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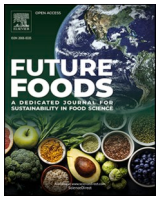

Phytochemical and metabolomics analysis of *Quercus ilex* acorns reveals substantial intraspecific variation, high nutritional and nutraceutical potential and rich associated microbiome

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ABSTRACT

There is a currently renewed interest in the use of *Q. ilex* acorns for alimentary purposes, including nuts, flour, and various derivatives. This aligns with the UN Sustainable Development Goals and is supported by their traditional use since prehistoric times. To support their safe and sustainable use, we conducted a comprehensive phytochemical and metabolomic analysis of acorns from 14 trees across different regions in Spain. Acorns were classified by size and bitterness. Multiple complementary techniques were used, including Near Infrared Spectroscopy (NIRS), High Performance Liquid Chromatography (HPLC), colorimetric assays, macro- and micronutrient analysis, and untargeted Ultra-high Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS). The analysis annotated 1189 metabolic features, along with detailed profiles of carbohydrates, amino acids, fats, phenolics, and minerals. High variability was found among trees, with significant differences independent of geographic origin. Two main groups of acorns emerged, corresponding to a low degree of bitterness and large size vs. a high degree of bitterness and medium size. Notable features included high starch content, predominance of unsaturated fatty acids, and elevated calcium and sodium levels. Metabolomic profiles were highly tree-specific, and several newly reported compounds with potential bioactivity were annotated. Furthermore, a substantial number of metabolites were of microbial origin, revealing a diverse seed-associated microbiome. These findings highlight the nutritional and functional potential of *Q. ilex* acorns and support their valorization in sustainable food systems. The metabolomic signatures also offer promising markers for tree genotype identification and acorn quality assessment.

1. Introduction

In recent years, plant-based diets have garnered increasing attention from both the global population and the food industry (FAO, 2022). Nevertheless, the current global food system is heavily reliant on a limited number of plant species, with only 150 out of approximately 7000 plant species cultivated being widely utilized (Waldron et al., 2017). For instance, just three crops, rice, wheat, and maize, account for over 50 % of global caloric intake (Ibba et al., 2025). In contrast, a vast number of potentially valuable food plants, such as forest trees and wild species (Laird et al., 2024). Therefore, the majority of food plants are either underutilized or ignored on a global scale. In general, these underutilized food plants are non-domesticated species that could play a crucial role in enhancing food security and resilience against biotic and abiotic stresses (Ahmed, 2021; Knez et al., 2024). Moreover, many of them possess unique functional and bioactive properties, associated to the antioxidants, essential fatty acids, and secondary metabolites content, making them valuable resources for both nutrition and health.

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Among these, *Quercus ilex* represents a noteworthy case due to its ecological relevance and valuable bioactive properties (Vinha et al., 2016a). With the alarming increase in human population and the rapid depletion of natural habitats, it has become essential to explore and develop sustainable plant-based resources for food, feed, energy, and industrial applications (Sabaté and Soret, 2014; Alae-Carew et al., 2022; Santos et al., 2022). In this context, urgent attention should be given to local and endemic plant species with high nutritional potential, as they represent underexplored yet valuable resources for promoting food security, dietary diversification, and environmental resilience.

The potential of the acorns of *Quercus ilex L.* subsp. *ballota* (Desf.) Samp., as a source of functional food is being underestimated relative to other plants, such as *Castanea sativa* Mill., *Juglans regia* L., and *Corylus avellana* L. (Li and Parry 2011; Barreira et al., 2012; Liao et al., 2016). *Quercus ilex* is the most representative species of Mediterranean forest areas, including the agrosilvopastoral ecosystem "*Dehesa*" in Spain and "*Montado*" in Portugal, with important economic and ecological roles (Plieninger et al., 2021). Since ancient times, *Quercus* species, *Q. ilex* in particular, have provided wood, food, and medicinal extracts, with acorns historically serving as a staple food in Mediterranean cultures and a key resource in times of scarcity (Hill, 1937, 1952; Mason, 1992; Pignone et al., 2010; Mohammadzadeh et al., 2013; Silva et al., 2016).

Even though there not much detailed work has been carried out on the use of the acorn as a food, several publications have reported on its nutritional and nutraceutical value. In general terms, 50 % of the total composition of acorns is starch; protein, gluten-free, contributes between 2 % and 5 %; their fat content is around 9 %, with oleic, palmitic and linoleic acids being the main fatty acids present in them (Vinha et al., 2016a, 2016b, 2020; López-Hidalgo et al., 2021a; Zarroug et al., 2021). Tocopherols and phenolic compounds (flavonoids and tannins) are considered the primary bioactive compounds in acorn fruits (Cantos et al., 2003; Rakić et al., 2006, 2007; Tejerina et al., 2011; López-Hidalgo et al., 2021a). These components are associated with biological functions, such as antitumoral, antiallergic, and anti-inflammatory activities (Ostertag et al., 2011; Heleno et al., 2015).

Given the growing interest in the nutritional and nutraceutical potential of *Q. ilex* acorns, their reintegration into the human diet is receiving increasing attention (Vinha et al., 2020). Although several studies have described the phytochemical composition of *Q. ilex* acorns (Vinha et al., 2016a; Inácio et al., 2024), comprehensive analyses involving well-characterized individuals and integrated analytical approaches are still lacking. Moreover, for acorn-derived products to be considered suitable for human consumption, they must comply with strict traceability and safety standards. In this context, the objective of this study is to carry out a comprehensive phytochemical and metabolomic characterization of acorn flour, in order to support its labelling, ensure traceability, and evaluate its nutritional and nutraceutical potential. To achieve this, acorns from fourteen specimens of *Q. ilex* located in Spain were analysed taking several approaches, including near-infrared spectroscopy (NIRS), biochemical colorimetric methods, liquid chromatography (LC) for simple sugars, antioxidant capacity assays, analysis of macro- and micronutrients, as well as non-targeted metabolomics using UHPLC-MS/MS. Moreover, the secondary metabolite profile was characterized, with a particular emphasis on phenolic compounds. This analysis will be the basis of the traceability of foods based on flour or containing acorn flour as an additive, being, at the same time, very useful in characterizing biodiversity and cataloguing *Q. ilex* provenances, ecotypes, and genotypes.

2. Material and methods

2.1. Acorn harvesting, morphometric parameters, and flour preparation

Acorns from fourteen different individuals of holm oak were harvested at the maturity stage during the 2022 – 2023 season (Table 1). Undamaged acorns were disinfected for 10 min in 10 % sodium hypochlorite solution, abundantly washed with tap water, and stored in the dark at 4 °C (Bonner and Vozzo, 1987; Simova-Stoilova et al., 2015) until flour preparation. The acorns were classified into three levels based on their bitterness levels (low, medium and high), according to the subjective assessment based on traditional knowledge provided by the local people who supplied the samples (Table 1). The weight, water content, volume, length and maximum width of eight acorns per group were measured (Valero-Galván et al., 2012; López-Hidalgo et al., 2021a). The acorns were scarified, and flour was prepared by grinding the seeds (without the coat) with liquid nitrogen, as reported in López-Hidalgo et al. (2021a). The flour was lyophilized and stored at 4 °C in a desiccator in the dark until NIRS analysis or metabolite extraction. All phytochemical analyses were carried out in three biological replicates, each biological replicate being a mix of 10 acorns.

Table 1
Geographical information on *Q. ilex* individuals harvested and bitterness associated with their acorns.

Individual	Localization	Coordinates	Altitude (MASL)	Bitterness
1	Hinojosa del Duque (Córdoba, Andalucía)	38°26′02"N, 5°08′32"W	607 m	Low
2	Torre de Miguel Sesmero (Badajoz, Extremadura)	38°38′48"N, 6°45′30"W	306 m	Low
3	Torre de Miguel Sesmero (Badajoz, Extremadura)	38°38′48"N, 6°45′30"W	306 m	Low
4	Aldea de Cuenca (Córdoba, Andalucía)	38°19′48''N, 5°32′14''W	579 m	Low
5	Villanueva de Córdoba (Córdoba, España)	38°14′57"N, 4°38′42"W	730 m	Low
6	Valle de los Pedroches (Córdoba, Andalucía)	38°26′00″N, 4°46′00″W	579 m	Low
7	Villanueva de Córdoba (Córdoba, España)	38°14′57"N, 4°38′42"W	730 m	Medium
8	Villanueva de Córdoba (Córdoba, España)	38°14′57"N, 4°38′42"W	730 m	Medium
9	Alcaracejos (Córdoba, Andalucía)	38°16′32"N, 4°58′11"W	631 m	Medium
10	Alcaracejos (Córdoba, Andalucía)	38°16′32"N, 4°58′11"W	631 m	Medium
11	Terra Alta (Tarragona, Cataluña)	41°03′00″N, 0°26′00″E	370 m	Medium
12	Aldea de Cuenca (Córdoba, Andalucía)	38°19′46"N, 5°33′15"W	574 m	Medium
13	Villanueva de Córdoba (Córdoba, España)	38°14′57"N, 4°38′42"W	730 m	High
14	Puebla de Guzmán (Huelva, Andalucía)	37°40′39"N, 7°25′14"W	152 m	High

Altitude (Meters Above Sea Level–MASL)

2.2. Near infrared spectroscopy analysis (NIRS) technology

Ash, total protein, fat, starch, phenolic compounds, digestibility, energy, fibre, palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) content were determined as previously described in Valero-Galván et al. (2012). Briefly, near infrared spectroscopy (NIRS) analysis of the flour was carried out with a Foss-NIR Systems 6500 System II spectrophotometer (Foss-NIR Systems Inc., Silver Spring, MD, USA) equipped with a transport module and autogain detector. Samples were analysed using WinISI software 1.50 (Infrasoft International, Port Matilda, USA).

