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Metabolic profiling of pea (*Pisum sativum*) cultivars in changing environments: Implications for nutritional quality in animal feed

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ABSTRACT

Field pea seeds have long been recognized as valuable feed ingredients for animal diets, due to their high-quality protein and starch digestibility. However, the chemical composition of pea cultivars can vary across different growing locations, consequently impacting their nutrient profiles. This study employs untargeted metabolomics in conjunction with the quantification of fatty acids and amino acids to explore the influence of three different growing locations in Spain (namely Andalusia, Aragon and Asturias), on the nutritional characteristics of seeds of various pea cultivars. Significant interactions between cultivar and environment were observed, with 121 metabolites distinguishing pea profiles. Lipids, lipid-like molecules, phenylpropanoids, polyketides, carbohydrates, and amino acids were the most affected metabolites. Fatty acid profiles varied across locations, with higher C16:0, C18:0, and 18:1 n-9 concentration in Aragón, while C18:2 n-6 predominated in Asturias and C18:3 n-3 in Andalusia. Amino acid content was also location-dependent, with higher levels in Asturias. These findings underscore the impact of environmental factors on pea metabolite profiles and emphasize the importance of selecting pea cultivars based on specific locations and animal requirements. Enhanced collaboration between research and industry is crucial for optimizing pea cultivation for animal feed production.

Keywords

Pea Seeds, FAME, Amino Acids, Animal feed, Metabolomics

Highlights

- There was a clear cultivar × environment interaction on pea metabolic profiles.

- Untargeted metabolomics revealed 121 significant metabolites, shaping the clustering of selected cultivars in a specific location.
- Lipids and lipid-like molecules, phenylpropanoids and polyketides (flavonoids, isoflavonoids), carbohydrates and amino acids are the most differential metabolites.
- Palmitic, stearic, oleic, linoleic, and α-linolenic are the most abundant fatty acids.
- Glutamic and aspartic acids were the most abundant amino acids. Methionine and cysteine demonstrated the lowest concentrations.
- Overall, cultivars grown in Asturias exhibit the highest amino acid content.

1. Introduction

Legumes, members of the Fabaceae family, are characterized by their notable ability to fix atmospheric nitrogen through symbiotic interactions with nitrogen-fixing bacteria, which are crucial for the sustainability of agricultural practices. Moreover, they offer a rich source of high-quality plant protein for human and animal consumption (Rubiales et al., 2021). Notably, there is a growing interest in dry peas as a functional ingredient in the food industry (Shanthakumar et al., 2022; Windsor et al., 2024).

Indeed, they are characterized by contents of moderately high crude protein (CP; 17.9 - 24.1%), low crude fat (CF; 1.5 to 3.7%), and neutral detergent fiber (NDF; 11-22 %) and a high starch content (42%), and they are highly digestible (Crépon, 2007). Like other legume grains, the protein is rich in lysine but deficient in sulfur-containing amino acids and tryptophan.

The characteristics of pea oil vary between genotypes, being some of them rich in poly-unsaturated fatty acids (Ω-3, Ω-6), that could become a dietary source contributing to animal health, physiological functions, and maintenance (Solis et al., 2013). Moreover, legumes, including peas, are rich in bioactive specialized metabolites, encompassing several classes of natural compounds. While beneficial for animal health, for instance, condensed tannins may positively impact animal health by preventing bloat, enhancing livestock protein utilization, and reducing the internal parasite burden in monogastric livestock.

Metabolomics has proven effective in exploring the metabolic features of food resources influenced by environmental and genetic factors, enabling the assessment of food quality (Harrigan et al., 2007; Kim et al., 2016; Utpott et al., 2022).

The metabolite composition of legumes and crops, in general, can be influenced both by the genotype and the growing conditions. There is some understanding of the influence of the

genotype on quality (Wang et al., 2004; Kwon et al., 2012; Hacisalihoglu et al., 2021), but less so on environmental effects and the genotype x environment interactions (Amarakoon et al., 2015; Maharjan et al., 2019; Pant et al., 2021; Wang, 2023). Recently, 107 Portuguese common bean accessions, cropped under contrasting environments, were analyzed by Mecha and co-authors using untargeted and targeted mass spectrometry approaches. The analysis revealed a clear genotype × environment interaction was also detected (Mecha et al., 2022). Addressing the impact of these factors on nutritional quality and, also, adaptation to climate change, especially in Europe is crucial for ensuring sustainable animal production, environmental stability, and farmers' economic prosperity (Solomon, 2022).

In this framework, this study aimed to understand how the nutritional values of different cultivars of peas could be affected by three contrasting growing locations in Spain. Insights in the post-harvest nutritional assessment are essential for aligning pea production with the specific nutritional requirements of feed manufacturers and optimizing formulations for animal nutrition to meet market demands. Hence, eight different pea cultivars were selected, and their chemical composition initially studied by an untargeted metabolomics approach utilizing liquid chromatography coupled with high-resolution tandem mass spectrometry to analyse a broad spectrum of metabolites. Subsequently, nutritionally significant fatty acids and amino acids were quantified using chromatography.

2. Material and Methods

2.1 Plant material and growth condition

The study included eight cultivars (cv.): Avenger, Chicana, Forana, Furious, Jarana, Karacter, Karpate, and Tirana. Samples of peas cultivated in three regions with highly diverse agro-climatic conditions were analyzed: Andalusia, Aragon and Asturias. The Andalusian samples were provided from field trials performed at the IAS-CSIC farm at Córdoba. During the growing period, the average temperature was 14.1 °C, reaching a maximum of 40.6 °C and a minimum of -1.2 °C, with a total rainfall of 313.0 mm. Pea samples from Aragon were collected from field trials performed at Lupiñén (Huesca) grown under an average temperature of 11.4 °C, a maximum of 39.7 °C, a minimum of -2.4 °C, and a total rainfall of 240.9 mm. The Asturian samples were provided from field trials performed at SERIDA experimental farm in Grado (Asturias). The average temperature during the growing period was 11.3 °C, with a maximum of 29.0 °C and a minimum of -1.7 °C, and a total rainfall of 410.5 mm. All the information about locations, crop management and yields obtained in the trials are available on the website of Go Inpulse project (https://goinpulse.com/wp-content/uploads/2022/09/Informe-Provisional-Tarea-4-vdef.pdf).

2.2. Extraction protocol for untargeted metabolomics analysis

Three mature, dried seeds from each selected cultivar in the different environments, taken from each biological replicate were collected and milled using a Ball Mill (MM 400 Retsch, Haan, Germany) to a particle size of 0.8 mm and stored at - 20 ◦C, until further analysis. The milled pea bean seeds were extracted in triplicate (technical replicates) following previously reported protocols, with slight modifications (Brigante et al., 2020; Mecha et al., 2022). Briefly, 2 mL of MeOH: water + 0.1% FA (70:30, v/v) solution was added to 400 mg of dry whole-seed flour, followed by a 30-second vortex and sonication for 30 min. After centrifugation at 420×g for 10 min, the supernatant was collected in a new tube, dried, and kept at - 20 ◦C, until analysis.

2.3. UHPLC- QTOF-HRMS apparatus and conditions

Before analysis, the dried extracts were reconstituted in 1 mL MeOH: water + 0.1% FA (70:30, v/v) and filtered through Millex syringe filters of 0.2 µm (Merck, Darmstadt, Germany). Umbelliferone, the internal standard (IS), was spiked into every solution at a final concentration of 10 µg/mL. Finally, 50 µL of each extract was taken to create three pooled quality control (QC) samples.

The UPLC-QTOF-HRMS analysis was carried out by the Metabolomics Platform of Agricultural Sciences, Food Science and Technology and Natural Resources (CEBAS)-CSIC, Murcia, Spain. In details, chromatographic separation was realized using Acquity UPLC- I-class system (Waters Corporations, USA). In QTOF-HRMS analysis, 7 µL of the organic extract was injected using a Sample Manager Fixed-Loop (SM-FL) (Waters Corporations, USA). Chromatographic separation was performed on Poroshell 120 EC-C18 Agilent column (100 × 3 mm, 2.7 µm, (Agilent Technologies, Waldbronn, Germany) operating at 30 °C and a flow rate of 0.4 mL/min in an Acquity I-Class column oven systems (Waters Corporations, USA). Compounds were separated using the following gradient conditions using H2O + 0.1% FA (A) and ACN + 0.1% FA (B): 0–10 min, 1–18 % phase-B; 10–16 min, 18–38 % phase-B; 16–22 min, 38–95 % phase-B. Finally, the phase Bcontent was returned to the initial conditions (1 %) for 1 min and the column re-equilibrated for five more min. Software Compass HyStar (version 3.2 Bruker Daltonics, Bremen, Germany) was used for the operation of the UHPLC systems. The pooled QCs were used for metabolomic analysis quality (**Fig. S1**). QCs, blanks and umbelliferone solution (1 µg/mL) were injected three times during the batch: beginning, middle and end. maXis Impact QTOF mass spectrometers

(Bruker Daltonics, Bremen, Germany) were utilized for QTOF-HRMS experiments. Ionization in the mass spectrometers was performed using an ESI source (Bruker Daltonics, Bremen, Germany), which were each operated under optimized conditions. The parameters for ESI source were set as follows: ionization was performed in the negative mode at −4.0 kV. Dry gas temperature was set to 200 °C at a flow rate of 9.0 L/min. Nebulizer gas pressure was 2 bar. The ESI ion source was operated at -4.0 kV with a probe gas temperature of 450 °C at a flow rate of 4 L/min. The dry gas temperature was set to 300 °C at a flow rate of 9.0 L/ min. The nebulizer gas pressure was 2 bar. A mass range of 50−1200 m/z was covered, and the full scan and MS2 data were recorded at a spectra rate of 2 Hz. Data-independent acquisition in the broadband collision-induced dissociation (bbCID) mode was chosen for MS/MS experiments. Fragmentation took place in a collision-induced dissociation cell using nitrogen. Spectral acquisition was performed at collision energy (CE) of 20 eV. In order to calibrate the mass axis, a 10 mM sodium formate cluster solution in 1:1 isopropanol-water was introduced into the ESI source at the beginning of each UHPLC run using a divert valve for instrument mass calibration and for re-calibration of individual raw data files. Software Compass otofControl (software version 3.4, Bruker Daltonics, Bremen, Germany) was used for the operation of the mass spectrometer and for data acquisition.

