The use of green technologies for processing lupin seeds (Lupinus angustifolius L.)

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The use of green technologies for processing lupin seeds (*Lupinus angustifolius* L.): Extraction of non-polar and polar compounds for concentrated-protein flour production

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ABSTRACT

This study aimed to promote the valorization of lupin seeds by extracting both non-polar and polar fractions to produce a protein-rich flour suitable for food applications. Green extraction methods such as Supercritical Fluid Extraction (SFE) and SFE followed by gas-expanded liquid extraction with ethanol/CO_2 mixtures were employed. SFE yielded lupin oil with extraction yields ranging from 2.27 ± 0.02 to 4.5 ± 0.2 %, significantly influenced by temperature (40 and 60 °C) and pressure (150–350 bar). SFE extracts exhibited higher tocopherol concentration, particularly α-tocopherol (116.7–296.9 μg/g oil) and γ-tocopherol (2006–4749 μg/g oil), compared to the Bligh and Dyer (B&D) method. The fatty acid profiles were similar, although they differed slightly in composition, with the extracts obtained by SFE having higher proportions of unsaturated fatty acids (UFA) and lower proportions of saturated fatty acids (SFA). Ethanol proportion positively correlated with extraction yield (r = 0.991), resulting in higher recovery of polar lipids (PL). However, increasing ethanol percentage decreased the phenolic compounds content and antioxidant activity assessed by DPPH radical scavenging method. SFE produced lupin flour with 36 % protein content, increased by 11 % post-extraction. Ethanolic extraction also increased protein concentration, albeit less pronounced (6.8–11 % increase post-sequential extraction). Essential amino acids consistently increased post-SFE, highlighting the potential of this sustainable method to yield protein-rich flour free of non-GRAS (Generally Recognized as Safe) solvents and containing compounds essential for human health. SDS-PAGE analysis showed consistent protein profiles across all extracted flours, while FTIR assessment revealed changes in the secondary structure of proteins induced by SFE and SFE followed by gas-expanded liquid extraction processes. These findings highlight the potential of this approach to enhance the nutritional and commercial value of lupin-based products while promoting sustainable food processing practices.

1. Introduction

There is a growing interest in incorporating plant-based ingredients into food formulations, driven by changes in industry production and consumer dietary preferences. In this context, it is crucial to explore as many natural sources as possible to enhance the recovery of macro and micronutrients, along with bioactive compounds, through the use of sustainable methods. This is essential to meet the increasing demand for high-quality products while preserving the environment.

Among the diverse sources of bioactive compounds, lupin seeds have gained attention in recent years due to scientific evidence supporting their health-promoting effects, making them suitable for producing special-purpose foods, such as those designed for people with diabetes, vegetarians, and individuals with celiac disease (Arnoldi et al., 2015). Lupin belongs to the *Fabaceae* or *Leguminosae* family and encompasses various species, but only a few are cultivated today for human or animal consumption. These include white lupin (*Lupinus albus*, mainly cultivated in Europe), yellow lupin (*Lupinus luteus*, mainly cultivated in the Mediterranean region), narrow-leaved lupin (*Lupinus angustifolius*, mainly cultivated in Australia and North Europe) and Tarrwi lupin (*Lupinus mutabilis*, mainly cultivated in South America) (Kurlovich & Kartuzova, 2002). The lupin seeds exist in two variants: sweet and bitter, differing in alkaloid content. Sweet lupin presents low alkaloid levels (<200 mg/kg) in accordance with food standards, while bitter varieties exhibit significantly higher alkaloid levels, ranging from 15,000 to 22,000 mg/kg (Lemus-Conejo et al., 2023).

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The nutritional composition of lupin varies significantly depending on the specific species and cultivars. These seeds are characterized by high protein levels (29.5–55 %), featuring a well-balanced profile of essential amino acids as well as an adequate protein efficiency ratio (2.17–2.67) (Shrestha et al., 2021; Sujak et al., 2006). Additionally, lupin is a notable source of dietary fiber (14–40 %), with moderate to high levels of oil (5.5–20 %) and ash (3.4–5.1 %), as well as a low carbohydrate content (4.5–10.4 %) (Boukid & Pasqualone, 2022; Shrestha et al., 2021; Yorgancilar & Bilgiçli, 2014). Beyond its macronutrient profile, lupin seeds contain essential phytochemicals such as polyphenols (19.4 mg GAE per 100 g fresh weight), carotenoids (101.19 mg/100 g oil), squalene (triterpene: 1.74 mg per 100 g fresh weight), phytosterols (53.6 mg per 100 g fresh weight), and tocopherols (166–225 mg per kg dried matter), offering various health benefits compared to other legume like black-eyed peas, pinto beans, split peas, and white beans (Al-Amrousi et al., 2022; Khan et al., 2015). Lupin is also a good source of essential fatty acids (linoleic and linolenic) (Lemus-Conejo et al., 2023), and essential micronutrients such as Mn, Fe, Zn, and Se (Bartkiene et al., 2016).

Considering its entire chemical composition, lupin emerges as an attractive plant material for incorporation into food formulations. However, the exploration of this raw material remains relatively limited, as most studies mainly focus on its protein fraction. This suggests that there is a rich fraction of bioactive compounds in this legume that requires better utilization for its complete valorization.

Supercritical fluid extraction (SFE) is a well-studied technology for maximizing plant material valorization. It offers a greener approach compared to traditional methods, with superior solvent penetration and faster extraction rates. Carbon dioxide (CO_2) is the preferred solvent due to its non-toxic, human, and environmentally safe, non-flammable, widely available, chemically inert, low viscosity, low surface tension, high diffusivity, and relatively low-cost nature. This technology has demonstrated its effectiveness in extracting oil from lupin beans (*Lupinus mutabilis*) (Yu et al., 2023), as well as lupin flowers, leaves, and roots (*Lupinus albescens*) (Confortin et al., 2017), and seeds (*Lupinus luteus*) (Buszewski et al., 2019), to obtain valuable compounds such as fatty acids, tocopherols, and phenolic compounds. However, since supercritical CO_2 ($scCO_2$) has limited affinity for polar solutes, polar co-solvents like ethanol or methanol (up to 15–20 % wt.) and water (up to 4–5 % wt.) are often added to improve the extraction of polar compounds. Alternatively, CO_2 can be used in combination with high amount of water and/or an alcohol (forming a gas-expanded liquid) to extract polar compounds like anthocyanins as a post extraction process after the initial $scCO_2$ extraction step (Seabra et al., 2010).

The aim of this study was to enhance the utilization of lupin seeds (*Lupinus angustifolius*) by extracting both non-polar and polar fractions, yielding a protein-concentrated plant flour suitable for food applications. As previously mentioned, although lupin has recently gained attention for its protein content, research exploring its oil fraction and phenolic compounds remains limited, particularly regarding lipid class composition, tocopherol content, and the phenolic profile of the *Lupinus angustifolius* species. It is important to note that even studies focusing on the protein fraction of plant materials have emphasized the necessity of removing its oil content to enhance protein accessibility (Náthia-Neves et al., 2024; Souza Almeida et al., 2021). Additionally, many existing studies utilize non-GRAS (Generally Recognized as Safe) organic solvents to obtain defatted lupin flour (Czubinski et al., 2019; Tahmasian et al., 2022). In this study, we investigated the use of $scCO_2$ to obtain the lupin oil fraction followed by the extraction using different ethanol/CO_2 mixtures to obtain polar lipids and a phenolic fraction. All obtained extracts were analyzed to evaluate their composition. The concentrated protein flour derived from these extractions underwent thorough characterization to investigate potential changes in its physicochemical and structural properties induced by the extraction processes. We expected that the sustainable processes developed in this study would not only provide valuable insights into the lupin products extraction, but also yield a higher-quality protein lupin flour, free of non-GRAS solvents.

2. Material and methods

2.1. Raw material preparation and characterization

Hulled sweet lupin seeds (*Lupinus angustifolius*, Iris variety) were kindly provided by DLF Corporate on December 2023. The whole seeds were ground into fine flour using a laboratory grinder (KN 295 Knifetec™, FOSS, Hillerød, Denmark), resulting in a mean particle diameter of 0.374 ± 0.003 mm, as determined by the ASAE standard S424 (2004) method. The ground samples were carefully packed in impermeable plastic bags and stored at 3 °C until the extraction assays were conducted. The proximate composition of the lupin flour was assessed using the AOAC (2005) methods to determine the moisture content (method 920.151) and ash content (method 923.03). Protein content (N × 6.25) was measured based on Dumas principle (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 800 °C, using pure oxygen (149 mL/min) as the combustion gas and pure argon (780 mL/min) as the carrier gas. The lipid content was determined following the Bligh and Dyer (1959) (B&D) method. Carbohydrate content was calculated by difference. All assays were conducted in triplicate, and the results were expressed as the mean percentage (grams of each macronutrient per 100 g of raw material on a dry matter basis) ± standard deviation.

