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Comparative highlights of morphological, phytochemical and nutritional key characteristics of Mediterranean *Lupinus* species

Imen Akremi [1], Souhir Kabtni [1*], Hajer Ben Ammar[1], Manon Genva [2], Sondos Hejazi [3,4],Safia Elbok [5], Slim Rouz [6], Sonia Marghali [1], Marie-Laure Fauconnier [2]

1. Laboratory of microorganisms and active biomolecules (LR03ES03). Department of Biology, Faculty of Sciences of Tunis, University Campus, 2092, Tunis El-Manar University, Tunisia.

2.Laboratory of Chemistry of Natural Molecules, Gembloux Agro-Bio Tech, Université de Liège, Gembloux, Belgique

3 Dipartimento di Agraria, University of Napels "Federico II", Parco Gussone, I-80055 Portici, Italy.

4 Departement of nutrition and Food Technology, An-Najah National University, Nabuls P400, Palestine.

5. Laboratory of Biodiversity, Biotechnologies and Climate Change (LR11ES09). Department of Biology, Faculty of Sciences of Tunis, University Campus, 2092, Tunis El-Manar University, Tunisia.

6. Institution of Agricultural Research and Higher Education - Tunisia Department of Agronomy.

*****Corresponding author: souhir.kabtni@fst.utm.tn

Abstract

Lupinus species are valuable for sustainable agriculture due to their high protein content, adaptability, and bioactive compounds. This study assessed the morphological, nutritional, and phytochemical diversity of Lupinus albus, L. luteus, and L. pilosus using 27 agro-morphological traits and analytical techniques (GC-MS, GC-FID, HPLC, and spectrophotometry). L. pilosus, with its dense leaf pubescence, thrives in arid environments, whereas L. luteus and L. albus, with glabrous leaves, are adaptable to humid conditions. L. albus has the shortest ripening period (208.61 ± 12.79 days) and the highest protein (37.18 ± 2.37%) and nitrogen (5.95 ± 0.38%) content. L. pilosus exhibits the strongest antioxidant activity (ABTS: 84.5%) due to its rich flavonoid and phenolic profile, while L. albus differ with its nutraceutical potential. Additionally, 38.88% of L. albus seeds are "sweet", improving edibility. These findings highlight L. albus as an optimal candidate for genetic improvement and sustainable agriculture, particularly in Mediterranean regions.

Keywords

lupin, agro-morphological traits, Phytochemical compound, Antioxidant activities, HPLC, selection.

Introduction

The growing demand for plant-based protein sources has driven extensive research into the nutritional and biochemical properties of legumes, highlighting their potential health benefits and functional applications in food chemistry. Lupin, in particular, is emerging as a promising alternative to traditional protein sources such as soybean and maize (Szczepański et al., 2022). As global demand for these crops rises, so do their costs, largely due to competition for use in animal feed and biofuel production, with feed costs comprising 60% to 80% of overall livestock production expenses (Alqaisi et al., 2014). In light of these challenges, sustainable alternatives like lupin, a member of the Fabaceae family, offer a nutrient-rich profile, adaptability to diverse climates, and the potential to enhance food security and environmental sustainability (Abraham et al., 2019). This is particularly relevant in regions such as Tunisia, where climate variability and recurrent droughts exacerbate reliance on costly imported animal feed (Baptista et al., 2022), highlighting the urgent need for locally available, sustainable alternatives.

The genus *Lupinus* comprises approximately 200 species, broadly categorized into "Old World" species, native to the Mediterranean region, North Africa, and East Africa, and "New World" species, which are predominantly found in North and South America (Mane et al., 2018). Among them, only four species are currently cultivated for human and animal consumption*: Lupinus albus* L. (white lupin), *Lupinus angustifolius* L. (blue or narrow-leafed lupin), *Lupinus luteus* L. (yellow lupin), and *Lupinus mutabilis* L. (Andean lupin). *Lupinus albus*, traditionally cultivated in the Mediterranean region, is valued for its high nutritional content and diverse applications, including soil fertilization (Cu et al., 2005; Cheng et al., 2011; Cernay et al., 2018), phytoremediation (Vázquez et al., 2006), and medicinal purposes (Ragunathan et al., 2009). Lupin seeds, contained in naturally dehiscent pods, can be consumed as snacks or used in a variety of food products such as plant-based milks, cheese alternatives, meat substitutes, and baked goods (Abreu et al., 2023). In addition, lupin oil, rich in beneficial fatty acids, and its protein isolates, with exceptional functional properties, further contribute to its appeal. Its gluten-free nature also makes it suitable for individuals with coeliac disease (Bryant et al., 2022).

While *Lupinus albus* has been extensively studied for its agronomic traits and nutritional benefits, lesser-known species like *Lupinus pilosus* and *Lupinus luteus* remain underexplored, particularly in regions like Tunisia, where they could offer significant advantages. *L. pilosus* is well-adapted to

challenging climatic conditions such as drought and poor soil quality, making it a promising candidate for sustainable agriculture in semi-arid regions. Similarly, *L. luteus*, characterized by its distinctive yellow seed coat, is known for its higher protein content and greater pest resistance compared to other lupin species, which may provide added value to local agricultural systems (Lichtin et al., 2020). Furthermore, both species may possess unique nutritional profiles, including elevated concentrations of specific amino acids and bioactive compounds, enhancing their potential use in functional foods. Despite the well-documented high protein content (Jul et al., 2003) and antioxidant properties of lupins (Estivi et al., 2023), comprehensive data comparing these traits across different species and geographical regions remain limited, underscoring the need for further investigation.

This study aims to address these knowledge gaps by providing a detailed morphological and biochemical characterization of three lupin species: *Lupinus pilosus*, *Lupinus luteus*, and *Lupinus albus*. Wild Tunisian accessions of *L. pilosus* and *L. luteus* were collected during field expeditions in 2022–2023, while cultivated accessions of *L. albus* were sourced from Tunisia, Algeria, Egypt, Italy, and France, ensuring broad genetic representation. The study will evaluate the nutritional and bioactive compounds of these accessions, supporting the broader adoption of lupin as a sustainable, high-protein crop. By elucidating the biochemical diversity of lupin species, this research will contribute to agricultural sustainability and food security, particularly in Tunisia and other regions facing similar climatic challenges.

Materials and Methods

1. Plant material

The study focused on three Lupinus species: *Lupinus pilosus*, *Lupinus luteus*, and *Lupinus albus*. Seeds from wild Tunisian accessions of L. *pilosus* and L. *luteus* were collected during expeditions in 2022-2023. Cultivated L. *albus* accessions were sourced from Tunisia, Algeria, Egypt, Italy, and France, ensuring diverse genetic representation across environments (Table 1, Fig. 1). The number of biological replicates varied based on the type of analysis conducted, either morphological or biochemical. Detailed information regarding the number of replicates for each analysis is presented in the corresponding results tables.

2. Morphological characteristics

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For the morphological characterization of *Lupinus* species, a controlled study was conducted using trial pots at the Laboratory of Chemistry of Natural Molecules, University of Liège, Gembloux Agro-Bio Tech, Belgium. The seeds from each species were manually scarified to enhance germination and then sown in pots filled with a peat-sand mixture. Pots had a capacity of 3 liters and were systematically irrigated with 250 mL of water weekly to ensure consistent moisture levels.

This study focused on 27 morphological traits, comprising 15 quantitative and 12 qualitative characters, which covered both vegetative (e.g., leaf, stem) and reproductive (e.g., flower, pod) parts of the species. Measurements were taken at five key developmental stages: the vegetative stage, flowering stage, growth stage, green maturity stage, and full maturity stage. These observations were conducted following the guidelines outlined in the *Lupinus* descriptor IBPGR and the international descriptors established by UPOV. The evaluation data summarizing these observations are presented in Table 2.

3. Nutritional characteristics

3.1. Fatty Acid Analyses

Lupin seed oil was extracted using a Soxhlet apparatus **(**VELP Scientifica, Italy) following the method described by Saha et al. (2018). The oil yield was calculated as the percentage of extracted oil relative to the dried seed powder mass, with solvent evaporation performed using a BUCHI Rotavapor (R-114) and a vacuum controller (BUCHI V-850) (BUCHI, Switzerland). For each sample, the experiment was repeated three times.

The yield of oil content was calculated using this Equation:

Extraction yield (%) = (Mass of extracted oil (g)/ Mass of dried powder (g)) *100

3.1.1. Transesterification of the fatty acids

Fatty acid methyl esters (FAMEs) were prepared via transesterification using n-hexane and BF_3 reagent under controlled heating conditions. The resulting FAMEs were analysed by an Agilent HP 6890 gas chromatograph (Germany) equipped with a flame ionization detector (GC-FID) and identified based on retention times. Fatty acids were identified by retention times and quantified by peak area ratios.

3.1.2. Gas chromatography-mass spectrometry (GC-MS) analyses of fatty acid

GC–MS analysis was performed using an HP 6890 (II) gas chromatograph with an HP 5972 mass spectrometer operating at 70 eV electron impact ionization under the same conditions as GC-FID, with helium as the carrier gas at 1.2 ml/min. Samples (1 μl) were injected in split mode (1:20). Components were identified by retention times and mass spectra comparison with FAME standards and spectral libraries (Figure S1). GC-FID quantified compounds, while GC-MS confirmed their identification for reliable results.

3.2. Amino Acid Analyzed by Acid Hydrolysis and HPLC Separation

Acid hydrolysis of the samples, followed by HPLC separation, enables the quantification of proteinogenic amino acids. The samples are hydrolysed using 6N HCl with 0.1% phenol for 24 hours at 110°C. Following hydrolysis, the pH is adjusted to 2.2, and the amino acids are separated on a cation-exchange column. Elution is achieved through a stepwise increase in pH, ionic strength, and temperature. Post-column derivatization with ninhydrin allows detection at 570 nm, except for proline, which is measured at 440 nm. The results indicate the conversion of aspartate and glutamate into their respective acidic forms, while tryptophan is entirely degraded, necessitating an alternative quantification method (Figure S2). Sulfur-containing amino acids undergo partial degradation, limiting their accurate quantification. The final results are expressed in grams of amino acids per 100 grams of fresh sample material.

3.3. Protein Analysis

Lupin seeds from various varieties were oven-dried at 45°C for 24 hours, then ground using a 1 mm sieve for subsequent analysis. Nitrogen (N) content was determined using the Kjeldahl method (FOSS, Denmark) (Ezeagu et al., 2002), and crude protein (CP) was calculated by multiplying the nitrogen content by a factor of 6.25. Additionally, the samples were analyzed for their amino acid composition.

4. Antinutritional Traits

4.1. Extraction of Phenolic Compounds

Phenolic compounds were extracted from dried plant samples ground with liquid nitrogen, following a modified method based on Krakowska et al. (2017). A 0.5 g portion of the powdered

sample was macerated in 80:20 (v/v) methanol for 16 hours. After filtration, the extract was concentrated using a Heidolph Laborota 4003 rotary evaporator (Heidolph Instruments, Germany) at 40°C under reduced pressure. The concentrated extract was then dried, reconstituted in methanol to a final concentration of 20 mg/mL, and stored at 4°C in the dark. All extractions were performed in triplicate to ensure consistency.

