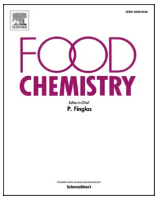
Food Chemistry 417 (2023) 135817

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Geographical region traceability of *Poria cocos* and correlation between environmental factors and biomarkers based on a metabolomic approach

Xiaoliu Liu [a,b], Can Zhong [b], Jing Xie [a,b], Hao Liu [b], Zhenni Xie [a,b], Shuihan Zhang [b], Jian Jin [b,*]

[a] *Hunan Academy of Chinese Medicine, Hunan University of Chinese Medicine, Changsha 410208, China*
[b] *Institute of Chinese Medicine Resources, Hunan Academy of Chinese Medicine, Changsha 410013, China*

ARTICLE INFO

Keywords:
Poria cocos
Geographical origin
Untargeted metabolomics
Biomarkers
Environmental factors

ABSTRACT

The edible values of *P. cocos* from different origins vary significantly, therefore, it is important to investigate the traceability of geographical regions and identify the geographical biomarkers of *P. cocos*. The metabolites of *P. cocos* of the different geographical origins were assessed using liquid chromatography tandem-mass spectrometry, principal component analysis and orthogonal partial least-squares discriminant analysis (OPLS-DA). The OPLS-DA could clearly discriminate the metabolites of *P. cocos* from the three cultivation regions (YN, Yunnan; AH, Anhui; JZ, Hunan). Finally, three carbohydrates, four amino acids, and four triterpenoids were selected as biomarkers for *P. cocos* origin tracing. Correlation matrix analysis revealed that the contents of biomarkers were closely related to geographical origin. Altitude, temperature, and soil fertility were the main factors responsible for the differences in biomarker profiles in *P. cocos*. The metabolomics approach provides an effective strategy for tracing and identifying the biomarkers of *P. cocos* from different geographical origins.

1. Introduction

Poria cocos is a Basidiomycetes saprophytic fungus that belongs to the Polyporaceae family (Ríos, 2011), distributed mainly in China, Korea, Japan, and North America (Li, Nie, & Tang, 2014). *P. cocos* has been used in China for more than 2000 years as one of the important edible and medicinal fungi, and a large area in Hunan, Yunnan and Anhui in China is dedicated to its cultivation nowadays (Esteban, 2009). *P. cocos* is rich in a variety of active compounds, mainly including polysaccharides and triterpenoids, and other compounds in small quantities, such as steroids, amino acids, choline, histidine, and potassium salts(Xiang, Wang, Bian, & Xu, 2016). Among the polysaccharides, β-(1 → 3)-ᴅ-glucan is the main one(G. Zhang et al., 2019). These triterpene acids can be classified into 4 subgroups according to their chemical structure, i. e., lanostane-8-ene type (type I), lanostane-7,9(11)-diene type (type II), 3,4-seco-lanostane-8-ene type (type III) and 3,4-secolanostane-7,9(11)-diene type (type IV) compounds(Jian et al., 2019).

As a traditional Chinese medicine, it is to be effective in the treatment of diuresis and dampness, strengthening the spleen and stomach, nourishing the heart and tranquilizing the mind (Yang et al., 2022). Meanwhile, modern pharmacology studies have shown that triterpenoids in *P. cocos* possess properties, including antitumor(Dong et al., 2015; Kikuchi, Uchiyama, Ukiya, Tabata, & Akihisa, 2011), enhance immunity(Chao, Huang, Su, Lin, & Wu, 2021), reduce blood sugar(Huang, Chang, Huang, Lin, Lin, & Chang, 2010), antioxidant (Zhou, Zhang, Gapter, Ling, Agarwal, & Ng, 2008), anti-inflammatory (Fuchs, Heinemann, Schliemann-Willers, Hrtl, & Elsner, 2006), antiproliferative(Zhou et al., 2008) and other effects, which has been valued by regional and foreign researchers, making it widely used in herb medicine and functional foods (Kikuchi et al., 2011). *P. cocos* is usually consumed directly, it is also popularly added to tea supplements, wine supplements, and functional foods (Wang, Tao, Zhao, Ji, & Liu, 2012).

The market demand for *P. cocos* is high in China owing to its unique edible value. In the supply and distribution chain, the price of *P. cocos* is determined by its quality and geographic origin, and consumers are more interested in authentically labeled *P. cocos* of a specific origin, and its quality depends on the geographical region of cultivation and

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Abbreviations: YN, Yunnan; AH, Anhui; JZ, Hunan; DTS, Dried leaves; UHPLC–MS/MS, Ultraperformance liquid chromatography dual mass spectrometry; GC–MS/MS, Gas chromatography-tandem dual mass spectrometry; TIC, Total ion chromatograph; PCA, Principal component analysis; HCA, Hierarchical clustering analysis; OPLS-DA, Orthogonal partial least squares discrimination analysis; FC, Fold change; VIP, Variable importance projection; EL, Altitude; Tmin, Minimum temperature; Tmax, Maximum temperature; DT, Differential temperature; RH, Relative humidity; API, Air pollution index; MSTR, Moisture.

[*] Corresponding author.
E-mail address: jinjian2016@163.com (J. Jin).

https://doi.org/10.1016/j.foodchem.2023.135817
Received 4 October 2022; Received in revised form 20 February 2023; Accepted 26 February 2023

climatic characteristics. Accordingly, it is important to identify *P. cocos* from different geographical origins to provide appropriate information to consumers. The polysaccharide and triterpenoid content in *P. cocos* polysaccharides and triterpenoids can be greatly influenced by their origin (Zan, Bin, & Xiao-Bo, 2010). Recently, some scholars propose a method was proposed for the evaluation of the quality of *P. cocos* by using near infrared (NIR) methods to determine the polysaccharides, water-soluble extracts, alcohol-soluble extracts and five triterpenoids in *P. cocos* to establish models for the differentiation of its origin(Xie et al., 2022). Although NIR has advantages over traditional methods, it can only provide information about specific compositions in *P. cocos*. Nonetheless, as an edible fungus, *P. cocos* contains many endogenous compounds, and relatively few studies on these endogenous compounds have been reported by advanced technology.

With the rise of metabolomics technology in recent years, it is being widely applied for the evaluation of food and Chinese herbal medicine quality evaluation, resource identification of the resource, plant classification, kinship and metabolic regulatory networks, etc.(Wei et al., 2020; Fiehn & Altmann, 2000). The analysis of metabolites to obtain biomarkers of different origins, species and tissue sites has become more of a hot topic of high interest in metabolomics research. It was reported that a variety of organisms have been successfully screened for biomarkers using metabolomics techniques to enable geographical origin differentiation recently, such as in red wines(Arapitsas, Ugliano, Marangon, Piombino, & Mattivi, 2020), beef(Kyma et al., 2020), oranges (Li, Liang, Xu, Yang, & Wang, 2020), coffee (Yue et al., 2022), licorice (Yla et al., 2022), and so on. Therefore, the creation of a metabolomics-based dataset of biomarkers associated with environmental factors in the production area to provide traceability and authenticity of *P. cocos* origin may be a useful tool to ensure maximum utilization of the value of its origin-specific products.