2.2. Extraction processes

The extraction procedures were carried out using a commercial laboratory-scale SFE unit (MV-10 ASFE System, Waters, Milford, MA, USA) in two steps. Initially, SFE with $ScCO_2$ was employed to extract non-polar compounds. Approximately 13.1 ± 0.2 g of lupin flour were placed into a 25 mL-extraction vessel. The system was then heated and pressurized to the desired conditions. The solvent flow rate was set at 4.17 g/min with a constant solvent (S) to feed (F) ratio of S/F = 25 (mass basis) and the effect of temperature (40 and 60 °C) and pressure (150, 250, and 300 bar) were evaluated. Extraction time and S/F were determined based on the kinetic curve obtained at 40 °C and 350 bar (Fig. S1 in the Supplementary material), while temperature and pressure values were selected according to literature studies (Buszewski et al., 2019; Confortin et al., 2017; Yu et al., 2023). Temperatures above 60 °C were not explored to avoid exceeding the thermal denaturation range of lupin proteins (63–72 °C) (Albe-Slabi et al., 2022). Extraction was performed in a single cycle in dynamic extraction mode for 78 min. The extracted oil was stored at −40 °C for further analysis. In the second step, the residue (partially defatted lupin flour) obtained from the preselected extraction condition (40 °C and 250 bar) for non-polar compounds (Section 3.2) was used as the raw material to extract polar compounds such as polar lipids and phenolics. For this, different solvent mixtures based on mass proportions of CO_2 (90–0 %, w/w) and ethanol (10–100 %, w/w, where 100 % refers to only absolute ethanol) were introduced into the system. These solvent mixtures were in a homogeneous liquid phase (Seabra et al., 2010). The remaining solvent in the extract was removed using a stream of nitrogen, and the weight of the solvent-free extract was recorded and stored at −40 °C for further analysis. Total extraction yield was determined by dividing the total extracted mass by the raw material mass on a dry basis and expressed as a percentage. All extractions were conducted in duplicate.

2.3. Extracts characterization

The non-polar and polar extracts, as well as the oil extract obtained by the B&D method, were characterized.

2.3.1. Fatty acid composition by gas chromatography (GC)

The fatty acids in the extracted oil were converted into methyl esters using a two-step direct methylation procedure described by Safafar et al.

(2019). The resulting methyl esters were then analyzed using a GC (HP 5890 A, Agilent Technology, Palo Alto, CA, USA) equipped with a DB-wax column (10 m × ID 0.1 mm × 0.1 μm film thickness, J&W Scientific, Folsom, CA, USA) according to the official method Ce 1b-89 (AOCS, 1998b). The samples were analyzed in quadruplicate, and the results were reported as the mean percentages of total fatty acids ± standard deviation.

2.3.2. Lipid class

The separation of lipid classes in the oil extracts was carried out using chromatographic separation on a solid phase consisting of aminopropyl-modified silica (Bond Elute column (Waters, Dublin, Ireland)), following the method described by Sørensen et al. (2022). A solvent with increasing polarity was employed for lipid separation, with chloroform and 2-propanol (2:1, v/v) used for neutral lipids (NL), diethyl ether and acetic acid (98:2, v/v) for free fatty acids (FFA), and methanol for polar lipids (PL). Fatty acids within each fraction were subsequently analyzed by GC, as described in Section 2.3.1. The samples were analyzed in quadruplicate, and the results were reported as the mean percentages of each lipid class ± standard deviation.

2.3.3. Tocopherol composition by high pressure liquid chromatography (HPLC)

The tocopherol content of the samples was analyzed by HPLC (Agilent 1100 Series Palo Alto, USA) using a silica column (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 μm silica film), following AOCS Official Method Ce 8-89 (AOCS, 1998a). Tocopherols were quantified by external tocopherol standards using single-point calibration (Sørensen et al., 2022). The samples were analyzed in quadruplicate, and the results were reported as the mean of μg tocopherol/g oil and μg tocopherol/g lupin flour, accompanied by their respective standard deviations.

2.3.4. Total phenolic content and phenolic profile by UHPLC-MS

For phenolic analysis, the dried ethanolic extracts were redissolved in 2 mL of methanol. Total phenolic content (TPC) was determined following the methodology proposed by Singleton et al. (1999) using the Folin–Ciocalteu reagent and measuring the absorbance of the solution at 765 nm (Shimadzu UV-1280, Duisburg, Germany). Gallic acid (Sigma Aldrich, St. Louis, USA) was used to plot the standard curve (7.8–250 μg/mL), and the TPC yield, and content of each sample were expressed as mg of gallic acid equivalent (GAE) per 100 g of lupin flour and mg GAE/g extract on a dry basis, respectively.

Ultra-high performance liquid chromatography (UHPLC) coupled with diode array detector followed by electrospray ion source and quadrupole time-of-flight mass spectrometry (UHPLC-DAD-ESI-QTOFMS) was performed on a Thermo Scientific Vanquish Horizon UHPLC + ISQEC system (Thermo Scientific, Waltham, Massachusetts, USA) to determine the profile of phenolic compounds. MS was performed at m/z 100–650 a full scan mode. All MS analyses were carried out in the negative ion mode with the following parameters: source voltage −2050 V, source current 20.00 μA, vaporizer temperature 288 °C, ion transfer tube temperature 300 °C, sheath gas (N_2) pressure 52.9 psig, aux gas (N_2) pressure was 5.3 psig, sweep gas pressure 0.5 psig, and foreline pressure of 1.57 Torr. Prior to injection, the dried phenolic extracts were diluted in methanol to achieve a concentration of approximately 3 mg/mL, and then passed through a 0.22 μm PTFE filter. Separation was performed according to Wollgast et al. (2001) with some modifications. The stationary phase was a Zorbax SB-C18 Rapid Resolution HT column (2.1 × 50 mm, 1.8 μm, 600 bar, Agilent, Santa Clara, California, USA). The column chamber was 45 °C with a post column cooler temperature of 40 °C. The mobile phase consisted of water (A) and methanol (B), both acidified with 0.1 % (v/v) formic acid and the following gradient: 0–0.5 min, 6 % (B); 0.5–8.5 min, increasing to 40 % (B); 8.5–9.5 min, increasing to 90 % (B); 9.5–12 min at 90 % (B); 12–14 min, decreasing to 6 % (B) at a flow rate of 0.330 mL/min and with a total run of 17 min (with 3 min post-time at a flow rate of 0.15 mL/min). The injected volume was 1.00 μL. For compounds identification, the isotopic profile obtained from the MS data was used to tentatively identify the phenolic compounds from lupin extracts. Standards were employed for quantification using Chromeleon 7 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All measurements were carried out in quadruplicate, and results were reported as μg/mL extract or μg/100 g lupin seeds.

2.3.5. Antioxidant activity by DPPH radical scavenging capacity

For antioxidant activity measurements, the dried ethanolic extracts were dissolved in methanol to obtain solutions with different concentrations. Subsequently, 100 μL of the methanolic extract was mixed with 100 μL of 0.1 mM DPPH (Sigma Aldrich, St. Louis, USA), also prepared in methanol. The full protocol to determine DPPH radical scavenging capacity and IC_{50} values can be found in a study conducted by Yang et al. (2008).

2.4. Extracted flour charaterization

The flours obtained after SFE extraction (40 °C and 250 bar), as well as after the extraction using different ethanol/CO_2 mixtures at 40 °C and 250 bar, were characterized for protein content using the Dumas method (Section 2.1), amino acid content, and protein structure through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Fourier-transform infrared spectroscopy (FTIR).

2.4.1. Amino acid profile

The amino acid profile of the flours was determined following the protocol described by Ghelichi et al. (2024). Briefly, the lupin flours were chemically hydrolyzed overnight with 6 M HCl (1:2) followed by neutralization with 0.2 M NaOH and ammonium acetate buffer (100 mM; pH 3.1 adjusted with formic acid). The resulting solution was analyzed by HPLC (Agilent 1260 Infinity II Series, LC/MSD Trap, Agilent technologies, USA) and the amino acid profile was expressed as mg of amino acid per g of lupin flour ± standard deviation on a dry basis. All measurements were performed in quadruplicate.

2.4.2. SDS-PAGE

The extracted lupin flours were analyzed under reducing conditions by SDS-PAGE, as described by Laemmli (1970). Protein fractions were separated on 4–12 % gradient Bis-Tris NuPAGE® gel (Invitrogen, San Diego, USA). The flours were dissolved in Laemmli buffer and kept under continuous stirring (67 rpm) at room temperature for 3 h to solubilize the protein fractions. Subsequently, the samples were boiled at 100 °C for 3 min, and the blended slurry was centrifuged at 12,000×g for 3 min (Biofuge pico centrifuge, Heraeus, Germany). Then, the same amount of protein (20 μg, 8 μL of the suspension) was loaded into each well and electrophoresis was performed using a Mini-Protean Cell II system (Bio-Rad Laboratories, Hercules, USA) at a voltage of 170 V for 60 min. The gel was then washed with distilled water and stained for 1 h under gentle shaking with Coomassie blue G-250 (Thermo Scientific, Pierce, USA) followed by a destaining procedure also under gentle shaking for 1 h with distilled water. The molecular weight of prominent bands was estimated by comparing them with See Blue TM Plus 2 Prestained Protein Marker standard (Invitrogen, San Diego, USA), ranging from 3 kDa to 198 kDa. SDS-PAGE electropherograms were generated using a ChemiDoc XRS + System (BioRad Laboratories, Hercules, USA).

2.4.3. FTIR

FTIR spectra of the native and extracted lupin flours were recorded by a FT-IR Nicolet iS50 spectrophotometer (Thermo Fisher Scientific, USA) equipped with a crystal diamond attenuated total reflectance (ATR) sampling accessory. Measurements were acquired in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} and an accumulation of 32 scans in the absorbance mode. FTIR spectra within the amide I band region (1700–1600 cm^{-1}) were used for analyzing the secondary

structure of proteins. Prior to curve fitting, FTIR spectra underwent auto-baseline correction, followed by Fourier self-deconvolution spectra using the OMNIC™ software (Thermo Fisher Scientific Co., Waltham, MA, USA). The peak fitting process was conducted within the same software program using Gaussian peak shapes with a low sensitivity setting, a full width at half height (FWHH) of 0.9, a noise target value of 20, and a constant baseline. Curve fitting was then performed using OriginPro software 2023 (OriginLab Co., Northampton, MA, USA). Identified peaks were assigned to specific structures based on their frequencies: high frequency (HF) β-sheet (1700–1690 cm^{-1}), β-turns (1690–1665 cm^{-1}), random coil structure and α-helix (1665–1640 cm^{-1}), and low frequency (LF) β-sheet (1640–1615 cm^{-1}) (Calix-Rivera et al., 2023). Measurements were performed in triplicate.

