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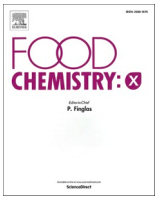

The key metabolite of fruit flavor change in different ripening stages of *Baccaure ramiflora*

Chongcheng Yang [a,1], Huachen Wang [a,1], Jiaqi Chen [a], Yang Zhang [b], Jianjian Huang [c,*], Jie Chen [a,*]

[a] College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, China
[b] Jiangxi Ganzhou Eco-environmental Monitoring Center, Ganzhou 341000, China
[c] School of Food Engineering and Biotechnology, Hanshan Normal University, Chaozhou 521041, China

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ABSTRACT

Baccaurea ramiflora has an unstable ripening period. Herein, five typical periods of fruit ripening of 'LR' *Baccaurea ramiflora* were analyzed by non-targeted metabolomics techniques. The results showed that ripening started 73 days after flowering and reached the ripening criterion at 93 days, a total of 451 differential metabolites were identified for the five periods. KEGG enrichment pathway showed that significant changes in citric acid were significantly correlated with changes in the downstream substance spermine ($R^2 = 0.9068$, $y = -5.49 + 0.66\times$), while citric acid ($R^2 = 0.9982$) and spermine ($R^2 = 0.9841$) were negatively correlated with the sugar-acid ratio. Citric acid was the main component of titratable acid and spermine ($R^2 = 0.9991$) was positively correlated with titratable acid. We speculated that citric acid is a key taste marker for fruit ripening in 'LR' *B. ramiflora*. The results of the study provide new metabolic evidence for flavor changes and scientific basis for their quality improvement and exploitation in *B. ramiflora*.

1. Introduction

Baccaurea ramiflora Lour. is a small evergreen tree in the genus *Baccaurea* in the family Phyllanthaceae (J. Huang et al., 2024). *B. ramiflora* produce abundant clusters of fruit that hang onlong strings from the twigs, main branches, and to a lesser extent, from the upper part of the trunk, mainly found in mountain forests and valleys in southern China (Guangdong, Guangxi, Hainan, Yunnan) as well as in Nepal, Bangladesh, Thailand, Myanmar, Indonesia, India and Malaysia, which is an important source of supplementary food (Hossain et al., 2017). Generally, the pulp yield of an adult tree can reach 100–150 kg, bringing many economic benefits to farmers (Pandey et al., 2018). *B. ramiflora* has a good edible, with a single fruit mass of 14.72–15.71 g, hardness of 3.442–3.353 kg/cm^2, soluble solids content of 13.67–14.37 %, titratable acid content of 1.25–1.39 %, and vitamin C content of 3.65–3.93 mg/100 g, which is also a good source of vitamin C (Kong et al., 2024). The fruit is delicious and juicy, delicate taste, rich in nutrients, and can be eaten fresh, but it also can be processed into juice, dried fruit, jam, and fruit wine, which are loved by local residents. Studies have found that *B. ramiflora* also has high medicinal value, including anti-inflammatory, antioxidant, hypoglycemi, hypolipidemic, etc. (Usha et al., 2014).

With the improvement of people's living standards and health consciousness, people's demand for nutritious and health-care fruits is becoming more and more prominent, and there is an urgent need to domesticate and cultivate new types of fruits with high nutritional and therapeutic values from wild fruit resources (H. Huang et al., 2021). *B. ramiflora* is a new type of fruit with good taste, nutrition and health care, but it is limited by various factors, including the great difference in taste between different strains (Chen et al., 2023), the uncertainty of the picking and harvesting period, which leads to very acidic fruit picked too early (Peiris, 2007), and overripe fruit picked at the late stage of ripening is not conducive to storage and transportation, which leads to the *B. ramiflora* has not been well commercialized and promoted (Banerjee et al., 2022).

Developments in metabolomics can reveal the composition and changes in compounds of fruit quality during ripening including fruit flavor quality, fruit coloring, storage after picking and the analysis of

* Corresponding author.
E-mail addresses: 3668602@qq.com (J. Huang), 812242515@qq.com (J. Chen).
[1] These authors contributed equally to this work.