In this study, endogenous metabolites in *P. cocos* samples from different origins were comprehensively and systematically identified and characterized using an unbiased metabolomics approach to screen for biomarkers that can facilitate tracing and distinguishing the geographical origin of *P. cocos*. This strategy provides a new perspective for the study of *P. cocos* from different regions and provides a basis for subsequent in-depth study and analysis of metabolic molecular markers.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium acetate was procured from Sigma-Aldrich (St Louis, MO, USA). Methanol, acetonitrile and formic acid were obtained from the Milli-Q purification system (Millipore, Bedford, MA, USA). Ammonia for high-performance liquid chromatography (HPLC) was purchased from Merck (Darmstadt, Hesse, Germany). 16α-Hydroxydehydrotrametenolic acid (HDTRA), poricoic acid B (PAB, CAS No.137551-39-4), poricoic acid A (PAA, CAS No. 137551-38-3), polyporenicacid C (PAC, CAS No. 465-18-9), poricoic acid AM (PAAM, CAS No.151200-92-9), 3-O-acetyl-16α-hydroxydehydrotrametenolic acid (AHDTRA, CAS No. 168293-14-9), 3-O-acetyl-16α-hydroxytrametenolic acid (AHTRA, CAS No. 168293-13-8), dehydropachymic acid (DPA, CAS No. 77012-31-8), pachymic acid (PA, CAS No. 29070-92-6), dehydrotrametenolic acid (DTRA, CAS No. 29220-16-4), dehydrotumulosic acid(DTUA, CAS No. 6754-16-1) were obtained from Chroma Biotechnology Co., Ltd (Chengdu, China). All other chemicals and solvents were of analytical reagent grade.

2.2. Collection of Poria cocos samples and environmental factors data

P.cocos samples were obtained in three production origins in China, i. e, Ning'er County (30°57′N, 115°3′E), Pu'er City, Yunnan Province (YN); Jinzhai County (30°57′N, 115°3′E), Lu'an City, Anhui Province (AH); and Jingzhou County (26°12′N, 109°16′E), Huaihua City, Hunan Province (ZJ). The same strain and agricultural operation process were performed for the cultivation of these samples in the three regions. Planting took placed in June and harvested in December 2021. Nine samples were randomly harvested in each production area for metabolism analysis.

The environmental factors, including minimum and maximum temperatures, pollution index, PM10, PM2.5, NO_2, O_3, CO, and air humidity were recorded for each day during the cultivation period. The values of the altitude of each cultivation base were determined by the global positioning system. The N, P, K, and pH of the soil were measured at five random points of each site at the cultivation base using a soil measuring instrument.

2.3. Sample preparation for metabolomic analysis

P.cocos samples were ground in liquid nitrogen, and 200 μL of pre-cooled water and 800 μL a mixture of pre-cooled methanol and acetonitrile (1:1, v/v) were added to 50 mg powder of each *P. cocos* sample and then placed for 1 h ultrasonic shaking in ice bath. Subsequently, the mixture was placed at −20 °C for 2 h and centrifuged at 16,000 g for 20 min at 4 °C. The supernatants were recovered and concentrated to dryness in vacuum. For mass spectrometry (MS), 100 μL of methanol--water solution (1:1, v/v) was added and the supernatant was centrifuged at 20,000g for 40 min at 4 °C. Then immediately collect the supernatant for liquid chromatography-mass spectrometry (LC-MS) analysis. In addition, to ensure data quality for metabolic profiling, quality control (QC) samples were prepared by pooling aliquots of all analyzed samples and used for data normalization. Reagent blank (water) and QC samples were injected every three samples during data acquisition. Each sample was filtered using a disposable cellulose acetate membrane (0.22 μm), transferred into 2 mL HPLC vials and stored at −80 °C until further analysis.

2.4. UHPLC-ESI-Q-Orbitrap-MS analyses

During the whole UHPLC-ESI-Q-Orbitrap-MS analysis, the samples were placed in an automatic sampler at 4 °C, and metabolomics profiling was performed using a UHPLC-ESI-Q-Orbitrap-MS system (UHPLC, Shimadzu Nexera X2 LC-30AD, Shimadzu, Japan) coupled with Q-Exactive Plus (Thermo Scientific, San Jose, USA). The hydrophilic interaction liquid chromatography (HILIC) separation, the chromatographic separation was achieved on an ACQUITY UPLC BEH column Amide (2.1 mm × 100 mm, 1.7 mm, Waters Technology Co. Ltd.) at a flow rate of 0.3 mL/min. Each sample was injected in a 2 μL volume after equilibration loaded at 25 °C. For the mobile phase, solvent A was water and 25 mM ammonium acetate, and solvent B was 100% acetonitrile. Chromatographic conditions of gradient elution were 95% B for 1 min, reduced to 65% B in 7 min, and then reduced to 35% B in 2 min and maintained for 0.5 min, followed by a sharp increase of B from 35% B up to 95% B in 0.5 min, and then a stable period at the volume ratio of 95% B for 4 min. The total procedure time for each sample was 15 min.

Both ESI positive and negative mode were applied for MS data acquisition. *P. cocos* was detected in positive (+) and negative (-) ion modes by ESI, and the minimum number of fragment ions were three. The ionization conditions were as follows: spray voltage: 3.8 kV (+) and 3.2 kV (-); capillary temperature: 320 °C (±); sheath gas: 30 psi (±); aux gas: 5 psi (±); probe heater temp: 350 °C (±); S-lens RF level: 50. The MS acquisition settings were as follows: MS acquisition time: 12 min. Parent ion scan range: 80–1200 *m/z*, primary MS resolution: 70,000@*m/z* 200, automatic gain control (AGC) target: level 1 maximum it: 100 MS. The secondary MS data were acquired as follows: full scan followed by triggered acquisition of 10 secondary mass spectra of the highest intensity parent ions (MS2 scan), secondary mass spectral resolution: 17,500 @ *m/z* 200, AGC target: level 2 maximum it: 100 MS, MS2 activation type: higher-energy collisional dissociation, isolation window: 2 *m/z*, normalized collision energy: 10, 20, and 30 eV.

2.5. UHPLC-QTOF-MS/MS analysis

Referring to the methodology constructed for the determination of *P. cocos* triterpene acids in the previous study of our team (Jian et al., 2019), the chemical markers of 12 triterpene acids, namely HDTRA, PAB, PAA, PAC, PAAM, AHDTRA, AHTRA, DPA, PA, DTRA and DTUA were accurately weighed and dissolved in ethanol to make standard solutions of triterpene acids at appropriate concentrations. After grinding the dried sample, 0.50 g of the ground sample was precisely weighed into a 10 mL centrifuge tube and extracted with 5 mL of ethanol in an ultrasonic water bath (25 Hz) at 60 °C for 2 h with shaking every 15 min. The extracted solution was filtered through a 0.22-m polytetrafluoroethylene injection filter and the filtrate was used for identification.