2.5. Statistical analysis

The analysis of variance (ANOVA) of the results was conducted using the commercial Minitab v.16® software (Minitab Inc., State College, PA, USA) with a 95 % confidence level (p-value ≤ 0.05). Significant differences between means were assessed by Fisher's test at a 5 % significance level. For the extraction using only $ScCO_2$ (First step), a randomized full factorial design (2 × 3) was employed to evaluate the effect of temperature (40 and 60 °C) and pressure (150, 250, and 350 bar) on total extraction yield, fatty acid composition, tocopherol content, and lipid class. Additionally, the effect ethanol:CO_2 mixtures based on mass proportions (Second step) on total extraction yield, fatty acid composition, lipid class, total phenolic content (TPC), and antioxidant activity were also evaluated. Furthermore, Pearson correlation coefficients (r) were calculated to describe the relationship between the responses and process parameters.

3. Results and discussion

3.1. Raw material (Lupin seeds flour) characterization

The proximate composition of lupin seed flour was found to be 10.70 ± 0.20 % moisture, 3.39 ± 0.02 % ash, 5.30 ± 0.10 % lipids, 32.65 ± 0.03 % proteins, and 62.10 ± 0.10 % carbohydrates (calculated by difference) on a dry basis. These values are consistent with those reported in literature for different lupin varieties, which show moisture content ranging from 8.9 % to 12.3 % (Lemus-Conejo et al., 2023), protein content from 20.9 % to 36.49 %, ash content from 2.27 % to 6.78 % (Ferchichi et al., 2021), and lipid content from 6 % to 12 % (Arnoldi et al., 2015; Arzami et al., 2022; Lemus-Conejo et al., 2023).

3.2. Characterization of the non-polar extract

The global extraction yields from the SFE process ranged from 2.27 ± 0.02 to 4.5 ± 0.2 oil g per 100 g of lupin seeds on a dry matter basis (Fig. 1), depending on the studied conditions. Temperature (40 and 60 °C) and pressure (150–350 bar), as well as their interaction, significantly influenced lupin oil extraction yield (p-value < 0.001). The lowest yields were observed at 150 bar for both 40 and 60 °C, which can be attributed to the lowest CO_2 density values under these conditions (604.09 kg/m^3 and 780.23 kg/m^3, respectively). Increasing pressure from 150 to 350 bar at a constant temperature increased the extraction yield. These findings are consistent with previous research showing that higher CO_2 density enhances the solubility of lupin oil (Yu et al., 2023) and other vegetable oils, such as those from monguba seeds (Rodrigues et al., 2021), sesame seeds (Buranachokpaisan et al., 2021), and favela seeds (Santos et al., 2020). Higher CO_2 density increases solvent power, facilitating better penetration of the solvent into the solid matrix. Despite higher CO_2 densities at 40 °C and pressures of 250 (879.49 kg/m^3) and 350 bar (934.81 kg/m^3) compared to the same pressures at 60 °C (786.55 kg/m^3 and 862.94 kg/m^3, respectively), no statistical difference was found in extraction yield among these conditions. This suggests that the increase in solute vapor pressure with temperature overcomes the reduction in density of $ScCO_2$, a phenomenon also observed by other authors (Santos et al., 2020). Moura et al. (2005) and Men et al. (2021) also reported no increase in the extraction yield of fennel oil and *Lycium barbarum* seeds oil when pressure was raised from 250 to 300 bar. There is limited literature information on oil extraction from lupin seeds using $ScCO_2$. Yu et al. (2023) reported extraction yields from *Lupinus mutabilis* ranging from 9.1 % to 16.2 %, with S/F ratios

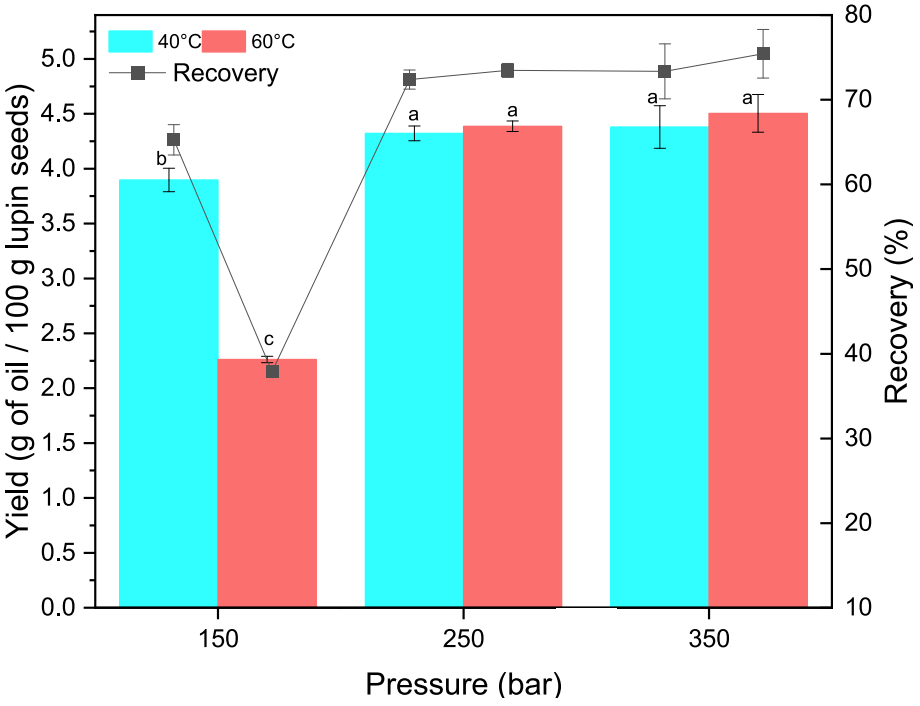

Fig. 1. Extraction yield of the extract obtained from lupin seeds by supercritical CO_2 extraction at different pressures and temperature. Oil recovery was calculated relative to the Bligh and Dyer method.

between 12 and 60 at 40 °C and pressures from 150 to 550 bar. For these authors, the oil recovery by SFE accounted for 45 to 82 % of the total oil extracted using Soxhlet (19.8 %). In our study, Fig. 1, using *Lupinus angustifolius* as raw material, we recovered between 37.86 ± 0.48 % (150 bar and 60 °C) and 75.43 ± 2.86 % (350 bar and 60 °C) of the total oil obtained by the B&D method (5.3 %).

Table 1 shows the fatty acid composition of the oils extracted by $ScCO_2$ and B&D methods. Both oils contained a total of 9 fatty acids, with a significant proportion being unsaturated (mono + poly-unsaturated) fatty acids. The fatty acid profiles were highly similar in both type and proportion for oils extracted using the two methods. However, the oil obtained via the B&D method exhibited a higher proportion of saturated fatty acids (SFA; 20 %) compared to those obtained by SFE (~17 %). In contrast, SFE extraction appeared to have a slight preference for unsaturated fatty acids (UFA; ~81 %) compared to the B&D method (~79 %), as the oils extracted by SFE contained a relatively higher percentage of UFA. It is important to note that these comparisons were made based on the relative fatty acid profiles, as determined by the area percentage in the chromatogram, rather than absolute quantities of the total oil recovered by each method. The results in Table 1, align with the literature, which indicates that the most predominant SFA in lupin seeds is palmitic acid (4.5–11.7 %), followed by stearic acid (1.9–7.4 %), while the most predominant monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) are oleic acid (24.6–62.7 %) and linoleic acid (12.6–52.7 %), respectively (Siger et al., 2023; Yu et al., 2023).

Table 2 presents the lipid class composition of lupin oils obtained by $ScCO_2$ extraction and the B&D method. The lupin lipid was mainly composed of NL and FFA. To the best of our knowledge, this is the first study to investigate lipid class composition in lupin oil, making direct comparisons with existing literature challenging. However, these results can be comparable with those from other seeds; for instance, raspberry seed oil showed 93.7 % NL, 3.5 % FFA and 2.7 % PL (Oomah et al., 2000), similarly to *Baphia nitida* and *Gliricidia sepium* seed oils, which showed 95–98 % NL, 1.9–4.1 % FFA, and 0.1–0.5 % PL (Adewuyi & Oderinde, 2013). As shown in Table 2, extraction conditions significantly influenced lipid composition (p-value < 0.001). For instance, the oil extracted at 40 °C and 350 bar exhibited the lowest proportion of NL, alongside the highest levels FFA and PL. Generally, higher levels of FFA were observed in samples extracted at 40 °C, possibly due to increased enzymatic activity at this temperature, leading to greater hydrolysis of lipid triglycerides into glycerol and FFA. In line with this, Ferreira et al. (2019) identified 40 °C as the optimal temperature for the hydrolysis of cottonseed oil. These results suggest that temperature may significantly influence enzymatic activity in lupin oil; however, further studies are required to better understand and confirm this effect, as well as to clarify the pronounced impact of increasing the pressure from 250 to 350 bar on FFA levels at 40 °C. According to Catchpole et al. (2018), water can also be co-extracted during the SFE process at high pressures, which may compromise the final oil quality and lead to increased FFA formation. The higher solvent density at 40 °C and 350 bar (934.81 kg/m^3), compared to 60 °C and 350 bar (862.94 kg/m^3), may further enhance water co-extraction. Although no visible phase separation was observed, the co-extraction of small quantities of water could have occurred, potentially influencing the FFA analysis results. No phospholipids were detected in oils extracted at pressures below 350 bar. The oil obtained by the B&D method exhibited lower NL content (75.7 %) compared to SFE extracts (84–98.6 %), while displaying higher levels of polar lipids (PL) (22 %) compared to SFE (0.1–3.2 %). This discrepancy may be partly attributed to the extraction process of the B&D method, which uses methanol, chloroform, and water. Although lipids are primarily quantified in the chloroform (non-polar) phase after phase separation, the initial use of methanol and water facilitates the extraction of polar lipid components that are typically not recovered in the SFE process.

