. The amphiphilic nature of saponins reduces water's surface tension, promoting increased protein adsorption at the air–water interface and enhancing air bubble formation [5,6]. Consequently, the high foaming properties of LPI and LPC from PLF (HPU, 30 °C, water solvent) may be partially attributed to its higher TSC compared to the LPI and LPC from PLF whose techno-functional properties have been analyzed (Table 2). Despite the LPC exhibited significantly ($p < 0.05$) lower FCA than LPI, most of them still exhibited great FCA values. Moreover, FS showed not significant ($p > 0.05$) differences between LPC and LPI, indicating that LPC may be also an effective functional ingredient for food applications.

3.4.3. Emulsifying properties

Most of the LPI from PLF exhibited higher emulsifying activity index (EAI) values than the LPI from LF, with an average of 19.1 m^2/g LPI, representing a 14 % increase over the LPI from LF (16.7 m^2/g LPI). EAI of LPI ranged from 14.6 to 21.1 m^2/g LPI and, like the other techno-functional properties, was affected by the process variables. All measurements were conducted at pH 7. Thus, the application of HPU during LF pretreatment resulted in LPI with significantly ($p < 0.05$) improved EAI (9 %), while pretreating the LF at 60 °C resulted in a 15 % enhancement in LPI's EAI (Table 4). On the other hand, LPI from PLF showed an average ESI value 276 % higher than LPI from LF (22.6 min). The emulsion stability index (ESI) of LPI from PLF ranged from 43.3 to 114.6 min (Table 4). Regarding the process variables, HPU significantly ($p < 0.05$) enhanced the ESI of LPI (89 %), while temperature and solvent had not significant effect ($p > 0.05$). Similar findings were reported by Tao et al. [15], who found that protein isolates from okara flakes pretreated by HPU showed the highest ESI. These results contrast with those obtained by Karki et al. [14], who found that soy protein isolates from soy flour pretreated by HPU exhibited lower EAI and ESI values than the control sample. As noted in Section 3.4.1, the variations in HPU

Table 3
Water and fat absorption indexes of concentrates (LPC) and protein isolates (LPI) from pretreated lupin flours.

	T (°C)		WAI (g water/g dm)		FAI (g oil/g dm)	
			LPC	LPI	LPC	LPI
Water	30	HPU	1.69 ± 0.01^{e}	1.80 ± 0.11^{a}	2.15 ± 0.12^{c}	2.33 ± 0.08^{a}
	60		2.24 ± 0.12^{bc}	1.33 ± 0.04^{c}	2.54 ± 0.26^{b}	2.17 ± 0.04^{ab}
Ethanol-water	30	no-HPU	2.51 ± 0.09^{a}	1.67 ± 0.01^{b}	3.19 ± 0.01^{a}	2.10 ± 0.01^{b}
		HPU	1.96 ± 0.01^{d}	1.43 ± 0.01^{c}	2.74 ± 0.17^{b}	2.17 ± 0.02^{ab}
	60	no-HPU	2.46 ± 0.05^{ab}	1.56 ± 0.04^{b}	3.28 ± 0.02^{a}	2.03 ± 0.16^{b}
		HPU	2.16 ± 0.17^{cd}	1.59 ± 0.04^{b}	2.72 ± 0.08^{b}	2.29 ± 0.01^{a}

WAI, water absorption index; FAI, fat absorption index; HPU, refers to sonicated pretreatments; no-HPU, refers to unsonicated pretreatments.
Values are presented as average ± SD. Different lowercase letters in columns indicate significant differences ($p < 0.05$).

Table 4
Foaming and emulsifying properties of concentrates (LPC) and protein isolates (LPI) from pretreated lupin flours.