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active ingredients of special medicinal value (Dickson et al., 2020; Mamat et al., 2020). Chen et al. studied the key substances that determine the fruit flavor of *B. ramiflora* by non-targeted metabolomics, and discussed the amount and types of the main metabolites, such as sugars, organic acids, and amino acids(Chen et al., 2023). Yang et al. investigated the key substances determining the flavor of BR *B. ramiflora* by non-targeted metabolomics techniques and studied the changes of major metabolites such as sugars, organic acids, and amino acids during the ripening process of 'BR' *B. ramiflora* (Yang et al., 2024). The study of metabolic pathways and key metabolites during fruit ripening of *B. ramiflora* is important for the quality improvement, optimal fruit harvesting period, and storage conditions of *B. ramiflora*. However, the flavor of different strains *B. ramiflora* varies greatly, the optimal harvesting period is uncertain, the flavor variations between strains and the key taste-differentiating metabolites are not known, and the limited data limit the development and utilization of high-quality strains of *B. ramiflora.*

In this study, we chose the fruits of the five stages of maturity of the 'LR' *B. ramiflora* as the study material. The 'LR' *B. ramiflora* is a germplasm resource preserved by the local people through years of screening, with good palatability, large yield, and better storage resistance, and is now widely cultivated in the area of Guangxi from Fangchenggang to Chongzuo (Chen et al., 2023). The metabolites of 'LR' *B. ramiflora* at five periods were screened by non-targeted metabolomics, and the characteristic metabolites and potential flavor biomarkers were identified in combination with the metabolic pathways, with a view to revealing the flavor changes of *B. ramiflora* at different ripening stages, and to provide a scientific basis for the improvement and commercialization of *B. ramiflora* varieties.

2. Materials and methods

2.1. Plant materials

'LR' *B. ramiflora* (green pericarp and milky white flesh) materials were selected to sample fruits at five typical periods of fruit maturation and development (30 d, 52 d, 73 d, 93 d, 112 d after full bloom) (Fig. 1). The experimental materials were collected from *B. ramiflora* trees planted by villagers next to the Nashuo Middle School in Nashuo Town, Fangcheng District, Fangcheng Port, Guangxi, China (N 21°42′33″, E 108°6′29″, alt 20 m), and the age of the trees was more than 10 years. The trees were more than 10 years old, healthy, and in full fruit.

2.2. Sample preparation and metabolite extraction

Three well-grown and balance 'LR' *B. ramiflora* trees were selected, and six fruits of uniform and good maturity were collected from each tree at each of the five periods of fruit ripening and development (LR1-LR5, respectively 30d, 52d, 73d, 93d, and 112d after full blossom). A total of 18 fruits were mixed together in each period, and then 4 fruit flesh mixes were randomly taken as one biological sample with 3 biological replications. Immediately after collection from the tree, the sample was stored in liquid nitrogen, and then returned to the laboratory and stored in the freezer at −80 °C for spare parts.

Fig. 1. Fruits of 'LR' *B. ramiflora.* at 5 typical stages of maturity.

Firstly, 50 mg of the 'LR' *B. ramiflora* sample was weighed and placed in a 1.5 mL centrifuge tube, and then two grinding beads were added, followed by the addition of 800 μL of the extraction solution (methanol: water = 7:3, v:v, pre-cooled at −20 °C) and 20 μL of internal standards (d3-Leucine, 13C9-Phenylalanine, d5-Tryptophan, 13C3-Progesterone, 13C3-Luteinizing hormone), the centrifuge tube was put into a tissue grinder (JXFSTPRP, Shanghai Jingxin, China) for grinding (frequency 50 Hz, 5 min); then, after sonication in a water bath at 4 °C for 30 min, the centrifuge tube was rested in a refrigerator at −20 °C for 1 h; and then centrifuged at 14000 rpm for 15 min at 4 °C on a low-temperature high-speed centrifuge (Centrifuge 5430, Eppendorf). After centrifugation, 600 μL of supernatant was extracted and passed through a 0.22 μm filter membrane, and then the filtered sample was placed in a vial for UPLC-MS/MS analysis. (Chen et al., 2023).

2.3. UPLC-MS/MS analysis

Non-targeted metabolomics analysis of the pulp of *B. ramiflora* was performed using UPLC-MS/MS, with ultra-high performance liquid chromatography (Waters 2D UPLC, Waters, USA) for metabolite separation and a tandem Q Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA) for metabolite detection, and the data were collected separately in both positive and negative ion modes. Data in both positive and negative ion modes to improve metabolite coverage (Di Guida et al., 2016).