4.2. Determination of The Total phenolic contents (TPC)

The total phenolic content (TPC) of *Lupinus* seeds was determined using the Folin-Ciocalteu colorimetric method, which quantifies phenolic compounds based on their ability to reduce a phosphomolybdate-phosphotungstate complex, resulting in a blue chromophore that can be measured spectrophotometrically (UltraSpec 400, Pharmacia Biotech, England). A 500 µl aliquot of the sample extract (diluted 1:10) was mixed with 1 ml of Folin-Ciocalteu reagent (pre-diluted 1:10) and incubated in the dark for 8 minutes. Subsequently, 1 ml of 7.5% sodium carbonate solution was added, and the mixture was incubated for 30 minutes at room temperature, in the dark. Absorbance was recorded at 765 nm, and TPC was expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) using a gallic acid standard curve (ranging from 0 to 100 µg/ml) (Table S1). All analyses were performed in triplicate to ensure precision.

4.3. Determination of The Total Flavonoid contents (TFC)

The Total Flavonoid Content (TFC) of Lupinus seeds was determined using a modified colorimetric assay with aluminum chloride, as outlined by Stanković (2011). In this method, 0.5 mL of each seed extract was mixed with 0.5 mL of a 2% AlCl3 solution dissolved in methanol. The mixture was incubated for 30 minutes at room temperature to allow the complex to form. The absorbance of the resulting solution was then measured at 420 nm using a spectrophotometer. The TPC was expressed as milligrams of Quercetin equivalents per gram of dry weight (mg QE/g seed). Flavonoid concentrations were quantified based on a calibration curve constructed using Rutin as the standard (Table S1). All measurements were performed in triplicate to ensure accuracy and reproducibility.

5. Biological activities

5.1. Antioxidant Activities

The antioxidant activities of seeds produced from *Lupinus* species were evaluated using in *vitro* by four methods:

5.1.1 Free radical scavenging (DPPH)

The free radical scavenging activity of lupin seed extracts was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following the method described by Sanchez-Moreno et al. (2005), with slight modifications to optimize concentration parameters. A 0.5 mL aliquot of a methanolic Trolox solution (0.1 mM) was mixed with 0.5 mL of 80% methanol and 0.5 ml of a methanolic DPPH solution (0.2 mM). The mixtures were vortexed vigorously to ensure homogeneity and subsequently incubated in the dark for 30 minutes at room temperature. Absorbance was then measured at 517 nm using a UV-visible spectrophotometer (Ultra Spec 400, Pharmacia Biotech, England) against a blank containing 80% methanol. The experiment was performed three times.

The DPPH radical scavenging activity, expressed as percentage inhibition, was calculated using the following equation:

$$\% \text{ Inhibition} = ((A_0 - A_T)/A_0) \times 100$$

With A_0 = absorbance of the blank sample

A_T = absorbance of the test sample

All experiments were performed in triplicate to ensure reproducibility and accuracy of the results. To quantify the antioxidant potential of each extract, a calibration curve was constructed using various concentrations of Trolox, plotting the percentage inhibition of DPPH radicals against Trolox concentrations. The antioxidant activity of the lupin seed extracts was expressed in terms of Trolox Equivalent Antioxidant Capacity (TEAC), with results reported in mg Trolox equivalents per gram of seed extract (mg TEAC/g) (Table S1).

5.1.2 Ferric-Reducing Power Determination (FRAP)

The reducing power of the samples and the standard (Trolox) was assessed using the method described by Singleton and Hseu (Lee et al., 2011; Hseu et al., 2008). Samples were prepared in methanol at four concentrations (25, 50, 75, and 100 µg/ml) and analyzed in triplicate. Each sample

was mixed with 1 ml of phosphate buffer (0.2 M, pH 6.6) and 1 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$], then incubated at 50°C for 20 minutes. Following incubation, 1 ml of 10% (v/v) trichloroacetic acid (TCA) was added, and the mixture was centrifuged at 3000 g for 10 minutes. The resulting supernatant was combined with 1.5 ml of distilled water and 150 µl of 0.1% (v/v) $FeCl_3$ solution. The absorbance of the final mixture was measured at 700 nm, with an increase in absorbance relative to the blank indicating enhanced reducing power (Table S1). To ensure accuracy, the absorbance values of the essential oils at each concentration were subtracted from the final readings. The experiment was performed in triplicate to ensure reliability.

5.1.3. Trolox equivalent antioxidant capacity (ABTS)

The ABTS radical scavenging assay was performed using Trolox as a reference antioxidant, based on protocols by Loizzo et al. (2019). The ABTS·+ radical cation was generated by mixing equal volumes of 7 mM ABTS·+ solution and 2.45 mM potassium persulfate, incubated in the dark for 16 hours. The solution was diluted with methanol to achieve an absorbance of 0.7–0.75 at 734 nm. Sample and Trolox solutions were prepared in methanol at various concentrations. For the assay, 20 µl of each sample was added to 170 µl ABTS+ working solution, and after 6 minutes at 30°C, absorbance was measured at 734 nm. Antioxidant activity was determined by plotting the percentage of remaining ABTS·+ and calculating the IC50 value. Results were expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in mg Trolox equivalents per gram of seed (mg Trolox eq/g) (Table S1). The experiment was repeated three times.

5.1.4. Test β-carotene bleaching

The β-carotene bleaching test is a spectrophotometric method that evaluates antioxidant activity based on the discoloration of β-carotene at 470 nm. In this assay, the oxidation of linoleic acid produces peroxide radicals and conjugated diene hydroperoxides, which subsequently oxidize β-carotene, resulting in a loss of its characteristic red colour (Kulisic et al., 2004; Tepe et al., 2005).

The procedure begins by dissolving 2 mg of β-carotene in 1 ml of chloroform. This solution is then combined in a flask with 2 mg of linoleic acid and 200 mg of Tween 40. Following the complete evaporation of chloroform, 100 ml of oxygen-saturated distilled water is added while stirring vigorously to form a stable emulsion. Subsequently, 2.5 ml of this emulsion is transferred into test tubes, to which 350 µl of each extract (at a concentration of 2 g/l) and a butylated

hydroxytoluene (BHT) control are added (Tepe et al., 2005). Absorbance measurements are taken at 490 nm at predetermined time intervals to monitor the bleaching of β-carotene (Table S1). The experiment was repeated three times

The antioxidant activity is quantified using the following equation:

AAR = (Abs Sample / Abs BHT)*100

where AAR represents the relative antioxidant activity, Abs Sample is the absorbance of the sample after 48 hours, and Abs BHT is the absorbance of the BHT control after the same duration.

5.2. Anti-inflammatory activity: Lipoxygenase Inhibitory Activity

Enzymatic extracts were prepared following the protocols of Clémente et al. (2000), Ridolfi et al. (2002), Schweiggert et al. (2005), and Yoshie-Stark & Wäsche (2004), with modifications. Briefly, seeds were ground, and a 2.5 g sample was extracted in a sodium phosphate buffer (50 mmol/l, pH 6.8) containing PVPP, DTT, EDTA, sodium metabisulfite, and Triton X-100. After stirring at 4 °C and centrifugation, the supernatant was dialyzed against the same buffer for 24 hours. Dialysates were collected as crude enzyme extracts, aliquoted, and stored at −50 °C

The supernatant was dialyzed (3.5 kDa cellulose membranes) against sodium phosphate buffer (50 mmol/l, pH 6.8) for 24 hours with buffer change, then collected, aliquoted, and stored at −50 °C. All extractions were performed in triplicate

Lipoxygenase (LOX) activity was assessed using Yoshie-Stark & Wäsche's (2004) modified method. Extracts at 100, 75, 50, and 25 µg/ml were incubated with commercial LOX (Sigma) in borate buffer (0.2 M, pH 9.0) for 15 minutes at 25 °C. The reaction was initiated with 35 µl linoleic acid (250 µM), and absorbance at 234 nm was recorded. Quercetin (Sigma) served as a standard inhibitor (Table S1).

The inhibition percentage of lipoxygenase activity was calculated as follows:

$$\textbf{Inhibition percentage (\%)} = 100 \times (OD_{blank} - OD_{sample})/O_{Dblank}$$

where OD_{blank} is the Optical Density (OD) of the reaction medium without Seed extract, and OD_{sample} is the OD of the reaction medium with Seed extract.

The inhibition of DPPH and lipoxygenase activity by the essential oils was quantified by determining the concentration required to achieve 50% inhibition (IC50), expressed as the percentage of the essential oil concentration.

6. Statistical analysis

Statistical analysis was conducted to identify the most discriminant morphological and biochemical traits among *Lupinus* species and to determine parameters that exhibited significant variation. *ANOVA* was performed at a significance level of 0.05, complemented by Tukey test and post-hoc analysis using Fisher's Least Significant Difference (LSD) test for all quantitative data means. To further discriminate between local *Lupinus* populations, the average values of both morphological and biochemical parameters were subjected to principal component analysis (PCA) and *Pearson* correlation tests to reveal associated traits. All statistical analyses and data visualization was computed using R software (version 4.2.1; R Core Team, 2022) along with the following packages: Rcmd (Fox, 2005, 2016), Agricolae (De Mendiburu, 2017), and FactoMineR (Lê et al., 2008), factoextra, ade4, ggplot2, corrplot, ggcorrplot.

Results and Discussion

1. Morphological Variation

Morphological characteristics, both qualitative and quantitative, play a key role in assessing the adaptability and agronomic potential of different species. These traits provide valuable information on species responses to environmental pressures, such as water availability, light, and temperature, as well as their evolutionary survival strategies. In this study, we examined various morphological parameters to compare the three *Lupinus* species. These characteristics are directly related to the ability of the species to thrive in their respective habitats and their potential uses in agricultural systems.

1.1 Qualitative traits

The analysis of qualitative traits revealed significant morphological differences among the three Lupinus species, except for their plant growth type (GT), which was indeterminate (GTIndet) in all examined plants (Table 3), which reflect the species diverse adaptations and agronomic potential. Thus, this growth pattern enables them to adapt to environmental variations, adjusting their development based on available resources like light, water, and nutrients (Annicchiarico et

al., 2010). Such flexibility is particularly beneficial in Mediterranean and semi-arid climates, where rainfall is irregular and unpredictable (Gresta et al., 2023).

Vegetative growth traits also exhibited distinct variations among species. Cotyledon surface texture differed significantly: *L. pilosus* displayed a medium-smooth surface (ACms), *L. luteus* had a rough surface (ACr), and *L. albus* exhibited smooth cotyledons (ACs). These differences may influence seedling emergence and early growth, affecting overall plant establishment and survival. Similarly, anthocyanin coloration (ACP) and leaf green color intensity (IGR) varied before bud emergence, with *L. pilosus* and *L. luteus* exhibiting medium anthocyanin presence (ACPm) and dark green leaves (IGRd), while *L. albus* had weak anthocyanin expression (ACPw) with both dark (IGRd) and medium green leaf intensity (IGRm) (Table 3). These variations suggest differential adaptation to environmental conditions (Wang et al., 2022).