	T (°C)		FCA (%)		FS_{1h} (%)		FS_{2h} (%)		EAI (m^2/g)		ESI (min)	
			LPC	LPI	LPC	LPI	LPC	LPI	LPC	LPI	LPC	LPI
Water	30	HPU	110.1 ± 2.9^{a}	117.7 ± 3.6^{a}	79.0 ± 6.1^{a}	74.3 ± 2.7^{a}	68.8 ± 2.9^{a}	66.5 ± 2.5^{a}	21.4 ± 1.5^{a}	21.1 ± 0.5^{a}	136.6 ± 5.5^{a}	114.6 ± 8.5^{a}
	60		91.2 ± 4.3^{c}	96.7 ± 3.3^{c}	61.2 ± 3.8^{b}	44.5 ± 0.5^{c}	41.1 ± 2.5^{d}	33.9 ± 2.7^{d}	16.0 ± 1.4^{c}	19.7 ± 1.3^{b}	108.3 ± 2.5^{b}	73.6 ± 9.0^{c}
Ethanol-water	30	no-HPU	93.0 ± 1.2^{c}	113.4 ± 2.5^{ab}	59.9 ± 1.0^{b}	58.6 ± 1.2^{b}	46.6 ± 0.9^{c}	26.8 ± 2.0^{e}	16.2 ± 2.0^{c}	14.6 ± 1.0^{d}	101.5 ± 7.6^{c}	43.3 ± 4.6^{d}
		HPU	103.9 ± 1.8^{b}	115.3 ± 2.4^{ab}	76.2 ± 3.1^{a}	46.2 ± 4.7^{c}	50.1 ± 0.8^{b}	34.9 ± 0.9^{d}	16.1 ± 2.5^{c}	17.6 ± 0.6^{c}	108.7 ± 4.8^{b}	95.3 ± 9.1^{b}
	60	no-HPU	52.0 ± 2.1^{d}	106.7 ± 9.8^{b}	37.4 ± 2.0^{c}	55.3 ± 3.3^{b}	21.3 ± 1.4^{e}	41.3 ± 2.7^{c}	16.4 ± 0.4^{c}	20.7 ± 0.3^{a}	43.6 ± 3.2^{e}	70.7 ± 2.7^{c}
		HPU	40.4 ± 2.9^{e}	111.8 ± 3.2^{ab}	28.8 ± 4.1^{d}	74.5 ± 3.3^{a}	9.3 ± 1.1^{f}	60.8 ± 2.2^{b}	18.5 ± 0.2^{b}	21.0 ± 0.1^{a}	54.9 ± 3.0^{d}	111.9 ± 6.4^{a}

FCA, foaming capacity; FS, foaming stability for 1 and 2 h; EAI, emulsifying activity index; ESI, emulsifying stability. HPU, refers to sonicated pretreatments; no-HPU, refers to unsonicated pretreatments.
Values are presented as average ± SD. Different lowercase letters in columns indicate significant differences ($p < 0.05$).

could be attributed to the specific properties of the plant source, as its effectiveness is product-dependent [51].

Concerning the LPC, none of the variables showed a significant ($p < 0.05$) effect on the EAI of LPC, with values ranging from 16.1 to 21.4 m^2/g LPC (Table 4). However, the ESI of LPC, which ranged from 43.6 to 136.6 min, was significantly ($p < 0.05$) affected by sonication, temperature and solvent. Concretely, LF sonication resulted in LPC with significantly ($p < 0.05$) improved ESI (10 %), while 60 °C or ethanol–water solvent significantly ($p < 0.05$) decreased the ESI of LPC (39 and 25 %, respectively, Table 4). The results suggested that cavitation and microagitation due to sonication may have induced conformational changes in proteins, improving their flexibility and capacity to unfold at the interface. This phenomenon promotes stronger protein-lipid interactions and enhances surface activity, leading to improved EAI and ESI of LPI and LPC [54]. It is important to note that emulsifying properties of LPC exhibited negligible differences compared to LPI, suggesting that LPC could also be a valuable functional ingredient in food formulation.

The results demonstrated that pretreatment conditions, such as ultrasound, temperature, and solvent choice, noticeable impacted the techno-functional properties of LPI and LPC. In overall terms, pretreatments improved WAI, FCA and EAI while maintaining great stability levels. In particular, HPU enhanced FAI and emulsifying properties of LPI. Thereby, both LPC and LPI showed functional attributes suitable for diverse food applications.