Quantitative analysis of twelve triterpene acids using an Agilent 6530 UHPLC-QTOF-MS/MS system (Agilent Technologies, Wilmington, DE, USA). This was coupled with a Poroshell 120 SB-AQ Column (4.6 mm × 100 mm, 2.7 μm) at 25℃. The mobile phase were 0.1% (v/v) formic acid/water (A) and acetonitrile (B). For identification, samples were separated using the following optimized linear gradient elution program: 0–5 min, 49% A; 5–30 min, 49 %-28% A; 30–37 min, 28 %-14% A; 37–47 min, 100% B; 47–60 min, 100% B. The flow rate was 1.0 mL/min, the injection volume was 20 μL and the detection wavelengths were 210 nm and 245 nm. The condition of Q-TOF was as follows: scan range 100–1000 *m/z*, drying gas temperature, 320 °C; sheath gas temperature, 320 °C; drying gas (N_2) flow rate, 8.0 L/min; capillary voltage, 3.5 kV; fragmentor, 110 V; collision energy at 30, 40 and 50 eV. Data analysis was done by the soft Qualitative Analysis B.05.00. The chemical constituents of *P. cocos* triterpenoids were analyzed and identified based on retention time and MS data.

2.6. Metabolomics data preprocessing, filtering and analysis

The raw MS data were processed using MS-DIAL for peak alignment, retention time correction and peak area extraction. The metabolites were identified by accuracy mass (mass tolerance < 0.01 Da) and MS/MS data (mass tolerance < 0.02 Da) which were matched with HMDB, MassBank and self-built metabolite standard library which was generated by Shanghai Bioprofile Biological Technology Co., LTD.

In the extracted-ion features, only those variables with more than 50% of the nonzero measurement values in at least one group were retained. The peaks of positive and negative ions were integrated, and R software was used for pattern recognition. The data matrix was uploaded to MetaboAnalyst 3.0 for principal components analysis (PCA), orthogonal partial least-squares discriminant analysis (OPLS-DA), hierarchical cluster analysis (HCA) and other data analysis including K-means clustering, volcano plot, Venn diagrams analysis, and correlation analysis. The remaining data were analyzed by using GraphPad Prism 6.0. The data were statistically evaluated using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). According to a guideline regarding the univariate statistical analysis of metabolomics-derived data, the metabolites identified through a multivariate analysis were subjected to analysis of variance (ANOVA)(Qza et al., 2022).

3. Results

3.1. Metabolomics analysis using UHPLC-ESI-Q-Orbitrap-MS

UHPLC-ESI-Q-Orbitrap-MS technology is widely used in metabolomics research because of its high sensitivity and separation efficiency (Zhong et al., 2022). Primary and secondary metabolites in *P. cocos* samples were identified by UHPLC-ESI-Q-Orbitrap-MS technique to have a better understanding of the variation pattern of metabolites in different origins. Total ion chromatograms of *P. cocos* samples from different geographical origins were obtained using UHPLC-ESI-Q-Orbitrap-MS in positive and negative ion mode. From the total ion flow chromatograms (TIC, Fig. S1,2,3,4) of one sample and QC samples, it was found that a large amount of compound information could be detected in either mode, indicating that the UHPLC-ESI-Q-Orbitrap-MS method is suitable for *P. cocos* sample data analysis. In this study, MS-DIAL software was used to extract the metabolite ion peaks. After peaks alignment and filtration, we could identify 19,470 positive ion features and 17,427 negative ion features. More characteristic ions were obtained for ESI+ than ESI- in different ionization modes, indicating that the material was covered better by the ESI+ ionization mode. In the ESI+/ ESI- mode, the total ion chromatograph (TIC) of *P. cocos* samples from different species in three regions of YN, AH and JZ were detected, and a total of 3137 metabolites were identified.

3.2. PCA and OPLS-DA of samples from the three regions

PCA is an unsupervised statistical approach capable of reflecting inter- and intra-group differences through several principal components representing metabolomic features under multidimensional data (Cubero-Leon, Penalver, & Maquet, 2014). The PCA model parameters are shown in Table S1. PCA analysis showed a close clustering of the QC in both positive and negative modes (Fig. 1A-B), indicating the efficient reproducibility of the experiment is well reproducible. In the score plot of PCA, among the three regions, JZ and AH regions were closely clustered together and the YN region displayed a certain degree of separation, indicating that PCA was not suitable for the differentiation of the metabolites in *P. cocos* sampled from different origins.

The *P. cocos* samples from the three regions were not well clustered in the PCA analysis, in order to achieve prediction of sample types screening out potential biomarkers that cause differences between three groups, therefore, OPLS-DA was applied to identify the major difference in metabolic profiles among groups and facilitate the identification of unique metabolites. To screen for biomarkers of *P. cocos* from the three regions of cultivation (YN, AH, and JZ), we established a stable and reliable model with 200 reciprocal tests so that both R2 and Q2 were close to 1 and the intercept of the Q2 regression line was <0, which did not produce overfitting. The model parameters are listed in Table 1. By contrast with the PCA, the clusters of *P. cocos* samples from the three regions of AH, YN, and JZ clusters exhibited a high degree of discrimination (Fig. 1C-D). This finding suggested that *P. cocos* from three different origins could be classified through differential metabolites, indicating the potential of these components to be developed as quality markers for *P. cocos*.

3.3. HCA of Poria cocos samples from the three regions

Hierarchical clustering of sample groups was performed using qualitative differences in metabolite expression levels. For this, we applied the heatmap of HCA to show the distribution patterns of important metabolites. Metabolites clustered in the same cluster indicate that they are in a relatively close reaction step in the metabolic process, with redder colors indicating higher relative expression and bluer colors indicating lower relative expression. As shown in Fig. 2A-B, all *P. cocos* samples could be well categorized into three groups by their geographical origin in positive and negative modes, and clustered into two branches according to the high and low content of metabolites. AH and JZ clustered together and YN clustered as a separate branch, this was consistent with results of the PCA results, indicating relatively similar metabolite expression patterns in samples from AH and JZ.