Leaf morphology exhibits significant interspecific variation in Lupinus, serving as a key adaptive trait for optimizing water use and photosynthetic efficiency in response to distinct environmental pressures. *Lupinus pilosus*, with its hairy leaves, is particularly adapted to withstand low-moisture environments, reducing water loss and providing protection in dry climates. In contrast, the smooth leaves of *Lupinus luteus* and *Lupinus albus* facilitate greater light capture and photosynthetic efficiency, a strategy well-suited for temperate climates with consistent moisture availability. These adaptations underscore how leaf morphology supports each species' ability to thrive in its respective ecological niche (Wang et al., 2022).

The floral coloration observed among the *Lupinus* species under study demonstrates distinct, species-specific patterns that play an essential role in ecological adaptation and interspecies interactions, particularly in terms of pollinator attraction and resource partitioning. In *Lupinus pilosus*, for example, the wings exhibit a vivid violet coloration (CWv), while *Lupinus luteus* displays bright yellow wings (CWy); *Lupinus albus* presents a distinctive combination of white and violet on its wings (CWwv) (Table S1). Additionally, the carina tip varies significantly among these species: both *Lupinus pilosus* and *Lupinus albus* display a blue-black tip (CTCbb), whereas *Lupinus luteus* exhibits a contrasting yellow tip (CTCy). These floral color variations are not merely ornamental; they represent key adaptive traits that enhance ecological fitness within the Lupinus genus. Floral pigmentation is a well-established determinant of pollinator preference, directly influencing species-specific pollination dynamics and reproductive success (Trunschke et

al., 2021). By promoting specialized pollinator interactions, these adaptations reinforce reproductive isolation, thereby minimizing interspecific competition for pollination resources in overlapping habitats (Gori, 1989). As a result, the distinct floral characteristics observed in Lupinus species exemplify how morphological differentiation drives ecological specialization and maintains genetic divergence within the genus (Reid, 2019).

The morphological analysis of *Lupinus* seeds reveals significant interspecies differences in both coloration and ornamentation, highlighting unique characteristics within each species. Both *Lupinus pilosus* and *Lupinus luteus* exhibit ornamented seeds (GOpres), though with distinct patterns and colour variations: *Lupinus pilosus* has black ornamentation (GCObl), while *Lupinus luteus* is marked by beige ornamentation (GCOb). In contrast, *Lupinus albus* shows no seed ornamentation (GOabs).

The pattern and density of ornamentation vary significantly between species. In *Lupinus pilosus*, ornamentation is restricted solely to the "eyebrow" region of the seed (DOeye-only), whereas *Lupinus luteus* features a broader distribution, with ornamentation covering the entire seed surface except for the eyebrow region (DOtot-eye). Furthermore, *Lupinus luteus* exhibits a medium density of ornamentation (Dens GOm), while the density of ornamentation in *Lupinus pilosus* remains unclassified due to its restricted pattern. These variations in seed ornamentation and colour likely reflect adaptations to differing environmental conditions and genetic diversity across the species (Gnieszka et al., 2005; Kroc et al., 2021).

The presence of bitterness, a key trait affecting palatability, is a distinguishing characteristic in both *Lupinus pilosus* (GBa) and *Lupinus luteus* (GBa). In contrast, *Lupinus albus* exhibits a notable variation, with 38.88% of its seeds lacking the bitter component (GBd). This absence of bitterness in a significant proportion of *L. albus* seeds suggests that this species may offer enhanced palatability, making it a more promising candidate for food applications compared to the other species (Natalia et al., 2018). Historically, the presence of bitter alkaloids in wild lupins has limited their development as food grains. However, contemporary agricultural advancements have led to the emergence of "sweet" lupin varieties, specifically bred to eliminate bitterness. This strategic breeding initiative has paved the way for the cultivation of lupins as acceptable and nutritious food sources in numerous countries (El Maadoudi et al., 2004). Such developments not only enhance

the culinary versatility of lupins but also hold promise for improving their marketability and acceptance in the food industry.

1.2 Quantitative traits

The analysis of variance (ANOVA) for quantitative traits revealed highly significant differences (p-value < 0.001) among the three Lupinus species across nearly all measured parameters, as presented in Table 4.

Quantitative traits provide an objective and robust framework for evaluating productivity differences among genotypes by directly measuring key phenotypic characteristics. This method enables a systematic comparison of genetic materials from diverse backgrounds using standardized evaluation criteria, facilitating the identification of superior traits across varying environmental and genetic contexts (Tsenov et al., 2014).

1.2.1 Phenological Development

The analysis of phenological traits, including Time of Ripening (TR), Time of Green Ripening (TGR), Date of First Bud Appearance (DFBA), and Time of Flowering (TF), reveals significant differences among the three *Lupinus* species, highlighting their distinct developmental patterns.

Lupinus albus (La) demonstrates the earliest ripening, with an average Time of Ripening (TR) of 208.61 ± 12.79 days, suggesting a relatively faster maturation process compared to the other species. The Time of Green Ripening (TGR) in *Lupinus albus* is also notably early, at 181.22 ± 25.05 days, indicating that this species transitions rapidly to its mature green phase. The Date of First Bud Appearance (DFBA) for *Lupinus albus* occurs at 70.07 ± 12.61 days, and flowering follows at 90.99 ± 12.73 days. These values suggest that *Lupinus albus* develops both its vegetative and reproductive phases earlier than the other species. This short growth cycle is a key factor to consider when determining the optimal spacing for selecting lupine cultivars. Proper spacing can enhance plant development and productivity, making it essential for successful lupine cultivation (Gresta et al., 2023).

On the other hand, *Lupinus luteus* (Ll) shows a markedly slower development, as reflected by its prolonged Time of Ripening (212 ± 0.00 days) and Time of Green Ripening (191.00 ± 0.00

days). Additionally, *Lupinus luteus* has a significantly delayed Date of First Bud Appearance, occurring at 141.85 ± 0.36 days, and flowering is similarly late, at 156.00 ± 0.00 days. This extended phenological timeline suggests that *Lupinus luteus* may be adapted to different environmental conditions that favour a longer developmental period.

Lupinus pilosus (Lp) occupies an intermediate position between *Lupinus albus* and *Lupinus luteus*. Its Time of Ripening (TR) is shorter than *Lupinus luteus* at 194.66 ± 6.55 days, and its Time of Green Ripening (178.47 ± 2.80 days) is also earlier, indicating a more accelerated maturation process compared to *Lupinus luteus*, through slower than *Lupinus albus*. The Date of first Bud Appearance in *Lupinus pilosus* occurs at 116.11 ± 7.18 days, and it reaches the Time of Flowering at 146.11 ± 9.44 days. These values suggest that *Lupinus pilosus* has a more extended reproductive phase than *Lupinus albus*, but a more rapid onset of flowering compared to *Lupinus luteus.* The variability, which depends on both the species and the growing conditions, is linked to the cultivar's potential and its level of adaptation (Gataulina, 2014).

1.2.2 Vegetative Growth Characteristics

The analysis of vegetative growth characteristics among the three *Lupinus* species reveals significant differences in traits critical for plant structure and biomass production potential. The cotyledon length/width ratio (Lslcotyl) remained similar across all species, with *L. pilosus* showing a slightly more rounded shape (1.005 ± 0.076), potentially enhancing early seedling vigor.

Lupinus albus demonstrated the largest central leaflet area (SF) at 8.094 ± 2.90 cm^2, indicating a superior photosynthetic capacity compared to *Lupinus luteus* (2.745 ± 0.61 cm^2) and *Lupinus pilosus* (6.632 ± 2.34 cm^2). The central leaflet length/width ratio (LslF) further distinguishes the species, with *Lupinus luteus* exhibiting the highest ratio (5.396 ± 0.77), signifying narrower, elongated leaflets. In contrast, *L. albus* (3.039 ± 0.75) and *L. pilosus* (3.674 ± 0.52) presented broader leaflets.

In terms of plant height during the vegetative stage (HVS), *Lupinus pilosus* reached the greatest height (21.653 ± 4.24 cm), closely followed by *L. albus* (20.045 ± 6.73 cm), highlighting their investment in vertical growth for optimal light capture. Conversely, *Lupinus luteus* was shorter (13.783 ± 1.57 cm), reflecting a more compact growth form that may be better suited for energy conservation in resource-limited environments. Plant height is a critical indicator of

resistance to lodging, which indirectly affects overall yield. Genotypes with greater height are particularly susceptible to stress, especially under intensive farming conditions. This increased vulnerability can lead to a disruption in the grain-filling process, ultimately resulting in inaccurate assessments of their properties and performance. Understanding the relationship between plant height and lodging resistance is essential for optimizing crop management strategies and enhancing yield potential (Wu et al., 2022).

1.2.3 Reproductive Development and Inflorescence

The reproductive development parameters highlighted distinct reproductive strategies across the species (Table 4). *Lupinus albus* had the longest inflorescence (19.05 ± 4.94 cm) and the greatest plant height at the vegetative stage (20.04 ± 6.73 cm), signifying more substantial reproductive structures compared to the other species. *Lupinus pilosus,* while shorter during the vegetative stage, had a significantly higher plant height at green ripening (35.02 ± 6.23 cm) and the beginning of flowering (46.21 ± 6.63 cm), reflecting its strategy of late but robust reproductive growth. In contrast, *Lupinus luteus* was the shortest in all reproductive traits, with inflorescence length (10.68 ± 1.77 cm) and height at green ripening (32.18 ± 3.11 cm) being the lowest. This indicates a lesser investment in reproductive biomass, suggesting a different evolutionary strategy that prioritizes growth over reproductive development.

1.2.4 Pod and Seed Characteristics

Plant productivity is significantly affected by factors such as pod count, pod length, and seed weight. Enhancing the yield potential of genotypes requires efforts to boost both the number of pods and the seed production per plant (Gresta et al., 2023).

The analysis reveals significant variability in pod and seed traits among the three lupin species studied, which are critical for evaluating yield potential (Table 4). *Lupinus albus* exhibited the largest pod length (7.59 ± 0.92 cm) and a relatively high pod number per plant (3.80 ± 0.62), indicating a promising potential for higher yield. Furthermore, the 100-seed weight of *L. albus* was recorded at 39.25 ± 16.29 g, surpassing the quantity reported by Mavromatis et al. (2023). The larger pod and seed size in *L. albus* not only reinforces the importance of these traits as determinants of yield but also positions this species as a favourable candidate for breeding programs aimed at increasing productivity.

In contrast, *Lupinus pilosus* demonstrated a high pod number per plant (3.60 ± 1.42) but with shorter pod lengths (4.65 ± 1.01 cm) and lower seed weights (17.04 ± 1.49 g). This suggests a strategy of producing numerous smaller pods, which may not be sufficient to enhance overall yield as effectively as the traits exhibited by *L. albus*. This observation aligns with the findings of Mavromatis et al., which highlighted similar trade-offs between pod number and seed size. Additionally, *Lupinus luteus* produced the fewest pods per plant (2.65 ± 0.90) and exhibited the lowest 100-seed weight (14.50 ± 0.50 g), confirming its lower yield potential.