2.3. Colorimetric reactions analysis

Total amino acids, sugars, starch, phenolics, and flavonoids were

quantified in acorn flour ethanol:water extracts by using classic colorimetric assays as described by López-Hidalgo et al. (2021b) with minor modifications. Additionally, the total content of condensed tannin was analysed (Herald et al., 2014). One ml of ice-cold ethanol:water (50:50, v/v) was added to 30 mg of lyophilized acorn flour, which was mixed by vortexing, and then sonicated for 10 min (ultrasonic bath, 40 kHz). After centrifugation (4 °C, 5 min, 26,300 g), the pellet was vacuum dried at 30 °C (Speedvac, Eppendorf vacuum concentrator Plus/5301, Eppendorf, Leicestershire, U.K.) and the remaining supernatant used for the analysis. The determination of free amino acids was performed using the ninhydrin reagent (Seracu, 1987). The absorbance was measured at 570 nm, and the content was expressed as %, mg L-glycine equivalents/100 mg dry weight (DW). Free sugars and starch were determined by the anthrone method (Chow and Landhäusser, 2004) in, respectively, the pellet and supernatant. For starch quantification, the pellet was first hydrolysed in 30 % perchloric acid at 60 ºC for 60 min. The acid-hydrolysed starch solution and the alcoholic extract were incubated with the anthrone reagent for 10 min at 100 °C. Absorbance was measured at 625 nm and content was expressed %, as mg D-glucose equivalent/100 mg DW. Total phenolic compounds were determined using the Folin-Ciocalteu reagent (Ainsworth and Gillespie, 2007); absorbance was measured at 720 nm and the amount was expressed as %, mg gallic acid equivalents/100 mg DW. Total flavonoids were determined by the aluminium chloride method (Huang et al., 2018); absorbance was measured at 415 nm and the amount was expressed %, as mg quercetin equivalents/100 mg DW. Condensed tannin determination was carried out using vanillin as a reagent, absorbance was measured at 500 nm, and content was expressed as %, mg catechin equivalent/100 mg DW (%).

2.4. HPLC analysis of sugars

Glucose, fructose, and sucrose were quantified in flour extracts of acorn by HPLC as previously described in Ordóñez-Díaz et al. (2024). Briefly, ice-cold ethanol:water (80:20) was added to 200 mg of lyophilized acorn flour, which was mixed by vortexing, and then sonicated for 10 min (ultrasonic bath, 40 kHz). After centrifugation (4 °C, 15 min, 15, 000 rpm), the pellet was collected and re-extracted twice. Supernatants were combined and subjected to high performance liquid chromatography with a refractive index detector (HPLC-RID). Simple sugars were separated using a Luna NH_2 column (250 × 4.6 mm i.d., 5 μm; Phenomenex) maintained at 40 °C. Isocratic elution was performed with a mobile phase consisting of deionized water (A) and acetonitrile (B) in a ratio of 20:80 (v/v), at a flow rate of 1.5 mL/min for 15 min. Retention times with pure reference standards were employed to identify the sugars, and the quantification was carried out referencing to the 0.3–50.0 g/L calibration curves of fructose, glucose and sucrose.

2.5. Antioxidant activity

The antioxidant activities were determined by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and the 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), respectively, as previously described in Ordóñez-Díaz et al. (2024), in ethanol:water (80:20) extracts of acorn flour. The antioxidant activities were represented as mmol of Trolox equivalents per g of dry weight (mmol TE/g DW).

2.6. Mineral analysis

Macro- (Ca, Mg, K, N, and P) and micronutrient (Fe, Zn, Mn and Cu) content was determined in 200 mg of lyophilised acorn flour extract digested in a mixture of 60 % nitric acid and 60 % perchloric acid (ratio 1:3) at 105 °C. The mixture was cooled down and the extracts were reconstituted to 10 ml of distilled water. P was determined spectrophotometrically using the vanadate-molybdate method (Murphy and Riley, 1962), K by flame photometry and Ca, Mg, Fe, Mn, Zn and Cu by atomic absorption spectrophotometry (Perkin Elmer /AA800, PerkinElmer, Inc., CA, USA) (Jakovljevic and Blagojevic 1998). Macronutrient concentrations were expressed in g/kg DW, and micronutrient concentrations were expressed in mg/kg DW, except Na, which was expressed in g/kg DW.

2.7. Non-targeted metabolomics analysis using UHPLC-MS/MS

Non-targeted metabolomic analysis was performed using a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole-orbitrap Q Exactive hybrid mass spectrometer (UHPLC-MS/MS, Thermo Fisher Scientific, Bremen, Germany), which was equipped with a heated-electrospray ionization probe (HESI-II). Ice-cold ethanol:water (50:50, v/v) was added to 30 mg of lyophilized acorn flour, which was mixed by vortexing, and then sonicated for 10 min (ultrasonic bath, 40 kHz). After centrifugation (4 °C, 5 min, 14,000 rpm), the pellet was vacuum dried at 30 °C (Speedvac, Eppendorf vacuum concentrator Plus/5301, Eppendorf, Leicestershire, U.K.). Dried extracts were re-dissolved in 0.5 mL of 50 % methanol containing 0.1 % of formic acid, centrifuged at 20,000×g for 10 min, and filtered through 0.22 μm polytetrafluoroethylene (PTFE) membranes (Thermo Fisher Scientific, Courtaboeuf, France). The filtrate was collected in 1.5-mL LC/MS certified sample vials. Chromatographic separation was carried out using an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm) (Waters, Manchester, U.K.), maintaining it at 40 °C. The injection volume was 5 μl and the flow rate was set at 0.5 mL/min. Mobile phases consisted of 0.1 % formic acid in water (Eluent A) and 0.1 % formic acid in methanol (Eluent B). Elution conditions were as follows: 5 % B for 1 min, linear gradient from 5 % to 100 % in solvent B for 9 min, isocratic at 100 % B for 2 min, and return to initial conditions, 5 % B for 3 min.

MS detection was performed with the Q Exactive Orbitrap mass spectrometer operating in positive and negative polarities. HESI source parameters in positive mode were spray voltage, 3.5 kV; S-lens RF level, 50; capillary temperature, 320 °C; sheath and auxiliary gas flow, 60 and 25, respectively (arbitrary units); and probe heater temperature, 400 ºC. For negative ion mode, all parameters remained the same, except that the spray voltage was set to −3.0 kV. Xcalibur v.4.3 software was used for instrument control and data acquisition. A Full Scan MS method was acquired at a resolution of 70,000 (full width half maximum, FWHM at m/z 200) and a data dependent acquisition MS_2 method was acquired at resolution 70,000 and 17,500 (FWHM at m/z 200) for Full Scan and Product Ion Scan, respectively, fragmenting the five most abundant precursor ions per MS scan (Top5). Full Scan MS and data dependent acquisition MS2 methods were acquired in positive and negative modes, and the mass range used for both experiments was 70 – 1050 m/z. Additionally, for continuous quality assurance and to promote confidence in the data, the quality control (QC) mix was prepared using equal volumes of all samples, and was injected after every six samples during the batch processing, along with methanol as a blank run to correct for a drift of the raw signal intensity during the analysis. Moreover, the QC samples were analysed in a data-dependent (dd-MS2/dd-SIM) manner for feature annotation. All the data acquired data were exported by Xcalibur software to be analysed by Compound Discoverer v3.2 software (Thermo Fisher Scientific, Bremen, Germany). The raw data were deposited on the Metabolomics Workbench (https://www.metabolomicsworkbench.org) with the assigned Project ID PR002302. The data can be accessed directly via its Project DOI: https://doi.org/10.21228/M82R84.

UHPLC-MS/MS raw data were pre-processed using Compound Discoverer software, applying alignment, peak selection, deconvolution, and normalization. The alignment was performed with a retention time with a maximum shift of 0.3 min and a mass tolerance of 3 ppm. The feature identification across all data was then made possible via peak selection and deconvolution. Furthermore, elemental compositions (chemical formula hypothesis) were predicted for all compounds, and

blank samples were used to hide the chemical background. Metabolite annotation was performed using a ddMS2 similarity search in mzCloud (https://www.mzcloud.org), which was supported by the agreement between theoretical and experimental isotopic patterns. Additionally, a search for the formula or exact mass (mass error ≤ 5 ppm) was conducted in ChemSpider (https://www.chem-spider.com/), which was also utilized for the literature references. By considering the areas of the corresponding chromatographic peaks of the MS1 precursor ion, metabolites were quantified in relative terms. The neutral mass in each ionization mode was merged separately to avoid duplicities (similar neutral mass and retention time), preserving the strongest peaks. Peak areas were processed using R package *pRocessomics* (https://github.com/Valledor/pRocessomics). Missing value imputation was not performed to ensure true post-filtering based on consistency. The data were filtered using a consistency criterion by establishing a threshold value of 0.5 and abundance balancing (the values were sample-centric, and then each value was multiplied by the average intensity; the sum of the peak area of all variables within a sample from among all of them). The resulting output data matrix (ID metabolite, retention time, *m/z*, peak area) was subjected to statistical analysis. Consensus InChIKey structural information for selected compounds was submitted to the ClassyFire server (http://classyfire.wishartlab.com/) for hierarchical chemical classification. The most abundant super chemical classes were represented on a donut chart using the *ggplot2* and *dyplyr* R package. According to currently accepted confidence levels in metabolomic compound identification (Reisdorph et al., 2019), most of the metabolites described here have a putative annotation, with confidence level 2. Compounds with confidence level 3 have not been considered for discussion.

2.8. Statistical analysis

The statistical analyses and plot representation were performed in R Studio, version 2023.12.0.369, running under R version 4.3.1. The morphometry, NIRS, colorimetry, HPLC, antioxidant capacity and mineral analysis data were subjected to the non-parametric Kruskal-Wallis test, with $p \leq 0.05$, using the agricolae R package (https://cran.r-project.org/web/packages/agricolae/index.html). Pearson's correlation matrices were plotted using *cor ()* function and *ggpubr* R package. Venn analysis was generated using *ggven* R package. Metabolomic data were subjected to multivariate analysis (Principal Component Analysis, PCA) using the *factoextra* and *ggplot2* R packages and the non-parametric Kruskal-Wallis test.