2.4. Analytical protocols for fatty acids and amino acids analysis

The mature dried seeds of the viable plants were collected and milled using a Rotary Mill (ZM200 Retsch, Germany) at 0.2 mm. All analyses of the chemical composition of pea grains were conducted in duplicate. Fatty acids were determined as fatty acid methyl esters using gas chromatography with flame ionization detection based on the method of Sukhija & Palmquist (1988) and Lee and coauthors (2012), after optimization of the process (Baila et al., 2023). For this purpose, the Bruker Scion 436-GC-FID gas chromatograph (Bruker, Billerica, USA) equipped with the CP-8400 autosampler (Bruker), HP-88 capillary column (100 m x 0.25 mm x 0.2 μm, Agilent, Santa Clara, USA), and all controlled by CompassCDS software (Bruker) were used. The FAME identifications were based on their retention times that were compared with those of the standard FAME mixtures GLC-532, GLC-401, GLC-643 and GLC-642 (Nu-Chek Prep, Elysian, MN, USA) and quantification was performed using C19:0 as an internal standard as described in ISO 12966-4:2015.

The amino acid profile (except for tryptophan) was carried out by the CIB, Margarita Salas-CSIC, Madrid (Spain). It was determined by ion exchange liquid chromatography with absorbance detection. Samples underwent protein hydrolysis with hydrochloric acid, subsequent post-column derivatization with ninhydrin, and finally, detection by absorbance at 570 and 440 nm (proline) using the Biochrom 30 analyzer (Biochrom Ltd, Holliston, USA). Regarding cysteine

determination, this amino acid is destroyed during acid hydrolysis, then cys value corresponds to cystine in this analysis. The quantification of the amino acids was performed using norleucine as an internal standard. Tryptophan was determined by high-resolution liquid chromatography (CITA, Zaragoza, Spain) with absorbance detection based on a previously reported method (La Couret al. 2019). Samples underwent protein hydrolysis with lithium hydroxide, chromatographic separation and detection at 289 nm. Tryptophan quantification was performed using a commercial reference standard (T2610000, Merck). The Waters Acquity UPLC H-Class liquid chromatograph (Waters, Milford, USA) equipped with an Acquity UPLC HSS PFP column (100 mm x 2.1 mm x 1.8 µm, Waters), a photodiode array detector (PDA eλ Detector, Waters), a fluorescence detector (2475 Multi λ Fluorescence Detector, Waters), all controlled by Empower3 software (Waters), was used.

2.5. Metabolites annotation and identification

To fully exploit the differences in the metabolite profile obtained, three bioinformatic tools were integrated: 1) the raw LC-HRMS were converted in mzML files using MSConvert software (Proteowizard, Palo Alto, US) and then analyzed by the web-based tools MetaboAnalyst 5.0 (Pang et al., 2021); 2) MS-DIAL (Version 5.10)/ MS-FINDER (Version 3.5) (Tsugawa et al., 2015), the computational approach which helps to characterize the structure of the metabolites rapidly; 3) MetFreg (Ruttkies et al., 2019), freely available web software to assist the annotation of high-precision tandem mass spectra of metabolites by *in silico* fragmentation.

This approach includes three steps: Step 1: spectra processing and peak annotation with MetaboAnalyst 5.0. All the parameters are reported in **Table S1**. Features with higher 25% per cent of RSD in QC samples were filtered out. Feature with more than 50% of missing values in the samples were removed while missing values were estimated by sample wisesample-wise k-nearest neighbours (KNN) algorithm. Step 2: univariate and multivariate analysis of the global metabolites profile with MetaboAnalyst 5.0. Full details about this step are reported in section 2.6 (Data Analysis and Visualization). Step 3: structural annotation of the metabolites was performed with MS-DIAL/MS-FINDER and MetFrag. The metabolite ions were converted into structural information with MS-DIAL/MS-FINDER linked to MS/MS databases. The MS-DIAL parameters were MS1 tolerance of 0.01 Da; MS2 tolerance of 0.05 Da; minimum peak height of 1000 (amplitude); for alignment, a QC sample was used as a reference file, and the retention time tolerance was set at 0.05 min. The MS/MS public databases used for peak identification were MSMS-Neg-MassBankEU, MSMS-Neg-GNPS, MSMS-Neg-MassBank, MSMS_Public_EXP_NEG_VS17, MSMS_Public_ExpBioInsilico_NEG_VS17, MSMS-Neg-

Vaniya-Fiehn_Natural_Products_Library, the identification score cutoff value was selected as 8. Significant metabolites with monoisotopic mass error within ±5 mDa with no proper match in the selected databases were manually screened for mass spectral peak matching. The molecular formulas were searched on COCONUT (Sorokina et al., 2021), LOTUS (Rutz et al., 2022) and HMDB (Wishart et al., 2022) database, SDF files were generated from the above-cited database and uploaded to MetFrag to identify the metabolites within silico fragmentation. The complete list of identified/annotated metabolites and confidence levels are reported in the **Table S2**. Careful manual curation of all assigned peaks was carried out, and the metabolites were annotated according to confidence levels A-D, as described in **Table S2** (Alseekh et al., 2021; Bulut et al., 2023; Sumner et al., 2007).

2.6. Data analysis and visualization

Datasets from the untargeted metabolomics analysis were analysed using the statistical module of MetaboAnalyst 5.0 (Pang et al., 2021). Data were normalised by the internal standard umbelliferone, and specific transformation and scaling conditions for the data sets are reported in **Table S3**. The dataset was analyzed with principal component analysis (PCA) to explore potential patterns and heatmaps that could show clustering of the features and visualize the differences between cultivars. The top 20 (ANOVA $p < 0.05$) most significant features were selected for structural annotation. Potential differences in the dataset, using environments as a discriminant class, were investigated using Volcano plot using p-value corrected for FDR < 0.05 and Fold Change (FC) > 5 as thresholds. Moreover, PLS-DA was carried out to sharpen groups' separation and reveal the global profile changes and potential application for biomarkers discovery according to metabolite composition. The VIP score cutoff value was 1.5. Cross-validation was carried out by 5-fold CV; Accuracy, R2 and Q2 values are reported in **Table S3**.

The contents of fatty acids and amino acids were analyzed using SAS software (v.9.3; SAS Inst. Inc., Cary, NC, USA). Analysis of variance with a general linear model considering the cultivar, the growing region and their interaction as fixed effects, was performed. Analysis of variance considering the cultivar as fixed effect was repeated separately for each growing region. The least-squares means and the standard errors were obtained, and Tukey's correction was used for pairwise comparisons. Differences were considered significant at $p < 0.05$. Heatmaps showing the clustering of fatty acids and amino acids and visualizing the differences between growing regions were generated by MetaboAnalyst 5.0.

3. Results

3.1. Metabolic diversity of dry seeds of different pea cultivars by untargeted metabolomics analysis

After processing the spectra, peak annotation and filtering of the LC-MS/MS data, both univariate and multivariate statistical analyses were carried out to identify the metabolites that exhibited significant alterations among the cultivars grown in specific regions and the metabolites that were significantly affected by the location factor.

3.1.1 Metabolic diversity of cultivars grown in Andalusia

The dataset was explored using PCA. The score plot, highlighting cultivar clustering is presented in **Fig. 1A**, while the box plots showing the metabolites that predominantly contribute to the clustering of cultivars, are presented in **Fig. S2**. The PCA analysis accounted for a total explained variation of 48.1%, (**Fig. 1A**). Along PC2, cv. Tirana and, to a lesser extent, cv. Jarana were separated from the other cultivars, indicating distinct metabolic profiles. The metabolites whose coefficients along PC2 primarily contribute to the differentiation of these cultivars include isopropyl citrate, a putative phenolic glycoside, a putative tetrahydroisoquinoline, naringenin, a putative isoflavone, and a putative pentose phosphate compound (**Fig. S2**). Higher levels of the first three metabolites were detected in cvs. Tirana and Jarana, while the latter three were downregulated in these cvs. Differences in metabolite content determined through analysis of variance of the top 20 metabolites contributing to the variations among cultivars are visualized in **Fig. 1B** using a heatmap. The heatmap also reveals distinct clustering patterns among the cultivars: Tirana and Jarana did not cluster with the other cultivars, while cvs. Avenger and Karpete were grouped. Cvs. Karacter, Chicana, Forana, and Furius constituted another cluster, with cv. Karacter positioned on the periphery of this group. Most differential metabolites belong to the carbohydrates and carbohydrate conjugate class. Putative hexosamines were found to have higher levels in cvs. Karacter, Chicana, Forana, and Furius. Cv. Tirana exhibited significantly higher concentrations of 3,4,5-trihydroxy-6-[(5-methoxy-1H-indol-6-yl)oxy]oxane-2-carboxylic acid and two phenolic glycosides. This cultivar also displayed the highest concentrations of the most significant metabolites across various metabolite classes including flavonoids, isoflavonoids, and coumarins. Lastly, cvs Avenger and Karpate exhibited higher relative concentrations of Chebulic Acid, UDP-L-threo-4-pentosulose, Epigallocatechin, and a putative coumarin, distinguishing them from the other cultivars.