The oils obtained by SFE exhibited significantly higher tocopherol concentration compared to that obtained via B&D method (4.9 ± 0.1, 4.6 ± 0.1, and 84 ± 1 μg of α-, β-, and γ-tocopherols per gram of oil,

Table 2
Lipid classes of the lupin oil extracted by supercritical CO_2 and Bligh and Dyer method.

T (°C)	P (bar)	Neutral lipids (%)	Free fatty acids (%)	Phospholipids (%)
40	150	97.8 ± 0.1^{a}	2.1 ± 0.1^{cd}	ND
	250	96.0 ± 1.0^{a}	3.9 ± 0.4^{b}	ND
	350	84.0 ± 0.1^{b}	12.7 ± 0.1^{a}	3.2 ± 0.0^{b}
60	150	97.7 ± 0.5^{a}	2.2 ± 0.5^{c}	ND
	250	98.6 ± 0.2^{a}	1.3 ± 0.2^{d}	ND
	350	97.5 ± 0.1^{a}	2.4 ± 0.1^{c}	0.2 ± 0.0^{c}
Bligh and Dyer		76.0 ± 5.0^{c}	1.7 ± 0.1^{cd}	22.0 ± 1.0^{a}
Analysis of variance and significance (p-values)				
T (F1)		***	***	***
P (F2)		***	***	***
F1 × F2		***	***	***

F1: Temperature (°C); F2: Pressure (bar); * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; ns: not significant; ND: not detectable. The different letters in the same column indicate statistically significant differences between means at $p < 0.05$.

Table 1
Fatty acid composition of lupin extracts obtained using supercritical CO_2 and Bligh and Dyer method.

Fatty acids (%)	40 °C			60 °C			Bligh and Dyer	ANOVA and significance (p-values)		
	150 bar	250 bar	350 bar	150 bar	250 bar	350 bar		T (F1)	P (F2)	F1 × F2
16:00	11.43 ± 0.08^{b}	11.47 ± 0.07^{b}	11.54 ± 0.15^{b}	12.50 ± 0.30 ^{a}	11.39 ± 0.05^{b}	11.40 ± 0.20^{b}	10.81 ± 0.01^{c}	*	**	**
18:00	2.59 ± 0.03^{d}	2.80 ± 0.10^{c}	2.85 ± 0.01^{c}	2.45 ± 0.01 ^{e}	2.844 ± 0.00^{c}	2.95 ± 0.02^{b}	4.39 ± 0.06 ^{a}	ns	***	**
18:1 (n−9)	26.57 ± 0.02^{b}	27.10 ± 0.90^{b}	26.94 ± 0.53^{b}	26.90 ± 0.20^{b}	26.40 ± 0.20^{b}	26.50 ± 0.30^{b}	28.74 ± 0.16 ^{a}	ns	ns	ns
18:1 (n−7)	0.86 ± 0.00^{a}	0.66 ± 0.02^{b}	0.59 ± 0.00^{c}	0.43 ± 0.02 ^{e}	0.44 ± 0.01 ^{e}	0.47 ± 0.00 ^{d}	0.36 ± 0.02^{f}	***	***	***
18:2 (n−6)	46.40 ± 0.80^{ab}	46.50 ± 0.40^{ab}	46.49 ± 0.01^{ab}	45.92 ± 0.08^{b}	46.32 ± 0.08 ^{ab}	47.01 ± 0.20 ^{a}	43.70 ± 0.10^{c}	ns	ns	ns
18:3 (n−3)	6.50 ± 0.01^{bc}	6.70 ± 0.20^{a}	6.66 ± 0.08^{ab}	6.36 ± 0.01^{c}	6.46 ± 0.03 ^{bc}	6.64 ± 0.07 ^{ab}	5.82 ± 0.02 ^{d}	*	*	ns
20:00	0.63 ± 0.01^{b}	0.58 ± 0.01^{bcd}	0.61 ± 0.02^{bcd}	0.56 ± 0.03 ^{d}	0.62 ± 0.02 ^{bc}	0.56 ± 0.04 ^{cd}	0.70 ± 0.01 ^{a}	ns	ns	*
22:00	1.50 ± 0.07^{b}	1.28 ± 0.03^{b}	1.35 ± 0.01^{b}	1.40 ± 0.50^{b}	1.44 ± 0.08^{b}	1.21 ± 0.20^{b}	3.10 ± 0.10 ^{a}	ns	ns	ns
24:00	0.36 ± 0.05^{cde}	0.32 ± 0.02^{e}	0.34 ± 0.01^{de}	0.411 ± 0.00 ^{bcd}	0.44 ± 0.03 ^{bc}	0.471 ± 0.00^{b}	1.39 ± 0.07 ^{a}	***	ns	ns
Sum of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and unsaturated fatty acids (MUFA + PUFA):										
Σ SFA	16.90 ± 0.10^{c}	16.90 ± 0.10^{c}	17.01 ± 0.11^{c}	17.81 ± 0.21^{b}	17.01 ± 0.11^{c}	17.09 ± 0.01^{c}	20.70 ± 0.30 ^{a}	**	*	**
Σ MUFA	27.89 ± 0.02^{b}	28.31 ± 0.81 ^{ab}	28.20 ± 0.60^{ab}	28.31 ± 0.51 ^{ab}	27.31 ± 0.11^{b}	27.10 ± 0.40^{b}	29.30 ± 0.10 ^{a}	ns	ns	ns
Σ PUFA	53.40 ± 0.70^{ab}	53.40 ± 0.51 ^{ab}	53.31 ± 0.11^{ab}	52.80 ± 0.10^{b}	53.36 ± 0.02 ^{ab}	53.80 ± 0.10 ^{a}	49.60 ± 0.11^{c}	ns	ns	ns
Σ UFA	81.30 ± 0.71^{a}	81.70 ± 0.20 ^{a}	81.60 ± 0.50^{a}	81.20 ± 0.60 ^{a}	80.70 ± 0.10 ^{a}	81.10 ± 0.20 ^{a}	79.10 ± 0.30^{b}	ns	ns	ns

The values represent the relative proportion of fatty acids based on the chromatogram areas. F1: Temperature (°C); F2: Pressure (bar); * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$; ns: not significant. The different letters in the same row indicate statistically significant differences between means at $p < 0.05$. The proportion of non-identified fatty acids is not shown.

respectively). In the SFE extract, the α-tocopherol concentration ranged from 116.7 to 296.9 μg/g oil, while the γ-tocopherol concentration ranged from 2006 to 4749 μg/g oil (Fig. 2). In contrast, the B&D method yielded 0.2 ± 0.01, 87 ± 1, and 4.5 ± 0.1 μg of α-, β-, and γ-tocopherols per gram of lupin seeds, respectively. Yu et al. (2023) also reported higher tocopherol yields in lupin oils obtained via SFE compared to those obtained by Soxhlet using hexane as a solvent. This indicates the selectivity of $ScCO_2$ under the temperature and pressure conditions employed, allowing for the recovery of significantly higher amounts of valuable compounds without the use of hazardous solvents. Interestingly, the oil obtained by $ScCO_2$ did not contain quantifiable amounts of β-tocopherol, suggesting a specific selectivity of $ScCO_2$ for α- and γ-tocopherols or potentially indicating a partial conversion of β-tocopherols into α- and/or γ-tocopherols (Estivi et al., 2023), an observation that requires further investigation. α-tocopherol is the only tocopherol homologue with proven efficacy in preventing vitamin E deficiency disease; therefore, its increased content in oils obtained by SFE enhances the nutritional quality of lupin oil obtained through this technology (Estivi et al., 2023). Comparisons of Fig. 1 and Fig. 2 showed that the extraction conditions providing the lowest extraction yield (60 °C and 40 °C at 150 bar) produced an extract with the highest concentration of α-tocopherol (297 μg/g oil) and γ-tocopherol (4749 μg/g oil). This finding aligns with Yu et al. (2023), who observed that oils extracted at 150 bar were richer in tocopherols compared to those extracted at higher pressures. Increasing the pressure at a constant temperature led to a significant reduction in both α- (Pearson's coefficient, r = −0.74) and γ- (r = −0.77) tocopherol concentration, while increasing the temperature at a constant pressure resulted in a positive, but nonsignificant, increase in both α- and γ-tocopherols (r = +0.40). Gustinelli et al. (2018) similarly found that increasing pressure reduced tocopherol concentration, while increasing temperature enhanced the extraction of these compounds in bilberry seed oil. The yields of α- and γ-tocopherols ranged from 5.1 to 6.8 μg/g lupin seeds and from 88.3 to 117.2 μg/g lupin seeds, respectively (Fig. 2). The amount of tocopherol in lupin seeds varies widely depending on the variety and cultivars. For instance, in *Lupinus angustifolius*, the α-tocopherol content ranged from 2.6 to 5.1 μg/g lupin seeds, while γ-tocopherol content ranged from 61.2 to 91 μg/g lupin seeds across four different cultivars (Siger et al., 2023). *Lupinus albus* and *Lupinus luteus* studied by Siger et al. (2023) exhibited no α-tocopherol content and a γ-tocopherol ranging from 85.7 to 130.2 μg/g lupin seeds.