The K-means clustering was performed on selected differential metabolites to further investigate the differences of metabolites from different origins. The differential metabolites were grouped into four clusters; in the positive mode (Fig. 2C), cluster 1 included 10 metabolites, cluster 2 included 23 metabolites, and these 33 metabolites had high relative expression in the samples from JZ origin, followed by samples from AH and YN, in the same order. Cluster 3 included 15 metabolites, which had highly expressed in AH, and cluster 4 included

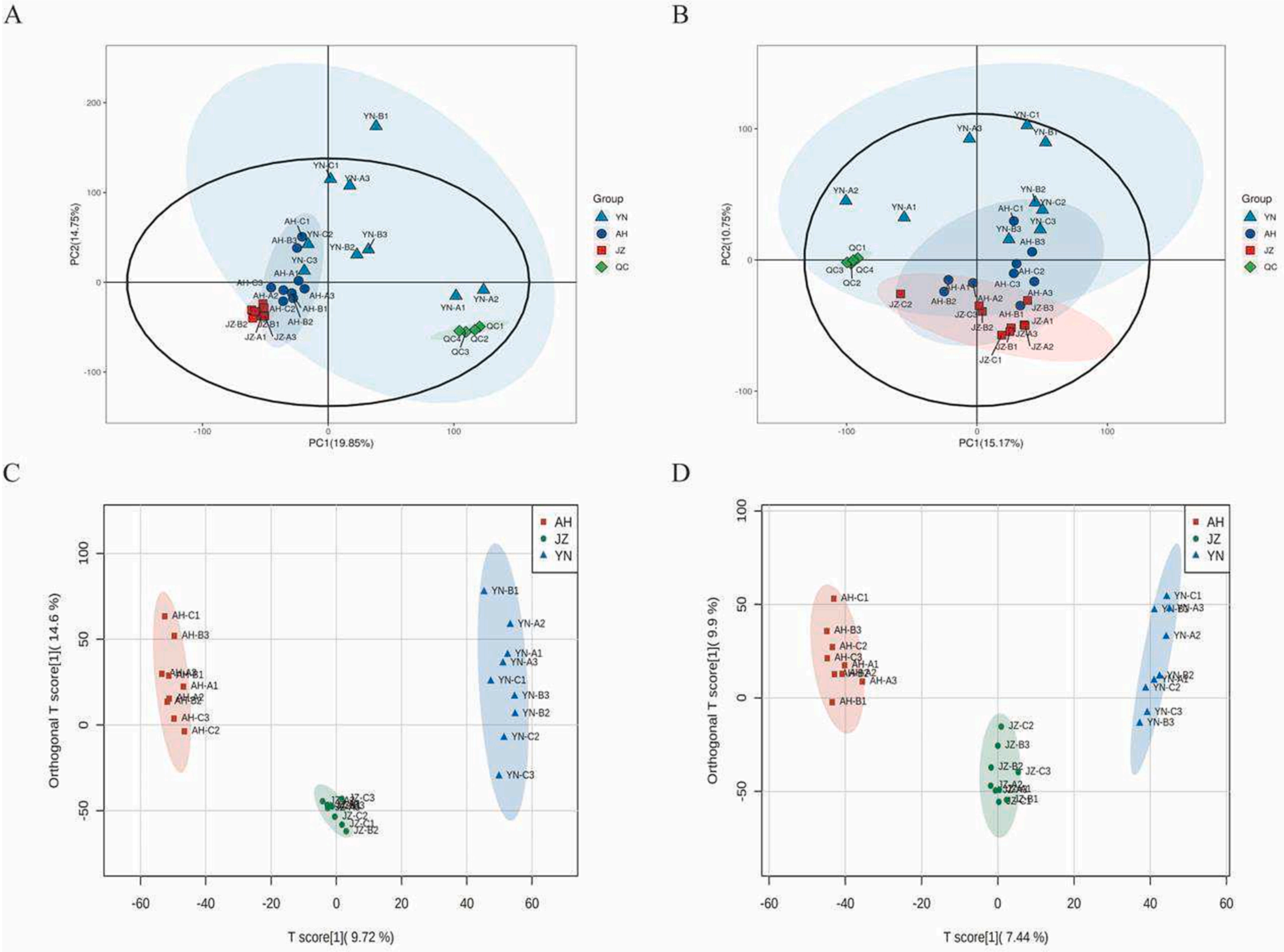

Fig. 1. The principal component analysis (PCA) score plots of *Poria cocos* samples in electrospray ionization (ESI)+ mode (A) and ESI– mode (B). The orthogonal partial least-squares discriminant analysis (OPLS-DA) score plots of *P. cocos* samples in ESI+ mode (C) and ESI– mode (D).

Table 1
The evaluation parameters of OPLS-DA models of *Poria cocos* from three regions.

Group	ESI+			ESI–		
	R2Y (cum)	R2X (cum)	Q2 (cum)	R2Y (cum)	R2X (cum)	Q2 (cum)
JZ.vs.YN	0.992	0.337	0.914	0.992	0.249	0.747
AH.vs.JZ	0.997	0.39	0.906	0.995	0.191	0.696
AH.vs.YN	0.985	0.241	0.747	0.993	0.198	0.700
AH.vs.ZJ. vs YN	0.996	0.389	0.861	0.995	0.240	0.825

60 metabolites, with high relative expression in YN and the lowest expression in JZ. In the negative mode (Fig. 2D), cluster 1 included 10 metabolites, cluster 2 included 18 metabolites, and cluster 4 included 24 metabolites; these 52 metabolites were expressed at relatively high levels in samples from YN origin and their lowest expression was observed in samples from JZ. Cluster 3 included 28 metabolites, which had relatively high expression in AH.

3.4. Identification and analysis of candidate differential metabolites

The positive mode and the negative ion modes were used to construct the OPLS-DA model, and the pairwise and combined comparison of the three regions were performed. Metabolites with VIP values of the OPLS-DA greater than 1.0 and p-values < 0.05 obtained from ANOVA were considered as potential marker compounds contributing to the class discrimination (Zhang, Yu, Li, Zhong, & Liu, 2020). The combination of fold change (FC) ≥ 1.5 or ≤ 0.667 and $P < 0.05$ was used to screen for differential metabolites with differential expressions based on their p-value and VIP value knots. In ESI + mode, 98 significantly different metabolites were identified between AH and YN (60 upregulated, 38 down-regulated) (Fig. 3A), 157 between AH and ZJ (96 upregulated, 61 down-regulated) (Fig. 3C), and 180 between ZJ and YN (85 upregulated, 95 down-regulated) (Fig. 3E). In ESI - mode, there were 78 significantly different metabolites between AH and YN (38 upregulated, 40 down-regulated) (Fig. 3B), 61 between AH and ZJ (41 upregulated, 20 down-regulated) (Fig. 3D), and 125 between ZJ and YN (55 upregulated, 70 down-regulated) (Fig. 3F). The shared differential metabolite and unique differential metabolite profiles of the different comparison groups were demonstrated through Venn diagrams (Daniore, Nittas, Moser, Höglinger, & von Wyl, 2021). For each comparison group, intersections in a Venn diagram were assessed; amongst these identified differential metabolites, there were a total of 46 differential metabolites in the three origins, with 46, 14, and 43 unique differential metabolites in the AH and JZ comparison groups, AH and YN, and JZ and YN comparison groups, respectively, in the positive mode (Fig. 3G). In the negative mode, there were a total of 12 differential metabolites in the three origins and 13, 15, and 40 unique differential metabolites in the AH and JZ, AH and YN, and JZ and YN comparison groups, respectively