3. Results

3.1. Phytochemical analysis

Phytochemical analysis was conducted on acorns from 14 individual trees from various Spanish provenances (Table 1). The acorns, provided by local owners, were categorized based on the following bitterness levels: low (individuals 1–6), medium (individuals 7–12), and high (individuals 13 and 14). Significant differences in the acorns' morphometric parameters were observed between the trees, with acorn width ranging from 1.19 to 2.28 cm, length from 3.06 to 4.58 cm, fresh weight from 4.63 to 14.19 g, volume from 2.63 to 7.50 mL, and water content from 23.59 % to 45.81 % (Table S1). Larger acorns (volume >5 mL) were generally associated with low bitterness, whereas two of the three smallest acorns—those from individuals 13 and 14—were among the most bitter (Table 2).

Various techniques were employed to characterize the phytochemical profiles of the acorns, including NIRS technology, classical biochemical methods based on colorimetric reactions, HPLC-RID, and analysis of macro- and micronutrients. These analyses were complemented by an assessment of the antioxidant activity of the ethanolic extracts.

The profiles of ash, protein, fat, starch, phenolics, energy, digestibility, fibre, and fatty acids were determined using NIRS technology

Table 2
Morphometric parameters measured on the acorns of different *Q. ilex* individuals.

Individual	Morphology	Width	Length	Fresh_Weight	Dry_Weight	Volume	Water_Content
1		2.08 ± 0.06 ^{ab}	3.50 ± 0.07 ^{ef}	8.56 ± 0.5 ^{b}	5.18 ± 0.36 ^{c}	4.88 ± 0.25 ^{c}	39.17 ± 3.1 ^{ab}
2		2.16 ± 0.03 ^{a}	4.08 ± 0.06 ^{bc}	10.15 ± 0.13 ^{a}	6.12 ± 0.08 ^{ab}	5.91 ± 0.08 ^{a}	42.83 ± 0.55 ^{a}
3		1.90 ± 0.06 ^{bc}	3.88 ± 0.07 ^{cd}	8.95 ± 0.28 ^{b}	5.39 ± 0.17 ^{bc}	5.21 ± 0.16 ^{bc}	37.74 ± 1.19 ^{ab}
4		2.28 ± 0.09 ^{a}	3.90 ± 0.08 ^{cd}	14.19 ± 0.54 ^{a}	7.99 ± 0.23 ^{a}	7.50 ± 0.28 ^{a}	43.10 ± 2.87 ^{a}
5		1.90 ± 0.03 ^{bc}	4.30 ± 0.06 ^{ab}	8.95 ± 0.15 ^{b}	5.39 ± 0.09 ^{bc}	5.21 ± 0.09 ^{bc}	37.74 ± 0.65 ^{ab}
6		1.81 ± 0.04 ^{cd}	3.77 ± 0.06 ^{cde}	8.53 ± 0.16 ^{b}	5.14 ± 0.1 ^{c}	4.97 ± 0.1 ^{c}	36.00 ± 0.7 ^{abc}
7		1.41 ± 0.02 ^{fg}	3.13 ± 0.06 ^{g}	6.65 ± 0.11 ^{cd}	4.01 ± 0.06 ^{d}	3.87 ± 0.06 ^{d}	28.06 ± 0.45 ^{bc}
8		1.91 ± 0.05 ^{bc}	4.58 ± 0.06 ^{a}	9.00 ± 0.24 ^{b}	5.43 ± 0.15 ^{bc}	5.24 ± 0.14 ^{bc}	37.99 ± 1.02 ^{ab}
9		1.46 ± 0.03 ^{fg}	3.43 ± 0.05 ^{fg}	4.63 ± 0.2 ^{e}	2.82 ± 0.13 ^{de}	2.75 ± 0.21 ^{de}	37.81 ± 5.2 ^{ab}
10		1.64 ± 0.05 ^{def}	3.59 ± 0.07 ^{def}	5.72 ± 0.33 ^{de}	3.59 ± 0.23 ^{de}	3.44 ± 0.22 ^{de}	35.43 ± 5.61 ^{abc}
11		1.80 ± 0.03 ^{cde}	3.30 ± 0.06 ^{fg}	8.47 ± 0.13 ^{bc}	5.11 ± 0.08 ^{c}	4.94 ± 0.07 ^{c}	35.75 ± 0.53 ^{abc}
12		1.85 ± 0.06 ^{c}	4.33 ± 0.08 ^{ab}	8.75 ± 0.48 ^{b}	5.92 ± 0.29 ^{bc}	5.81 ± 0.21 ^{ab}	30.59 ± 5.7 ^{abc}
13		1.19 ± 0.04 ^{g}	3.55 ± 0.12 ^{ef}	5.59 ± 0.16 ^{de}	3.37 ± 0.1 ^{de}	3.26 ± 0.1 ^{de}	23.59 ± 0.7 ^{c}
14		1.60 ± 0.03 ^{ef}	3.06 ± 0.06 ^{g}	4.63 ± 0.3 ^{e}	2.60 ± 0.19 ^{e}	2.63 ± 0.23 ^{e}	42.92 ± 4.59 ^{a}
p-value		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Values are mean of three replicates, with the SE (Standard Error). Superscript letters indicate significant differences between groups based on Dunn's test with Bonferroni correction ($p < 0.05$). Groups sharing the same letter are not significantly different.

Table 3
Chemical and fatty acid profiling for acorns analysed by NIRS technology.

Individual	Ash (%)	Protein (%)	Fat (%)	Starch (%)	Phenolics (%)	Energy (kcal/kg)	Digestibility (%)	Fibre (%)
1	2.97 ± 0.04 ^{ab}	4.37 ± 0.18 ^{hi}	16.37 ± 0.30 ^{a}	60.47 ± 0.20 ^{h}	0.11 ± 0.03 ^{hi}	5013.26 ± 18.52 ^{a}	81.65 ± 1.62 ^{ab}	2.43 ± 0.05 ^{bc}
2	2.53 ± 0.02 ^{ef}	6.27 ± 0.07 ^{bc}	12.55 ± 0.48 ^{d}	62.44 ± 0.16 ^{fg}	0.63 ± 0.01 ^{g}	4829.78 ± 29.34 ^{cd}	76.66 ± 2.14 ^{cdef}	2.43 ± 0.02 ^{bc}
3	2.74 ± 0.07 ^{bcde}	5.71 ± 0.10 ^{de}	11.57 ± 0.37 ^{e}	61.99 ± 0.69 ^{fg}	0.88 ± 0.02 ^{cd}	4775.38 ± 18.97 ^{de}	71.78 ± 0.81 ^{fgh}	2.43 ± 0.01 ^{bc}
4	2.70 ± 0.06 ^{cde}	5.68 ± 0.05 ^{de}	9.77 ± 0.04 ^{f}	64.10 ± 0.24 ^{bcde}	0.74 ± 0.01 ^{f}	4663.11 ± 4.17 ^{fg}	75.95 ± 0.48 ^{bcde}	2.37 ± 0.02 ^{cde}
5	2.52 ± 0.07 ^{ef}	6.50 ± 0.28 ^{bc}	14.34 ± 0.21 ^{ab}	63.34 ± 0.37 ^{def}	0.76 ± 0.03 ^{ef}	4905.52 ± 15.74 ^{ab}	79.85 ± 1.68 ^{bcd}	2.58 ± 0.01 ^{a}
6	2.86 ± 0.04 ^{bc}	6.59 ± 0.04 ^{b}	13.43 ± 0.03 ^{bc}	60.37 ± 0.20 ^{h}	0.81 ± 0.01 ^{de}	4881.01 ± 2.26 ^{b}	73.41 ± 0.66 ^{efg}	2.42 ± 0.01 ^{bcd}
7	2.55 ± 0.03 ^{ef}	6.60 ± 0.10 ^{ab}	11.84 ± 0.04 ^{e}	62.64 ± 0.30 ^{fg}	1.22 ± 0.01 ^{a}	4832.07 ± 4.00 ^{de}	80.13 ± 0.58 ^{abc}	2.46 ± 0.01 ^{ab}
8	2.69 ± 0.05 ^{cde}	7.43 ± 0.03 ^{a}	13.06 ± 0.12 ^{cd}	61.49 ± 0.13 ^{gh}	0.95 ± 0.01 ^{bc}	4869.11 ± 6.20 ^{bc}	87.38 ± 0.58 ^{a}	2.57 ± 0.01 ^{a}
9	2.93 ± 0.03 ^{ab}	5.45 ± 0.07 ^{ef}	13.65 ± 0.11 ^{bc}	64.37 ± 0.12 ^{bcd}	0.00 ± 0.01 ^{i}	4836.82 ± 4.92 ^{cd}	69.71 ± 0.63 ^{ghi}	2.38 ± 0.01 ^{cde}
10	2.81 ± 0.04 ^{bcd}	2.76 ± 0.05 ^{i}	11.86 ± 0.06 ^{e}	68.60 ± 0.16 ^{a}	0.27 ± 0.03 ^{gh}	4735.63 ± 2.92 ^{ef}	44.64 ± 0.84 ^{i}	2.17 ± 0.01 ^{f}
11	2.33 ± 0.04 ^{f}	5.86 ± 0.03 ^{cd}	12.80 ± 0.05 ^{d}	64.65 ± 0.17 ^{abc}	0.80 ± 0.01 ^{e}	4843.88 ± 3.29 ^{cd}	75.62 ± 0.76 ^{def}	2.45 ± 0.01 ^{bc}
12	2.62 ± 0.03 ^{de}	5.01 ± 0.02 ^{gh}	12.82 ± 0.08^{d}	62.85 ± 0.26 ^{efg}	0.74 ± 0.03 ^{ef}	4886.36 ± 3.41 ^{ab}	77.42 ± 0.38 ^{bcd}	2.38 ± 0.01 ^{cde}
13	2.72 ± 0.07 ^{bcde}	5.23 ± 0.11 ^{fg}	5.20 ± 0.33 ^{f}	66.42 ± 0.06 ^{ab}	1.12 ± 0.03 ^{ab}	4480.25 ± 15.82 ^{g}	72.74 ± 0.34 ^{efgh}	2.35 ± 0.00 ^{def}
14	3.34 ± 0.07 ^{a}	5.01 ± 0.04 ^{gh}	7.98 ± 0.40 ^{f}	63.52 ± 0.51 ^{cdef}	1.28 ± 0.03 ^{a}	4609.06 ± 20.41 ^{fg}	60.38 ± 1.19 ^{hi}	2.31 ± 0.02 ^{ef}
p-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Individual	Palmitic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (%)
1	26.93 ± 0.31 ^{b}	0.79 ± 0.07 ^{f}	68.6 ± 0.52 ^{abc}	12.44 ± 1.09 ^{ef}
2	22.49 ± 0.32 ^{de}	1.35 ± 0.05 ^{de}	65.27 ± 0.76 ^{cde}	17.23 ± 0.27 ^{abc}
3	23.53 ± 0.2 ^{cd}	1.77 ± 0.06 ^{bc}	67.29 ± 1.25 ^{bcd}	17.16 ± 0.35 ^{bc}
4	26.60 ± 0.28 ^{b}	2.00 ± 0.03 ^{ab}	62.16 ± 0.97 ^{ef}	15.15 ± 0.46 ^{de}
5	24.27 ± 0.46 ^{c}	1.03 ± 0.03 ^{ef}	69.71 ± 0.33 ^{ab}	15.20 ± 0.36 ^{de}
6	23.06 ± 0.2 ^{de}	0.84 ± 0.05 ^{f}	62.87 ± 0.51 ^{ef}	18.82 ± 0.30 ^{ab}
7	22.09 ± 0.18 ^{e}	1.93 ± 0.05 ^{b}	66.51 ± 0.76 ^{bcd}	16.65 ± 0.10 ^{c}
8	23.10 ± 0.14 ^{cde}	1.96 ± 0.10 ^{b}	65.46 ± 0.69 ^{cde}	15.21 ± 0.32 ^{de}
9	30.28 ± 0.35 ^{a}	0.31 ± 0.05 ^{g}	64.02 ± 0.46 ^{de}	10.36 ± 0.74 ^{fg}
10	30.93 ± 0.22 ^{a}	0.81 ± 0.08 ^{f}	75.43 ± 0.88 ^{a}	4.82 ± 0.35 ^{g}
11	26.45 ± 0.32 ^{b}	0.87 ± 0.05 ^{f}	65.99 ± 1.10 ^{cde}	13.60 ± 0.08 ^{ef}
12	21.68 ± 0.43 ^{e}	1.66 ± 0.04 ^{cd}	64.62 ± 0.34 ^{de}	16.37 ± 0.19 ^{cd}
13	23.49 ± 0.33 ^{cd}	2.33 ± 0.05 ^{a}	57.27 ± 0.62 ^{f}	16.59 ± 0.46 ^{cd}
14	26.22 ± 0.47 ^{b}	1.95 ± 0.07 ^{b}	56.96 ± 0.56 ^{f}	19.28 ± 0.39 ^{a}
p-value	<0.01	<0.01	<0.01	<0.01