3.3. Extraction of polar compounds

Based on results described in Section 3.2, trials were conducted at 250 bar and 40 °C to evaluate the impact of Ethanol:CO_2 mixtures (based on mass proportions) on lupin extract yield and quality. Fig. 3 shows a positive correlation (r = 0.991) between extraction yield and ethanol percentage, with yield increasing from 1 % to 5 % as ethanol proportion rose from 10 % to 100 %. This result aligns with similar trends observed by Seabra et al. (2010) in the extraction of defatted elderberry pomace using different Ethanol:CO_2 solvent mixtures. Their study revealed that increasing ethanol percentage from 10 % to 100 % led to an extraction yield increase from 2.4 % to 13.9 %. This enhancement was attributed to the increased polarity of the solvent, facilitating the extraction of polar compounds. Buszewski et al. (2019) reported extraction yields from *Lupinus luteus* ranging between 0.35 % and 2.35 %, with temperatures ranging from 40 to 80 °C, pressures between 100 and 300 bar and

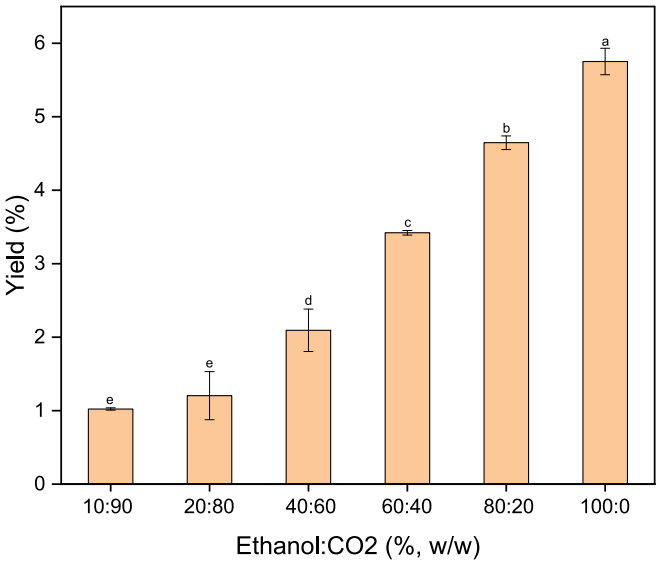

Fig. 3. Extraction yield of the extract obtained from defatted lupin seeds at 250 bar and 40 °C using different mixtures of Ethanol:CO_2 (in mass proportions).

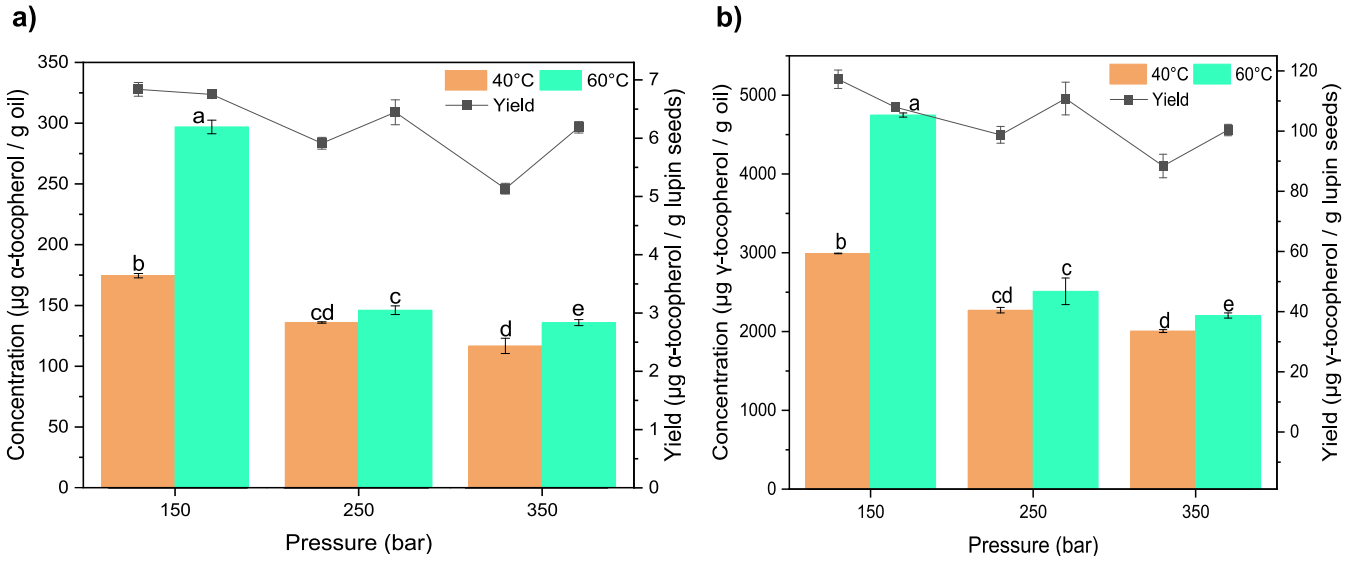

Fig. 2. Results of tocopherol content obtained by supercritical CO_2 extraction at different pressures and temperature: (a) concentration and yield of α-tocopherol; (b) concentration and yield of γ-tocopherol.

ethanol percentage ranging from 10-20 %.

Table 3 presents the fatty acid composition of the extracts obtained using different Ethanol:CO_2 mixtures at 250 bar and 40 °C. Consistent with observations in the oils obtained with $ScCO_2$, linoleic acid (polyunsaturated) was the most abundant fatty acid in the ethanolic extracts, followed by oleic acid (monounsaturated), palmitic acid (saturated), linolenic acid (polyunsaturated) and stearic acid (saturated) in equal proportions. Increasing the ethanol proportion led to a significant rise in SFA recovery, while MUFA recovery showed the opposite trend. A higher extraction of PL was observed in the ethanolic extracts compared to those obtained with $ScCO_2$, suggesting that using ethanol to recover polar lipids from defatted samples is an effective strategy. Indeed, Getachew et al. (2024) demonstrated that increasing the ethanol flow rate from 1 to 3 mL/min (while keeping the CO_2 flow rate constant at 4 mL/min) led to almost a threefold increase in PL content in starfish extract. To the best of our knowledge, there are no studies in the literature on the extract composition of defatted lupin seeds obtained through ethanol and CO_2 mixtures. The lipid class composition (Table 3) did not exhibit a clear trend with respect to the extraction solvent composition. The highest NL was observed in the extracts obtained with 20 % and 100 % Ethanol:CO_2, while the remaining solvent mixtures provided the highest recovery of PL.

The ethanolic extracts were characterized for their total phenolic content (TPC), phenolic profile by UHPLC, and antioxidant activity assessed by DPPH radical scavenging (Table 4). The extract obtained using 20 % ethanol exhibited the highest TPC content (36.2 ± 0.4 mg GAE/g extract), indicating a higher concentration of phenolic compounds compared to extracts obtained under other conditions. A decrease in extract concentration was observed with increasing ethanol percentage ($r = -0.892$), attributed to the tendency of higher ethanol proportions to extract more polar compounds besides phenolics. Conversely, an increase in TPC yield was observed with increasing ethanol content ($r = +0.967$), definitely due to the overall increase in extraction yield discussed previously (see Fig. 3). However, it is essential to consider that the use of high amounts of ethanol in the extraction process may escalate manufacturing costs. The lower content of phenolic in the obtained extracts when ≥40 % ethanol was added could potentially increase the cost of isolating/purifying these compounds in industrial applications. The TPC results obtained in this study (38–101 mg GAE/100 g lupin seeds) align with those reported by Lampart-Szczapa et al. (2003), who observed TPC values ranging from 16 to 41 mg/100 g for various sweet lupin seed varieties and cultivars extracted with 80 % aqueous ethanol. However, Siger et al. (2012) reported higher TPC values (ranging from 212 to 317 mg/100 g) compared to our study, potentially attributed to the use of methanol as an extraction solvent and a different lupin variety.

As shown in Table 4, five phenolic compounds were identified in the lupin extracts, with gentisic acid being the most abundant (3.3–15.4 μg/g lupin seeds). The phenolic profile of lupin seeds appears to vary

Table 3
Fatty acid composition and lipid class of lupin extracts obtained at 40 °C and 250 bar using different ethanol and CO_2 mixtures.