3.4.4. Color properties

The variations in LPI color parameters were minimal and no clear trend was identified (Table 5). Additionally, the total color differences (ΔE) between LPI from PLF and LPI from LF (Fig. 3) were perceptible to the human eye, as they exceeded 5 units of color change [55]. Despite these color differences, our findings revealed that both LPI from LF (L*: 66.6, a*: 8.6, b*: 64.2) and PLF exhibited a brownish-yellow hue (avg. L*: 51.9, a*: 7.9, b*: 49.2; Table 5). This brownish color, similar to cooked meat, suggests that LPI could be a promising ingredient for plant-based meat alternatives. Regarding LPC, significant ($p < 0.05$) effects on color parameters were observed with variations in temperature, HPU application and choice of solvent during LF pretreatment. Nevertheless, all LPC exhibited a trend towards lighter and whiter tones (avg. L*: 78.3, a*: 1.7, b*: 29.6) when compared to the LPI (Table 5 and Fig. 3), suggesting its suitability for new formulations such as fortified pasta or bakery products. Interestingly, the LPC and LPI from ethanol-water PLF showed significantly ($p < 0.05$) darker tones when applying HPU, compared to the LPI and LPC from unsonicated PLF (Table 5 and Fig. 3). Although, future studies should elucidate the color changes caused by heating during thermal treatment or cooking since they can induce Maillard reactions, lipid oxidation, pigment degradation or polymerization that influence the visual aspects of the final product [56,57].

Table 5
Color parameters of lupin protein isolates (LPI) and lupin protein concentrates (LPC) from pretreated lupin flours (L*, lightness value; a*, green–red; b*, blue-yellow; h* hue angle; C*chroma).

	T (°C)		L*		a*		b*		h*		C*	
			LPC	LPI	LPC	LPI	LPC	LPI	LPC	LPI	LPC	LPI
Water	30	HPU	69.9 ± 0.3^{c}	49.8 ± 0.3^{d}	2.5 ± 0.1^{b}	7.9 ± 0.1^{c}	32.9 ± 0.6^{b}	47.0 ± 0.5^{d}	85.7 ± 0.2^{c}	80.5 ± 0.2^{d}	33.0 ± 0.6^{b}	47.7 ± 0.5^{d}
	60		68.6 ± 0.3^{d}	57.3 ± 0.4^{a}	4.1 ± 1.1^{a}	5.3 ± 0.1^{d}	39.8 ± 0.2^{a}	29.1 ± 0.3^{e}	84.1 ± 1.5^{d}	79.7 ± 0.2^{f}	40.0 ± 0.3^{a}	29.6 ± 0.3^{e}
Ethanol-water	30	no-HPU	83.1 ± 0.3^{a}	56.8 ± 0.4^{b}	0.9 ± 0.1^{cd}	8.7 ± 0.2^{b}	26.8 ± 0.2^{d}	52.7 ± 0.7^{c}	88.0 ± 0.2^{b}	80.6 ± 0.2^{c}	26.8 ± 0.2^{d}	53.4 ± 0.7^{c}
		HPU	82.7 ± 0.2^{b}	37.2 ± 0.4^{e}	0.5 ± 0.1^{d}	9.2 ± 0.1^{a}	24.0 ± 0.2^{f}	52.9 ± 0.7^{c}	88.8 ± 0.1^{a}	80.1 ± 0.1^{e}	24.0 ± 0.2^{f}	53.7 ± 0.7^{c}
	60	no-HPU	82.9 ± 0.2^{ab}	56.9 ± 0.2^{b}	1.1 ± 0.1^{c}	7.9 ± 0.1^{c}	28.2 ± 0.4^{c}	55.5 ± 0.5^{b}	87.7 ± 0.1^{b}	81.9 ± 0.1^{a}	28.2 ± 0.4^{c}	56.1 ± 0.5^{b}
		HPU	82.7 ± 0.1^{b}	53.6 ± 0.4^{c}	1.1 ± 0.1^{c}	8.6 ± 0.1^{b}	26.3 ± 0.2^{e}	57.8 ± 0.6^{a}	87.8 ± 0.2^{b}	81.5 ± 0.1^{b}	26.4 ± 0.2^{e}	58.5 ± 0.6^{a}

L*, lightness; a*, green–red; b*, blue-yellow; h*, °hue; C*, chroma. Values are presented as average ± SD. Different lowercase letters in columns indicate significant differences ($p < 0.05$).