The chromatographic column was a Hypersil GOLD aQ column (100 × 2.1 mm, 1.9 μm, Thermo Fisher Scientific, USA), and the liquid composition consisted of an aqueous solution containing 0.1 % formic acid (liquid A) and 100 % acetonitrile containing 0.1 % formic acid (liquid B). The elution was performed using the following gradient: first 0–2 min, 5 % A liquid; then 2–22 min, 5 % ~ 95 % A liquid; then 22–27 min, 95 % B liquid; and finally 27–30 min, 5 % B liquid. The flow rate was 0.3 mL/min, the column temperature was 40 °C, and the injection volume was 5 μL/time.

Primary and secondary mass spectrometry data were collected for the metabolites using a Q Exactive mass spectrometer, respectively. The mass spectrometry was performed in the range of 150–1500, with a primary resolution of 70,000, AGC of 1e6, and a maximum injection time of 100 ms. According to the intensity of the parent ions, Top3 was selected for fragmentation, and the secondary information was collected with a secondary resolution of 35,000, AGC of 2e5, and a maximum injection time of 50 ms, and fragmentation energies were set at 20, 40, and 60 eV. The parameters of the ion source (ESI) were set as follows: sheath gas flow rate of 40, auxiliary gas flow rate of 10, spray voltage (|KV|) of 3.80 and 3.20 for positive and negative ion modes, respectively, the temperature of the ion transport tube of 320 °C, and the heating temperature of the auxiliary gas of 350 °C (Yang et al., 2024). Methanol (A454–4), acetonitrile (A996–4) were at LC-MS level (Thermo Fisher Scientific, USA); ammonia formate (17843-250G, Honeywell Fluka, USA), formic acid (50144-50 mL, DIMKA, the USA), and water was provided by a water purifier (Milli-Q Integral, Millipore Corporation, USA).

2.4. Quality control measurement

Data quality control (QC) samples were mixed from 20 μL of each sample from the LR pulp of the *B. ramiflora*, with three samples per period for a total of 15 QC samples, were mixed and three randomly selected to assess the reproducibility and stability of the experiment. The QC samples included the number of peak lifts, peak response intensity differences, PCA (Principle Component Analysis), and chromatogram overlap. The base peak chromatogram (BPC) of all the QC samples

overlapped, which is a continuous depiction of the intensity of the strongest ions in the mass spectrometry at each time point. The BPC overlap of the QC samples is shown in Fig. S1, and it was found that the overlap of the chromatograms was good, the retention time was basically the same and the fluctuation of the peak intensity of the peak response was small, which indicated that the instrument was in good condition during the whole sample detection and analysis process. This indicates that the instrument is in good condition and the signal is stable during the whole sample detection and analysis process.

We performed Principal component analysis (PCA) analysis by $\log_2$ processing of metabolite peak areas in positive and negative ion modes, respectively. PCA separated the five developmental stages and quality control (QC) samples significantly with significant differences ($P < 0.01$) (Fig. 2). LR1 was clearly separated from the others, LR2 and LR3 were very close to each other, and LR4 and LR5 were very close to each other. This analysis revealed five typical developmental stage characteristics of 'LR' *B. ramiflora*, respectively (Fig. 1). Thus, the principal component analysis indicated that these five periods had different metabolic characteristics.

2.5. MS data and statistical analysis

The raw data of the mass spectrometry of M. papaya pulp collected by ultra performance liquid tandem mass spectrometry (UPLC-MS/MS) were imported into Compound Discoverer 3.1 software for data processing and analysis, including peak extraction, background peak labeling, retention time correction, missing value filling, addition ion merging, and metabolite identification, etc., to obtain the information of molecular weights of the compounds, retention times, peak areas, and identification results. Metabolite identification was performed with reference to Berry's own standard library, mzCloud, and ChemSpider (HMDB, KEGG, LipidMaps) (Yang et al., 2024).