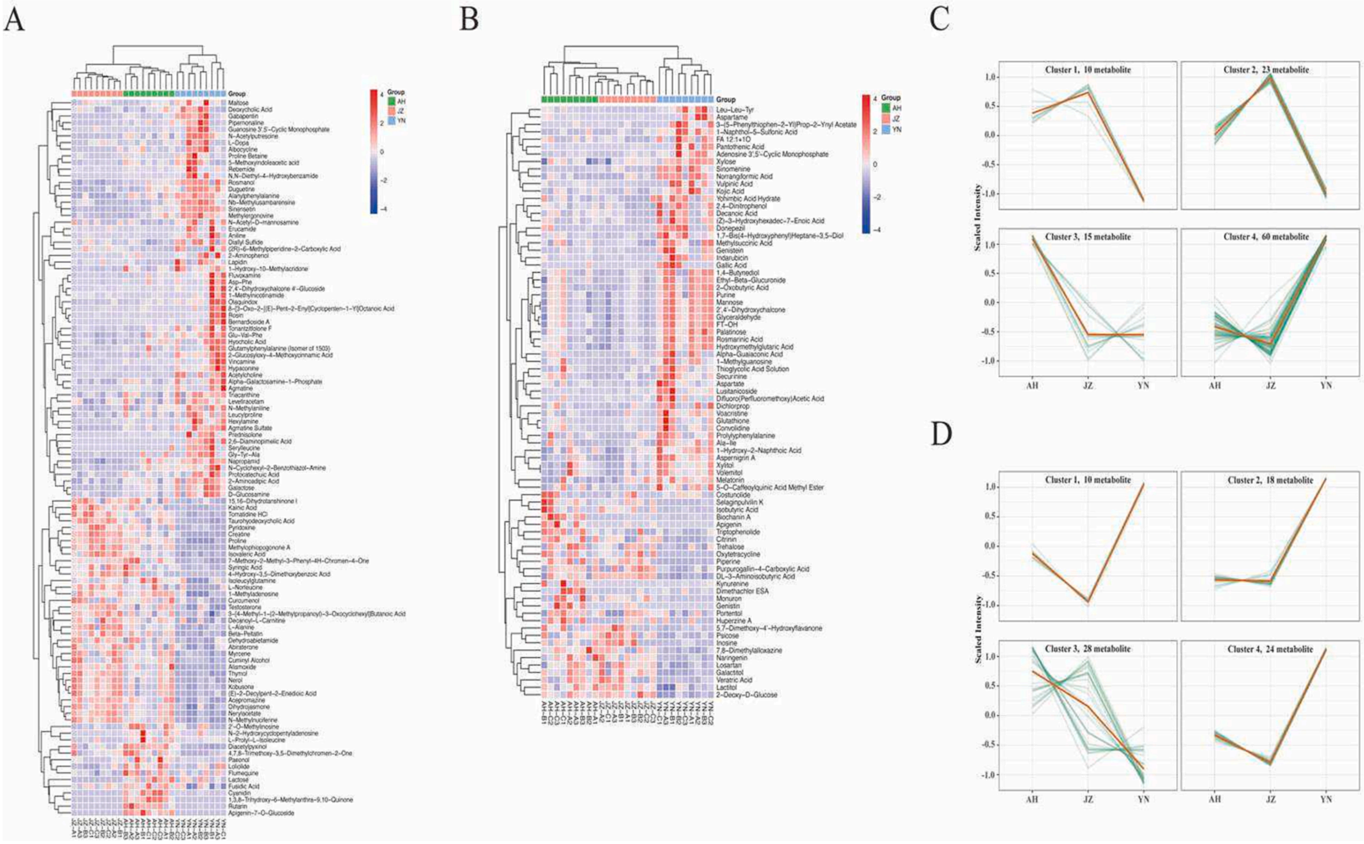

Fig. 2. Differential profile of metabolites of *Poria cocos* samples from geographical origins. Heatmap visualization of the identified differential metabolites in *Poria cocos* samples from Yunnan Province (YN), Anhui Province (AH), and Jingzhou County (ZJ) regions for electrospray ionization (ESI) + mode (A) and electrospray ionization (ESI)– mode (B). Red and blue colors, respectively represent the high and low content of target compounds. K-means cluster analysis of differential metabolites for ESI+ mode (C) and ESI– mode (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3H). Further multi-group analysis revealed that a total of 188 metabolites were significantly different in positive and negative modes when the samples from three origins of *P. cocos* samples were compared simultaneously. Among these 188 metabolites, 112 were significantly higher in the samples from YN origin than the other two origins, 33 were significantly higher in the samples from AH origin than the other two origins, and 43 were significantly higher in the samples from JZ origin than the other two origins. This indicates the promising potential of these metabolites to distinguish of *P. cocos* from different origins of cultivation.

3.5. Metabolite biomarkers differentiating geographical origins

Then, we investigated the differential metabolites of *P. cocos* samples from three geographical origins. In differential metabolite profiles, carbohydrates, amino acids, organic acids, nucleic acids, peptides, flavonoids, nitrogenous organic compounds, antibiotics and hormones dominated. The information on the profiles of metabolites of carbohydrates and amino acids is shown in Supplementary Data Table S2. Also based on the fact that carbohydrates and amino acids are the main nutrients of *P. cocos* (Xiang et al., 2016), these metabolites were selected for relative abundance and origin significance analysis. As shown Fig. 4, we can see that these three carbohydrates had the highest relative abundance in samples from YN, followed by AH, and the lowest in JZ. The relative abundance of the above-mentioned four amino acids was the highest in samples from JZ, followed by those from AH, and the lowest in samples from YN. Therefore, three carbohydrates (mannose, galactose, and palatinose) and four amino acids (proline, L-alanine, L-norleucine, and kainic acid) were identified as important biomarkers for tracing the origins of *P. cocos*. Thus, we could achieve the differentiation of the relative levels of these biomarkers in *P. cocos* from different origins.

3.6. UHPLC-QTOF-MS/MS analysis of 11 triterpene acids

The contents of 11 triterpene acids in these *P.cocos* samples were determined through HPLC analysis. Eleven peaks, namely those for HDTRA, PAB, PAA, PAC, PAAM, AHDTRA, AHTRA, DPA, PA, DTRA and DTUA, were clearly and accurately identified based on their retention time, molecular and fragment ions generated by QTOF-MS/MS, and comparison of the MS data to their reference standards. The results showed that the ionic peaks of 11 triterpenoids were detected in samples from all three origins, among which the contents of PAA, PAB, PAAM and PAC were significantly higher in samples from JZ than in those from YN, and these four triterpenoids showed significant differential expression in the samples from the three origins (Fig. 4). The other seven triterpenoids also showed the highest relative content in the samples from JZ origin; suggesting that the content of triterpenoids varied greatly among the geographic origins and can be used as biomarkers to differentiate the growth origins of *P. cocos*.