In summary, the results emphasize that *Lupinus albus* possesses advantageous traits that significantly enhance its yield potential compared to *L. pilosus* and *L. luteus*. However, the smaller seed size in *Lupinus luteus* may provide advantages in specific agronomic practices, potentially favouring certain cultivation methods or market preferences.

2. Nutritional characteristics

The nutritional composition analysis among the three *Lupinus* species was conducted to evaluate key parameters essential for exploring their potential as nutritional resources as exposed in table5.

The nitrogen percentage (%N) varied significantly between species, with *Lupinus albus* showing the highest nitrogen content at 5.948 ± 0.380, compared to *Lupinus luteus*, which had the lowest value (5.020 ± 0.00). In contrast, Porres et al., (2007) reported the highest nitrogen levels in *L. luteus* (8.3%) followed by *L. albus* (4%). These discrepancies could arise from genetic variation, environmental conditions, or methodological differences. Local adaptations might lead to varying nitrogen accumulation patterns in different accessions. Additionally, environmental factors such as soil type, climate, and cultivation practices likely influence nitrogen content. Finally, methodological differences in nitrogen measurement could contribute to these variations.

The protein percentage was notably higher in *Lupinus albus*, reaching 37.175 ± 2.37, which significantly surpassed the values observed for *Lupinus luteus* (31.37±0.00) and *Lupinus pilosus* (33.78±1.28). Furthermore, the protein content in these lupins exceeded that of other commercial legumes, such as chickpea, which contains 24.7% protein (Sánchez-Vioque et al., 1999). The findings of Jha et al. (2022) underline the importance of increasing protein levels in legumes as a means of supporting food security initiatives. Within this framework, the results for *Lupinus albus*

are particularly significant; its elevated protein content not only meets essential nutritional needs but also contributes to broader strategies aimed at improving food availability and sustainability. This is especially pertinent in resource-constrained regions, where enhancing the nutritional quality of crops can play a vital role in addressing food insecurity and promoting sustainable agricultural practices.

The comparative analysis of amino acid composition across *Lupinus* species reveals significant variations in their nutritional profiles. All species show an amino acid pattern characteristic of legumes (Pastor-Cavada et al., 2009). *L. albus* consistently exhibits the highest concentrations of key amino acids, including alanine (1.213 ± 0.04), arginine (3.599 ± 0.21), aspartic acid (4.025 ± 0.19), and glutamic acid (9.168 ± 0.75). These elevated levels underscore its potential as a superior protein source, as they are integral to metabolic processes and protein synthesis. Notably, the concentrations of essential amino acids such as isoleucine (1.683 ± 0.04), leucine (2.616 ± 0.07), and lysine (1.685 ± 0.05) in *L. albus* significantly exceed those found in *L. luteus* and *L. pilosus.* Furthermore, *L. albus* also demonstrates elevated levels of phenylalanine (1.405 ± 0.05), proline (1.560 ± 0.08), and serine (1.831 ± 0.08), highlighting its comprehensive role in supporting cellular functions and overall growth. In contrast, *Lupinus luteus* and *Lupinus pilosus* display lower concentrations of amino acids, yet they maintain a relatively stable amino acid profile, though with diminished nutritional value. These results highlight that *Lupinus albus* not only provides a higher amino acid content but also stands out as a highly valuable candidate for enhancing protein intake in both human and animal diets. This emphasizes its critical role in agricultural practices and nutritional strategies aimed at meeting dietary needs and improving food security.

3. Comparative Fatty Acid Profile and Oil Content Analysis

The analysis of oil content and fatty acid composition among the three *Lupinus* species reveals significant variability, as displayed in Table 6. *Lupinus albus* demonstrates the highest oil content at 9.161 ± 1.43%, indicating its potential as a valuable oilseed crop. In contrast, *Lupinus pilosus* exhibits a notably lower oil content of only 2.025 ± 0.91 %. This stark difference suggests that L. albus may be more suitable for oil extraction and food industry applications.

The analysis of specific fatty acids further emphasizes the distinct profiles among the species. *L. albus* exhibited elevated levels of palmitic acid (C16:0) at 6.898 ± 0.66% and oleic acid (C18:1) with 42.372 ± 3.59%, suggesting its potential utility in food and industrial applications due to these

beneficial fatty acids. Additionally, *L. albus* contained substantial linoleic acid (C18:2) with 21.055 ± 1.07%, supporting its role in promoting cardiovascular health. The fatty acid profile of *L. albus* is particularly noteworthy. The high concentrations of palmitic acid (C16:0) at 6.898 ± 0.66% and oleic acid (C18:1) at 42.372 ± 3.59% indicate its considerable potential for applications in both food and industrial sectors. These findings align with the research conducted by Sbihi et al. (2013), who similarly reported oil yields within this range under optimal agronomic conditions. The predominance of oleic acid is of considerable significance, as numerous studies, including those by Al-Amrousi et al. (2022), have elucidated its crucial role in promoting cardiovascular health and enhancing the oxidative stability of oils. Thus, the fatty acid composition of *L. albus* not only supports its viability as a valuable oilseed crop but also underscores its potential contributions to health and nutrition. Moreover, the analysis of fatty acid composition among the *Lupinus* species in this study revealed significant differences, highlighting their unique nutritional profiles. This may provide enhanced nutritional benefits due to its beneficial role in supporting cardiovascular health. Additionally, stearic acid (C18:0) levels varied notably across the species, with *L. luteus* presenting a concentration of 1.226 ± 0.07%, compared to a markedly higher concentration of 7.224 ± 1.05% in *L. pilosus*. This variation suggests the presence of distinct fatty acid metabolism pathways among the species. *Lupinus pilosus* is distinguished by its elevated concentration of α-linolenic acid (C18:3), an essential omega-3 fatty acid, measured at 54.063 ± 8.70%. This substantial α-linolenic acid content is linked to numerous health benefits, including anti-inflammatory effects. Additionally, *L. pilosus* demonstrates lower levels of saturated fatty acids, such as palmitic acid (C16:0), highlighting its unique lipid composition. This favorable profile may render *L. pilosus* an advantageous candidate for functional foods designed to reduce saturated fat intake, thereby supporting healthier dietary practices.

The *ANOVA* results (p-value <0.001) confirmed the statistical significance of the observed differences in oil content and fatty acid profiles among the species, indicating a strong variation across the studied *Lupinus* taxa. Significant variations were also noted in specific fatty acids like erucic acid (C22:1n9), where *L. luteus* (0.714 ± 0.02%) differed from *L. pilosus* (0.761 ± 0.85%), while lignoceric acid (C24) levels remained similar between the species.

These findings diverge from the fatty acid composition reported by Erbaş et al., (2005), who characterized lupin oil as having 13.5% saturated, 55.4% monounsaturated, and 31.1%

polyunsaturated fatty acids, with oleic acid being the dominant component. In contrast, our results indicate that *L. luteus* and *L. pilosus* possess a higher proportion of polyunsaturated fatty acids, particularly linoleic acid. This difference suggests potential functional and nutritional advantages of these species for diverse food applications, where the composition of polyunsaturated fats plays a crucial role. The distinct lipid profiles observed across species may be attributed to both genetic factors and environmental influences, including temperature, soil composition, and moisture levels. Previous studies have demonstrated that climatic conditions can significantly impact fatty acid desaturation, thereby altering the balance between monounsaturated and polyunsaturated fatty acids (Boschin et al.,2007). Understanding these interactions can provide valuable insights for optimizing lupin cultivation to enhance oil quality and nutritional value.

4. Antioxidant activities

The antioxidant activity and phenolic content of three *Lupinus* species (*Lupinus albus*, *Lupinus luteus*, and *Lupinus pilosus*) were evaluated using several assays, revealing distinct differences among the species (p-value <0.001) (Fig. 2). *Lupinus albus* exhibited the highest ABTS radical scavenging activity (15.817 ± 1.26 mg Trolox eq/g of seed), suggesting it has strong antioxidant potential in this assay, followed by *Lupinus pilosus* (10.475 ± 0.56 mg Trolox eq/g of seed) and *Lupinus luteus* (4.295 ± 0.21 mg Trolox eq/g of seed)). In contrast, *Lupinus pilosus* showed the highest activity in the DPPH assay (3.322 ± 0.23 mg TE/g of seed), indicating its superior ability to neutralize free radicals, while *Lupinus luteus* had the lowest DPPH activity (0.541 ± 0.10 mg TE/g of see). These findings align with those reported by Siger et al. (2012), who noted similar levels of antioxidant activity in lupin seeds, underscoring comparable radical scavenging capabilities across different studies.

For the FRAP assay, which measures reducing power, *Lupinus pilosus* again outperformed the other species (6.431 ± 0.51 mg Trolox eq/g of seed), followed by *Lupinus luteus* (5.115 ± 0.22mg Trolox eq/g of seed), indicating a greater ability to reduce ferric ions (Fig. 2).

Lupinus luteus exhibited the highest levels of β-carotene (0.325 ± 0.03 mg/g), suggesting a potential for enhanced protection against oxidative stress. Additionally, its total phenolic content (TPC) was significantly elevated at 249.896 ± 16.66 mg GAE/g DW, indicating a richer concentration of phenolic compounds. In contrast, *Lupinus albus* displayed the greatest total

flavonoid content (TFC) at 4.470 ± 2.61 mg QE/g; however, the differences in TFC among the species were not statistically significant (Fig. 2).

The observed variations in antioxidant activities among Lupinus species correspond with the established effects of specific phenolic and flavonoid profiles on antioxidant mechanisms. Previous studies have shown that the choice of assay can significantly impact the ranking of antioxidant capacities, with ABTS being less sensitive in distinguishing certain plant extracts compared to DPPH and FRAP assays (Munteanu et al., 2021). The observed variations in antioxidant activities among the *Lupinus* species could be linked to the different types of antioxidants present in the samples, which may react differently with the radicals used in each assay. Each analytical method has distinct benefits and limitations, such as differences in cost, chemical availability, procedural complexity, preparation time, and reproducibility. These factors can significantly influence the results and sensitivity of the assays, potentially affecting the detection of specific antioxidant activities (Karadag et al., 2009). For instance, while the ABTS assay was particularly effective in identifying high scavenging activity in *Lupinus albus*, the DPPH assay showed higher sensitivity to the compounds present in *Lupinus pilosus*.

5. Anti-inflammatory activities

The significant variation in lipoxygenase activity among the *Lupinus* species, with *Lupinus luteus* demonstrating the highest activity (938.61 ± 252.49 U/g), suggests a substantial difference in the enzymatic antioxidant potential across the species ($p < 0.001$) (Fig. 2). This higher lipoxygenase activity in *L. luteus* indicates a greater capacity for producing pro-inflammatory mediators through the enzymatic action of lipoxygenase. In contrast, the substantially lower activity observed in both *Lupinus pilosus* (303.91 ± 197.96 U/g) and *Lupinus albus* (312.47 ± 135.00 U/g) suggests a possible species-specific variation in the enzymatic capacity of lipoxygenase (Fig. 2).