Values are mean of three replicates, with the SE (Standard Error). Superscript letters indicate significant differences between groups based on Dunn's test with Bonferroni correction ($p < 0.05$). Groups sharing the same letter are not significantly different. NIRS indicates near infrared spectroscopy.

(Table 3). Starch was the most abundant substance (60.37–68.60 %), followed by fat (5.20–16.37 %) and protein (2.76–7.43 %). Phenolic content was below 1.50 %. Additional parameters included ash (2.33–3.34 %), energy (4480.25–5013.26 kcal/kg), digestibility (44.64–87.38 %), and fibre (2.17–2.58 %). Substantial variability was observed between the individual trees, with differences being statistically significant for most or all of the variables. Notably, small-sized, highly bitter acorns exhibited the lowest fat content and the highest phenolic concentrations—up to five-fold and ten-fold differences, respectively, compared to other samples. These acorns also had the lowest energy, digestibility, and fibre values. Unsaturated oleic and linoleic acids were the most abundant fatty acids, comprising 57–75 % and 5–20 % of the total fatty acids, respectively. Saturated fatty acids, palmitic and stearic, were present, at 21.68–30.93 % and 0.31–2.33 %, respectively. Among all the samples analysed, the acorns from individuals 13 and 14 (small and bitter) exhibited the lowest oleic acid levels and the highest stearic acid content (Table 3).

In order to implement the NIRS analysis, colorimetric methods were used to quantify starch, soluble sugars, free amino acids, phenolic compounds, flavonoids, and tannins following the sequential protocol previously published by López-Hidalgo et al. (2021b) (Table 4). The anthrone method yielded slightly lower starch content values compared with those obtained through NIRS analysis, falling within the 40–70 % DW range. Soluble sugar content, also determined by this method, ranged from 5–30 % DW. The highest and lowest starch values were observed in individuals 11 and 7, respectively, while individuals 5 and 10 showed the maximum and minimum soluble sugar levels. Regarding soluble sugars, sucrose, glucose, and fructose were quantified by HPLC-RID (Fig. 1), accounting for 5–15 % DW. Sucrose was the most abundant in most individuals (2.03 to 9.73 g/100 g DW), followed by glucose (1.51 to 5.24 g/100 g DW), while fructose levels remained below 0.5 g/100 g DW. The highest sucrose and glucose values were found in individuals 8 and 13, respectively, whereas the lowest values were observed in individuals 10 and 1, respectively. Statistically significant differences were found between individuals, but not correlating to size or bitterness.

The amino acid content, as determined using the ninhydrin reagent, ranged from 3.09 mg glycine/g DW (individual 10) to 10.55 mg glycine/g DW (individual 4), showing statistically significant variation between individuals.

The phenolic content, as determined by the Folin–Ciocalteu method, ranged from 9.14 (individual 9) to 25.85 (individual 14) mg gallic acid equivalents (GAE)/g DW (Table S4). Both NIRS and colorimetric data highlighted individuals 13 and 14 (high bitterness) as having the highest total phenolic content. Flavonoid levels ranged from 0.76 (individual 7) to 3.70 (individual 1) mg quercetin equivalents/g DW (Table 4). Condensed tannin content varied from 0.21 mg/g DW (individual 14) to 1.36 mg/g DW (individual 1). Interestingly, a general trend was observed in which low-bitterness acorns exhibited higher levels of condensed tannins compared to high-bitterness ones (Table S4). As for phenolic compounds, the antioxidant activity of acorn extracts from the fourteen *Q. ilex* individuals was assessed using the DPPH and ABTS assays (Table 4). Mean values ranged from 0.03 to 0.08 mmol TE/g DW for ABTS, and from 0.04 to 0.12 mmol TE/g DW for DPPH. Significant differences were observed between individuals, with the highest antioxidant activities recorded in individuals 13 and 14, and the lowest in individuals 9 and 1 for ABTS and DPPH assays, respectively.

From the analysis of micro- and macronutrients, it was observed that the most abundant macronutrients were K (8.26–12.50 g/kg DW), P (4.49–7.77 g/kg DW), Ca (0.98–1.31 g/kg DW), and Mg (0.01–0.03 g/kg DW), while the most abundant micronutrients were Na (0.06–0.33 g/kg DW), followed by Mn (0.00–81.34 mg/kg DW), Fe (23.19–36.23 mg/kg

Table 4
Phytochemical profile analysis by colorimetric reactions, HPLC-RID, ABTS and DPPH on the acorns of fourteen *Q. ilex* individuals.

Individual	Total Phenolic Compounds (mg/g DW)	Flavonoids (mg/g DW)	Condensed Tannins (mg/g DW)	Free Amino acids (mg/g DW)	Free Sugars (mg/g DW)	Starch (mg/g DW)
1	9.46 ± 0.48 ^{f}	3.70 ± 0.44 ^{a}	1.36 ± 0.25 ^{a}	6.24 ± 0.27 ^{ab}	95.02 ± 1.07 ^{fg}	489.76 ± 31.26 ^{ab}
2	12.87 ± 0.60 ^{def}	1.75 ± 0.53 ^{ab}	0.70 ± 0.19 ^{abc}	6.21 ± 0.70 ^{ab}	117.90 ± 0.18 ^{efg}	407.98 ± 52.59 ^{b}
3	17.11 ± 0.87 ^{ab}	0.85 ± 0.15 ^{ab}	0.49 ± 0.03 ^{abc}	5.36 ± 0.77 ^{ab}	161.55 ± 2.10 ^{abc}	445.81 ± 7.11 ^{b}
4	12.81 ± 0.97 ^{def}	1.68 ± 0.50 ^{ab}	0.59 ± 0.13 ^{abc}	10.55 ± 0.06 ^{a}	134.68 ± 0.02 ^{cde}	476.10 ± 73.99 ^{ab}
5	14.00 ± 0.50 ^{bcde}	1.75 ± 0.49 ^{ab}	0.78 ± 0.15 ^{ab}	4.70 ± 0.67 ^{ab}	269.91 ± 42.48 ^{a}	441.35 ± 19.97 ^{b}
6	12.33 ± 0.86 ^{ef}	2.14 ± 0.81 ^{ab}	0.51 ± 0.10 ^{abc}	4.03 ± 0.98 ^{b}	143.90 ± 7.17 ^{bcd}	436.65 ± 69.27 ^{ab}
7	15.7 ± 0.29 ^{abcd}	0.76 ± 0.06 ^{b}	0.30 ± 0.03 ^{bc}	3.86 ± 0.51 ^{b}	135.81 ± 1.35 ^{bcd}	389.24 ± 53.95 ^{b}
8	16.72 ± 0.55 ^{abc}	1.57 ± 0.21 ^{ab}	0.56 ± 0.13 ^{abc}	6.16 ± 1.57 ^{ab}	126.58 ± 5.45 ^{def}	530.66 ± 35.78 ^{ab}
9	9.14 ± 0.32 ^{f}	1.57 ± 0.78 ^{ab}	0.72 ± 0.27 ^{abc}	6.92 ± 0.34 ^{ab}	58.14 ± 5.94 ^{h}	479.94 ± 51.74 ^{ab}
10	13.58 ± 0.62 ^{cdef}	0.80 ± 0.10 ^{ab}	0.27 ± 0.02 ^{bc}	3.09 ± 1.19 ^{b}	56.30 ± 7.99 ^{h}	552.46 ± 40.53 ^{ab}
11	17.51 ± 0.55 ^{ab}	1.69 ± 0.34 ^{ab}	0.69 ± 0.16 ^{abc}	5.59 ± 0.49 ^{ab}	129.20 ± 0.67 ^{def}	722.58 ± 29.92 ^{a}
12	15.19 ± 1.29 ^{bcde}	1.93 ± 0.28 ^{ab}	0.74 ± 0.09 ^{abc}	4.16 ± 0.39 ^{ab}	81.65 ± 5.29 ^{gh}	591.76 ± 45.70 ^{ab}
13	22.62 ± 0.57 ^{a}	3.66 ± 0.46 ^{a}	0.52 ± 0.08 ^{abc}	6.55 ± 1.41 ^{ab}	183.93 ± 15.31 ^{ab}	604.99 ± 22.41 ^{ab}
14	25.85 ± 2.61 ^{a}	0.86 ± 0.33 ^{ab}	0.21 ± 0.03 ^{c}	4.44 ± 0.70 ^{ab}	73.99 ± 0.25 ^{gh}	607.74 ± 46.73 ^{ab}
p-value	<0.01	0.03	0.01	0.02	<0.01	0.02