Data preprocessing was carried out via metaX software using Probabilistic Quotient Normalization (PQN) (Di Guida et al., 2016). Calculate the mean value of all QC samples to obtain the reference vector; calculate the median value between each sample and the reference vector to obtain the associated coefficient vector, and then divide each sample by the median value of the coefficient vector to obtain the relative peak area by data normalization; and then correct for the batch effect by fitting a local polynomial regression to the real sample signal based on the information of the QC samples to correct for the batch effect (Quality control-based robust LOESS signal correction, QC-RLSC) (Dunn et al., 2011). The compounds with a Coefficient of Variation (CV) greater than 30 % of the relative peak area in QC samples were deleted.

2.6. KEGG pathway enrichment analysis of metabolites

The above metabolites identified in 'LR' *B. ramiflora* were interpreted by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. KEGG is a well-known and reliable database for interpreting the molecular-level details of genomes, enzymes, and chemicals in living organisms. Pathway enrichment analysis is then performed to find key pathways based on the number of enriched factors and metabolites. Finally, metabolic pathways are mapped to describe the interpretation of key taste markers.

2.7. Statistical analysis

Statistical analysis of metabolites, including metabolite classification and functional annotation, using metaX, a metabolomics *R* package developed in-house by Berry and Wellcome Genetics, and the Metabolomics Information Analysis Process (Wen et al., 2017). The multivariate raw data were downscaled by Principal Component Analysis (PCA) to analyze similarities and differences within and between sample groups and outliers (the presence of abnormal samples). The Partial Least Squares Method-Discriminant Analysis (PLS-DA) model was used to calculate the Variable Important for the Projection (VIP) of the two principal components, and VIP was able to VIP can measure the strength of the influence of each metabolite expression pattern on the classification and discrimination of each group of samples, and can assist in the screening of metabolic marker. The data were first $\log_2$ log-transformed, and then the PLS-DA model was built, and the method used for scaling was Par; 7 times of cross-validation were performed, and 200 times of response permutation testing (RPT) were performed to determine the model quality of the model of PLS-DA. Differential metabolites were screened by combining the results of the multiplicity of change in