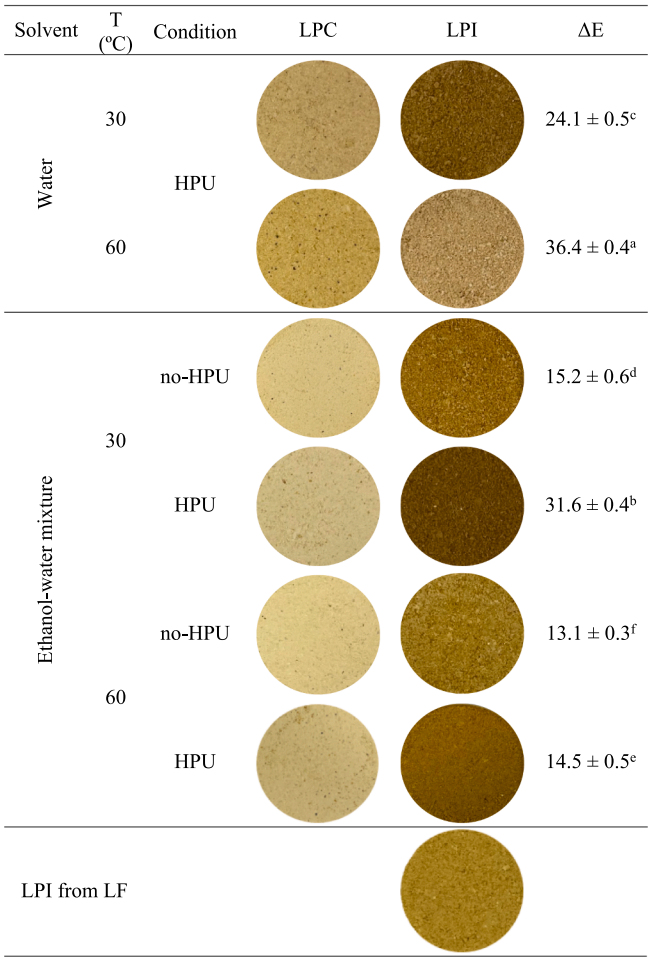

Fig. 3. Images of lupin protein isolates (LPI) and lupin protein concentrates (LPC) from pretreated lupin flours (PLF). Total color differences (AE) between LPI from PLF and LPI from LF. Different lowercase letters indicate homogeneous groups ($p < 0.05$).

4. Conclusions

Flour pretreatment represents a promising strategy for enhancing protein extraction from lupin seeds by reducing the content of undesirable compounds, such as fat, polyphenols, saponins and alkaloids. In addition to the protein isolate (LPI), pretreatment also facilitates the extraction of a valuable protein concentrate (65 g/100 g LPC), which represents the solubilized fraction during pretreatment and can be easily recovered through precipitation. By combining the LPC and LPI fractions, an average extraction of 54 % of the total protein present in lupin flour was achieved. Overall, the LPC and LPI from pretreated lupin flour exhibited lower levels of anti-nutritional factors (ANF) and anti-technological factors (ATF), while retaining excellent techno-functional properties. However, the effects were largely dependent on the specific pretreatment variables used. Sonication led to the highest protein yields while effectively reducing ANF and ATF and maintaining or even enhancing the techno-functional properties of both LPC and LPI. Notably, the effect of sonication was more effective at 30 °C and when water was used as the solvent, which was linked to higher acoustic pressure measured under these conditions. Therefore, the application of high-power ultrasound represents a promising approach for large-scale industrial applications, enabling efficient low-temperature operations while avoiding the use of organic solvents. These aspects are well aligned with the Sustainable Development Goals facing food industry, particularly those aimed at fostering sustainable production practices, promoting innovative technologies that enhance efficiency and advancing principles of the circular economy by reducing energy consumption and minimizing solvent use.

CRediT authorship contribution statement

Paola Navarro-Vozmediano: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Esperanza Dalmau:** Visualization, Validation, Methodology. **Jose Benedito:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Jose V. Garcia-Perez:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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