The increased lipoxygenase activity in *L. luteus* may be linked to a higher concentration of bioactive compounds, such as polyphenols and flavonoids, which may influence the enzymatic activity. This finding is consistent with studies by Ben Hassine et al. (2021), which reported variability in the secondary metabolite profiles of different *Lupinus* species, potentially impacting antioxidant activity levels. These findings suggest that the *Lupinus luteus* may play a more significant role in the production of inflammatory mediators, indicating potential for its use in the

development of functional foods or nutraceuticals aimed at modulating inflammatory responses. Further research into the specific compounds responsible for the higher lipoxygenase activity in *L. luteus* could provide valuable insights into its therapeutic potential.

6. Principal Component Analysis of Morphometric, Nutritional, and Biochemical Traits in Lupinus Species

Multivariate analysis through Principal Component Analysis (PCA) revealed substantial differentiation among the studied *Lupinus* species, with the first two principal components (PC1 and PC2) collectively explaining 59.45% of the total variance (PC1: 40.56%; PC2: 18.89%) (Fig. 3a). This level of explained variance indicates robust patterns of variation in the analysed traits across species.

The first principal component (PC1), primary axis of variation, demonstrated strong positive correlations with morphological characteristics, particularly pod length (PL), and several key fatty acids including palmitic (C16:0), stearic (C18:0), oleic (C18:1), and eicosadienoic acid (C20:2). Conversely, PC1 exhibited a negative association with behenic acid (C22:0), DPPH radical scavenging activity, and phenological traits such as time of ripening (TR). The second principal component (PC2) showed distinct positive correlations with α-linolenic acid (C18:3) and DPPH activity, while negatively correlating with total phenolic content (TPC), lipoxygenase (LOX) activity, carotenoid content, and total oil content (Fig. 3b).

The projection of variables in the PC1-PC2 biplot revealed three distinct species-specific clusters, highlighting significant interspecific variation in biochemical and morphological traits. *Lupinus albus* demonstrated clear separation along PC1, characterized by elevated oil and protein content, coupled with enhanced antioxidant capacity as measured by the ABTS assay (Fig. 3c). This distinctive positioning suggests that *L. albus* possesses a unique combination of nutritionally valuable traits, potentially reflecting its history of domestication and cultivation in the Mediterranean region.

Lupinus pilosus formed a discrete cluster primarily differentiated along PC2, exhibiting strong associations with specific fatty acid profiles and antioxidant activities (DPPH and FRAP). This clustering pattern indicates that *L. pilosus* possesses a distinct biochemical profile characterized by

enhanced levels of bioactive compounds, particularly those contributing to antioxidant capacity. These traits distinguish it from *L. albus*, which shows stronger correlations with growth-related parameters.

Lupinus luteus displayed a more diffuse distribution pattern across both principal components, partially overlapping with the clusters of other species. This intermediate positioning suggests a more balanced trait profile, combining elements of both morphological and biochemical diversity. Significant associations were observed with carotenoid content and lipoxygenase activity, along with moderate correlations to various antioxidant parameters. This pattern may reflect a broader adaptive capacity or genetic diversity within *L. luteus*.

The clear species-specific clustering patterns observed in this analysis provide valuable insights into the biochemical and morphological differentiation among these *Lupinus* species. These findings have important implications for breeding programs targeting specific nutritional or biochemical profiles, as well as for the selection of species for particular agricultural or nutritional applications. Furthermore, the distinct trait associations identified through this PCA could serve as valuable markers for species identification and quality assessment in lupin breeding and cultivation programs.

7. Correlations between the morphological and biochemical traits

Correlation analysis was conducted to explore the associations between nutritional compositions, biological activities, and agro-morphological traits in lupin species (Fig. 4). The results show a strong positive correlation (+ 0.71) between the white colour of wings (CWw) and the absence of bitterness in seeds (GBd). Additionally, the white-violet flower wings (CWwv) exhibit a positive correlation (0.73) with seed weight (G100sw). These findings suggest that wing colour could serve as an effective marker for varietal selection in lupin species (Fig. 4).

Additionally, the violet flower wings (CWv), the present of seeds ornamentation (GOpres), the black seeds ornamentation (GCObl) distributed in only eyebrow (DOeye-only), display a significant positive correlation among themselves and with the antioxidant activities (DPPH) and C18.0, C18.2, fatty acids amounts (Fig.4). This implies that plants with violet flowers and seeds adorned by black eyebrow-like ornamentation display low levels of antioxidant activity and a high

quantity of fatty acid, specially C18.0, C18.2. These plants could exhibit low oil content and reduced quantities of carotene, TPC and C20.0, C18.1, and C16 fatty acids, as evidenced by the strong negative correlation shown in figure 4.

Indeed, the yellow flower wings (CWy), the yellow tips of the carina flower (CTCy), the moderate density of seed ornamentation (Dens GOm), ornamentation on the entire seed surface except for the eyebrow region (DOtot-eye), and the beige seeds ornamentation (GCOb) all exhibit a strong positive correlation between each other and a moderate positive correlation with carotene, TPC, and anti-inflammatory activity (Lox). The seeds from this group of plants probably contain a low level of C18.3 and have substantial anti-inflammatory (Lox) and antioxidant (ABTS and DPPH) activities, which is supported by a strong association (Fig.4).

Moreover, the heatmap *Pearson* correlation reveals that seeds without ornamentation (Goab) are strongly associated with higher seeds weight (G100sw) (+0.94) and notably levels of total oil (+0.88), protein (+0.74), nitrogen (+0.74), and specific fatty acids, including C16.0 (+0.88), C18.1 (+0.83), C20.0 (+0.86). These seeds are typically found in longer pods (PL) (+0.87) and are produced by plants with white-violet flower wings (CWwv) (+0.67). Conversely, they exhibit low accumulation of fatty acids such as C18:0, C18:2, C20:1, C22, C22:1n9, and C22:2, with respective negative correlations of -0.80, -0.92, -0.74, -0.68, -0.67, and -0.80. On top of that, our findings suggest that plants with longer ripening time (TR) assimilate more oil and fatty acids, especially for C16.0, C18.1, and C20.0 (Fig.4). Notably, longer ripening time (TR) is positively linked to increased oil and fatty acid content, indicating that late-maturing varieties might be ideal for maximizing oil yield. This is a crucial consideration for lupin breeding aimed at enhancing oil productivity without compromising protein content.

The strong positive correlation between oil contents and fatty acid components, C16.0, for C18.1, and C20.0 (Fig.4), (+0.94, +0.94, and +0.92), suggests that these fatty acids are major contributors to overall oil composition in lupin species. However, the analysis revealed notably strong negative correlations between total oil content and specific fatty acids C18.0 (-0.90), C18.2 (-0.89), C20.1 (-0.81), and C22.0 (-0.73). This inverse relationship suggests a fundamental biological trade-off in resource allocation, where the biosynthesis of certain fatty acids is favoured at the expense of others. Such metabolic prioritization likely reflects enzymatic regulation and precursor availability within the lipid biosynthetic pathway. Understanding this trade-off is essential for optimizing both

oil yield and fatty acid composition in lupin, ensuring a balance between productivity and nutritional quality. Moreover, the observed correlations highlight the intricate regulation of lipid metabolism in lupins. The dynamic interplay between different fatty acids suggests a coordinated biosynthetic network, where fluctuations in total oil content influence the proportional accumulation of individual fatty acids (Al-Amrousi et al.,2022). This metabolic complexity underscores the need for a systems-level approach in breeding strategies.

The correlation patterns observed between morphological parameters, such as colour of the flower wings, seeds ornamentation, seeds bitterness, seeds weight, ripening time, and biochemical components were predominantly strong, reflecting a significant degree of interdependence between physical plant characteristics and their nutritional composition. In summary, identifying the relationships between parameters can potentially facilitate varieties selection in breeding programs. These findings have several important implications for lupin improvement programs. First, the moderate correlation between protein and oil content necessitates careful consideration in breeding strategies, particularly when attempting to optimize both components simultaneously. Second, the consistency in antioxidant activities correlations provides a reliable framework for quality assessment in breeding selections.

Conclusions

This study offers a comprehensive evaluation of three Lupinus species, revealing notable interspecies differences in morphology, phytochemical composition, and antioxidant activity. *Lupinus albus* stood out with its superior plant height, high total flavonoid content, and exceptional ABTS radical-scavenging activity, which aligns with previous reports highlighting its potential for nutritional and functional applications. Notably, our findings also reveal that Lupinus albus contains a protein content of 37%, which enhances its value as a high-quality dietary protein source, positioning it as a promising crop for both nutrition and crop improvement. On the other hand, *Lupinus pilosus* demonstrated lower levels of palmitic, oleic acids, presenting a distinct advantage in reducing antinutritional effects and improving nutrient bioavailability compared to other species. This finding is significant as it offers a potential for enhancing the nutritional profile of Lupinus-based products. These results not only reinforce existing knowledge but also provide new insights into the agricultural and nutritional potential of Lupinus species. By demonstrating the unique benefits of each species, our study contributes to a deeper understanding of their role in sustainable

agriculture and functional food development. The findings support the potential for targeted breeding strategies that enhance both the nutritional quality and functionality of Lupinus species in future agricultural and food applications.

CRediT authorship contribution statement

Akremi. I: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review and editing, Data curation, Formal analysis; **Kabtni. S:** Conceptualization, Methodology, Formal analysis**,** Software, Writing – original draft, Writing – review and editing; **Ben Ammar. H:** Writing – original draft**,** Writing – review and editing, Formal analysis; **Genva. M:** Methodology, **Hejazi. S:** Resources**, Elbok. S:** Resources**; Rouz.S:** Resources **Marghali. S:** Conceptualization, Visualization, Methodology, Supervision**,** Validation**; Fauconnier. M, L:** Visualization, Supervision, Funding acquisition, Project administration

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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Figure captions:

Figure 1: Geographic distribution of the three *Lupinus* species (*Lupinus albus, Lupinus luteus*, and *Lupinus pilosus*) analysed in this study, showing their collection sites across different regions. The map highlights the distinct ecological zones and geographical origins of each species, providing context for the comparative analysis of their morphological traits and biochemical compositions. Maps were created using the *maps* and *mapdata* packages of the R software.

Figure2: Comparative box plots illustrating the antioxidant activities (expressed as DPPH and ABTS scavenging activities) and oil content of seeds from three Lupinus species: *Lupinus albus* (La) in blue, *Lupinus luteus* (Ll) in green, and *Lupinus pilosus* (Lp) in red. The box plots represent the median, interquartile range (IQR), and potential outliers of the measured parameters.

Significant differences between species are denoted by different letters ($P < 0.05$), highlighting variability in antioxidant capacity and oil yield across the studied species.