3.7. Correlation analysis between metabolites and environmental factors

The interdependence between various metabolites and environmental factors was evaluated through correlation matrices for correlation analysis between carbohydrates, amino acids, triterpenoids and environmental factors. The correlation coefficients (R) between the differential metabolites are between −1 and +1. R > 0 indicates a

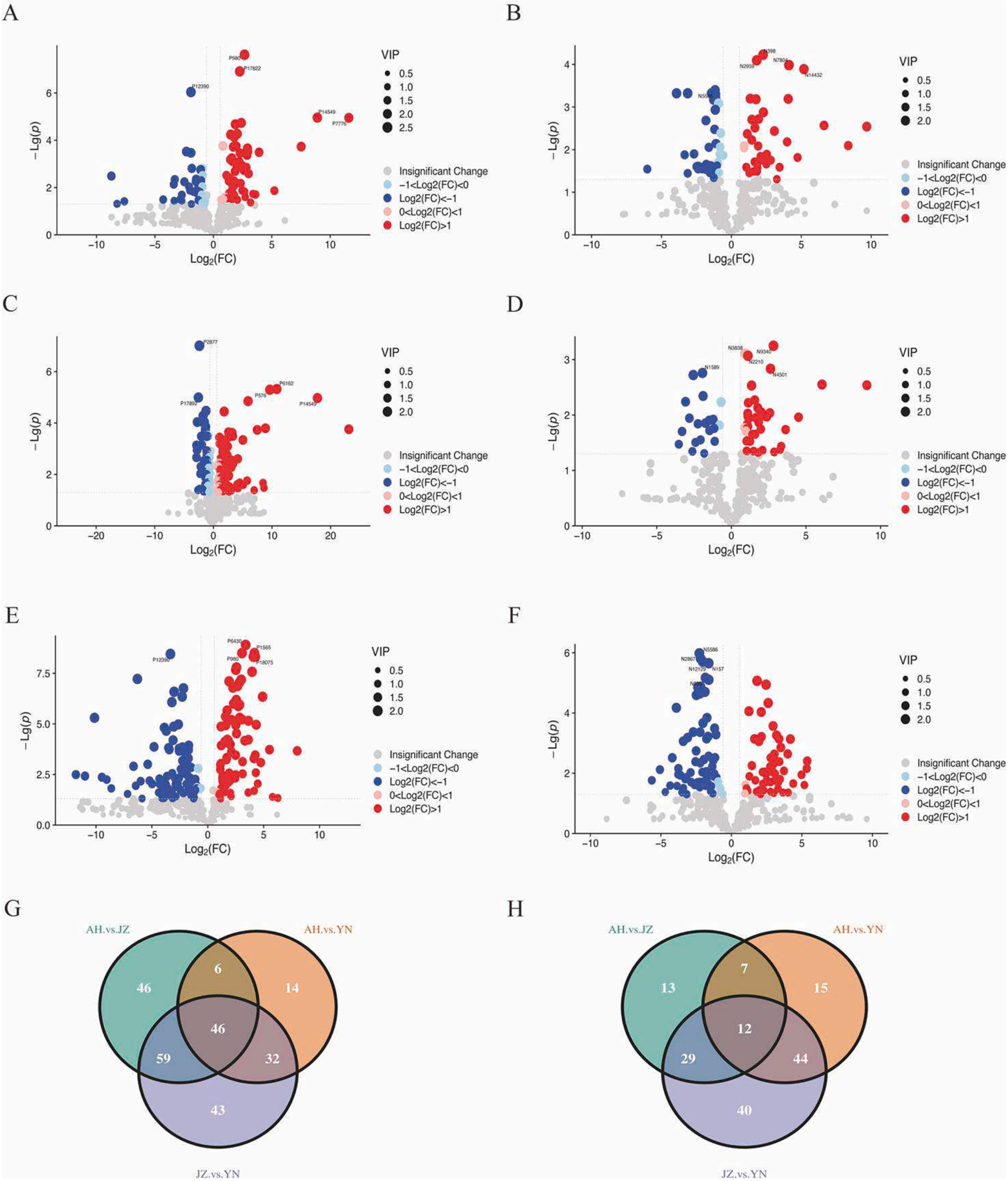

Fig. 3. Volcano plot of differential metabolites of *Poria cocos* from different origins in electrospray ionization (ESI)+ mode, (A) AH/YN, (C) AH/JZ, (E) JZ/YN, and in ESI − mode (B) AH/YN, (D) AH/JZ, (F) JZ/YN. The number of differential metabolites venn diagram analysis in ESI (G) + mode and in ESI − mode (H).

positive correlation and is shown in red; R < 0 indicates a negative correlation and is shown in blue. A larger percentage of the colored interval indicates a stronger positive/negative correlation (Fig. 5A-B). The size of the circle represents the significance of the corresponding correlation. The environmental factors of the origins of cultivation mainly include altitude, temperature (average daily highest temperature, average daily lowest temperature, and average daily temperature difference), relative humidity, air quality (air pollution index, PM10,