3.1.2. Metabolic diversity of cultivars grown in Aragon

The PCA score plot, depicted in **Fig. 2A**, revealed that the total explained variation was 48.7%. Notably, along PC2, cv. Jarana exhibited a distinct separation from the other cultivars, suggesting unique whole metabolic profiles. Box plots reporting metabolites whose coefficients along PC2 are responsible for distinguishing cv. Jarana grown in Aragon are also reported in **Fig. S3** Among these metabolites, three remained unidentified, while the relative concentrations of two metabolites belonging to the class of amino acid, peptides, and derivatives specifically, 2-[(3,4-dihydroxy phenyl)formamide]pentanedioic acid and a putative oligopeptide were higher in cv. Jarana. Conversely, a metabolite belonging to the class of isoflavones displayed a lower level in this cultivar. The heatmap of the top 20 metabolites contributing to the variations among cultivars (**Fig. 2B)** also revealed that cv. Jarana did not cluster with the other cultivars; cvs. Avenger and Karpate were grouped; cvs. Tirana, Forana and Karacter clustered together, while cvs. Chicana and Furius showed similar metabolic profiles. Unfortunately, most of the metabolites remain unknown. However, the other differential metabolites belong to two main superclasses: phenylpropanoids and polyketides, which include cinnamic acids and derivatives and flavonoids, and lipids and lipid-like molecules, which include prenol lipids.

3.1.3. Metabolic diversity of cultivars grown in Asturias

The PCA score plot, depicted in **Fig. 3A**, revealed that the total explained variation was 45%. Consistent with the previous environments, cv. Jarana exhibited a different behaviour from the other cultivars. However, cvs. Avenger and Karpate notably separated from the other cultivars, suggesting unique whole metabolic profiles. Among the metabolites whose coefficients along PC2 are responsible for distinguishing the cultivars reported in the box plots in **Fig. S4**, two remained unknown, two metabolites belonging to the superclass of phenylpropanoids and polyketides: epigallocatechin and a putative coumarin were upregulated, while a putative hexosamine and a pyranochromene were downregulated. The heatmap revealed distinct clustering patterns among the cultivars: Tirana and Karacter did not cluster with the other cultivars, while Avenger and Karpate, consistent with the previous environments, were grouped. Chicana and Forana formed another cluster, and Jarana and Furius grouped together **Fig. 3B**. Apart from the unknown metabolites, most differential metabolites belong to the superclass of phenylpropanoids and polyketides, followed by benzenoids, and lipids and lipid-like molecules. Four metabolites were present at higher concentrations, exclusively in cvs. Avenger and Karpate. These include two flavonoids, (-)-Epigallocatechin and a putative furanoflavone, chebulic acid, a derivative of gallic acid, and a putative hydantoin.

3.1.4. Effect of environments on pea dry grains' metabolomics diversity

A pairwise comparison of location effects on the cultivars was done using a volcano plot. **Fig. 4A** showcases the differences between the cultivars grown in Andalusia and those in Aragon. The volcano plot highlights a total of 133 metabolites that were detected at lower concentrations in Andalusia, while only eight metabolites were upregulated. **Table S4** presents the top 10 downregulated metabolites and the eight upregulated metabolites, which were dereplicated and annotated. Four of them remained unknown. These metabolites represent five superclasses: lipids and lipid-like molecules, benzenoids, organic acids and derivatives, organic oxygen compounds, phenylpropanoids and polyketides.

Fig. 4B reports the differences between the cultivars grown in Andalusia and those in Asturias. The volcano plot highlights a total of 105 metabolites that were detected at lower concentrations in Andalusia than in Asturias, while only ten metabolites were upregulated. **Table S5** showcases the top ten downregulated and upregulated metabolites, which have been successfully dereplicated and annotated, four remained unknown. These metabolites span six superclasses, including benzenoids, lipids and lipid-like molecules, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides. Notably, the most prevalent superclass in this case was organic oxygen compounds, comprising five metabolites. Among them, all five belonged to the carbohydrates and carbohydrate conjugate class, with four metabolites exhibiting upregulation.

Fig. 4C illustrates the differences between the cultivars grown in Aragon and those in Asturias. The volcano plot highlights only three altered metabolites, one downregulated and two upregulated. Showing low metabolic differences among the cultivars cropped in the two regions. **Table S6** presents the altered metabolites, which were dereplicated and annotated, one remained unknown, and the other two belonged to the lipids and lipid-like molecule superclass.

PLS-DA was carried out to sharpen groups' separation and reveal the global profile changes and potential application for biomarker discovery according to metabolite composition in the different environments. The score plot and the heatmap reporting the 60 metabolites showing VIP scores higher than 1.5, the value selected as threshold, are shown in **Fig. 5**. The total explained variation considering three components was 55.6 %. Accuracy, Q2 and R^2 values of the model in cross-validation are reported in **Table S3.** Along PC1 cultivars grown in Andalusia are clearly separated on the left of the quadrant from those cropped in Aragon and Asturias. The latter two were only slightly separated and almost overlapped along PC 1. However, exploring the 3D score plot, 5.1 % of the variance was due to differences between Aragon and Asturias (**Fig. S5**). Nevertheless, the almost overlapping of these two groups was due to cv. Jarana, highlighting

similar behaviour, and thus a similar metabolic profile, of this cultivar in the two environments. Regarding the identified metabolites, most of the differential compounds, excluding the unknown metabolites, belong to the superclass of lipids and lipid-like molecules. They are followed by carbohydrates and carbohydrate conjugates and amino acids peptides and derivatives. Regarding the group clustering, most of the contributing metabolites were detected at lower concentrations in Andalusia, except nine which exhibited higher levels in this region (**Fig.5B**). Specifically, the metabolites benzoyl malic acid, N-acetyl phenylalanine, a flavonoid identified as Methyl 3,4,5-trihydroxy-6-[(5-hydroxy-6-methoxy-4-oxo-2-phenyl-3,4-dihydro-2H-1-benzopyran-7-yl)oxy]oxane-2-carboxylate were found to be higher in Andalusia. Additionally, a putative N-acyl-alpha amino acid and a putative glycoside compound demonstrated elevated levels in this region. Unfortunately, four metabolites could not be identified. Furthermore, three metabolites displayed higher concentrations in Asturias than in Andalusia and Aragon. These include 3-methyl hexane dioic acid, a putative fatty alcohol with the molecular formula $C_{18}H_{32}O_6$ belonging to the class of fatty acids and derivatives, and malic acid from the class of carboxylic acids and derivatives.

3.2. Fatty acid and amino acid composition

Thirteen fatty acids were identified however, only those with content greater than 1 mg/g on dry matter (DM) base, are presented in **Table 1**. The most abundant fatty acids were C18:2 n-6, C16:0 and C18:1 9c while C18:0 and C18:3 n-3 had lower concentrations. Among the studied cultivars, in Andalusia, cv. Chicana exhibited the highest contents of C16:0, C18:0, C18:2 n6 and C18:3 n3 whereas cv. Tirana showed the highest content of C18:1 9c. Cv. Karacter had the lowest contents of the five fatty acids. In Aragon, cv. Avenger had higher C16:0 and C18:2 n6 than the other cultivars ($p<0.05$) which had similar contents ($p>0.05$); higher C18:0 content than cvs. Tirana and Karpate ($p<0.05$), which had the lowest content; and had the highest C18:1 9c content ($p<0.05$) whereas cv. Jarana had the lowest content. Regarding C18:3 n3 content, cv. Chicana had the highest content and Tirana the lowest. In Asturias, cv. Tirana had the highest C16:0 and C18:1 9c contents, intermediate C18:2 n6 and C18:3 n3 contents whereas cv. Karacter had the lowest contents of the four fatty acids ($p<0.05$).

Regarding the effect of location, the impact on these five fatty acids revealed distinctive trends (**Table 1**, **Fig. S6A**). Cultivars grown in Aragon showed higher C16:0 and C18:0 concentrations, Asturias intermediate contents and Andalusia the lowest ($p<0.001$). Similarly, cvs. grown in Aragon had the highest C18:1 9c contents ($p<0.001$), being similar in Asturias and Andalusia ($p>0.05$). Regarding C18:2 n6 contents, cvs. grown in Asturias and Aragon had higher

contents than in Andalusia ($p<0.001$). In contrast, cvs. grown in Andalusia had the greatest C18:3 n3 contents ($p<0.01$).

The interplay between location and cultivar markedly influenced the contents of 18 amino acids, presented separately for the three locations in **Table 2**. As a recurring pattern, glutamic and aspartic acids were the most abundant amino acids. Meanwhile, sulfur-containing amino acids, methionine and cysteine, demonstrated the lowest concentrations regardless of cultivars and growing locations. In Andalucía, cvs. Jarana and Tirana had the highest contents of most amino acids, while cvs. Chicana and Furious had the lowest. In Aragón, differences between cultivars were observed only for glutamic acid, isoleucine, phenylalanine, and tryptophan. Cv. Forana presented higher glutamic acid and phenylalanine than cv. Karacter ($p<0.05$), cv. Tirana greater Isoleucine than Karacter ($p<0.05$) and Karacter greater tryptophan than Furious and Tirana ($p<0.01$). In Asturias, most amino acids showed differences between cultivars ($p<0.05$). In most amino acids, cv. Karacter had the highest contents, followed by cv. Karpate, while Furious had the lowest contents.

The interaction between the cultivar and the location was significant for all the amino acids (AA). In these cases, we present the interaction not the overall main effects. In some cultivars there was no effect of location on AA content. Asturias vs Aragon: 47% of the AA levels were higher in Asturias than in Aragon. Cv. Avenger showed no differences whereas 17 AA out of 18 differed in cvs. Karacter and Karpate. Asturias vs Andalusia: only 22% of AA differed. AA in cvs. Avenger and Jarana were not affected by location whereas those of cv. Karpate were. Andalusia vs Aragon: 25% of AA were affected by location. AA of Avenger, Forana and Furious were similar whereas 55% AA of Jarana and Karpate were higher in Andalusia than in Aragon. Overall, the contents of AA were higher in Andalucía than in Aragón, except for cysteine (**Fig. S6B**).