Figure 3: Principal Component Analysis (PCA) of morphometric, nutritional, and biochemical traits across the three *Lupinus* species (*Lupinus albus*, *Lupinus luteus*, and *Lupinus pilosus*). **(a)** the amount of variation retained by each principal component (Dimension). **(b)**: The quality of representation of the variables on factor map: Variables with low cos^2 values are represented with light colours, indicating a weak representation by the principal components. On the other hand, variables with moderate cos^2 values are shown in dark brown, signifying a strong representation by the principal components. **(c)**: The PCA biplot displays the variance in traits among species, with each point representing an individual accession. The direction and length of the trait vectors indicate the contribution of each trait to the principal components, illustrating distinct clustering patterns and highlighting key factors driving differentiation among the species.

Figure 4: Heatmap *Pearson*'s Correlation Analysis of Morphological and Biochemical Parameters in *Lupinus* Species. Colour intensity represents the strength of the correlation: red for strong positive correlation, blue for strong negative correlation, and white for no correlation or a weak correlation. The dendrograms on the X and Y axes represent the results of hierarchical clustering applied to the variables. These dendrograms show how the variables are grouped based on their similarity, which is measured using the Pearson correlation coefficient.

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Table 1: Geographical origins of Lupinus species sampled for the study, including specific coordinates, altitude, and regional provenance.

Species	Origin	Seed Type
Lupinus pilosus	Tunisia	Wild
Lupinus luteus	Tunisia	Wild
Lupinus albus	Tunisia	Cultivated
Lupinus albus	Egypt	Commercial cultivar
Lupinus albus	Egypt	Commercial cultivar
Lupinus albus	France	Commercial cultivar
Lupinus albus	Algeria	Commercial cultivar
Lupinus albus	Italy	Commercial cultivar

Table 2: The agro-morphological parameters assessed, selected from the UPOV guidelines for *Lupinus*

	Quantitative traits	Qualitative traits
Phenological Development	Date of first bud apparence (DFBA) Height at beginning of flowering (HDF) Time of flowering (TF) Time of green ripening (TGR) Height at green ripening (HGR) Time of ripening (TR)	Plant growth type (GT) :determinate (GTDet) or indeterminate (GTIndet)
Vegetative Growth Characteristics	Cotyledons :length/width (LSl cotyl) Central leaflet: length/ width (LslF) Central leaflet : area (SF) Height at vegetative stage (HSV)	Cotyledons Appearance (AC): smooth (ACs) or medium smooth (ACms) or rough (ACr) Anthocyanin coloration of stem prior to bud emergence (ACP): weak (ACPw) or medium (ACPM) or strong (ACPs) Green color intensity of Leaf prior to bud emergence (IGR): medium (IGRm) or dark (IGRd) Leaf above texcture (Lab): hairy (LabH) or smooth (LabS) Leaf below texcture (Lbw): hairy (LbwH) or smooth (LbwS)

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Reproductive Development and Inflorescence	Height of insertion of first inflorescence at green ripening (from ground level to insertion of first inflorescence) (HIFI)	Flowers: color of wings (CW): white (CWw) or violet (CWv) or yellow (CWy) or white/violet (CWwy)
Pod and seed	Length of inflorescence (LINF) Pod: length (PL) Pod number per plant (Npinf) Seed 100 seed weight (G100sw)	Flower: color of tip of carina (CTC): yellow (CTCy) or blue black (CTCbb) Seed : ornamentation (GO): absent (GOabs) or present (GOpres) Seed : color of ornamentation (GCO) beige (GCOb)or black (GCObl) Seed : distribution of ornamentation (Dis O): total except eyebrow (DOtot-eye) or eyebrow only (DOeye-only) Excluding varieties with eyebrow only: Seed : density of ornamentation (Dens GO) : medium (Dens GOm) Seed : bitter principle (GB): absent (GBd) or present (GBa)

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Table 3: Qualitative parameters differentiating lupines species: **N**: number of plants, **GT**: Plant growth type: determinate (GTDet) or indeterminate (GTIndet), **AC**: Cotyledons Appearance: smooth (ACs) or medium smooth (ACms) or rough (ACr), **ACP**: Anthocyanin coloration of stem prior to bud emergence: weak (ACPw) or medium (ACPM) or strong (ACPs), **IGR**: Green color intensity of Leaf prior to bud emergence: medium (IGRm) or dark (IGRd), **Lab**: Leaf above texcture: hairy (LabH) or smooth (LabS), **Lbw**: Leaf below texcture: hairy (LbwH) or smooth (LbwS), **CW**: Flowers: color of wings: white (CWw) or violet (CWv) or yellow (CWy) or white/violet (CWwy), **CTC**: Flower: color of tip of carina: yellow (CTCy) or blue black (CTCbb), **GO**: Seed : ornamentation: absent (GOabs) or present (GOpres), **GCO**: Seed : color of ornamentation () beige (GCOb)or black (GCObl), **Dis O**: Seed : distribution of ornamentation (): total except eyebrow (DOtot-eye) or eyebrow only (DOeye-only), **Dens GO**: Seed : density of ornamentation: medium (Dens GOm), **GB**: Seed : bitter principle: absent (GBd) or present (GBa)

Species	Frequency per modality (%)													
	N	GT	AC	ACP	IGR	Lab	Lbw	CW	CTC	GO	GCO	Dis O	Dens GO	GB
L. pilosus	30	3 10 0% GTIndet	10 0% ACms	10 0% ACPm	100 % IGRd	10 0% LabH	10 0% LbwH	100 % CWv	10 0% CTCbb	10 0% GOpres	10 0% GCObl	10 0% DOeye-only	0%	86, 36% GBa (285) 13, 63% GBd (45)
L. luteus	60	10 0% GTIndet	10 0% ACr	10 0% ACPm	100 % IGRd	10 0% LabS	10 0% LbwS	100 % CWy	10 0% CTCy	10 0% GOpres	10 0% GCOb	10 0% DOtot-eye	100% Dens GOm	100 % GBa
L. albus	270	2 10 0% GTIndet	10 0% ACs	10 0% ACPw	44, 44% IGRd 66, 56% IGRm	10 0% LabH	10 0% LbwS	61, 88% CWwv 38, 88% CWw	10 0% CTCbb	10 0% GOabs	0 %	0 %	0%	61, 88% GBa (165) 38, 88% GBd (105)

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Table 4: Means value and standard deviation of the morphological traits measured in *Lupinus* species: **N**: number of plants, **P value**: the significance probability value, Df: degrees of freedom.

Species		mean± sd													
		TR	TGR	TFBA	TF	SF	LF	Npinf	lSlF	LsIcotyl	LINF	HVS	HIFI	HGR	HFS	G100sw
L. albus	40	208.60 ± 12.79	181.22 ± 25.05	70.06 ±12.60	90.99 ±12.73	8.09 ±2.90	7.59 ±0.91	3.80 ±0.62	3.03 ±0.75	1.21 ±0.20	19.05 ±4.93	20.04 ±6.73	4 5.95 ±9.04	6 5.15 ±12.8	3 5.38 ±11.19	39.25 ±16.28
L. luteus	0	212 ±0.00	191 ± 0.00	141.80±0.36	156.00 ± 0.00	2.74 ±0.61	4.14 ±0.28	2.65 ±0.89	5.39 ±0.77	1.19 ±0.14	10.68 ±1.77	13.78 ±1.57	3 2.18 ±3.11	4 2.95 ±2.68	2 5.30 ±1.10	14.50 ± 0.50
L. pilosus	28	194.66 ±6.55	178.47 ±2.80	116.1±7.175	146.11 ± 9.44	6.63 ± 2.34	4.65 ±1.009	3.59 ± 1.42	3.67 ±0.52	1.005 ±0.07	11.195± 2.80	21.65±4.242	3 5.018 ±6.22	4 6.20 ± 6.632	3 6.49 ± 7.36	17.04 ± 1.49
ANOVA	f						2									
	value	< 2e-16 ***	0.000000102 ***	<2e-16 ***	< 2e-16 ***	< 2e-16 ***	< 2e-16 ***	4.11e-11 ***	< 2e-16 ***	<2e-16 ***	<2e-16 ***	<2e-16 ***	< 2e-16 ***	< 2e-16 ***	< 2e-16 ***	<2e-16 ***

Signifiant. codes : 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1**

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Table 5: Nutritional Profiling of Lupinus Species: Insights into Protein and Amino Acid Composition. This table compares the mean ± standard deviation for key nutritional parameters (%N, %MS, %Protein) and amino acid content (g/100g) among *Lupinus albus* (La), *Lupinus luteus* (Ll), and *Lupinus pilosus* (Lp), highlighting significant differences identified through ANOVA analysis.

Species		**Mean ± Sd**			**Anova test**
		L. albus	***L. luteus***	***L. pilosus***	**Pr (>F)**
%N		5.94±0.38	5.02±0.00	5.40±0.205	0.0103 *
%MS		91.38±0.26	91.04±0.00	91.51±1.27	0.575
%Protein		37.17±2.37	31.37±0.00	33.78±1.28	0.0103 *
Amino Acid	**Ala**	1.21±0.04	1.15±0.00	1.06±0.08	0.0262 *
	Arg	3.59±0.21	2.84±0.00	3.12±0.52	0.0139 *
	Asp	4.02±0.19	3.48±0.00	3.36±0.35	0.00713 **
	Cys	0.42±0.01	0.41±0.00	0.51±0.05	0.00682 **
	Glu	9.16±0.75	7.67±0.00	9.25±1.22	0.0561
	Gly	1.48±0.07	1.33±0.00	1.28±0.11	0.0142 *
	His	0.80±0.03	0.74±0.00	0.80±0.08	0.178
	Ile	1.68±0.04	1.43± 0.00	1.27± 0.03	0.00000978 ***
	Leu	2.61±0.07	2.34±0.00	2.21±0.21	0.00295 **
	Lys	1.68±0.05	1.57±0.00	1.39±0.08	0.00129 **
	Met	0.20±0.03	0.21±0.00	0.18±0.01	0.491
	Phe	1.40± 0.05	1.22±0.00	1.16±0.10	0.00254 **
	Pro	1.56±0.08	1.36±0.00	1.30±0.19	0.0229 *
	Ser	1.83±0.08	1.61±0.00	1.42±0.16	0.00245 **
	Thr	1.28±0.03	1.19±0.00	1.02±0.08	0.000688 ***
	Tyr	1.48± 0.08	1.20± 0.00	0.88±0.07	0.0000458***
	Val	1.50±0.01	1.39± 0.00	1.22±0.10	0.000262 ***

Asterisks indicate levels of statistical significance: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

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Table 6. Comparative Analysis of Fatty Acid Composition and Oil Content in *Lupinus* species. Values represent the mean ± standard deviation (SD) of the percentage of total fatty acids. *ANOVA* results indicate highly significant differences (P< 0.001) among species for all measured traits, highlighting the variability in oil content and fatty acid profiles among the *Lupinus* species.