Fatty acids (%)	Ethanol:CO_2 (%)					
	10:90	20:80	40:60	60:40	80:20	100:0
16:0	11.50 ± 0.10^{e}	12.10 ± 0.10^{d}	13.29 ± 0.03^{c}	13.84 ± 0.01^{b}	14.11 ± 0.11^{a}	14.33 ± 0.01^{a}
18:0	4.82 ± 0.00^{e}	5.10 ± 0.10^{d}	5.95 ± 0.01^{c}	6.39 ± 0.03^{b}	6.51 ± 0.11^{b}	6.81 ± 0.02^{a}
18:1 (n−9)	35.41 ± 0.01^{a}	34.02 ± 0.01^{b}	32.50 ± 0.10^{c}	31.29 ± 0.02 ^{e}	31.40 ± 0.10 ^{e}	32.31 ± 0.01^{c}
18:1 (n−7)	0.25 ± 0.00^{d}	0.63 ± 0.00^{a}	0.55 ± 0.00^{b}	0.55 ± 0.00^{b}	0.61 ± 0.02^{a}	0.27 ± 0.01^{c}
18:2 (n−6)	39.85 ± 0.05^{a}	40.01 ± 0.21^{a}	39.75 ± 0.01^{a}	39.21 ± 0.11^{b}	39.24 ± 0.02^{b}	38.60 ± 0.10^{c}
18:3 (n−3)	4.86 ± 0.02^{c}	5.01 ± 0.10^{a}	5.06 ± 0.01^{a}	4.98 ± 0.00^{b}	4.95 ± 0.03^{b}	4.77 ± 0.02^{d}
22:0	0.78 ± 0.01^{a}	0.76 ± 0.02^{a}	0.63 ± 0.01^{b}	0.59 ± 0.00^{b}	0.57 ± 0.02^{b}	0.57 ± 0.01^{b}
24:0	0.34 ± 0.01^{ab}	0.36 ± 0.01^{a}	0.28 ± 0.00^{ab}	0.27 ± 0.00 ^{bc}	0.18 ± 0.08^{c}	0.28 ± 0.01^{ab}
Sum of saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and unsaturated fatty acids (MUFA + PUFA):						
Σ SFA	18.35 ± 0.15^{e}	19.21 ± 0.18^{d}	20.79 ± 0.04^{c}	21.87 ± 0.05^{b}	22.17 ± 0.42^{b}	23.37 ± 0.10^{a}
Σ MUFA	35.65 ± 0.15^{a}	35.03 ± 0.30^{b}	33.10 ± 0.10^{c}	32.17 ± 0.03^{e}	32.02 ± 0.07^{ef}	32.59 ± 0.10^{d}
Σ PUFA	44.90 ± 0.06 ^{bc}	45.33 ± 0.40^{b}	45.11 ± 0.03^{b}	49.57 ± 0.17^{a}	44.52 ± 0.02^{c}	43.64 ± 0.13^{d}
Σ UFA	80.56 ± 0.07^{b}	80.37 ± 0.41^{b}	78.21 ± 0.06^{c}	81.70 ± 0.12^{a}	76.54 ± 0.04^{d}	76.23 ± 0.13^{d}
Lipid Class: Neutral Lipids (NL), Free Fatty acids (FFA) and Phospholipids (PL):						
NL (%)	33.0 ± 3.0^{b}	45.6 ± 0.5^{a}	29.8 ± 0.5^{b}	30.7 ± 0.8^{b}	32.9 ± 0.1^{b}	43.0 ± 2.0^{a}
FFA (%)	1.9 ± 0.1^{b}	1.2 ± 0.2^{c}	1.0 ± 0.1^{c}	1.1 ± 0.1^{c}	0.80 ± 0.0^{c}	2.6 ± 0.1^{a}
PL (%)	65.0 ± 3.0^{a}	53.1 ± 0.7^{b}	69.1 ± 0.6^{a}	68.1 ± 0.7^{a}	66.2 ± 0.2^{a}	55.1 ± 2.1^{b}

The values represent the relative proportion of fatty acids based on the chromatogram areas. Different letters in the same row indicate statistically significant differences between means at $p < 0.05$.

Table 4
Phenolic compounds profile, total phenolic content, and antioxidant activity by DPPH method of lupin extracts obtained at 40 °C and 250 bar using different ethanol and CO_2 mixtures in mass proportions.

Phenolic Compounds	Ethanol:CO_2 (%)					
	10:90	20:80	40:60	60:40	80:20	100:0
Content (μg/g extract)						
Phloroglucinol	3.1 ± 0.1^{a}	2.1 ± 0.1^{b}	2.2 ± 0.1^{b}	1.6 ± 0.1^{d}	2.0 ± 0.1^{c}	2.2 ± 0.1^{b}
Gallic acid	3.8 ± 0.1^{c}	6.8 ± 0.1^{a}	3.5 ± 0.1^{c}	3.8 ± 0.1^{c}	5.0 ± 0.8^{b}	4.6 ± 0.7^{bc}
Gentisic acid	302.8 ± 0.1^{b}	308.5 ± 0.3^{a}	150.8 ± 0.2^{e}	119.0 ± 3.0^{f}	274.0 ± 6.0^{c}	242.0 ± 2.0^{d}
Chlorogenic acid	ND	4.8 ± 0.2^{d}	7.8 ± 0.1^{b}	8.7 ± 0.2^{a}	6.6 ± 0.5^{c}	7.2 ± 0.3^{bc}
p-Coumaric acid	3.2 ± 0.6^{a}	2.9 ± 0.1^{a}	0.9 ± 0.0^{b}	0.4 ± 0.0^{bc}	ND	ND
Yield (μg/100 g lupin seeds*)						
Phloroglucinol	3.4 ± 0.1^{d}	2.2 ± 0.1^{e}	5.1 ± 0.7^{c}	5.9 ± 0.2^{c}	10.1 ± 0.4^{b}	13.8 ± 0.7^{a}
Gallic acid	4.3 ± 0.1^{d}	7.3 ± 0.1^{c}	8.1 ± 1.0^{c}	14.4 ± 0.2^{b}	25.9 ± 2.1^{a}	29.1 ± 2.0^{a}
Gentisic acid	334.9 ± 0.5^{e}	333.0 ± 3.0^{e}	374.0 ± 6.0^{d}	450.1 ± 3.0^{c}	1432.0 ± 3.1^{b}	1545.0 ± 7.0^{a}
Chlorogenic acid	ND	5.1 ± 0.2^{d}	18.0 ± 2.1^{c}	32.7 ± 0.6^{b}	34.0 ± 3.0^{b}	46.0 ± 3.0^{a}
p-Coumaric acid	3.6 ± 0.6^{a}	3.1 ± 0.1^{a}	2.0 ± 0.2^{b}	1.7 ± 0.1^{bc}	ND	ND
Total Phenolic Compounds (TPC):						
Content (mg GAE/g extract)	34.3 ± 0.1^{b}	36.2 ± 0.4^{a}	22.1 ± 0.1^{c}	15.1 ± 0.1^{d}	14.6 ± 0.1^{e}	16.1 ± 0.2^{d}
Yield (mg GAE/100 g lupin seeds*)	38.4 ± 0.4^{e}	38.7 ± 0.6^{e}	51.0 ± 6.0^{d}	59.4 ± 0.1^{c}	75.1 ± 0.2^{b}	101.0 ± 1.0^{a}
Antioxidant Activity:						
DPPH (%) at 1 mg/mL	38.0 ± 1.0^{a}	31.1 ± 1.0^{b}	24.4 ± 0.1^{c}	25.0 ± 1.0^{c}	25.9 ± 0.6^{c}	27.0 ± 1.0^{c}
EC 50 (mg/mL)	1.5 ± 0.1^{d}	2.3 ± 0.1^{c}	3.2 ± 0.1^{b}	2.1 ± 0.1^{c}	4.4 ± 0.2^{a}	4.4 ± 0.4^{a}

* Refers to defatted flours. Different letters in the same row indicate statistically significant differences between means at $p < 0.05$.

significantly depending on the studied variety, the extraction method, and the analytical procedure. For instance, Siger et al. (2012) reported p-hydroxybenzoic acid (22.7–43.7 mg/kg lupin seeds) as the most abundant phenolic acid in methanolic extracts of different cultivars of *Lupinus albus* and *Lupinus angustifolius*, while Król et al. (2018) found ferulic acid as the most abundant phenolic acid (1.2–2.1 mg/g extract) in four different varieties of *Lupinus angustifolius*. Karamać et al. (2018), identified 3 different phenolic compounds in white lupin (*Lupinus albus* L.) seeds named as p-coumaric acid derivative (1), p-coumaric acid derivative (2) and apigenin-6,8-di-C-glucoside. Consistent with observations for TPC, the content of phenolic compounds decreased as the ethanol proportion increased during the extraction process, except for gallic and chlorogenic acids, which could be attributed to the highest polarity of these compounds (ChemSpider, 2024; Galanakis et al., 2013). However, it is important to note that the quantitative data on individual phenolic compounds did not align with the TPC values, as higher TPC values were observed compared to the sum of the individual measured phenolic compounds. This discrepancy can be attributed to the different principles of each method: the individual compounds were quantified by UHPLC-MS, while TPC was measured using a spectrophotometric method where other compounds than phenolic compounds can react with the Folin–Ciocalteau reagent. A similar inconsistency was reported by Buszewski et al. (2019), who found TPC values ranging from 9.44 to 13.58 mg/g, whereas the sum of individual phenolics was only 0.037971 mg/g.

The DPPH radical scavenging assay revealed the highest antioxidant activity in the extract (at 1 mg/mL) obtained with 10 % ethanol, likely attributable to the presence of less polar compounds in this extract. Similar to TPC, as the ethanol percentage increased in the solvent mixture, the DPPH activity also significantly decreased ($r = -0.670$), suggesting that other compounds with lower DPPH radical scavenging activity are being recovered in extracts obtained with high ethanol proportions. The IC_{50} values (1.5–4.4 mg/mL) of the extracts (Table 4) were lower than those reported by Karamać et al. (2018) (3.98–9.7 mg/mL), who found higher TPC values (4.36–7.24 mg GAE/g seed) compared to those found in this study, suggesting that the antioxidant activity is not exclusively related to the amount of phenolic compounds. Indeed, Karamać et al. (2018) suggested that the reduced antioxidant activity observed in extracts rich in phenolic compounds could be attributed to the specific chemical structure of lupin phenolics, which predominantly exist as glycosides and di-glycoside derivatives. The presence of reducing sugar moieties may interfere with the reaction of phenol with Folin–Ciocalteu's reagent, potentially leading to inflated values of TPC in lupin extracts. Additionally, in O-glycoside derivative structures, electron-donating –OH groups may be blocked, limiting their ability to participate in radical scavenging. As a consequence, glycosides typically exhibit weaker antioxidant properties compared to aglycones (Karamać et al., 2018).