4. Discussion

Our study aimed to explore the post-harvest nutritional assessment of pea cultivars grown in varying climate locations in Spain. We selected eight pea cultivars and employed an untargeted metabolomics approach to analyse a broad spectrum of metabolites comprehensively. We then quantified nutritionally relevant fatty acids and AA for animal feeding.

The untargeted metabolomics analysis, followed by univariate and multivariate statistical analysis, identified 121 significantly altered metabolites that cause the clustering of the selected cultivars based on their metabolic profiles in a specific location. These metabolites were classified into seven different super-classes: benzenoids, lignans, neolignans and related compounds, lipids

and lipid-like molecules, nucleosides, nucleotides and analogues, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds and phenylpropanoids and polyketides, and some remain unknown. Previous studies highlighted that multiple loci across the genome collectively influence pea metabolites' abundance and structural varieties. Untangling this network of interactions illustrates how individual loci may impact more than one compound and vice versa, which might help with the selection of specific cultivars for defined quality traits and commercially valuable compounds (Ellis et al., 2018; Pandey et al., 2021). Our results showed that the cvs. Avenger and Karpate consistently clustered together while cv. Jarana formed a distinct and independent group in the PCA, irrespective of the environmental conditions. This fact suggests that genetic factors and inherent biochemical differences in these cultivars may contribute to the observed similarities in metabolite composition. Cv. Tirana, cropped in Andalusia, also exhibited an independent group in the PCA score plot, suggesting that environmental conditions in this region distinctly affect its metabolic profile. This highlights the adaptability and responsiveness of cv. Tirana to the specific environmental factors present in Andalusia. Similarly, environmental conditions in Asturias likely contribute to the observed differences in metabolite composition of cvs. Avenger and Karpate that form a separate cluster in the PCA score plot.

From the metabolic point of view, most of the metabolites that allow for cultivar differentiation in the study belong to the superclass of phenylpropanoids and polyketides, which includes flavonoids and isoflavonoids class. Phenylpropanoids and polyketides, crucial plant secondary metabolites, are pivotal in bolstering plant defence against abiotic stressors like drought, salinity, and heavy metal toxicity (Dewick, 2002; Ziani et al., 2023). Phenylpropanoids scavenge reactive oxygen species (ROS), fostering antimicrobial activity, inducing defence genes, and providing UV protection (Deng & Lu, 2017). Polyketides, constituting a vast superclass of compounds and representing 20% of the biosphere's total carbon, showcase antimicrobial, antioxidant, hormone, and anti-herbivore activities (Dewick, 2002; Ziani et al., 2023). Enhancing the production of phenylpropanoids and polyketides in cultivated crops is a focal point of breeding programs, aiming to fortify plant stress tolerance and increase crop yields under abiotic stress, especially amidst the challenges of climate change (Schulz, 2020; Verpoorte & Memelink, 2002). A notable subset within this superclass is flavonoids, including isoflavonoids, which significantly contribute to crop stress tolerance. These compounds play a crucial role in establishing symbiotic relationships between plants and microorganisms, acting as vital agents in plant survival by repelling insects and herbivores, underscoring the multifaceted significance of flavonoids and isoflavonoids in fortifying plants against environmental stressors.

Nevertheless, among the other significant metabolites that allowed for cultivar clustering, regardless of the environment, there were hexosamines; they are involved in synthesising cell wall components, glycoproteins, and glycolipids, which are crucial for plant structure, function, and defence (Hartweck et al., 2002; Love & Hanover, 2005). Additionally, hexosamines are precursors for the biosynthesis of essential biomolecules like vitamins, hormones, and signalling molecules (Hartweck et al., 2002; Love & Hanover, 2005). Environmental factors can significantly influence hexosamine metabolism in plants. Abiotic stresses such as drought, salinity, and heavy metal toxicity can alter the expression of genes involved in hexosamine biosynthesis and degradation (Jennings, 1978; Pusztai, 1964). Further studies understanding the regulation of hexosamine metabolism in response to environmental are essential for developing strategies to enhance pea cultivar stress tolerance and improve yields under challenging conditions.

Current knowledge regarding the influence of environmental factors and cultivation areas on the nutritional composition of field peas remains limited. Evidence suggests a strong association between pea composition and growing location, prevailing climatic conditions, and soil characteristics. (Nikolopoulou et al, 2007; Wang et al., 2010). In our study, the environmental conditions impacted the metabolic profiles of the studied pea cultivars according to the Volcano plots and PLS-DA analysis. The top 98 metabolites were attempted to be dereplicated. Nevertheless, they exhibited consistently lower concentrations in peas cultivated in Andalusia, irrespective of their metabolic superclass or class. This outcome may be attributed to the pronounced heat stress experienced by the pea cultivars in Andalusia. The growth period, marked by an average temperature of 14.1 °C, is 2.7 °C higher than the average temperature recorded in Aragon and Asturias. Additionally, the maximum temperature in Andalusia, reaching 40.6 °C, is 0.9 °C higher than that recorded in Aragon and 11.6 °C higher than the maximum temperature in Asturias. This warm climate likely affected the pea seeds. Previous research indicates that elevated temperatures during pea growth, especially in the seed developmental stage, decrease seed weight. This reduction could be attributed to forced maturation, leading to a hastened rate of seed development and a shortened seed-filling period (Lamichaney et al., 2021). Consequently, such environmental stressors may adversely impact the overall nutrient quality of pea flour. The multivariate analysis using the growing locations as class, indicated that the most altered metabolites belong to the superclass of lipids and lipid-like molecules, followed by the class of carbohydrates and carbohydrates conjugates and amino acids peptides and derivatives. A total of 18 compounds were identified among the modified lipids and lipid-like metabolites. Within this superclass, prenol lipids, including triterpene saponins and triterpenoid compounds, were the most diverse classes, with a total of 8 putatively identified compounds. Prenol lipids, essential

components of cellular membranes, play critical roles in various physiological processes, including photosynthesis, signalling, and stress tolerance. Among them, triterpenes are ubiquitous compounds in the plant kingdom. These structurally diverse molecules play broad roles in plant biology, ranging from signalling to defence against pathogens (Dewick, 2002). Various environmental factors, including light and heat stress, influence the metabolic routes involved in triterpene biosynthesis. These stressors can alter the availability of carbon precursors, leading to competition between lipid and phenylpropanoid biosynthesis. Understanding this intricate regulation is crucial for uncovering the complex interplay between plants' triterpene biosynthesis and other metabolic processes (Surmacz et al., 2016; Surmacz & Swiezewska, 2011; Tholl, 2015). Further investigations are needed to elucidate these regulatory mechanisms to understand how these metabolites contribute to plant growth, development, and stress adaptation. Furthermore, two lipids and lipid-like metabolites displayed higher concentrations in Asturias than in Andalusia and Aragon. One of these compounds is 3-methyl hexane dioic acid, classified as a dicarboxylic acid. These metabolites and phosphoglycerolipids are crucial in regulating plant development and responding to biotic and abiotic stress conditions. They may also be involved in the biosynthesis of cutin and suberin, contributing to the formation of complex hydrophobic barriers. While the cutin compounds are located in the epidermis cell walls across all aerial tissues, the suberin is present in the cell walls of various internal and external tissue layers, such as the root endodermis, periderm, and seed coat. While their functions within plant cells are well-documented, their role in long-distance signalling requires further investigation (Barbaglia & Hoffmann-Benning, 2016). The other metabolite more upregulated in Asturias is malic acid from the class of carboxylic acids and derivative. Various studies indicated that malic enzymes play a crucial role in responding to environmental factors and facilitating the reversible oxidative decarboxylation of this metabolite. Moreover, they enhance water use efficiency, boost plant photosynthesis, and provide reduced power, among other functions (Sun et al., 2019). Additionally, a prior study observed that cold-sensitive chickpea plants and their mutants exhibited different seed set patterns at lower temperatures, with the mutants showing higher seed set based on the increase in malic acid concentration (Maqbool et al. 2010; Savithri et al., 1980).

Carbohydrates and carbohydrates conjugate are vital components of plants, cellular accumulation of soluble sugars during drought stress influences sugar transporter expression, facilitating sugar distribution from source to sink and aiding drought stress adaptation (Salvi et al., 2022). Recent metabolite profiling studies reignite interest in components within the 'temperature-stress metabolome' associated with induced stress tolerance, emphasizing their importance in plant resilience (Guy et al., 2008).

Amino acids and peptides play a fundamental role in plant biology. Their intricate regulation, governed by feedback inhibition loops, ensures a delicate balance between synthesis and degradation. However, in some crops, this balance can be disrupted by several factors, limiting the availability of critical amino acids and potentially hindering enzyme activity and metabolic pathways (Galili et al., 2016). Among them, N-acetyl-l-phenylalanine was found to be upregulated in the cultivars grown in Andalusia. Previous studies reported that this metabolite could be accumulated in plants under stress conditions to increase osmotic regulation and antioxidant activity to ensure the stability of cell structure and function (Han et al., 2023). Nevertheless, further studies are needed to investigate factors other than climate conditions, including soil composition and cultivar management.