Individual	Sacarose (g/100 g DW)	Glucose (g/100 g DW)	Fructose (mg/100 g DW)	ABTS (mmol/g DW TE)	DPPH (mmol/g DW TE)
1	2.82 ± 0.11 ^{gh}	1.51 ± 0.05 ^{h}	0.47 ± 0.04 ^{abcd}	0.04 ± 0.01 ^{bc}	0.04 ± 0.01 ^{h}
2	4.95 ± 0.09 ^{efg}	2.26 ± 0.07 ^{efg}	0.26 ± 0.02 ^{abc}	0.06 ± 0.00 ^{ab}	0.05 ± 0.00 ^{g}
3	8.67 ± 0.04 ^{abc}	1.87 ± 0.08 ^{fgh}	0.26 ± 0.05 ^{ab}	0.07 ± 0.00 ^{a}	0.07 ± 0.00 ^{f}
4	8.10 ± 0.14 ^{abcd}	1.84 ± 0.08 ^{fgh}	0.00 ± 0.02 ^{bcde}	0.07 ± 0.00 ^{a}	0.07 ± 0.01 ^{f}
5	7.09 ± 0.11 ^{cde}	2.63 ± 0.06 ^{de}	0.66 ± 0.03 ^{a}	0.07 ± 0.00 ^{a}	0.09 ± 0.01cd
6	5.93 ± 0.20 ^{def}	2.82 ± 0.23 ^{cde}	0.00 ± 0.02 ^{abc}	0.06 ± 0.00 ^{ab}	0.08 ± 0.00 ^{ef}
7	6.62 ± 0.21 ^{cde}	3.31 ± 0.17 ^{abc}	0.07 ± 0.02 ^{ab}	0.07 ± 0.00 ^{a}	0.12 ± 0.00 ^{ab}
8	9.73 ± 0.24 ^{a}	3.89 ± 0.04 ^{ab}	0.00 ± 0.01 ^{abcd}	0.07 ± 0.00 ^{a}	0.09 ± 0.01 ^{cd}
9	2.12 ± 0.04 ^{h}	3.04 ± 0.08 ^{bcd}	0.00 ± 0.00 ^{de}	0.03 ± 0.01 ^{c}	0.04 ± 0.00 ^{gh}
10	2.03 ± 0.11 ^{h}	2.27 ± 0.04^{ef}	0.00 ± 0.01 ^{e}	0.07 ± 0.01 ^{a}	0.08 ± 0.00 ^{ef}
11	7.83 ± 0.22 ^{bcd}	1.81 ± 0.14 ^{gh}	0.00 ± 0.01 ^{bcde}	0.06 ± 0.01 ^{a}	0.11 ± 0.01 ^{bc}
12	3.78 ± 0.08 ^{fgh}	2.48 ± 0.08 ^{de}	0.00 ± 0.01 ^{cde}	0.07 ± 0.01 ^{a}	0.08 ± 0.00 ^{ef}
13	9.45 ± 0.32 ^{ab}	5.24 ± 0.10 ^{a}	0.03 ± 0.02 ^{ab}	0.08 ± 0.00 ^{a}	0.09 ± 0.01 ^{de}
14	3.25 ± 0.11 ^{fgh}	3.64 ± 0.25 ^{ab}	0.00 ± 0.01 ^{de}	0.07 ± 0.02 ^{a}	0.12 ± 0.00 ^{a}
p-value	<0.01	<0.01	<0.01	<0.01	<0.01

Values are mean of three replicates, with the SE (Standard Error). Superscript letters indicate significant differences between groups based on Dunn's test with Bonferroni correction ($p < 0.05$). Groups sharing the same letter are not significantly different. GAE (Gallic Acid Equivalents), QE (Quercetin Equivalents), CE (Catechin Equivalents), GlyE (Glycine Equivalents), GluE (D-Glucose Equivalents) and TE (TROLOX Equivalents)
Values are mean of three replicates, with the SE (Standard Error). Superscript letters indicate significant differences between groups based on LSD test. Groups sharing the same letter are not significantly different. GAE (Gallic Acid Equivalents), QE (Quercetin Equivalents), CE (Catechin Equivalents), GlyE (Glycine Equivalents), GluE (D-Glucose Equivalents) and TE (TROLOX Equivalents)

DW), Zn (6.39–15.20 mg/kg DW) and Cu (4.91–11.30 mg/kg DW) (Table S1). All the nutrients, except Fe and Ca, showed significant differences between the individuals. Individual 14 showed the highest Mn and K values. Other maximum values corresponded to individuals 4 (Mn), 6 (Na), 8 (Zn and P), 9 (Mg), and 12 (Cu).

Phytochemical data, together with morphometric values (a total of 38 variables), were subjected to PCA to assess existing variability, reduce dimensionality, identify the variables contributing most to variability, and establish groups and correlations with phenotypic characteristics. The PCA structure reflected variation in Principal Component 1 (PC1, 23.40 %) and PC2 (18.00 %), revealing three distinct groups (Fig. 1a). PC1 distinguished two main groups of acorns. The first group (purple ellipse), composed of individuals 13 and 14, was characterized by the smallest and most bitter fruits. The second group (yellow ellipse) included individuals with large and sweet acorns, with individual 1 standing out as the most distant from the rest of the sweet acorn individuals. A third group (blue ellipse), positioned between the two main clusters, comprised acorns of intermediate bitterness. Within this group, individuals 9 and 10 were distinctly separated from the rest along PC2. Additionally, the PCA biplot (Fig. 1b) indicated that the variables contributing most to the separation along PC1 were those related to acorn morphometry as well as energy, fibre, digestibility, fat content, tannins and sucrose, all of which showed higher values in the sweetest acorns. Individual 1 stands out for its high fat content, especially oleic acid, and elevated tannin levels, despite being one of the sweetest acorns. In PC2, phenolic compounds, glucose and antioxidant capacity stand out for their high values in the two bitter acorns, while neutral individuals 9 and 10 are characterised by their high palmitic acid content. These marked differences among acorn groups could serve as the basis for defining the first phytochemical and morphometric parameters to classify acorn phenotypes according to their nutritional qualities. Such classification could have practical applications in the selection and breeding of superior varieties, as well as in conservation strategies and quality control for acorn-derived products.

A clustering dendrogram (Fig. 1c) supports the above results, revealing the presence of two main clusters in the tree sets. The first group included individuals 9, 10, 13, and 14, and the second one, the rest of the provenances. This grouping was not dependent on the trees' locations, but rather was related to the acorns' size and bitterness, separating small, bitter fruits from medium to large ones of medium to low bitterness.

3.2. Untargeted metabolomic profiling

The metabolome of water:ethanolic acorn extracts was analysed using an LC-Q-Orbitrap, operating in the positive and negative ion modes. A total of 7401 and 4192 features were resolved in the positive and negative ionization modes, respectively. The raw dataset was filtered based on consistency criterion (present in all replicates of at least one individual, or in 50 % of all individuals), obtaining a processed dataset of 4811 metabolic features (Table S2). A PCA analysis was performed to decrease the dimensionality of the data and display the

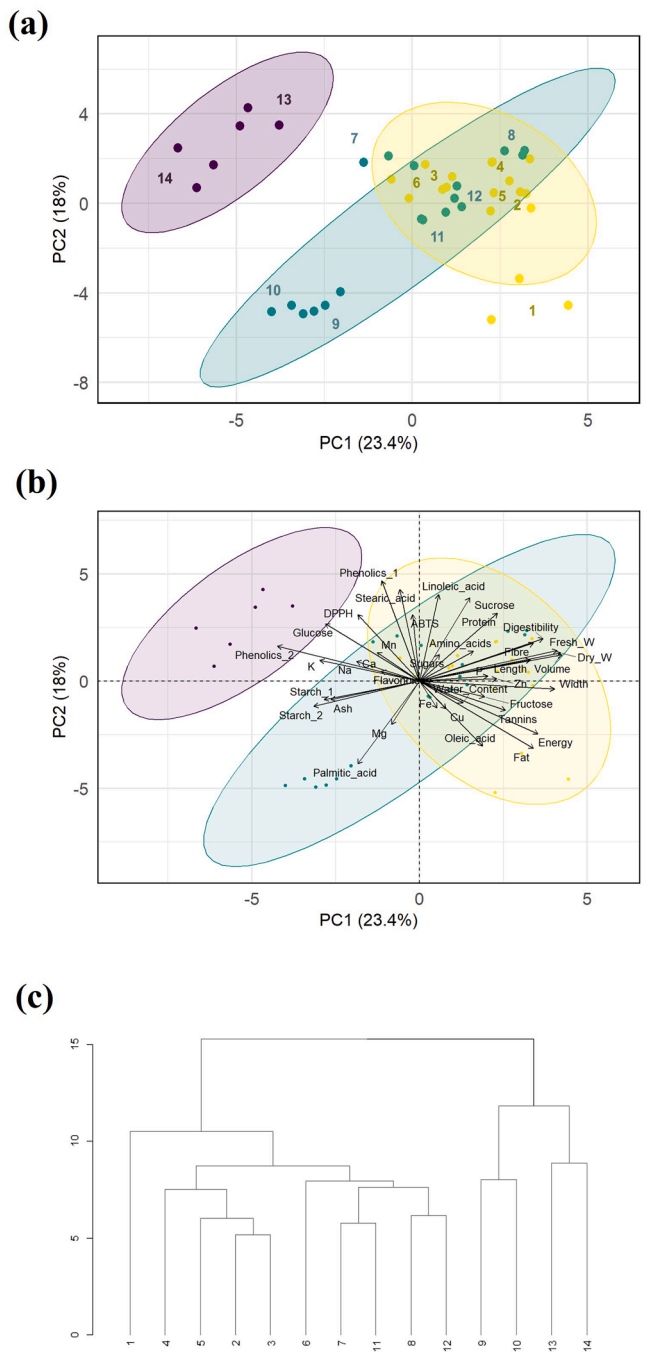

Fig. 1. (a) Principal component analysis plot, (b) biplot, and (c) dendrogram of morphometric variables, NIRS, colorimetric methods, minerals, soluble sugar and antioxidant capacity in fourteen individuals of *Q. ilex*. Bitterness levels are represented by colors, with high bitterness indicated in purple, medium bitterness in blue, and low bitterness in yellow.