3.4. Extracted flours characterization

Extracting non-polar compounds from lupin seeds using $ScCO_2$ resulted in flour with a protein content of 36.28 ± 0.05 %, indicating an 11 % increase compared to the initial content (Table 5). Subsequent extraction with various Ethanol/CO_2 mixtures also concentrated proteins in the flour, with protein contents ranging from 34.9 % to 36.3 % (6.8–11.2 % increase). Table 5 presents the amino acid profile of lupin seeds before and after the studied extraction processes. The extraction of both non-polar and polar compounds did not change the amino acid profile; consistent profiles were observed across all samples. However, certain amino acids, such as phenylalanine (up to +19.9 %), leucine (up to +18.4 %), isoleucine (up to +19.0 %), threonine (up to +15 %), cysteine (up to +37 %), and aspartic acid (up to 8.7 %), exhibited increases after $ScCO_2$ extraction, possibly due to their concentration in the defatted flour associated with selective lipid extraction. Domínguez-Valencia et al. (2024) also observed an increase in certain essential amino acids, such as isoleucine and leucine, following the application of supercritical CO_2 to lupin protein isolate at 40 °C and 400 bar. Upon extraction of defatted flour with Ethanol/CO_2 mixtures, certain amino acids showed varying trends, with some increasing and others decreasing. Notably, reductions were observed for histidine (up to −15 %), lysine (up to −12 %), alanine (up to −9.6 %), and glutamic acid (up to −8.1 %). Protein-phenolic interactions may contribute to these observations (Czubinski & Feder, 2019). Samples extracted with 100 % ethanol showed the highest reductions in amino acid content and lowest protein content, supporting the possibility of protein extraction along with phenolics such as protein–phenolic complexes under this condition. Although significant, the differences observed were minor, suggesting that the extraction processes may not substantially affect the nutritional quality of lupin flour. However, the green processes used in this study preserved the amino acid profile, highlighting their potential for producing protein- and amino acid-concentrated products. A similar amino acid profile was identified by Starkute et al. (2016), who found glutamic acid to be the major amino acid in *Lupinus angustifolius* seeds (24–27 g/

Table 5
Amino acid profile and protein content of the studied flours on a dry basis.

Aminoacids (mg/g lupin flour)	Native	SFE	Ethanol:CO_2 (%)					
			10:90	20:80	40:60	60:40	80:20	100:0
Phenylalanine	9.4 ± 0.3^{e}	11.3 ± 0.1^{a}	10.6 ± 0.2^{bc}	10.1 ± 0.1^{abc}	10.6 ± 0.1^{bc}	10.3 ± 0.1^{cd}	11.0 ± 0.1^{ab}	9.9 ± 0.5^{de}
Leucine	17.8 ± 0.2^{e}	21.1 ± 0.2^{a}	19.2 ± 0.0^{cd}	19.6 ± 0.1^{c}	19.9 ± 0.5^{bc}	19.3 ± 0.2^{cd}	20.7 ± 0.1^{ab}	18.6 ± 0.8^{d}
Isoleucine	10.8 ± 0.5^{d}	12.9 ± 0.1^{a}	12.7 ± 0.2^{a}	12.5 ± 0.1^{ab}	12.4 ± 0.1^{abc}	11.7 ± 0.1^{c}	12.6 ± 0.1^{a}	11.7 ± 0.6^{bc}
Methionine	0.1 ± 0.0^{d}	0.7 ± 0.1^{a}	0.36 ± 0.01^{c}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}	0.6 ± 0.0^{b}	0.5 ± 0.1^{b}
Tyrosine	8.4 ± 0.2^{ab}	8.5 ± 0.2^{a}	8.4 ± 0.2^{ab}	8.0 ± 0.2^{ab}	8.4 ± 0.1^{ab}	7.9 ± 0.1^{ab}	8.5 ± 0.1^{a}	7.7 ± 0.6^{b}
Proline	11.6 ± 0.4^{cd}	12.9 ± 0.1^{a}	11.8 ± 0.1^{bc}	12.1 ± 0.1^{cd}	11.7 ± 0.1^{cd}	11.6 ± 0.1^{cd}	12.4 ± 0.1^{ab}	11.3 ± 0.5^{d}
Valine	12.3 ± 0.4^{bc}	13.1 ± 0.1^{a}	12.4 ± 0.3^{bc}	12.4 ± 0.1^{bc}	12.1 ± 0.1^{cd}	11.7 ± 0.4^{d}	12.8 ± 0.1^{ab}	11.8 ± 0.1^{cd}
Alanine	10.3 ± 0.6^{ab}	10.6 ± 0.1^{ab}	10.1 ± 0.7^{abc}	10.2 ± 0.2^{abc}	11.1 ± 0.2^{a}	9.7 ± 0.4^{bc}	10.0 ± 0.1^{bc}	9.3 ± 0.1^{c}
Threonine	9.8 ± 0.3^{d}	11.3 ± 0.5^{abc}	10.8 ± 0.1^{abc}	11.2 ± 0.4^{abc}	10.5 ± 0.6^{bcd}	11.4 ± 0.5^{ab}	11.4 ± 0.1^{a}	10.4 ± 0.2^{cd}
Glycine	13.8 ± 0.1^{b}	14.3 ± 0.1^{a}	13.7 ± 0.2^{b}	14.1 ± 0.1^{a}	14.3 ± 0.1^{a}	13.6 ± 0.1^{bc}	14.6 ± 0.3^{a}	13.0 ± 0.1^{c}
Serine	15.9 ± 0.5^{ab}	16.2 ± 0.1^{a}	15.1 ± 0.1^{ab}	15.1 ± 0.1^{ab}	15.5 ± 0.2^{ab}	14.8 ± 0.0^{b}	16.3 ± 0.2^{a}	15.1 ± 1.0^{b}
Arginine	31.2 ± 0.2^{a}	30.5 ± 0.4^{ab}	30.5 ± 0.1^{ab}	30.6 ± 0.1^{ab}	30.1 ± 0.1^{b}	30.4 ± 0.5^{ab}	30.4 ± 0.3^{ab}	30.5 ± 0.5^{ab}
Histidine	8.1 ± 0.2^{a}	7.9 ± 0.2^{a}	7.3 ± 0.6^{ab}	7.2 ± 0.1^{ab}	6.9 ± 0.3^{b}	7.1 ± 0.3^{b}	7.5 ± 0.3^{ab}	6.8 ± 0.4^{b}
Lysine	13.6 ± 0.7^{a}	14.0 ± 0.6^{a}	12.6 ± 0.1^{bc}	13.3 ± 0.3^{ab}	12.5 ± 0.2^{bc}	12.2 ± 0.2^{c}	13.3 ± 0.1^{ab}	11.8 ± 0.2^{c}
Glutamic acid	71.0 ± 1.0^{a}	70.4 ± 0.1^{a}	70.1 ± 0.5^{a}	65.5 ± 0.2^{b}	70.0 ± 1.0^{a}	70.0 ± 1.0^{a}	69.3 ± 0.1^{ab}	65.0 ± 4.0^{b}
Cysteine	1.4 ± 0.1^{de}	1.8 ± 0.1^{cde}	3.1 ± 0.2^{a}	2.6 ± 0.1^{ab}	2.0 ± 0.2^{bc}	1.8 ± 0.1^{cd}	2.6 ± 0.3^{ab}	1.3 ± 0.3^{e}
Aspartic acid	33.2 ± 0.1^{d}	36.2 ± 0.4^{ab}	34 ± 0.2^{cd}	36.5 ± 0.6^{ab}	37.0 ± 1.0^{a}	34.0 ± 1.0^{cd}	35.0 ± 0.5^{bc}	31.1 ± 1.0^{e}
Total Aminoacids (mg/g lupin flour)	279.0 ± 2.0^{bc}	294.1 ± 1.0^{a}	283.3 ± 0.5^{bc}	283.0 ± 1.0^{bc}	286.0 ± 2.0^{abc}	278.2 ± 0.3^{c}	289.5 ± 0.5^{ab}	267.0 ± 12.1^{d}
Protein (g/100 g lupin flour)	32.6 ± 0.3^{d}	36.3 ± 0.1^{a}	35.3 ± 0.1^{bc}	35.9 ± 0.1^{ab}	36.0 ± 0.2^{ab}	35.6 ± 0.4^{abc}	36.3 ± 0.1^{a}	34.9 ± 0.9^{c}

Native refers to flours that undergo no extraction process. SFE refers to flour extracted using supercritical CO_2 at 40 °C and 250 bar pressure. Results are expressed as mg amino acids/g defatted flours. Different letters in the same row indicate statistically significant differences between means at $p < 0.05$.

100 g), followed by aspartic acid (11–12 g/100 g).

Changes in protein structure resulting from the extraction processes were evaluated using SDS-PAGE and FTIR analysis (Fig. 4). Since it is recommended to remove the lipid fraction before SDS-PAGE analysis, in this study, the SDS-PAGE analysis was performed only on the extracted flours (Náthia-Neves et al., 2024). Fig. 4a illustrates that the extraction processes did not significantly alter the protein profile, maintaining consistent profiles (ranging from 6 to 98 kDa) across all extracted flours. To our knowledge, no study has investigated the effect of extraction processes using $ScCO_2$ followed by gas-expanded liquid extraction on lupin protein fractions. Clear protein bands were observed at various molecular weights (95–62 kDa, ~63 kDa, ~50 kDa, ~33 kDa, ~17 kDa, and 14 kDa) on the reducing SDS-PAGE gel. These molecular weights align with most protein bands (ranging from 20 to 100 kDa) reported in previous studies on lupin proteins (Burgos-Díaz et al., 2019; Olukomaiya et al., 2020). Lupin proteins typically consist of four fractions based on their electrophoretic mobility, with α- and β-conglutin being the predominant globulins (80 %), followed by γ-conglutin (15–20 %). According to some studies, α-conglutin fractions and β-conglutin fractions appear at 74–36.5 kDa and 75–16 kDa, respectively, while smaller fragments between 30 and 17 kDa have been associated with γ-conglutin (Chamone et al., 2023; Czubinski & Feder, 2019; Devkota et al., 2023).