At this stage, addressing a bottleneck in untargeted metabolomics using LC-MS/MS is crucial. Challenges in untargeted metabolomics using mass spectrometry include selecting ionization modes, encompassing polarity switching, and optimizing extraction methods. These issues lead to signal variability and data complexity. Due to the lack of standardized protocols, scientists must extensively describe their methods and highlight potential limitations (Alseekh et al., 2021; Bulut et al., 2023; Sumner et al., 2007). Moreover, our results should be validated through targeted metabolomics approaches by developing quantitative methods using proper internal standards for quantifying specific metabolite classes. By quantifying key metabolites, researchers gain molecular insights into desired traits like stress tolerance, nutritional value, and crop quality, facilitating crop development. Targeted metabolomics also proves beneficial in systems biology, aiding the creation of detailed models that capture specific crop biological systems and overcoming limitations associated with model organisms (Allwood et al., 2021; Anzano et al., 2021; Guy et al., 2008). Analytical chemists have made significant efforts to merge untargeted and targeted metabolomics methods using mass spectrometry (Caija & Fiehn, 2016). Finally, the extensive chemical diversity and complexity often hinder unbiased structure assignment for metabolites of interest. Despite significant progress in expanding metabolite databases, many signals in metabolomics experiments cannot be directly linked to specific metabolites due to missing spectra in databases (Bittremieux et al., 2022). Establishing specialized databases could enhance metabolomics-assisted crop breeding and enable metabolite-based genome-wide association studies in legume species (Bulut et al., 2023). Various dereplication strategies, including *in silico* fragmentation, and molecular networking, have been developed to tackle this issue (Allard et al., 2016; Gauglitz et al., 2022; Guo et al., 2022). However, a challenge remains in distinguishing potential stereo and regioisomers using standard spectral library matching or *in silico* fragmentation. This results in a level C annotation or unknown classification based on similar fragmentation profiles across multiple

classes. To address this, orthogonal analytical methods like NMR are indispensable for metabolite validation, achieving level A identification, and dereplicating unknown or novel metabolites (Garcia-Perez et al., 2020).

Our study highlights the complex interplay between location and cultivar, also significantly impacting the contents of fatty acids and amino acids, in the same line of that observed in metabolic profiles. Significant variations in fatty acid composition were observed among cultivars in all locations, and the individual cultivars had different behaviour according to the location. The study of the amino acids revealed recurring patterns. Glutamic and aspartic acids consistently being the most abundant. Glutamate, glutamine and aspartate are major metabolic fuels for the small intestine to maintain its digestive function and to protect the integrity of the intestinal mucosa. Thus, diets for animals must contain all of these to optimize their survival, growth, development, reproduction, and health (Deng et al., 2023). Amino acid contents were generally higher in Asturias than in Aragon and Andalusia, except for some specific amino acids. Furthermore, the contents of amino acids were higher in Andalusia than in Aragon, except for cysteine. Our findings agree with Witten et al. (2015) and Zhou et al. (2023), who found differences between the pea cultivars in yields of amino acids per hectare, affecting the cultivation site. This might be due to site-specific characteristics of the soil, plus soil fertility management, tillage, time of seeding and harvesting, row spacing, seed rate, pest and disease infestations, and further factors combined with weather conditions.

5. Conclusion

Our findings show that metabolic profiles and fatty acid and amino acid levels vary across different regions, providing valuable insights into the environmental influences on the selected cultivars. Moreover, the insights from these studies have practical implications for industrial applications, emphasizing the necessity for enhanced collaboration between research and industry. For instance, in animal feed production, the knowledge acquired is crucial due to variations in nutritional requirements based on the specific animal or its life stage. Our results could help producers choose the optimal pea cultivar for the given environmental conditions, ensuring the desired nutritional value in pea cultivation for feed production. Finally, beyond their role in animal feed production, dry peas have been recognized as crucial components of human food. Indeed, their usage aligns with the increasing demand for sustainable and nutrient-rich food sources, enhancing the economic value of this crop and contributing to farmers' efforts to improve their social conditions.

CRediT authorship contribution statement

Pierluigi Reveglia: Conceptualization, Methodology, Investigation, Software, Data curation, Formal analysis, Validation, Visualization, Writing original draft, Review & editing. **Mireia Blanco**: Methodology, Investigation, Software, Formal analysis, Data curation, Writing - review & editing. **Maria Josè Cobos**: Methodology, Investigation, Software, Review & editing. **Maryke Labuschagne**: Conceptualization, Investigation, Writing - review & editing. **Margalida Joy**: Project administration, Supervision, Funding acquisition, Resources, Writing - review & editing. **Diego Rubiales**: Project administration, Supervision, Funding acquisition, Resources, Writing - review & editing.

Declaration of Competing Interest

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

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Figures and Tables

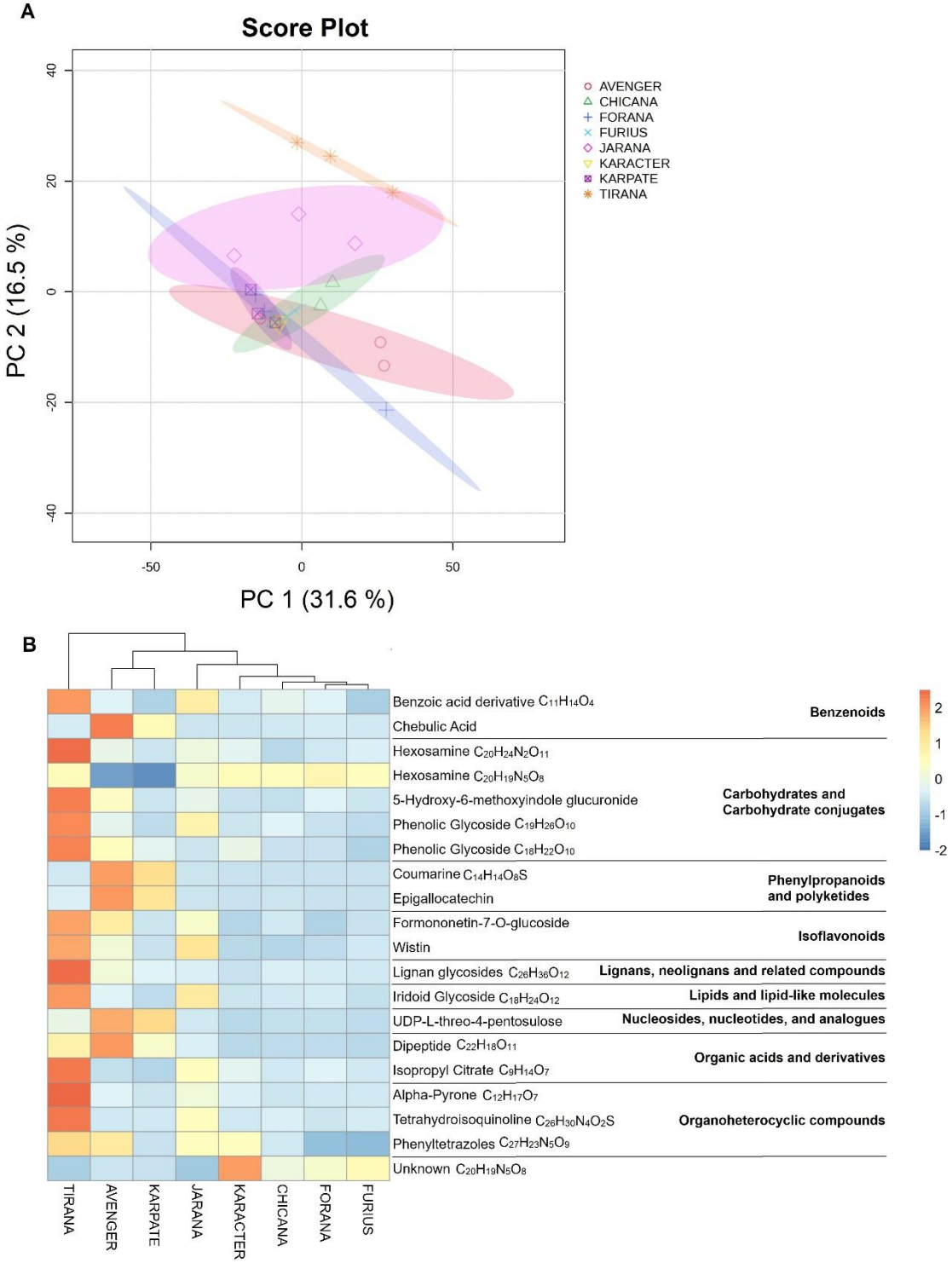

Fig. 1. (A) Score plot obtained by principal component analysis (PCA), showing the eight pea cultivars cropped in Andalusia**. (B)** Heatmap reporting the top 20 metabolites contributing to the variations among pea cultivars cropped in Andalusia, organized by natural product superclass or class.