relationships and differences among individuals (Fig. 2a). The first two principal components (PC1 and PC2) explained a low percentage of the variability: 22.50 %. Out of 4811 features, 1189 features were putative, annotated based on spectral information (dMS2) using Compound Discoverer software (Table S3). The putative annotated dataset comprised 98 compounds detected in all samples with no statistically significant differences, 759 compounds showing qualitative differences (the absence of a compound in the three biological replicates of an individual), 308 compounds with statistically significant differences between individuals, 138 compounds varying based on bitterness level, and 110 compounds exhibiting differences at both the individual and bitterness levels (Table S4).

When the PCA analysis was performed with the annotated compounds, PC1 and PC2 explained 12.90 % and 8.00 %, respectively (Fig. 2b). Individual 13, the smallest in size and the most bitter, was clearly separated from the rest of the *Q. ilex* individuals analysed. No clear differences were observed among the rest of individuals. Of the 1191 annotated compounds, 227 were detected in the fourteen individuals (18.40 %), which can be considered characteristics of the *Q. ilex* metabolome (Table S5). The 227 common compounds were classified into chemical families (Fig. 3). Organic acids and derivatives (21.70 %) represented the largest family of compounds, followed by lipids and lipid-like molecules (18.20 %) and organoheterocyclic compounds (17.30 %). Within the secondary metabolism, phenolic compounds were the most abundant (benzenoids (10.40 %) and phenylpropanoids and polyketides (6.10 %)). Others were restricted to a subset of individuals, cyrtominetin (individual 4), nothofagin (individuals 8, 11, 12 and 13), p-cymene (individuals 3 and 11), scoparone (individual 13) (Table S4). Some of the annotated compounds are, to our knowledge, firstly reported in *Q. ilex*, such as apocynin, secologanin, 3,4,2′,4′,6′-Pentahydroxydihydrochalcone, and brevifolin carboxylic acid.

Some other structural or functional groups based on plant characteristics were identified, including phytohormones and signalling compounds (gamma-aminobutyric acid, salicylic acid, abscisic acid, jasmonic acid), fatty acids (alpha-linoleic acid, oleamide, palmitoleic acid, adipic acid), amino acids and their derivatives (asparagine, aspartic acid, glutamic acid, phenylalanine, and valine), and vitamins (nicotinic acid and nicotinamide, thiamine, pyridoxine, and pantothenic acid).

In addition to plant-derived compounds, 102 compounds were annotated as non-plant-derived, including those associated with fungi and bacteria, based on PubChem database and scientific literature (Table S6). These compounds have been previously reported in fungi and bacteria. Among fungi, they are mainly associated with the genus *Aspergillus* (Asperpanoid B, Wortmanamide B, Acetyl Sumiki's acid, Annulohypoxylomanol B), *Penicillium* (Penicibenzoxepinol), *Xylaria* (Xylapyrone F, Xylarinol A), *Chaetomium* (Chaetomellic acid B), *Monascus* (Monascustin, Monascuspirolide B), and *Pestalotiopsis* (Neopestalotin D, Pestalotiopsone B). Among bacteria, they were reported in *Bacillus* (Bacillamidin G) and actinobacteria (*Streptomyces*-derived compounds such as Salfredin A7). Of the 102 compounds of microbial origin, 28 were detected in all acorns, so we could be dealing with a part of the endophytic microbiome of *Q. ilex* acorns (Table S6).

For some of the annotated compounds, biological activity has been reported in the literature (Table S7). These metabolites include a wide range of simple phenolic compounds, tannins, flavonoids, and terpenoids, known for their potential nutritional and therapeutic benefits. Notable examples include ellagic acid, resveratrol, taxifolin, epigallocatechin, caffeic acid and derivatives. Some of them were detected in all samples (vanillin, syringic acid, ellagic acid, brevifolin carboxylic acid, (-)-Epigallocatechin, 3,4,2′,4′,6′-Pentahydroxydihydrochalcone, cuminaldehyde, oleamide, and secologanin), but some of them were specific to some individuals, as we mentioned above and is also shown on Table S11.

4. Discussion

In the last decade, there has been a renewed interest in the alimentary and industrial uses of *Quercus* acorns (Vinha et al., 2016a, 2016b; Chao et al., 2017; Szabłowska and Tanska, 2024; Inácio et al., 2024). This trend is aligned with the United Nations Sustainable Development Goals (SDGs), which promote the search for food alternatives to conventional crops—a pursuit driven by key factors such as sustainability, nutrition, food security, and environmental protection. The use of *Q. ilex* for human consumption is justified and well-supported, considering its utilization since prehistoric times, its nutritional and nutraceutical

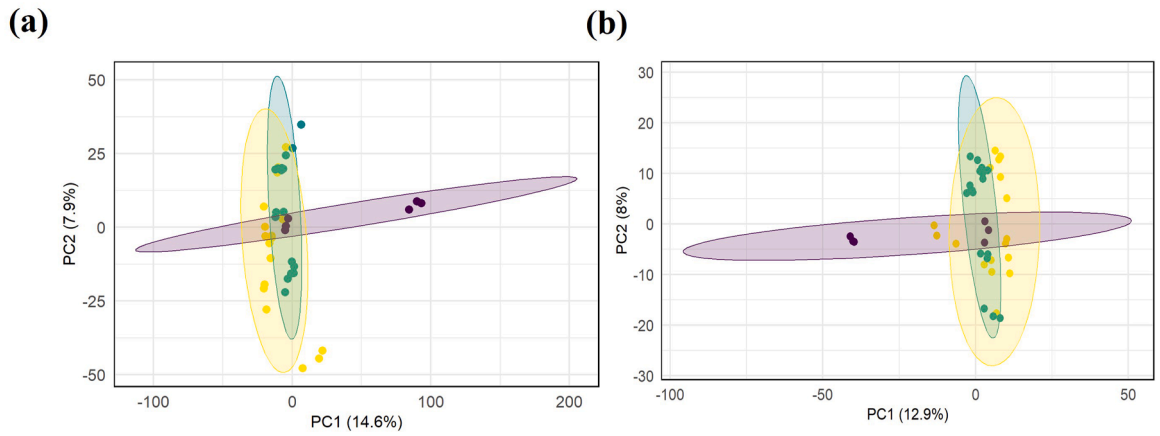

Fig. 2. Principal component analysis plot of proceed (a) and annotated (b) dataset obtained by UHPLC-MS/MS in fourteen individuals of *Q. ilex*. Bitterness levels are represented by colors: high bitterness is indicated in purple, medium bitterness in blue, and low bitterness in yellow.

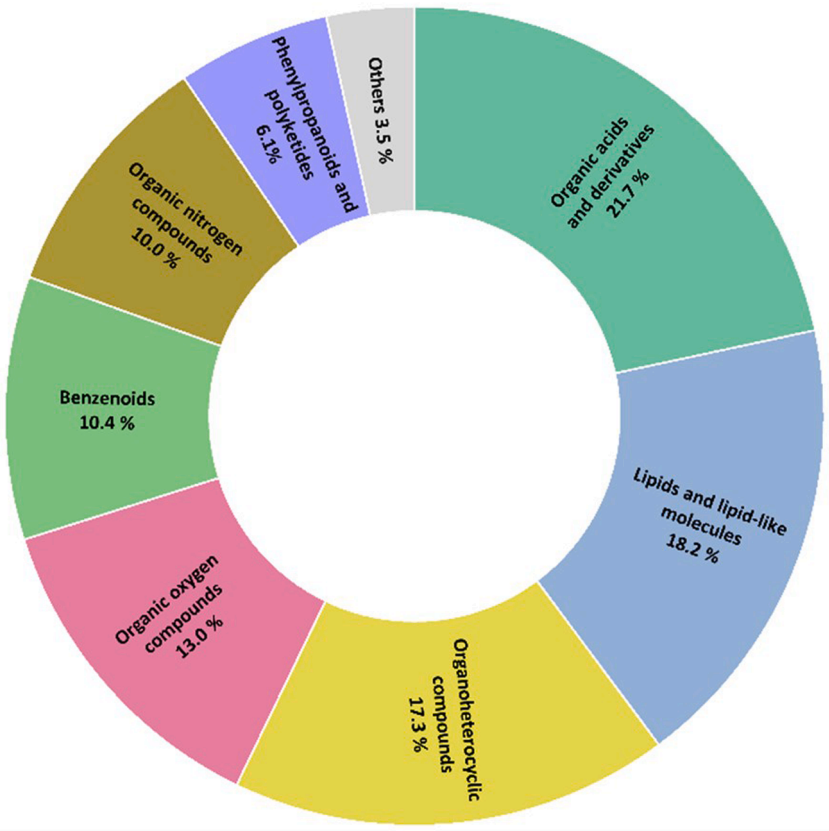

Fig. 3. Annotated compounds commonly observed in the fourteen *Q. ilex* individuals. All of them were classified into the main super chemical classes as established using the ClassyFire server.

properties, and its current underexploitation beyond its primary use in animal feed (García-Gomez et al., 2017; Burlacu et al., 2020; Martins et al., 2022).