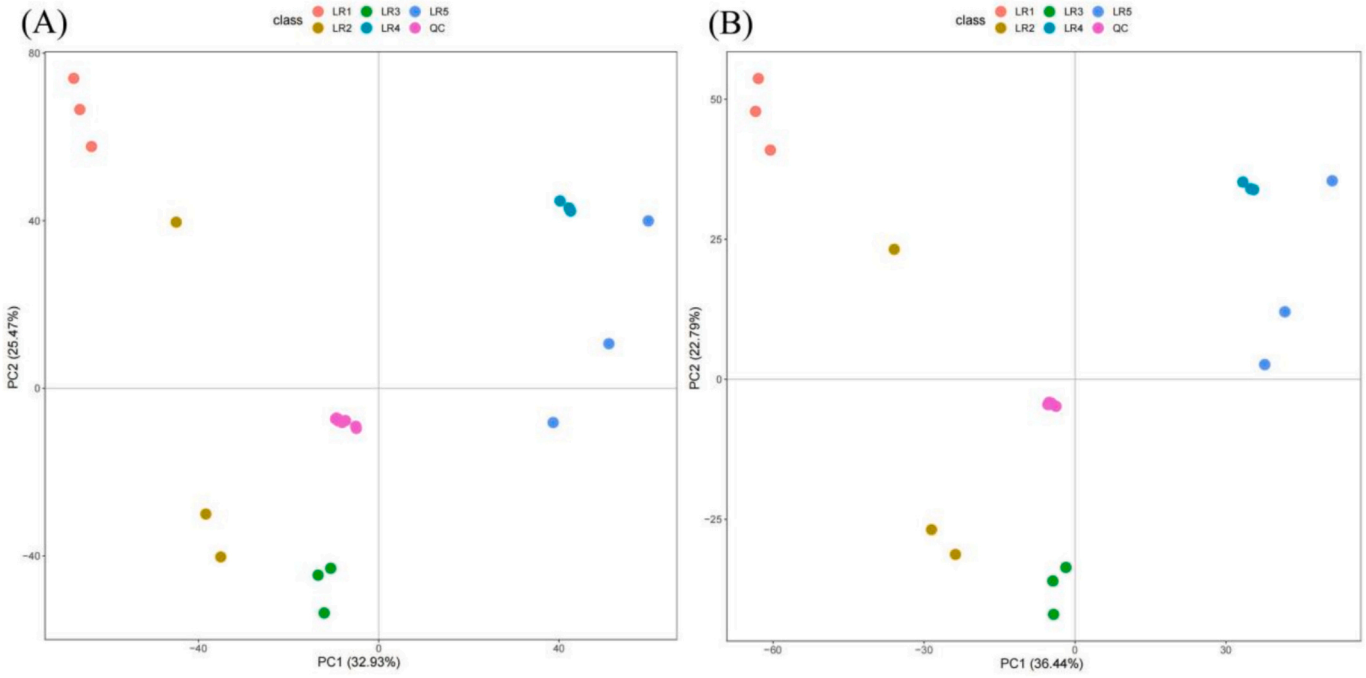

Fig. 2. Principal component analysis under positive and negative ion patterns at different maturity stages.

variance (FC, Fold change) and *t*-test (Student's t-test) obtained from univariate analysis.PCA and FC were log2 processed, and the criteria for screening were *p*-value <0.05, VIP ≥ 1, FC ≥ 1.2, or ≤ 0.83.

SPSS (22.0, IBM Corp., Armonk, NY, USA) software and OriginLab (2019, OriginLab Inc., Northampton, MA, USA) software were used for data statistical analysis and graphing and expressed as the mean ± standard deviation (SD). Data were evaluated by one-way analysis of variance (ANOVA) using Tukey's honestly significant difference (HSD) test ($p < 0.05$).

3. Results and discussion

3.1. Identification and classification of metabolites

In order to determine metabolite differences between different maturation stages of *B. ramiflora*, five periods of 'LR' *B. ramiflora*, LR1-LR5 (30d, 52d, 73d, 93d, and 112d after full bloom), were analyzed for non-target metabolites. A total of 530 metabolites were identified at different maturity stages of the 'LR' *B. ramiflora*, with 361 positive and 201 negative ion patterns, of which 32 were replicated (Table S1). The metabolites were clustered (Fig. 3), and the data were analyzed with log2-transformed zero-mean normalization, the clustering algorithm used was Hierarchical Cluster, and Euclidean Distance was used for distance calculation.

The main components affecting the flavor were the primary metabolites 42 fatty acids, 34 carbohydrates, 42 amino acids and its derivatives, 20 organic acids, 3 vitamins and the secondary metabolites 53 terpenoids, 40 flavonoids, 31 phenylpropanoids, 20 steroids and derivatives, 7 phenol, 4 phenolic acids, and the main components of the pulp metabolites during the ripening stage of 'LR' *B. ramiflora* are shown in Table S1. Compared to previous studies on the quality of *B. ramiflora* (Goyal et al., 2022; Kong et al., 2024), non-targeted metabolomics has been studied more systematically for the identification of 'LR' *B. ramiflora* pulp substances.

3.2. Differential metabolites (DMs) at 5 different maturity stages

The screening conditions for metabolite differences at different stages of the five periods were VIP ≥ 1, $Log_2FC \geq 1.2$, and $Log_2FC \leq 0.83$. A total of 451 differential metabolites were screened, and the major metabolites were terpenoids, fatty acyls [FA], flavonoids, and amino acid derivatives, carbohydrates (Fig. 4A), consistent with the recent finding by Chen et al. that the *B. ramiflora* has high levels of fatty acids and flavonoids, consistent with the abundance of carbohydrates and terpenoids (Chen et al., 2023). The highest number and diversity of differential metabolites during the LR4vsLR3 period was consistent with PCA validation and in line with previous studies on different maturity levels of *B. ramiflora* (Yang et al., 2024). It was shown that *B. ramiflora* from LR4vsLR3 was a critical period for fruit ripening in *B. ramiflora*. There were a total of 305 differential metabolites of LR4 vs LR3, and the percentage of different differential metabolites was shown in Fig. 4(B), mainly terpenoids, amino acid derivatives, heterocyclic compounds, fatty acyls [FA], flavonoids, phenylpropanoids, carbohydrates, benzene and derivatives, steroids and derivatives, alkaloids and derivatives, organic acids and Others. The LR2 vs LR1, LR3 vs LR2, LR4 vs LR3 and LR5 vs LR4 there were 213, 120, 305, 199 differential metabolites, respectively. Comparison of the differential metabolites in the five periods by means of Wenn plots (Fig. 4C) revealed a total of 11 differential metabolites, and the changes in their contents (Fig. 4D) indicated that *B. ramiflora* consistently affected fruit ripening during ripening by a greater variety of differential metabolites in lesser quantities. We focused on the flavor changes in ripening of *B. ramiflora*, including sugars, organic acids, and amino acids, and there were 24 Amino acid derivatives, 18 Carbohydrates, and 11 Organic acids, which accounted for 7.9 %, 5.9 %, and 3.6 % of the total differential metabolites, respectively, during the period of LR4 vs LR3, suggesting that this is an important stage of fruit ripening.