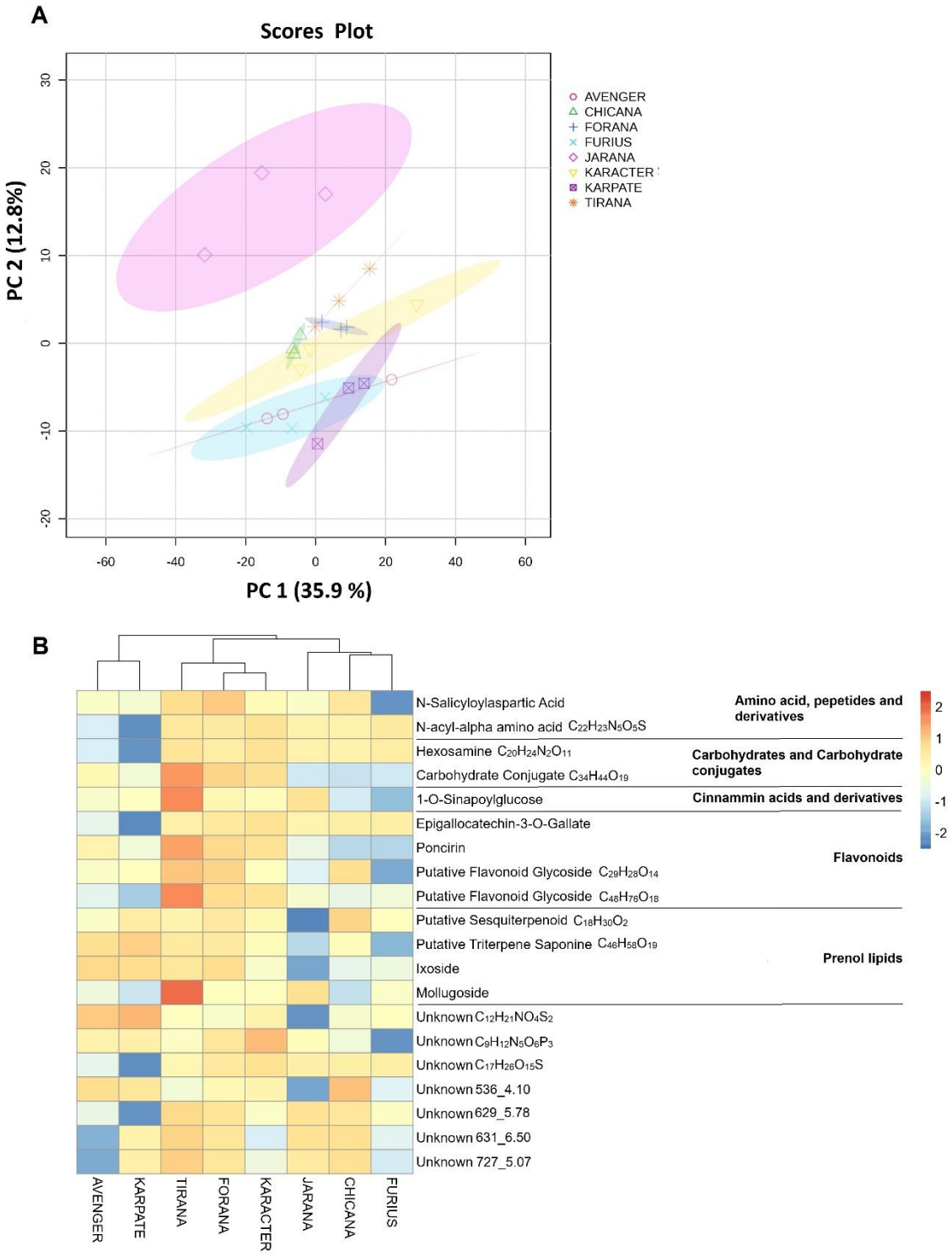

Fig. 2. (A) Score plot obtained by principal component analysis (PCA), showing the eight pea cultivars cropped in Aragon**. (B)** Heatmap reporting the top 20 metabolites and their contributing to the variations among pea cultivars cropped in Aragon, by natural product superclass or class. For Unknown metabolites where the molecular formula is not available, the m/z_Rt, available in Table S2, is added.

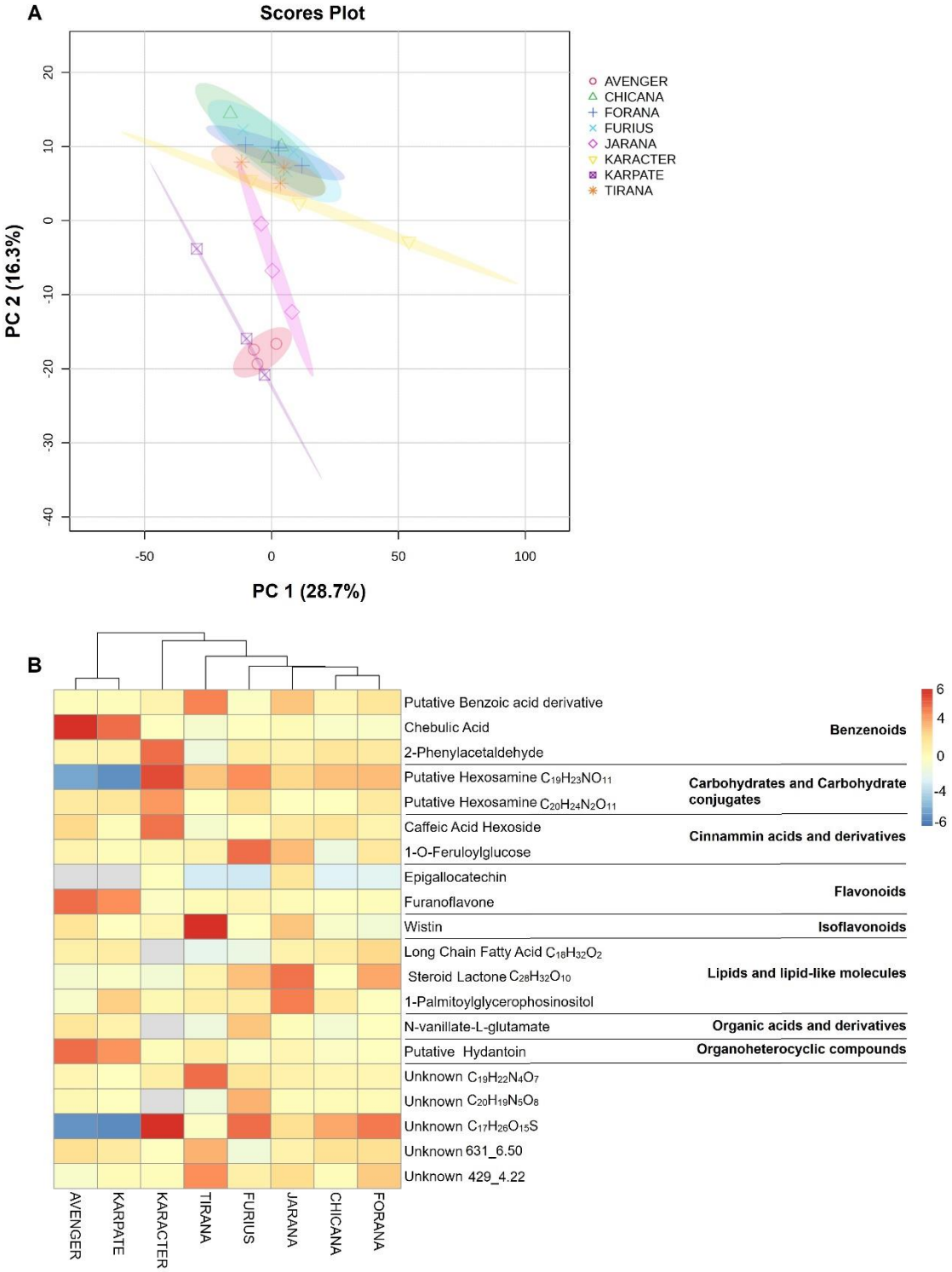

Fig. 3. (A) Score plot obtained by principal component analysis (PCA), showing the eight pea cultivars cropped in Asturias**. (B)** Heatmap reporting the top 20 metabolites and their contributing to the variations among pea cultivars cropped in Asturias, by natural product superclass or class. For Unknown metabolites where the molecular formula is not available, the m/z_Rt, available in Table S2, is added

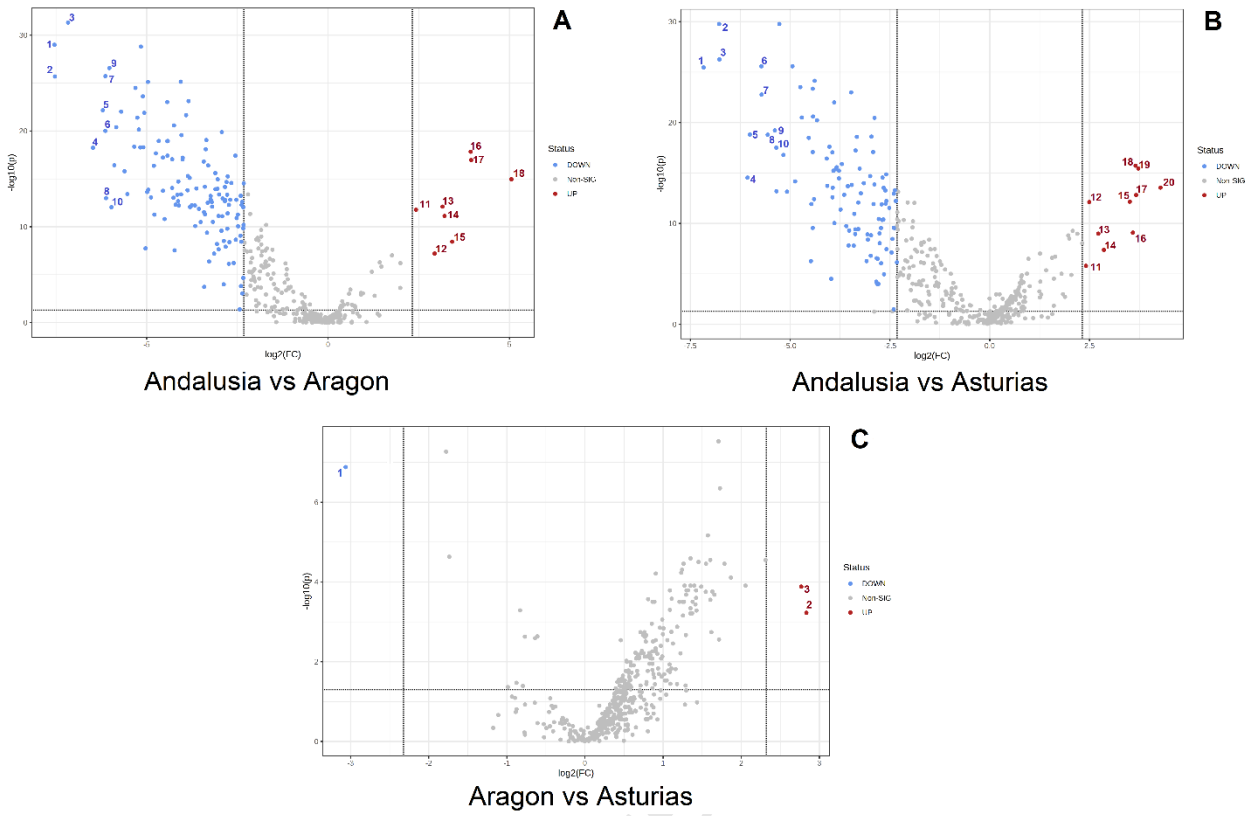

Fig. 4. Volcano plots reporting metabolic profiles differences of pea cultivars: **(A)** cropped in Andalusia versus the same cropped in Aragon; **(B)** cropped in Andalusia versus the same cropped in Asturias; **(C)** cropped in Aragon versus the same cropped in Asturias. Fold-change threshold (x) 5 and t-test threshold (y) of 0.05 FDR corrected. The blue (downregulated) and the red (upregulated) circles represent features above the threshold. Note that fold changes log2 (FC) and p values log10 (p) were log transformed. On the (x) axis, the further the position from (0,0), the more significant the feature, top significant features have numbers. and their identities reported in **Tables S4-S6**.