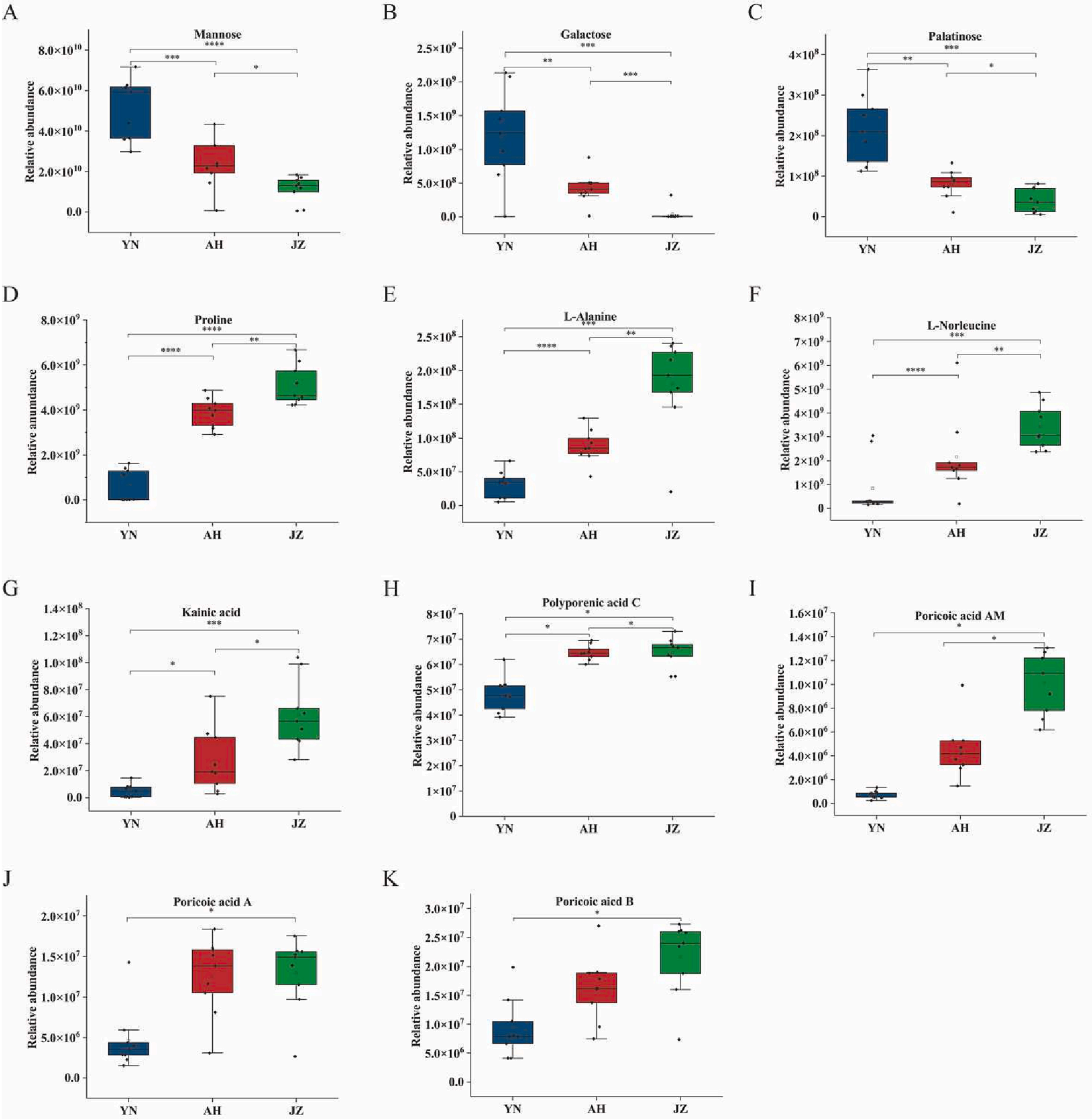

Fig. 4. Box plots of significantly different metabolites across *Poria cocos* samples obtained from three different geographical origins. (A-C) carbohydrates, (D-G) amino acids, and (H-K) triterpenoids. Data are mean values ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

PM2.5, NO_2, SO_2, O_3 and CO concentration), soil fertility (the content of soil trace elements N, P, K, moisture and pH in the soil). Among the screened biomarkers, four amino acid and four triterpenoid biomarkers showed significant negative correlations with altitude, daily maximum temperature, daily temperature difference, relative humidity and soil moisture, and significantly positive correlations with elements such as N, P, and K in soil and air pollution index, PM10, PM2.5, O_3, and CO in the air. In contrast, the eleven carbohydrate compounds showed opposite correlations with amino acids and triterpenoids, positive correlations with altitude, daily maximum temperature, daily temperature difference, relative air humidity, and soil moisture, and negative correlations with elements such as N, P, and K representing soil fertility and pollution indices, PM10, PM2.5, O_3, and CO representing air quality (Fig. 5C). However, particularly, the correlations between these 11 compounds and daily minimum temperature, NO_2, SO_2, concentration and soil pH were not significant. We also analyzed the correlations among the 11 metabolites, and found the results showed that amino acids were significantly positively correlated with triterpenoids and significantly negatively correlated with carbohydrates (Fig. 5D). Further, correlation analysis showed that environmental factors had similar effects on *P. cocos* amino acid metabolites and triterpenoids metabolites, while the opposite effect was observed for carbohydrate