	Lupinus albus	*Lupinus luteus*	*Lupinus pilosus*	**ANOVA**
Oil content	9.16 ± 1.43 a	6.26 ± 0.65 b	2.025 ± 0.91 c	$<2^{e-16}$ ***
Palmitic acid (C 16:0)	6.89 ± 0.66 a	5.06± 0.19 b	0.0 c	$<2^{e-16}$ ***
Stearic acid (C18:0)	1.07 ± 0.24 a	1.22± 0.07 a	7.22 ± 1.05 b	$<2^{e-16}$ ***
Oléic acid (C18:1)	42.37 ± 3.52 a	29.40 ± 0.72 b	3.00 ± 0.79 c	$<2^{e-16}$ ***
Linoleic (C18:2)	21.055 ± 1.07 a	44.38 ± 1.1 b	54.06 ± 8.70 c	$<2^{e-16}$ ***
A-linolenic (C18:3)	7.59 ± 1.04 a	4.01 ± 0.05 b	7.61 ± 0.87 a	3.57e-12 ***
Arachidic acid (C20:0)	1.13 ± 0.21 a	0.78 ± 0.04 b	0 c	$<2^{e-16}$ ***
Cis-11-eicosenoic (C20:1)	2.22 ± 0.61 a	2.23 ± 0.17 a	4.11 ± 0.75 b	2.32e-15 ***
Erucic acid (C22-1n9)	0.63 ± 0.07 a	1.20 ± 0.11 b	1.30 ± 0.50 b	0.0000000398 ***
Behinic acid (C22)	0.18 ± 0.07 a	0.71± 0.02 ab	1.47 ± 1.68	0.00107 **
Docosadienoic acid (C22:2)	0.0 a	2.34 ± 0.07 b	0.76 ± 0.85 c	5.83e-11 ***
Lignoceric acid (C24)	3.36 ± 0.65 a	2.44 ± 0.06a	3.19 ± 2.59 a	0.585

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Declaration of interest statement

☒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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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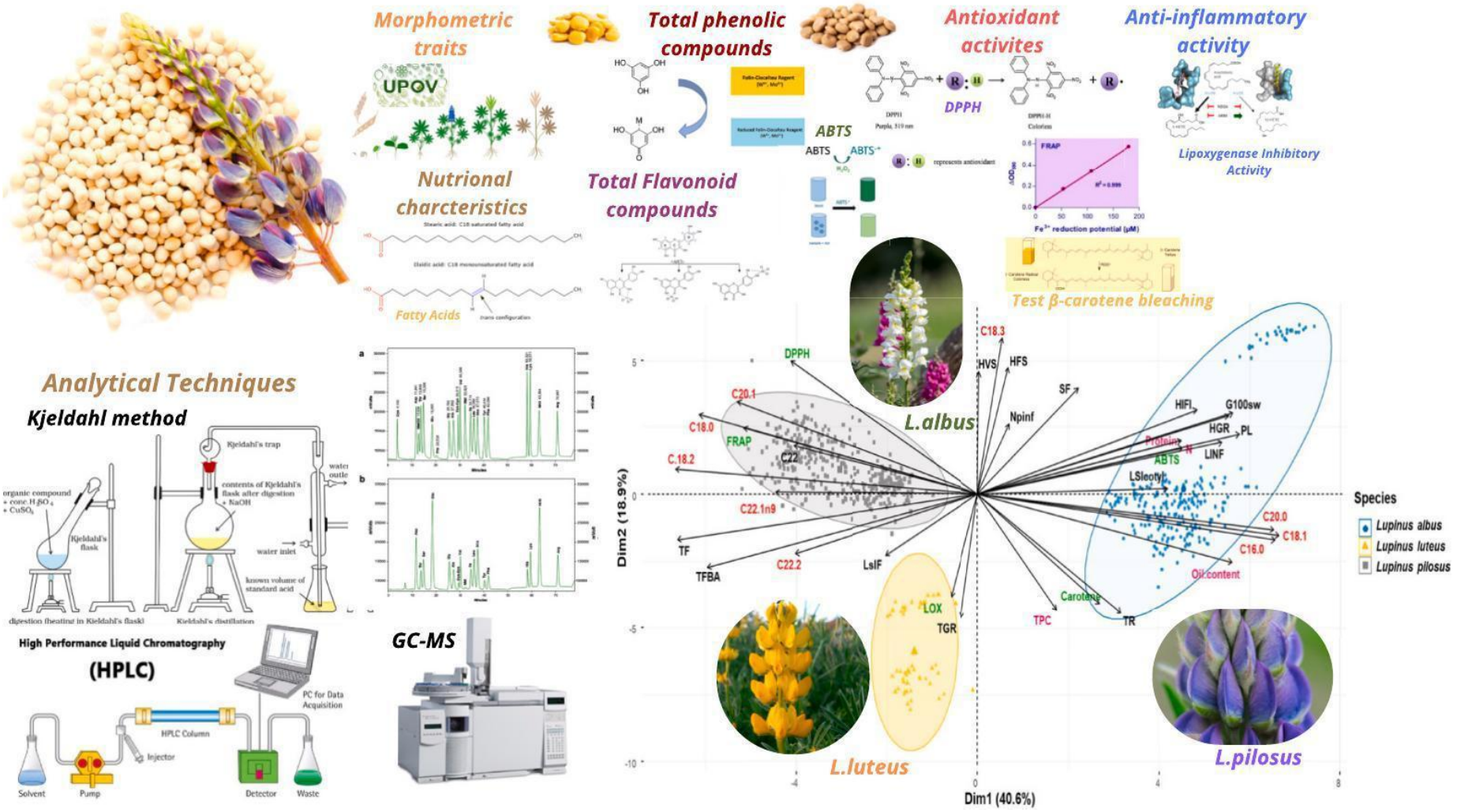
Graphical abstract

Highlights

- ✓ This study evaluated the nutrients and bioactive of three Lupinus species for food use.
- ✓ L. albus excelled in protein, while L. luteus had the highest phenolic content.
- ✓ L. albus excelled in ABTS, while L. pilosus showed higher DPPH and FRAP activities.
- ✓ L. pilosus had the lowest specific fatty acids, supporting healthier lipid profiles.

Journal Pre-proof



Graphics Abstract

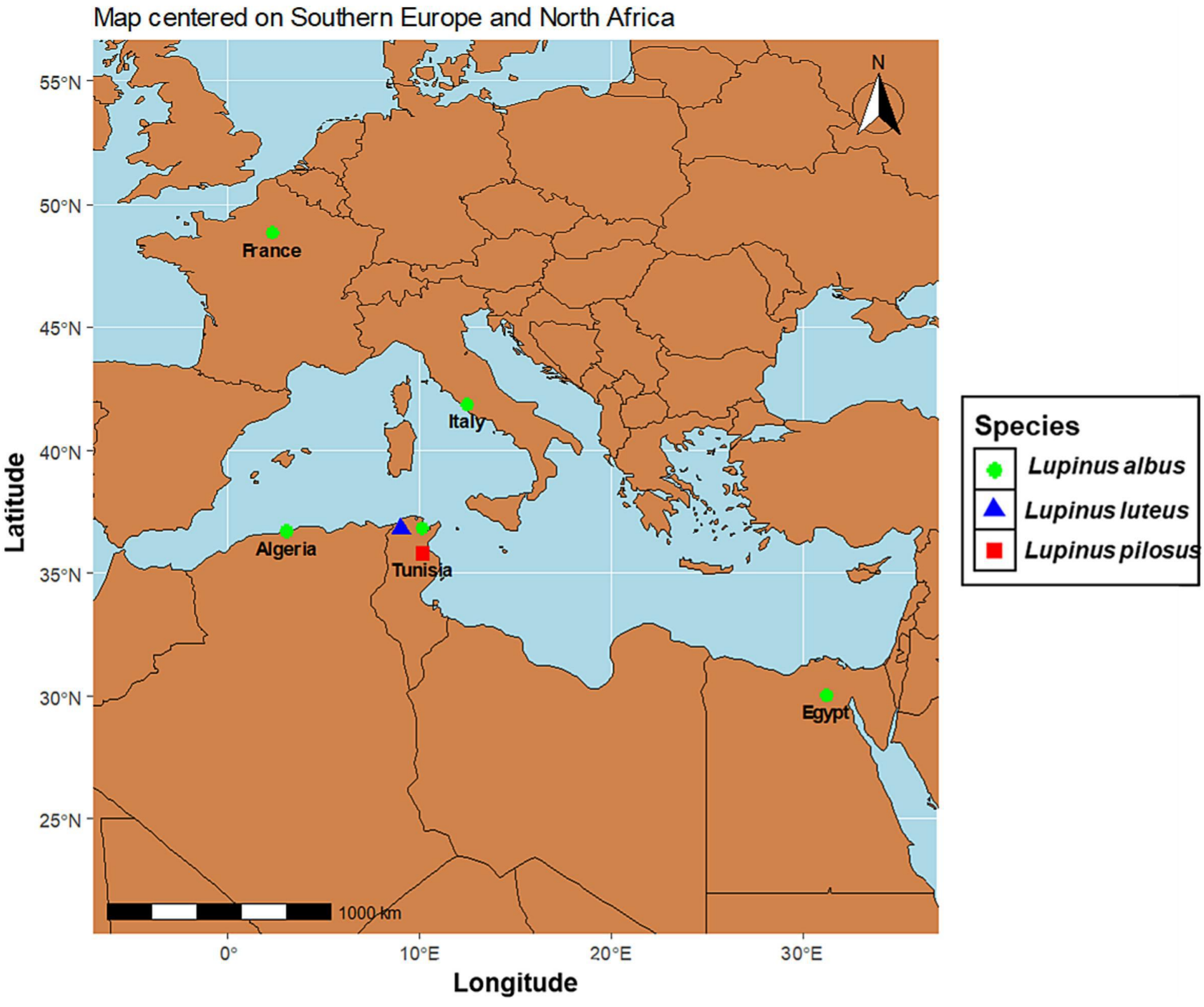

Map centered on Southern Europe and North Africa
N
55°N
50°N
45°N
40°N
35°N
30°N
25°N
Latitude
France
Italy
Algeria
Tunisia
Egypt
1000 km
0°
10°E
20°E
30°E
Longitude
Species
Lupinus albus
Lupinus luteus
Lupinus pilosus