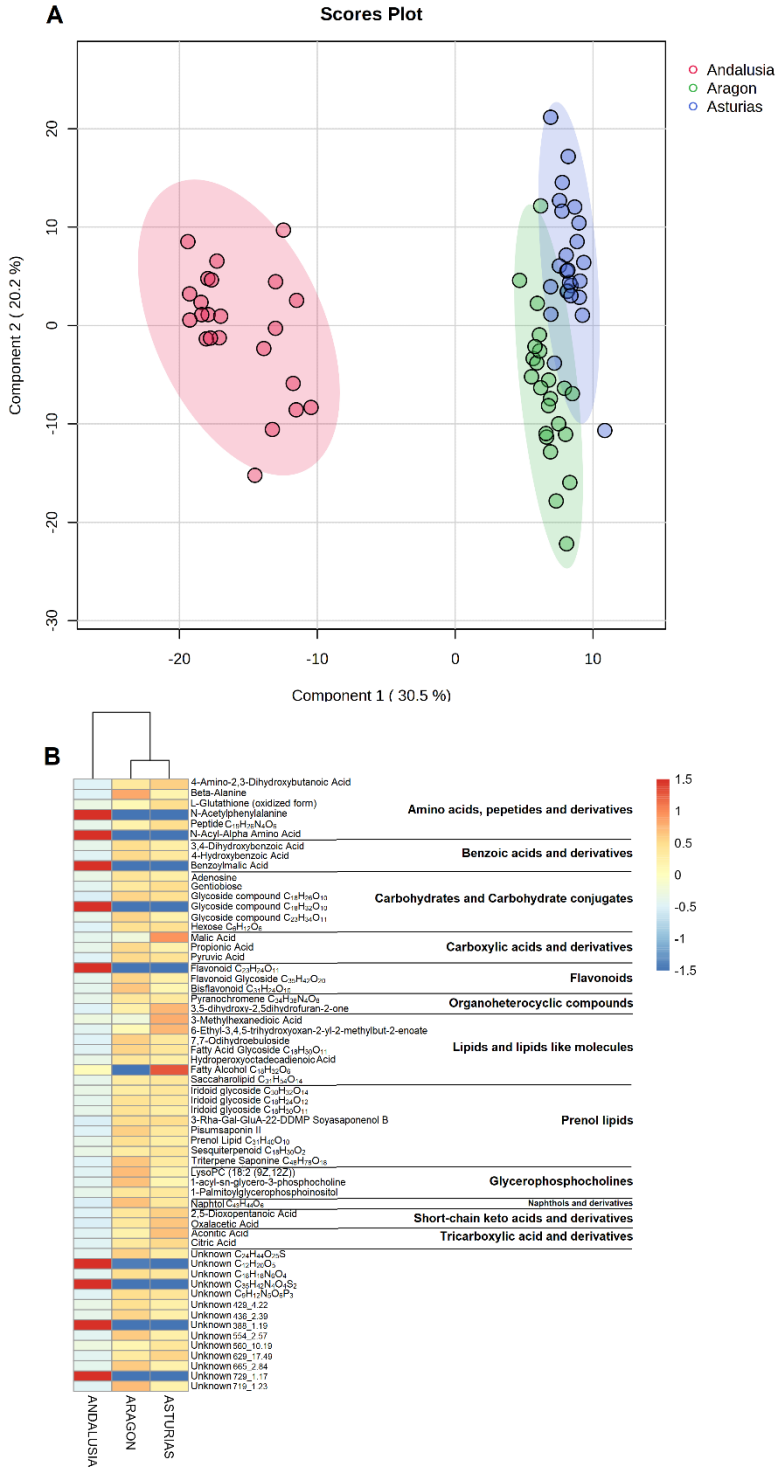

Fig. 5. **(A)** Score plot of pea cultivars, obtained by partial least square – discriminant analysis (PLS-DA), grouped into different clusters, according to environments: Andalusia (red), Aragon (green), Asturias (blue). The explained variance (%) attributed to the first and second component, are shown in the figure. **(B)** Heatmap showing 60 metabolites having VIP Score > 1.5, contributing to the cultivars grouping according to environments. For Unknown metabolites where the molecular formula is not available, the m/z_Rt, available in Table S2, is added

Table 1. Predominant Fatty Acids (FA, mg/g dry matter) in pea cultivars cropped across Andalusia, Aragon, and Asturias regions.

FA	Avenge	Chicana	Forana	Furious	Jarana	Karacte	Karpate	Tirana	SEM	p-value
Andalusia										
C16:0	5.34^{b}	5.91^{a}	4.94^{bcd}	5.17^{bc}	4.93^{bcd}	4.64^{d}	4.87^{cd}	5.33^{b}	0.03	<0.001
C18:0	3.09^{a}	3.19^{a}	3.05^{ab}	3.04^{abc}	2.86^{c}	2.86^{c}	2.90^{bc}	3.08^{ab}	0.01	<0.001
C18:1 9c	5.51^{b}	5.5^{b}	4.6^{cd}	5.1^{bc}	4.18^{ef}	3.61^{f}	4.2^{ef}	6.65^{a}	0.05	<0.001
C18:2 n6	9.55^{bcd}	12.44^{a}	9.92^{bc}	9.13^{cd}	10.21^{b}	8.93^{d}	9.98^{bc}	9.55^{bcd}	0.07	<0.001
C18:3 n-3	1.59^{bc}	1.95^{a}	2.04^{a}	1.89^{a}	1.51^{c}	1.46^{c}	1.68^{b}	1.68^{b}	0.01	<0.001
Aragon										
C16:0	7.93^{a}	6.87^{b}	6.23^{b}	6.36^{b}	6.25^{b}	6.15^{b}	6.28^{b}	6.4^{b}	0.08	<0.001
C18:0	4.08^{a}	3.86^{abc}	3.91^{ab}	3.83^{abc}	3.74^{abc}	3.85^{abc}	3.41^{c}	3.41^{bc}	0.04	<0.001
C18:1 9c	8.74^{a}	6.76^{bc}	5.79^{bcd}	5.43^{cd}	5.03^{d}	5.42^{cd}	6.52^{bcd}	7.12^{b}	0.11	<0.001
C18:2 n6	13.1^{a}	11.03^{b}	10.39^{b}	11.28^{b}	10.79^{b}	11.01^{b}	11.0^{b}	11.16^{b}	0.09	<0.001
C18:3 n-3	1.75^{a}	1.80^{a}	1.70^{ab}	1.49^{abc}	1.37^{bc}	1.48^{abc}	1.57^{abc}	1.31^{c}	0.03	0.001
Asturias										
C16:0	6.51^{a}	6.51^{a}	6.15^{ab}	6.09^{ab}	6.28^{ab}	5.31^{b}	5.93^{ab}	6.63^{a}	0.08	0.01
C18:0	3.38	3.38	3.37	3.3	3.24	3.19	3.39	3.46	0.03	0.26
C18:1 9c	5.54^{abc}	5.54^{abc}	4.62^{bcd}	5.58^{ab}	4.49^{cd}	2.64^{e}	4.35^{d}	6.47^{a}	0.08	<0.001
C18:2 n6	12.67^{a}	12.67^{a}	11.17^{ab}	10.52^{ab}	12.33^{a}	8.51^{b}	11.02^{ab}	11.82^{ab}	0.26	0.01
C18:3 n-3	1.50^{ab}	1.50^{ab}	1.91^{a}	1.71^{ab}	1.52^{ab}	1.10^{b}	1.38^{ab}	1.64^{ab}	0.05	0.03

Within a parameter, means with different letter differ at p<0.05

Table 2. Amino acids (AA) of pea cultivars cropped cultivated in Andalusia, Aragon, and Asturias regions.