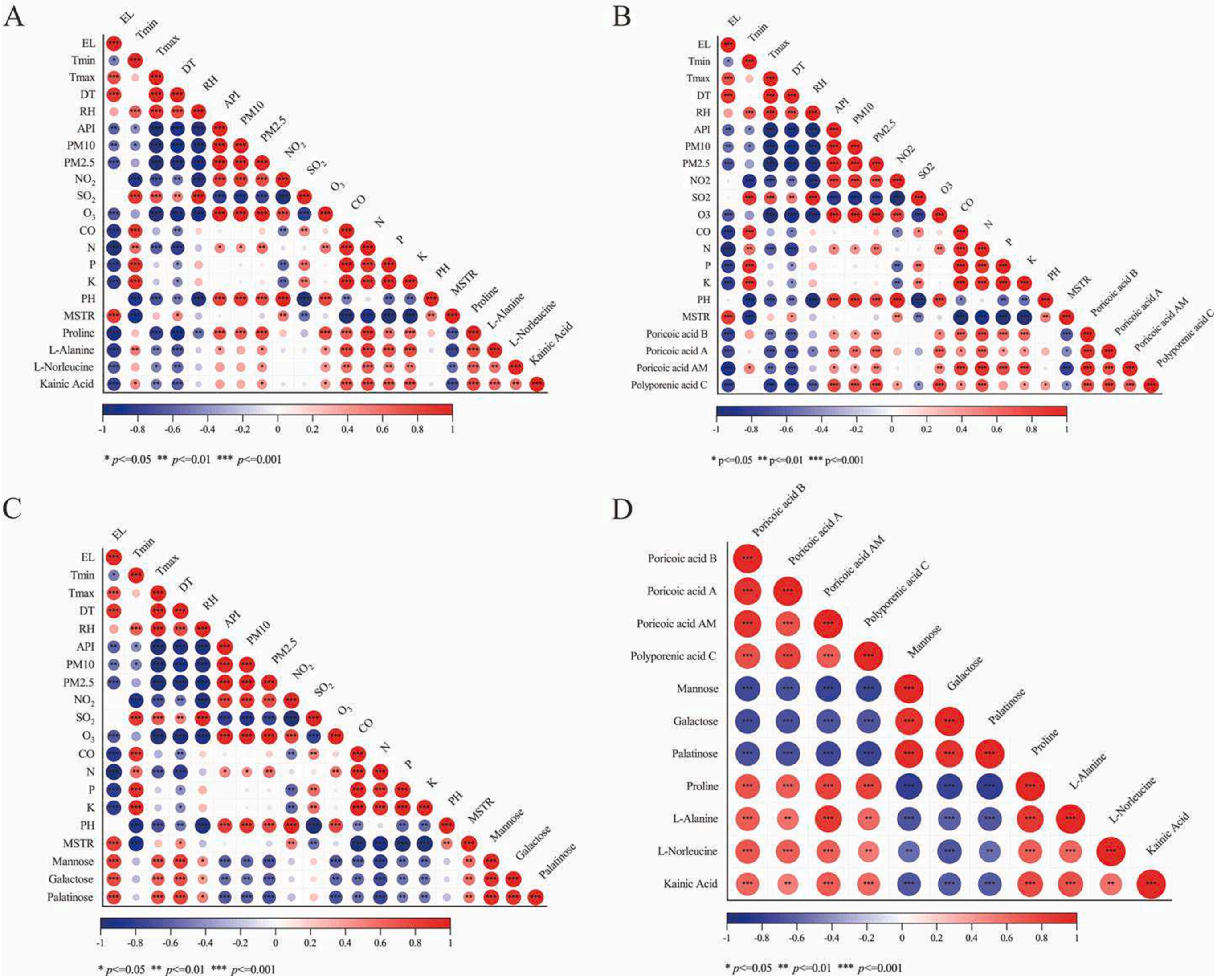

Fig. 5. Correlation among (A) amino acids, (B) triterpenes, (C) carbohydrate species biomarkers, and (D) environmental factors, and correlations between amino acids, triterpenes, and carbohydrates. EL: Altitude, Tmin: Minimum temperature, Tmax: Maximum temperature, DT: Differential temperature, RH: Relative humidity, API: Air pollution index, MSTR: Moisture.

metabolites.

4. Discussion

To comprehensively assess the differences in metabolic fingerprints of *P. cocos* samples from three different cultivation regions, qualitative and quantitative analysis by LC-MS/MS was conducted on the metabolites of 27 dried *P. cocos* samples, and a total of 3137 metabolites were identified, of which the expression of 188 metabolites were differed significantly. Then, unsupervised and supervised discrimination models were constructed using PCA and OPLS-DA, the most commonly used multivariate analysis techniques in metabolomics. Under unsupervised conditions, the samples from three groups samples could not be completely distinguished due to the limitations of the PCA model (Zhao, Zhao, Han, Ding, & Chang, 2020). According to the clustering analysis of the relative expression of metabolites, *P. cocos* samples from different origins were categorized into three regions based on their similarity, and most of the metabolites were highly expressed in samples from YN, while they showed low levels in samples from JZ.

Amino acids and carbohydrates are essential nutrients needed by the human body and play an important role in the physiological activities of the human body. However small molecule carbohydrates and amino acids in *P. cocos* have received little attention. Herein, amino acids and carbohydrates were selected as biomarkers for the differentiation of *P. cocos* origin through a comprehensive assay of non-targeted metabolomics. Consistent with previous studies, we observed significant differences in amino acids in samples from different origins (Zhao et al., 2022). We also analyzed carbohydrates in *P. cocos* rather than nucleosides and bases that are present alongside amino acids. In previous studies, researchers focused on the effect of the origin on *P. cocos* polysaccharides; this is the first report of the analysis of small carbohydrates molecules in *P. cocos*. This is an important finding in the understanding of the correlation relationship between the origin of *P. cocos* and its nutritional composition. The present findings confirm that the difference in nutrient content may lead to differences in the quality of *P. cocos* samples from different regions. As an important criterion to determine the quality of *P. cocos*, triterpenoids can be used for origin differentiation (Lixia et al., 2018). Ten triterpenoids were unequivocally or tentatively identified, and were found to play key roles in differentiating the *P. cocos* samples on the basis of their origins and can be thus used as biomarkers for origin identification and authenticity establishment of *P. cocos* (Bing, Yan, Hong, Li, & Hong, 2014; Zhu et al., 2018).

We found that the expressions of PAB, PAA, PAC and PAAM were significantly different among samples from different geographical origins, confirming the findings from previous studies and indicating that these markers could be used as good reference compounds for QC and origin differentiation of *P. cocos*.

The excellent quality of *P. cocos* from different origins is closely related to the local environmental factors. Significant differences were observed in the nutrient contents of *P. cocos* in samples from different geographical origins, owing to different geographical and climatic environments. At six sites in YN province, altitude is an important factor influencing the concentration of elements in *P. cocos*, and a relatively low altitude is conducive to the accumulation of elements (Yue, Li, Zuo, Liao, Huang, & Wang, 2021). In contrast, when we extended the geographical origins for comparative analysis, we found that the relative expression of metabolites was higher in *P. cocos* samples from the higher altitude, YN. We also analyzed the relationship between *P. cocos* metabolites and temperature, air quality and soil fertility. According to the significance of correlation, altitude, temperature, and soil fertility were found to be the main environmental factors leading to the differences in the expression of metabolites of *P. cocos* in different origins, while the effect of air quality was relatively small. Earlier studies have only speculated on the effects of environmental factors when assessing the relationship between metabolite differences and cultivation origin (Li, Zuo, & Wang, 2022). In contrast, we used data to support this and confirmed a robust link between environmental factors of the regions of cultivation and the expression of metabolites. The results will be benefit us in selecting a more suitable origin for the growth of *P. cocos* for breeding and cultivation, and obtaining high quality *P. cocos* samples.

5. Conclusions

P. cocos from different origins have similar biological characteristics and are difficult to be distinguished based on their physical appearance. The present study provides a systematic and comparative identification of the metabolites and their profiles using UHPLC-ESI-Q-Orbitrap-MS and UHPLC-QTOF-MS/MS in *P. cocos* from three cultivation origins in China, namely AH, JZ and YN. Multivariate analyses (PCA, OPLS-DA and HCA) revealed that the *P. cocos* samples from three origins could be clearly categorized into three groups according to their metabolite profiles. A total of 188 differential compounds were identified in positive and negative ionization modes. By metabolite significance analysis, three carbohydrates, and four amino acids, four triterpenoids, were identified as biomarkers for tracing *P. cocos* origin tracing. Correlation analysis showed that altitude, temperature and soil fertility were the main environmental leading to significant differences in metabolites profiles of *P. cocos* from different origins.

CRediT authorship contribution statement

Xiaoliu Liu: Investigation, Methodology, Writing – original draft. **Can Zhong:** Visualization, Supervision, Project administration. **Jing Xie:** Formal analysis, Project administration. **Hao Liu:** Supervision, Validation. **Zhenni Xie:** Resources, Data curation. **Shuihan Zhang:** Conceptualization, Supervision, Writing – review & editing. **Jian Jin:** Conceptualization, Supervision, 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.

Data availability

Data will be made available on request.

Acknowledgments

We greatly appreciate Hunan Butian Pharmaceutical Co., Ltd. for their help with sample collection and Shanghai Bioprofile Technology Co., Ltd. (Shanghai, China) for their help with data analysis. This work was supported by the National Natural Science Foundation of China (No. 81803664), China Hunan Provincial Science & Technology Department (2022NK2005, 2022GK4015 and 2022RC1224), Hunan Provincial Administration of Traditional Chinese Medicine (2021012), Training Program for Excellent Young Innovators of Changsha (kq2206064).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.135817.

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