A comprehensive phytochemical analysis is essential for any novel food product, for various scientific, regulatory, and health-related reasons, as well as for variability studies and traceability purposes. Such research is crucial not only to assess the nutritional value of the food, but also to identify nutraceutical compounds, as well as potentially toxic or allergenic substances. In this context, the present study reports on the phytochemical analysis and metabolomic profiling of acorns collected from 14 individual *Quercus* trees located in different Spanish areas and provided by the owners of *dehesa* lands (Table 1). The collection includes acorns exhibiting varying levels of bitterness (low, medium, and high), as well as different sizes (small, medium, and large) (Table 1 and Table 2). Multiple methodological approaches were employed, including Near-Infrared Spectroscopy (NIRS), classical biochemical assays based on colorimetric reactions, HPLC-RID, macro- and micronutrient analysis, and UHPLC-MS/MS. The analysis included the following parameters: carbohydrates (starch, total soluble-free sugars, soluble sugars, sucrose, glucose, and fructose), fats and fatty acids (oleic, linoleic, palmitic, stearic acids), proteins, total amino acids, phenolic compounds (total, flavonoids, condensed tannins), macro- and micronutrients, and whole metabolome analysis.

4.1. Variability in acorn morphometry and phytochemical parameters between individual trees

Although the phytochemical parameters reported for *Q. ilex* acorns in this study fall within the range previously documented in the literature (Valero-Galván et al., 2012; López-Hidalgo et al., 2021a; Martins et al., 2022), the general conclusion is the high variability observed in acorn morphometry and chemical composition. This is a common feature of non-domesticated, allogamous (cross-pollinated) species and is particularly evident in *Q. ilex* at morphometric, physiological, and molecular levels, with even greater variability found within populations than between them (Sánchez-Vilas and Retuerto, 2007; Valero-Galván et al., 2012; López-Hidalgo et al., 2021a; San Eufrasio et al., 2021).

The contribution of genetic, epigenetic, and environmental factors to this variability remains unknown and is still speculative. Experimentally addressing this question is challenging. *Q. ilex* has a long-life cycle, and paternal lineage cannot be determined, as only the maternal parent is known. Additionally, there are no optimized techniques for controlled self-pollination in this species.

There were statistically significant differences in all the phytochemical parameters, except for Fe and Ca. In this context, we can conclude that each individual tree exhibits a specific chemical signature, which appears to be more genetically than environmentally determined. Thus, there must be a specific genotype for different purposes, alimentary or industrial, based on the abundance of specific compounds, starch, fatty acids, phenolics, and so on. According to the PCA and cluster analysis (Fig. 1a-c), it is evident that the chemical profiles of acorns can be more similar between trees from different locations than between neighbouring trees. This finding suggests that chemical composition in acorns is influenced by factors beyond simple geographic proximity, such as genetic variation or microenvironmental conditions. Consequently, efforts to select superior trees for nutritional or bioactive properties should incorporate comprehensive chemical profiling rather than relying solely on location-based assumptions. Moreover, conservation strategies should consider this chemical diversity to maintain valuable traits across populations. PCA and the clustering dendrogram clearly establish two main groups corresponding to small size and high bitterness, and medium-large size and medium-low bitterness. In this regard, individuals 13 and 14—both small and highly bitter—stand out for their distinct characteristics, including the lowest fat content (characterized by low oleic and high stearic acid levels), the highest phenolic content, and the lowest energy, digestibility, and fibre values. While they may not be ideal for consumption, their characteristics make them potentially useful for the starch or tannin industries. In fact, the combination of both compounds makes it ideal for its exploitation in bioethanol production (Chao et al., 2017). Individual 10, with starch values close to 70 %, may also be of interest to the food industry to produce flour, thickeners, and fermented beverages such as kefir (Sardão et al., 2021).

The identification of trees producing sweet acorns is mostly known by owners and local people (Soddu Pirellas et al., 2024). In general, *Q. ilex* acorns are characterized by their bitterness, which is mainly associated with high tannin content, considered one of their most common antinutritional factors (Makkar and Becker, 1994; Bhat et al., 2013). While acorn sweetness is commonly associated with increased sugar content and reduced phenolic levels (Igueld et al., 2015), the results presented here do not confirm such a relationship. Thus, very sweet acorns do not always show the highest and lowest sugar and phenolic values, respectively. For example, individual 14, despite its high bitterness, showed one of the lowest tannin levels, whereas individual 1, with low bitterness, exhibited the highest tannin content.

4.2. Phytochemical analysis and nutritional value as compared with other nuts

Today, the inclusion of nuts in the human diet is widely endorsed for their nutritional and nutraceutical value (Gonçalves et al., 2023). When compared with other commercially valuable nuts, *Q. ilex* acorns exhibit some unique and interesting characteristics that highlight their nutritional value (Table 5). Their energy content (444–508 kcal) falls within the mid-range reported for other nuts (385–750 kcal), while their fibre content (2.2–2.6 %) is on the lower end (typically 4–8 %). Given that dietary fibre is inversely associated with obesity, diabetes, and cardiovascular diseases, breeding programs aimed at increasing the fibre content of *Q. ilex* acorns should be considered.

The total carbohydrate content of *Q. ilex* acorns (30–43 %) is higher than that reported for most other nuts, except for chestnuts, which have an exceptionally high carbohydrate content (around 80 %). Starch is the most abundant carbohydrate in nuts, although the proportions of amylose and amylopectin vary between species, influencing both digestibility and flour properties (Li et al., 2022). These specific starch characteristics have yet to be investigated in *Q. ilex* acorns. The content and relative proportions of sucrose, glucose, and fructose also vary by species, with typical ranges of 2–5 % for sucrose, 1–6 % for glucose, and 1–5 % for fructose (Kazankaya et al., 2008). In *Q. ilex* acorns, sucrose and fructose presented the highest and lowest values, respectively, at approximately 2.03–9.73 % and below 0.65 %. Interestingly, we found a correlation between higher glucose content in very bitter acorns, while sucrose and fructose content were associated with sweeter acorns.

The protein and total lipid content of *Quercus ilex* acorns are relatively low (5.6 % and 11.9 %, respectively) compared to that of other nuts, such as almonds (26.2 % protein and 50.2 % lipids) and peanuts (23.2 % protein and 43.3 % lipids) (Table 3). A notable advantage of *Q. ilex* acorns is that, in addition to being a gluten-free food, they differ from many other nuts in that allergic reactions to *Q. ilex* acorns are rarely reported (Hernández-Lao et al., 2024). Particularly noteworthy is their fatty acid profile, which closely resembles that of olive oil (Ferreira-Dias et al., 2003), with oleic acid and linoleic acid together accounting for >60 % of the total fatty acids.

Nuts provide a plant-based source of many essential macro- and micronutrients, making them especially valuable in plant-based diets (Donaldson, 2024). Generally, nuts contain high levels of K (0.5–1.8 g/100 g) and Mg (0.2–0.5 g/100 g (Wojdyło et al., 2022), with *Q. ilex* acorns falling within the mid-range for K, but below the typical range for Mg (Table S1). Remarkably, *Q. ilex* acorns have a relatively high Ca and Na content, approximately, 10 mg/100 g each. The elevated Ca content may confer benefits for bone health and metabolic functions, highlighting their potential as a valuable dietary source of this essential mineral (Vannucci et al., 2018). However, the Na content warrants caution, as excessive sodium intake is associated with increased cardiovascular risks (Wang et al., 2020). Therefore, while *Q. ilex* acorns represent a promising nutritional resource, their consumption should be balanced, particularly for individuals with hypertension or cardiovascular concerns, to maximize benefits without adverse effects. Levels of Fe, Cu, Zn, and Mn, meanwhile, are comparable to those found in other nuts (Table 5; Wojdyło et al., 2022).

4.3. Metabolomic analysis, variability and nutraceutical value

Metabolomics has emerged as a powerful technique for the analysis of nuts and other plant-based foods, offering comprehensive insights into their biochemical composition and relationship with nutritional and nutraceutical value, organoleptic properties, as well for safety and traceability purposes. Unlike other nuts (Kang and Suh, 2022), there are very few publications on the metabolomic analysis of *Quercus* acorns (López-Hidalgo et al., 2018, 2021a). Therefore, the present work is one of the most in-depth studies of the *Q. ilex* metabolome reported to date.

The raw dataset processed resulted in 4811 resolved metabolic features, of which 1189 were putatively annotated. As with phytochemical analysis, metabolomic analysis reveals the variability between individuals. A total of 759 and 308 annotated metabolites showed qualitative (absent in at least one individual) and quantitative (present in all

Table 5
Comparison of the nutritional profile of the most consumed nuts (data taken from the USDA, https://fdc.nal.usda.gov/, accessed 6 March 2025) and from the analysis of acorn frout carried out in the laboratory, using NIRS technology. Total phenolic compounds content has been taken from the Phenol-Explorer database (http://phenol-explorer.eu/foods, accessed 6 March 2025).