AA, mg/kg DM	Avenger	Chicana	Forana	Furious	Jarana	Karacter	Karpate	Tirana	SEM	p-value
Andalusia										
Aspartic acid	31.3^{abc}	29.2^{bc}	30.6^{bc}	29.0^{c}	34.7^{a}	32.4^{ab}	30.4^{bc}	32.6^{ab}	0.24	<0.001
Threonine*	10.4^{abc}	9.7^{c}	9.8^{bc}	9.9^{bc}	11.0^{a}	10.7^{ab}	10.1^{abc}	10.9^{a}	0.07	0.001
Serine	14.0^{ab}	13.1^{b}	13.8^{ab}	13.2^{b}	15.2^{a}	14.0^{ab}	13.0^{b}	14.2^{ab}	0.10	0.001
Glutamic acid	44.4^{bc}	42.4^{bc}	44.8^{bc}	41.5^{c}	49.9^{a}	46.0^{abc}	43.4^{bc}	47.0^{ab}	0.36	0.001
Proline	10.5^{abc}	10.1^{c}	11.0^{abc}	10.2^{bc}	11.4^{ab}	11.4^{ab}	11.0^{abc}	11.6^{a}	0.09	0.003
Glycine	11.6^{ab}	11.0^{b}	11.4^{ab}	11.1^{b}	12.3^{a}	12.0^{ab}	11.4^{ab}	11.9^{ab}	0.08	0.01
Alanine	11.9^{ab}	11.3^{b}	12.2^{ab}	11.7^{ab}	12.7^{a}	12.3^{ab}	11.7^{ab}	12.1^{ab}	0.09	0.04
Cystine	0.83^{ab}	0.60^{b}	0.80^{b}	0.93^{ab}	1.33^{a}	1.00^{ab}	0.87^{ab}	0.90^{ab}	0.04	0.009
Valine*	12.2^{abc}	11.5^{bc}	11.9^{abc}	11.4^{c}	13.0^{a}	12.2^{abc}	11.7^{bc}	12.7^{ab}	0.09	0.004
Methionine*	1.8^{ab}	1.3^{b}	1.7^{ab}	1.8^{ab}	2.2^{a}	2.0^{ab}	1.6^{ab}	2.0^{ab}	0.06	0.04
Isoleucine*	10.8^{ab}	10.1^{bc}	10.1^{bc}	9.6^{c}	11.2^{a}	10.3^{abc}	9.6^{c}	11.1^{ab}	0.08	<0.001
Leucine*	19.1^{abc}	17.9^{c}	18.5^{bc}	17.3^{c}	20.4^{a}	18.7^{abc}	18.0^{c}	19.9^{ab}	0.13	<0.001
Tyrosine	4.2^{ab}	2.3^{b}	2.6^{b}	3.8^{ab}	6.3^{a}	5.7^{a}	3.9^{ab}	5.4^{a}	0.20	0.001
Phenylalanine	13.2^{abc}	12.6^{bc}	12.9^{abc}	12.3^{c}	14.2^{a}	13.3^{abc}	13.0^{abc}	13.9^{ab}	0.10	0.002
Histidine*	6.5^{bcd}	6.2^{d}	6.4^{bcd}	6.2^{cd}	7.2^{a}	6.9^{ab}	6.5^{abcd}	6.9^{ab}	0.05	0.001
Lysine*	19.8^{ab}	18.8^{b}	19.7^{ab}	19.1^{b}	21.2^{a}	20.3^{ab}	19.5^{ab}	20.6^{ab}	0.15	0.02
Arginine	18.4^{bc}	14.0^{c}	14.2^{c}	15.6^{c}	25.4^{a}	22.6^{ab}	17.2^{c}	24.5^{a}	0.36	<0.001
Tryptophan*	2.2^{ab}	2.0^{b}	2.3^{ab}	2.3^{ab}	2.2^{ab}	2.3^{ab}	2.1^{ab}	2.4^{a}	0.03	0.03
Aragon										
Aspartic acid	30.4	27.0	30.6	28.0	31.8	26.8	29.7	29.1	0.53	0.26
Threonine *	10.3	9.8	10.5	9.3	9.3	8.0	9.4	9.7	0.21	0.18
Serine	15.5	14.1	15.8	13.4	11.9	11.6	13.2	13.2	0.36	0.09

Glutamic acid	48.9^{a}	43.1^{ab}	49.8^{a}	41.1^{ab}	43.0^{ab}	37.3^{b}	41.6^{ab}	41.5^{ab}	0.82	0.03
Proline	10.8	9.8	10.9	10.0	10.5	9.6	10.7	10.2	0.20	0.66
Glycine	11.5	10.8	11.8	10.8	11.2	9.8	11.2	11.1	0.21	0.44
Alanine	10.8	9.8	10.9	10.9	11.7	10.5	11.8	11.3	0.22	0.43
Cystine	1.0	1.2	1.6	1.2	1.1	0.8	0.7	0.8	0.06	0.06
Valine*	10.8	9.9	11.0	9.7	10.6	8.6	10.1	11.1	0.20	0.08
Methionine*	1.4	1.7	1.8	1.4	1.5	1.0	1.3	1.7	0.07	0.16
Isoleucine*	9.1^{ab}	8.4^{ab}	9.3^{ab}	7.7^{ab}	8.8^{ab}	6.8^{b}	8.2^{ab}	9.6^{a}	0.19	0.04
Leucine*	17.0	15.1	17.4	15.2	16.7	13.9	16.2	17.3	0.30	0.10
Tyrosine	2.2	3.6	4.1	2.3	2.6	1.3	1.8	3.9	0.30	0.25
Phenylalanine	12.5^{ab}	11.1^{ab}	12.7^{a}	10.7^{ab}	11.9^{ab}	9.6^{b}	11.0^{ab}	11.9^{ab}	0.22	0.049
Histidine*	6.2	5.7	6.4	5.9	6.3	5.4	6.1	6.1	0.11	0.44
Lysine*	18.9	17.0	18.9	17.9	19.1	16.6	18.8	18.8	0.34	0.46
Arginine	14.8	14.1	18.0	11.7	15.6	10.6	12.2	17.2	0.63	0.10
Tryptophan*	2.2^{ab}	2.2^{ab}	2.3^{ab}	2.0^{b}	2.2^{ab}	2.4^{a}	2.2^{ab}	2.0^{b}	0.02	0.004
Asturias										
Aspartic acid	31.4^{b}	31.1^{ab}	38.3^{a}	30.1^{b}	34.9^{ab}	39.3^{a}	36.2^{ab}	34.6^{ab}	0.57	0.007
Threonine *	10.2^{ab}	10.2^{ab}	11.7^{ab}	9.5^{b}	11.1^{ab}	12.8^{a}	11.8^{ab}	11.7^{ab}	0.20	0.01
Serine	14.1	13.6	15.8	13.0	14.6	17.2	15.9	15.9	0.31	0.06
Glutamic acid	44.7^{ab}	43.3^{ab}	50.7^{ab}	41.1^{b}	48.5^{ab}	55.3^{a}	51.3^{ab}	50.5^{ab}	0.84	0.01
Proline	10.6^{bcd}	9.2^{cd}	10.2^{bc} d	8.3^{d}	10.9^{bc}	13.3^{a}	12.1^{ab}	11.8^{ab} c	0.17	<0.001
Glycine	11.6^{b}	11.9^{ab}	13.2^{ab}	11.0^{b}	12.4^{ab}	14.8^{a}	13.6^{ab}	13.2^{ab}	0.23	0.02
Alanine	11.9^{ab}	11.5^{ab}	12.9^{ab}	10.8^{b}	12.7^{ab}	15.1^{a}	13.8^{ab}	13.5^{ab}	0.24	0.01
Cystine	1.07^{b}	1.20^{ab}	1.83^{ab}	2.00^{a}	1.17^{ab}	1.60^{ab}	1.13^{ab}	1.17^{ab}	0.07	0.02
Valine*	11.9^{abc}	11.1^{bc}	12.7^{ab}	9.2^{c}	12.6^{ab}	14.6^{a}	13.4^{ab}	13.4^{ab}	0.22	<0.001
Methionine*	2.0	2.1	2.3	1.8	2.1	2.5	2.2	2.2	0.08	0.43
Isoleucine*	10.3^{ab}	8.7^{bc}	9.9^{ab}	6.8^{c}	10.7^{ab}	12.3^{a}	11.2^{ab}	11.6^{ab}	0.23	<0.001
Leucine*	18.3^{abc}	15.7^{cd}	18.1^{bc}	12.7^{d}	19.7^{ab} c	22.7^{a}	20.5^{abc}	20.8^{ab}	0.33	<0.001
Tyrosine	4.23^{b}	7.55^{ab}	9.27^{a}	6.40^{ab}	6.43^{ab}	6.93^{ab}	5.27^{ab}	5.33^{ab}	0.30	0.03
Phenylalanine	12.7^{abc}	10.8^{cd}	12.2^{bc}	8.4^{d}	13.4^{ab} c	15.6^{a}	13.9^{abc}	14.7^{ab}	0.24	<0.001
Histidine*	6.6^{ab}	6.6^{ab}	7.8^{ab}	6.5^{b}	7.2^{ab}	8.5^{a}	7.8^{ab}	7.5^{ab}	0.14	0.02
Lysine*	19.7^{ab}	19.3^{ab}	22.9^{ab}	19.0^{b}	21.4^{ab}	25.0^{a}	22.6^{ab}	22.6^{ab}	0.43	0.04
Arginine	20.5^{b}	23.5^{ab}	30.7^{a}	20.2^{b}	27.0^{ab}	27.5^{ab}	24.1^{ab}	23.2^{ab}	0.65	0.01
Tryptophan*	2.9^{a}	2.3^{ab}	2.3^{ab}	2.2^{ab}	2.5^{ab}	2.6^{ab}	2.2^{ab}	2.1^{b}	0.05	0.04

* Essential AA.

Within a parameter, means with different superscript differ at $p < 0.05$.

Declaration of interests

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.

Highlights

- There was a clear genotype × environment interaction on pea metabolic profiles.

- Untargeted metabolomics revealed 121 significant metabolites, shaping the clustering of selected pea cultivars in a specific location.
- Lipids and lipid-like molecules, phenylpropanoids and polyketides (flavonoids, isoflavonoids), carbohydrates and amino acids are the most differential metabolites.
- Palmitic, stearic, oleic, linoleic, and α-linolenic are the most abundant fatty acids.
- Glutamic and aspartic acids were the most abundant amino acids. Methionine and cysteine demonstrated the lowest concentrations.
- Overall, varieties grown in Asturias exhibit the highest amino acid content.