Figure 1

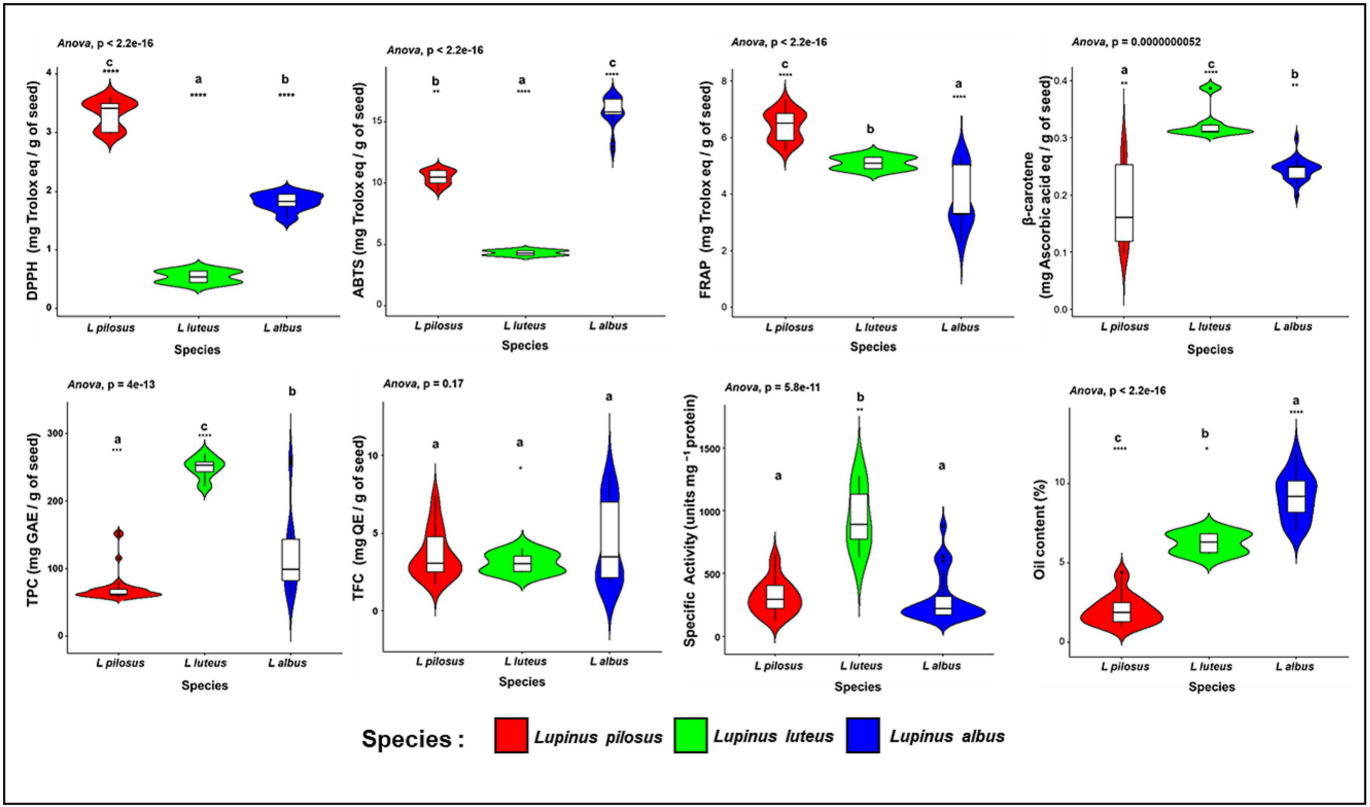

Anova, p < 2.2e-16
DPPH (mg Trolox eq / g of seed)
Anova, p < 2.2e-16
ABTS (mg Trolox eq / g of seed)
Anova, p < 2.2e-16
FRAP (mg Trolox eq / g of seed)
Anova, p = 0.0000000052
β-carotene (mg Ascorbic acid eq / g of seed)
Anova, p = 4e-13
TPC (mg GAE / g of seed)
Anova, p = 0.17
TFC (mg QE / g of seed)
Anova, p = 5.8e-11
Specific Activity (units mg −1 protein)
Anova, p < 2.2e-16
Oil content (%)
L pilosus
L luteus
L albus
Species
Species :
Lupinus pilosus
Lupinus luteus
Lupinus albus

Figure 2

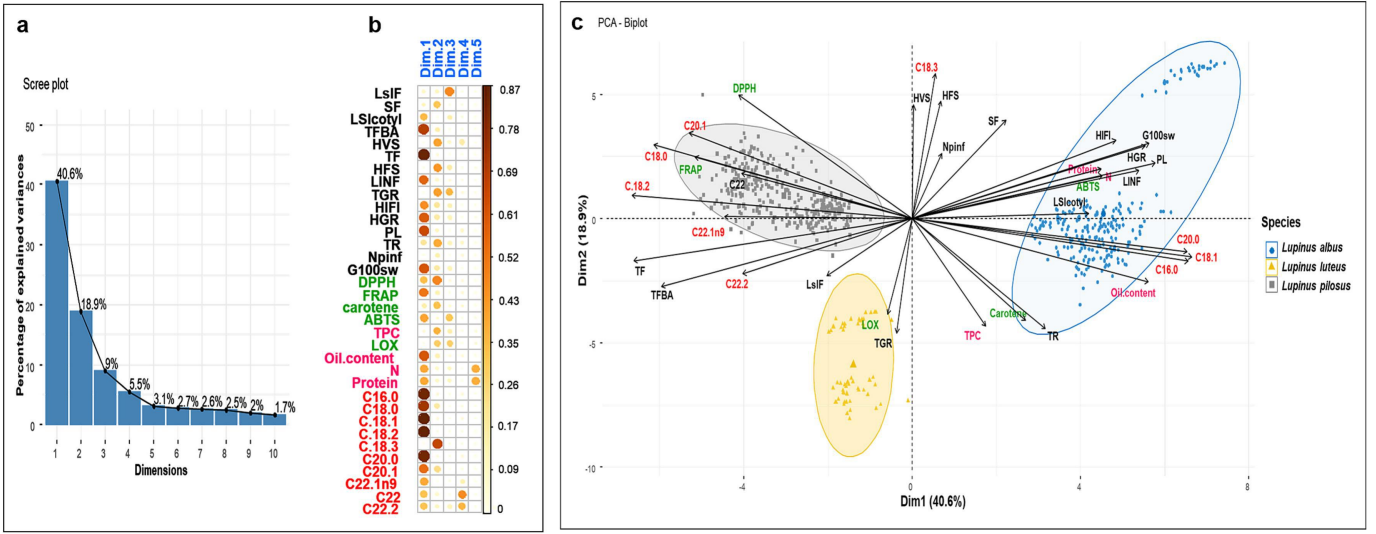

a
Scree plot
Percentage of explained variances
Dimensions
40.6%
18.9%
9%
5.5%
3.1%
2.7%
2.6%
2.5%
2%
1.7%
b
Dim.1
Dim.2
Dim.3
Dim.4
Dim.5
LsIF
SF
LSIcotyl
TFBA
HVS
TF
HFS
LINF
TGR
HIFI
HGR
PL
TR
Npinf
G100sw
DPPH
FRAP
carotene
ABTS
TPC
LOX
Oil.content
N
Protein
C16.0
C18.0
C18.1
C18.2
C18.3
C20.0
C20.1
C22.1n9
C22
C22.2
0.87
0.78
0.69
0.61
0.52
0.43
0.35
0.26
0.17
0.09
0
c
PCA - Biplot
Dim2 (18.9%)
Dim1 (40.6%)
Species
Lupinus albus
Lupinus luteus
Lupinus pilosus

Figure 3

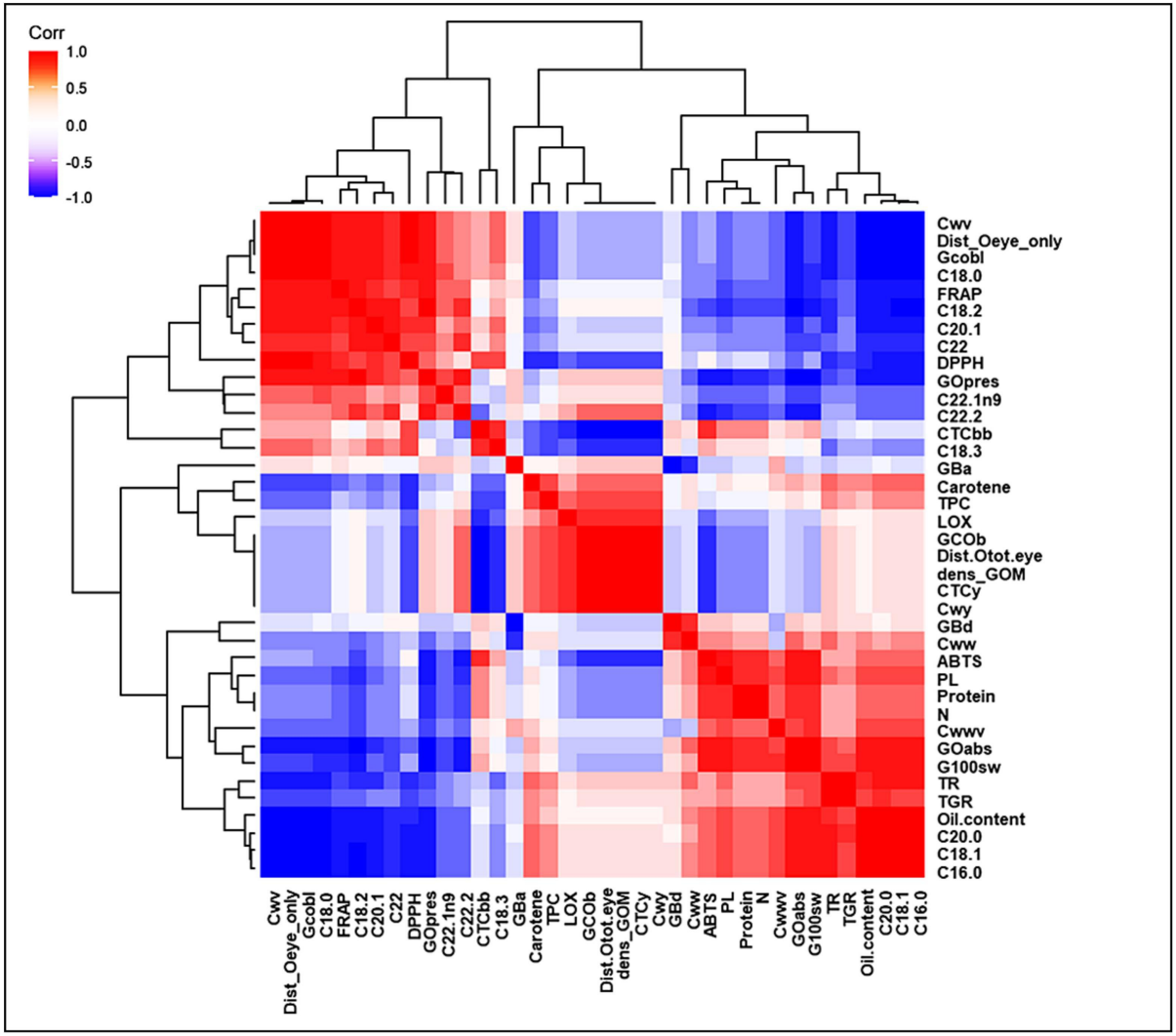

Corr
1.0
0.5
0.0
-0.5
-1.0
Cwv
Dist_Oeye_only
Gcobl
C18.0
FRAP
C18.2
C20.1
C22
DPPH
GOpres
C22.1n9
C22.2
CTCbb
C18.3
GBa
Carotene
TPC
LOX
GCOb
Dist.Otot.eye
dens_GOM
CTCy
Cwy
GBd
Cww
ABTS
PL
Protein
N
Cwwv
GOabs
G100sw
TR
TGR
Oil.content
C20.0
C18.1
C16.0

Figure 4