Portion: 100 g	Acorn	Peanut	Cashew	Chestnut	Hazelnut	Macadamia nut	Brazil nut	Pecan Nut	Pine nut	Pistachio	Walnut	Almond
Energy (Kcal)	444 - 508	588	565	385	641	712	664	750	689	598	730	622
Fiber, total dietary (g)	2.2 – 2.6	8.0	4.1	8.7	8.4	7.6	6.0	5.8	3.9	7.0	5.2	9.3
Carbohydrate, by difference (g)	30.5 – 42.7	26.5	36.3	80.4	26.5	24.1	21.6	12.7	18.6	27.7	10.9	16.2
Protein (g)	2.6 – 7.5	23.2	17.4	5.2	13.5	7.7	15	9.9	15.7	20.5	14.6	26.2
Total lipids (g)	4.5 – 17.5	43.3	38.9	4.64	53.5	64.9	57.4	73.3	61.3	45.0	69.7	50.2
Fatty acids, total saturated (g)	20.8 – 33.9	n.a	n.a	0.689	n.a	n.a	n.a	6.6	n.a	n.a	6.0	n.a
Fatty acids, total monounsaturated (g)	55.4 – 77.2	n.a	n.a	1.26	n.a	n.a	n.a	39.3	n.a	n.a	9.6	n.a
Fatty acids, total polyunsaturated (g)	3.7 – 20.7	n.a	n.a	1.52	n.a	n.a	n.a	22.9	n.a	n.a	49.3	n.a
Calcium, Ca (mg)	97.8 – 131.4	49	42	56	135	53	168	55	9	117	88	232
Iron, Fe (mg)	2.3 – 3.6	1.5	5.9	1.6	3.4	1.8	2.4	2.3	5.3	3.4	2.2	3.2
Magnesium, Mg (mg)	1.4 – 2.7	180	251	68.8	156	107	351	103	206	110	142	251
Phosphorus, P (mg)	448.8 – 776. 7	380	532	134	321	208	707	253	540	500	365	512
Potassium, K (mg)	817.9 – 1249.8	636	638	1030	636	373	592	360	655	947	424	667
Sodium, Na (mg)	5.6 – 32.9	<2.5	5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5
Zinc, Zn (mg)	0.6 – 1.5	2.7	5.0	1.0	2.3	1.2	3.8	3.9	5.7	2.1	2.7	2.8
Copper, Cu (mg)	0.4 – 1.1	0.4	2.2	0.5	1.4	0.4	1.7	0.9	1.1	1.0	1.2	0.9
Manganese, Mn (mg)	1.1 – 8.1	1.6	1.9	2.9	6.0	11.3	1.2	2.2	10.2	1.0	3.0	1.8
Total Phenolic Compounds Content (Folin method) (mg)	914 - 2585	406	232	2756	671	126	244	1284	58	1420	1574	287

n.a indicates data were not available.

individuals but with statistically significant differences, $p < 0.05$) variation between individuals, respectively. Only 227 of the metabolites annotated were found in all fourteen individuals (Table S5), constituting the characteristic *Q. ilex* metabolome under the analytical approach used. Some of metabolites annotated were previously reported in other species of the *Quercus* genus, in general, and in *Q. ilex*, in particular, such as epigallocatechin, ellagic acid, acetophenone, syringic acid, and piceol (Dar and Ikram 1979; Karioti et al., 2011; Nones et al., 2022; Hernández-Lao et al., 2024). To our knowledge, some of them were first detected in *Q. ilex*, such as apocynin, secologanin, 3,4,2′,4′,6′-pentahydroxydihydrochalcone (also known as 3-Hydroxyphloretin), and brevifolin carboxylic acid. (Boshtam et al., 2021; Li et al., 2024, 2012; Xu et al., 2023; Fan et al., 2024), supporting the enormous variability of secondary metabolites in *Q. ilex* species. Other compounds were individual-specific, such as cyrtominetin, which was observed in individual 4 nothofagin, in individuals 8, 11, 12 and 13; p-cymene, in individuals 3 and 11; and scoparone, in individual 13 (Table S7). Those tree-specific compounds could be proposed as molecular markers in plant breeding programs.

The PCA analysis was unable to explain the variability between individuals (variability of PC1 plus PC2 of 14.6, and 7.9 %) and did not result in a clear separation, except for in individual 13. The compounds contributing to the variability of individual 13 have not been yet annotated in available databases, and others were annotated as microorganism-specific metabolites, which will be discussed in the following section. A separation based on geographical coordinates has not been found, so it is supposed that genetic, more than the environment, determines the metabolome.

Due to its relevance to the nutraceutical value of acorns (Bolling et al., 2010; Yang et al., 2009), the following section focuses on the phenolic group of metabolites. The total phenolic content is characteristic of each nut species, even though great variability has been found between genotypes (Wojdylo et al., 2022). *Q. ilex* acorns, along with chestnuts, has the highest phenolic content, with values ranging from 0.9 % to 2.5 %. (Table 3) (Kornsteiner et al., 2006). As previously shown (Makhlouf et al., 2019), phenolic content is correlated with antioxidant activity, which is one of the most beneficial characteristics of foods in terms of their cardioprotective, antitumoral, antiallergic, antimicrobial, and antiinflammatory effects (Cook and Samman, 1996; Silva et al., 2016; Taib et al., 2020; Martins et al., 2022). The *Quercus* genus, in general, and *Q. ilex*, in particular, are species rich in flavonoids and condensed tannins, as also reported for other nuts (Wojdyło et al., 2022; Socaciu et al., 2023), which is confirmed in the present study. Thus, in between 0.5–4.0 (flavonoids) and 0.2–1.5 (condensed tannins) mg/g DW have been found. Both groups are relevant from an alimentary point of view because of their biological activities, flavonoids, and anti-nutrititive, and astringent properties. In terms of plant breeding programmes, and for alimentary purposes, sweet, big acorns with high levels of flavonoids and low tannin levels are desirables, as is the case with individuals 4 and 6. In contrast, for the tannin industry individual 1 seems more appropriate.

Several of the compounds annotated exhibit biological activities previously reported in the literature, as summarized in Table S7. These metabolites encompass a wide range of simple phenolic compounds, tannins, flavonoids, and terpenoids, all known for their potential nutritional and therapeutic benefits. Notable examples such as vanillin, trans-caffeic acid, ellagic acid, resveratrol, and trigonelline have demonstrated antioxidant, anti-inflammatory, or other bioactive effects in vitro or in vivo according to previous studies. However, these activities were not experimentally confirmed in the present work.

4.4. Metabolomics analysis and acorn microbiome

One of the most surprising findings in the metabolomic analysis was the identification of many complex metabolites, up to 102, originating from fungi or bacteria (Table S6). These compounds have been previously reported in species such as *Aspergillus* spp., *Penicillium* spp., *Bacillus* spp., and *Streptomyces* spp., among others. Their number and relative abundance varied between acorns from different trees. It is noteworthy that individual 13 exhibited a significantly different metabolomic

profile, marked by the prominent presence of these microbial metabolites. Thus, we can hypothesize that the presence of such compounds reveals the existence of a *Q. ilex* acorn endophytic microbiome, which could have an impact on the quality and nutritional properties of the fruit. Presumably, there was no contamination, as the acorns were sterilized twice, before and after eliminating the pericarp to prepare the flour. The endophytic seed microbiome has been studied in *Q. petraea* acorns from a heritability point of view, but not from a nutritional composition perspective (Fort et al., 2021). Also, Magaña Ugarte et al. (2024) reported that microorganisms present on the surface of *Q. ilex* seeds significantly influence germination, but nothing is known yet about the internal microbiome of the *Q. ilex* acorn. Bilous et al. (2023) demonstrated in *Q. robur* that the endophytic bacteria of the genus *Bacillus*, isolated from the internal tissues of unripe acorns, could inhibit the proliferation and transmission of phytopathogens, highlighting their potential as biological control agents in sustainable pest management strategies.

While the microbiomes of roots, mostly rhizosphere and phyllosphere, are well studied, that of seeds remains largely unexplored. The extent to which the acorn microbiome is actively or passively recruited, and the bases of the symbioses, calls for further research. It is well established that microorganisms play a crucial role in germination, growth, stress responses, and, ultimately, plant survival (War et al., 2023). The importance of the seed microbiome is revealed by recent studies revealing evidence of microbe inheritance across plant generations, including transmission from pollen and flower tissues to other growing organs, even seed to seed (Cardinale and Schnell, 2024; Abdelfattah et al., 2021, 2023).

5. Conclusions

Acorn flour from *Q. ilex* is considered a food with good nutritional and nutraceutical characteristics, warranting the revival of its use in human nutrition. A detailed phytochemical analysis using NIRS, colorimetric methods, simple sugars, antioxidant capacity, minerals, and non-targeted metabolomic analysis (UHPLC-MS/MS) was described in fourteen *Q. ilex* trees varying in size and bitterness. These techniques enable us to support their labelling for human consumption, as well as to facilitate their traceability. Phytochemical analysis, together with morphometric parameters, revealed groups of acorns with distinct characteristics of interest to the food industry. Despite the great variability observed between the individual trees, acorn flour was rich in starch, fats, proteins, and minerals (Ca, Fe, P, K, and Na) and exhibited a high antioxidant capacity. Their oleic acid content was similar to that of olive oil, while the flour remained free of celiac-related proteins. Regarding bitterness level, total phenolic content, stearic acid, glucose, antioxidant capacity, and certain nutrients (K, Na, Ca, and Mn) were positively correlated with higher bitterness in acorn flour. In contrast, lower bitterness levels were more closely associated with higher morphometric parameters, sucrose, oleic acid, tannins, fat and protein content. Complementing their phytochemical profiling, their metabolite profiling revealed similarities between all the individuals, except for one (individual 13), which was richer in fungi and bacteria content. This indicates the important role of the microbial community as a parameter to be considered in the classification of acorn flour. A total of 35 compounds with previously reported biological activity were annotated, highlighting the rich nutraceutical profile and potential health benefits of acorn flour. Although many studies have described the chemical profile of *Q. ilex* acorn flour, few have employed such a wide range of analytical techniques as this work. Therefore, this work could serve as the molecular basis for the cataloguing of acorn flour, establishing the first acorn flour database, with the potential to be expanded with data from other *Q. ilex* individuals, or even other *Quercus* species.

CRediT authorship contribution statement

Marta Tienda-Parrilla: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cristina López-Hidalgo:** Writing – review & editing, Methodology, Formal analysis. **Mónica Sánchez-Parra:** Writing – review & editing, Methodology. **José Luis Ordóñez-Díaz:** Writing – review & editing, Methodology. **José Manuel Moreno-Rojas:** Writing – review & editing, Methodology. **María-Ángeles Castillejo:** Writing – review & editing, Investigation, Funding acquisition. **Jesús V. Jorrín-Novo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization. **María-Dolores Rey:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

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Ethical statement

This work did not involve animal or human studies for experimentation.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fufo.2025.100754.

Data availability

Data will be made available on request.

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