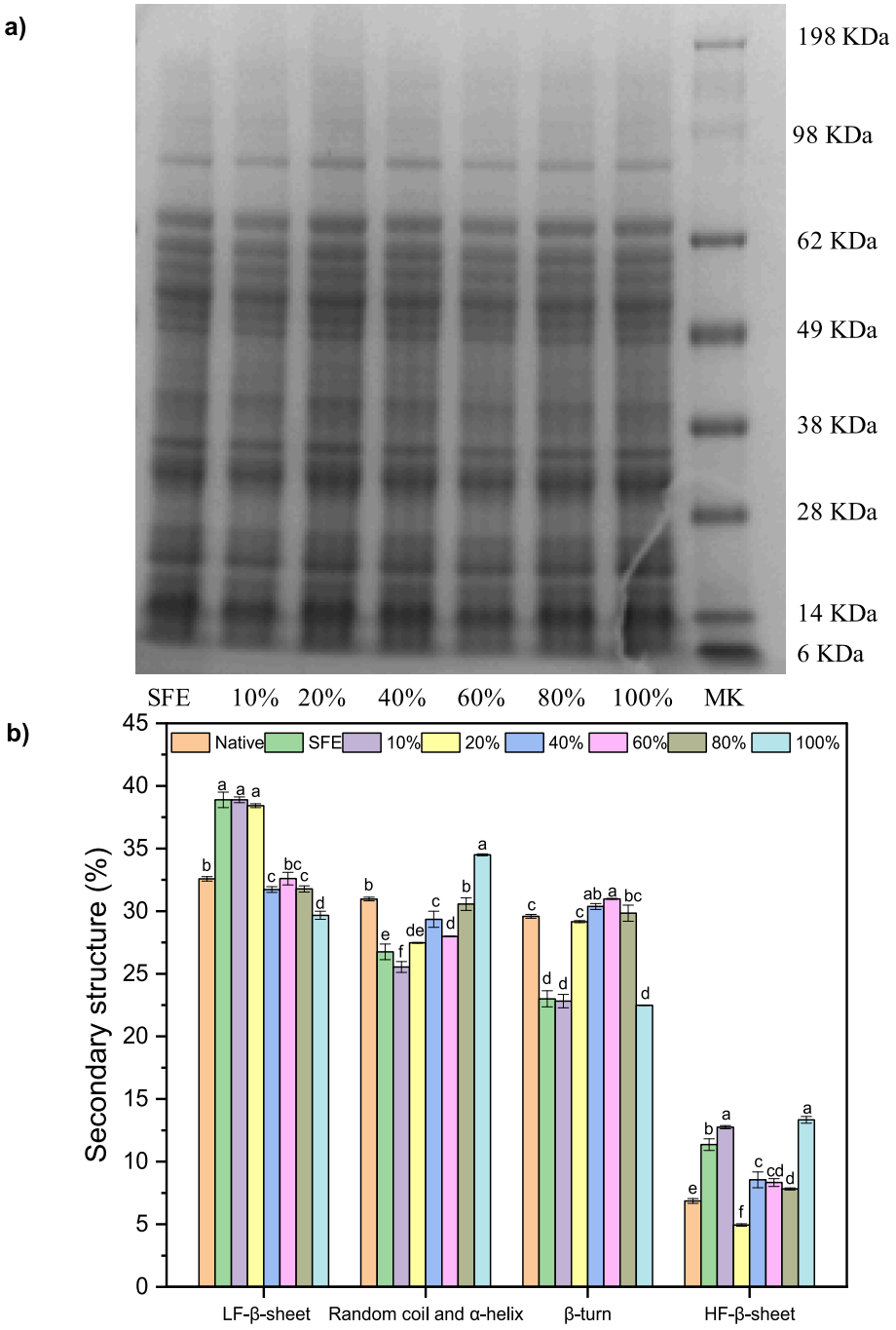

Fig. 4. (a) SDS-PAGE under reduced conditions for all extracted lupin flours; MK stands for molecular weight marker. (b): Secondary structure content of native and extracted lupin flours from FTIR analysis. SFE refers to the flour obtained after supercritical fluid extraction at 40 °C and 250 bar. LF and HF refer to low-frequency and high-frequency structures, respectively. The percentages from 10 % to 100 % indicate the mass proportion of ethanol in the CO_2 mixtures used for the gas-expanded liquid extractions of polar compounds.

The changes in protein secondary structure induced by extraction processes were evaluated by FTIR. The individual bands identified within the 1700–1600 cm^{-1} range, correspond to amide I band. This band has been widely used in infrared spectroscopy to study protein folding, unfolding, and aggregation because of its strong protein signal and minimal influence from side chains (Calix-Rivera et al., 2023). The individual peaks identified in the amide I after iterative fitting, assuming Gaussian band shapes, were classified as follows: high frequency (HF) β-sheet (1700–1690 cm^{-1}), β-turns (1690–1665 cm^{-1}), random coil structure and α-helix (1665–1640 cm^{-1}), and low frequency (LF) β-sheet (1640–1615 cm^{-1}) (Vela et al., 2023). The secondary structure distribution of lupin proteins before and after the extraction processes are shown in Fig. 4b and the deconvoluted spectra and their fit to gaussian band shapes are depicted in Fig. S3. Native lupin proteins exhibited a predominant low-frequency β-sheet structure (32.5 %), followed by α-helix and random coil structures (30.9 %), β-turn (29.5 %), and high-frequency β-sheet (6.8 %). Extraction of lipids using $ScCO_2$ at 40 °C and 250 bar induced significant changes in all evaluated structures, leading to a loss of their native state. Notably, there was an increase in β-sheet structures (both low and high frequency), while a decrease in α-helix, random coil, and β-turn structures was observed due to the defatting process. There is limited information on the effect of lipid extraction using $ScCO_2$ on vegetable proteins. However, it is known that $ScCO_2$ may alter the tertiary and secondary structures of proteins (Monhemi & Housaindokht, 2019). For instance, Zagrobelny and Bright (1992) have shown conformational changes in trypsin resulting from exposure to $ScCO_2$ solvent. The use of high hydrostatic pressure (HPP) was also found to affect secondary protein structure differently depending on the conditions. For example, Tabilo-Munizaga et al. (2014) reported an increase in α-helix, β-turn, and β-sheet structures in Sauvignon blanc wine treated at 400 MPa for 3 min, while Cepero-Betancourt et al. (2020) reported a decrease in α-helix structure and an increase in β-turn in red abalone muscle treated at 400 MPa for 5 min. Ngarize et al. (2004) also found that β-sheet structure decreased after pressurization, while β-turns structure increased in β-lactoglobulin and ovalbumin pressurized at 600 MPa for 20 min. These results reflect the differences in how proteins from different matrices respond to different high-pressure levels. The literature suggests that HHP alters the arrangement of intermolecular β-sheet structures, leading to the formation of new intramolecular interactions. HHP's impact on proteins involves the restructuring of bonds within and between protein molecules due to the disruption of non-covalent interactions within them. Depending on whether intramolecular or intermolecular interactions predominate, proteins tend to adopt either partial states or aggregate, respectively (Cepero-Betancourt et al., 2020). Moreover, the changes in electrostatic interactions and hydrogen bond stability could in turn contribute to the loss of α-helix structure under high pressure conditions (Tabilo-Munizaga et al., 2014). In this study, apart from the effect of pressure (250 bar for approximately 78 min), there is also the dynamic effect of $ScCO_2$ passing through the samples. Overall, the addition of ethanol during gas-expanded liquid extraction of the defatted samples showed varied effects on the evaluated structures depending on the ethanol percentage employed. It is worth highlighting that under specific conditions of expanded liquid extraction, there was a recovery of low-frequency β-sheet (60 % ethanol), α-helix, and random coil (80 % ethanol), and β-turn (20 % and 80 % ethanol) structures, suggesting a reversible behavior of lupin proteins under these conditions.

4. Conclusions

This study explored the use of green extraction technologies for extracting both non-polar and polar bioactive compounds from lupin seeds, and for producing high-protein flour that may have potential for food applications. The SFE process yielded lupin oil with significantly higher tocopherol content compared to B&D method, thereby enhancing the nutritional value of the resulting oil. Moreover, the intensified extraction process promoted the recovery of polar lipids alongside phenolic compounds, predominantly in the form of gentisic acid. Flours obtained from both the SFE process and the intensified process (using $ScCO_2$ to defatted lupin flours followed by the use Ethanol/CO_2 mixtures) exhibited concentrated protein content and increased levels of certain essential amino acids compared to native flour, indicating effective protein concentration without altering their SDS-PAGE profile. Analysis of protein secondary structures via FTIR revealed changes induced by the extraction processes, with lupin proteins exhibiting reversible behavior under certain ethanol percentage additions. Further research is needed to explore the impact of this sequential extraction process on the technological functionalities of the extracted flours by accessing the protein functionality, thus expanding its potential application in the development of products such as emulsions, gels, foaming agents, and bakery goods. We believe that the dual-focus approach proposed in this study, combining the extraction of bioactive compounds and the production of protein-concentrated flour, has the potential to enhance the nutritional and economic value of lupin-based products. However, an economic analysis is necessary to evaluate the viability of these processes and confirm their commercial potential.

CRediT authorship contribution statement

Grazielle Náthia-Neves: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Adane Tilahun Getachew:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Sakhi Ghelichi:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Charlotte Jacobsen:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, 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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.115434.

Data availability

Data will be made available on request.

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