3.3. Analysis of dynamic changes of sugars and organic acids

Although the *B. ramiflora* provides a great source of a variety of nutrients and health-promoting metabolites, such as sugars, minerals, vitamins, carbohydrates, and antioxidants (Haque et al., 2009), consumer preference is largely influenced by sensory quality characteristics (Iglesias et al., 2007; Obenland et al., 2009). The sugar-acid ratio of the fruit, defined as the maturity index, is a major determinant of the timing of commercial fruit harvest, fruit quality, and thus grower's income (Hussain et al., 2017). Comparison of metabolite abundance at five typical growth stages from young fruit to full maturity (30, 52, 73, 93 and 112 d) of *B. ramiflora* has been showed in Table S2. 34 carbohydrates and 20 organic acids were identified, the major sugars being L-Sorbose, D-(+)-glucose, sucrose and bis(methylbenzylidene)sorbitol, and the major organic acid being citric acid. The major sugars L-Sorbose (FC 3.5236,VIP 1.0352, P 0.0111), D-(+)-glucose (FC 2.0991, VIP 1.1679, P 0.0281) and sucrose (FC 2.6213,VIP 0.8103, P 0.0713) all changed insignificantly during LR4 vs LR3 (Table 1). Citric acid accumulated significantly higher by 73 days in LR2 vs LR1 (FC 1.1178, VIP 0.6247, P 0.7390), LR3 vs LR2 (FC 1.3673, VIP 0.1293, P 0.3080), and declined significantly during the LR4 vs LR3 period (FC 0.1532,VIP 0.5952, P 0.0014). The non-significant changes in major sugars and the significant decrease in organic acids indicated that the maturation of *B. ramiflora* began after 73 days, and that the LR4 vs LR3 period was an

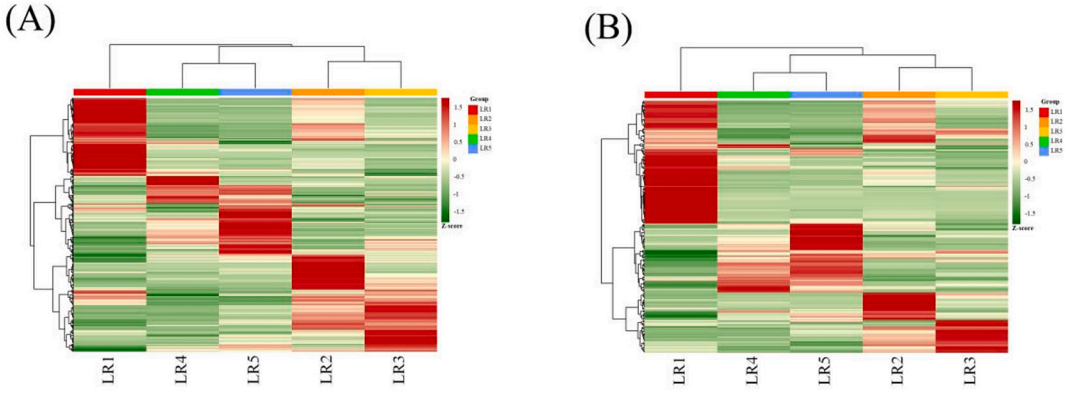

Fig. 3. Clustering analysis of metabolites in five typical periods of 'LR' *B. ramiflora*.
(A) Positive ion model; (B) Negative ion model (The same as below).
The color indicates the level of accumulation of each metabolite, from low (green) to high (red). The score represents the deviation from the mean by standard deviation units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

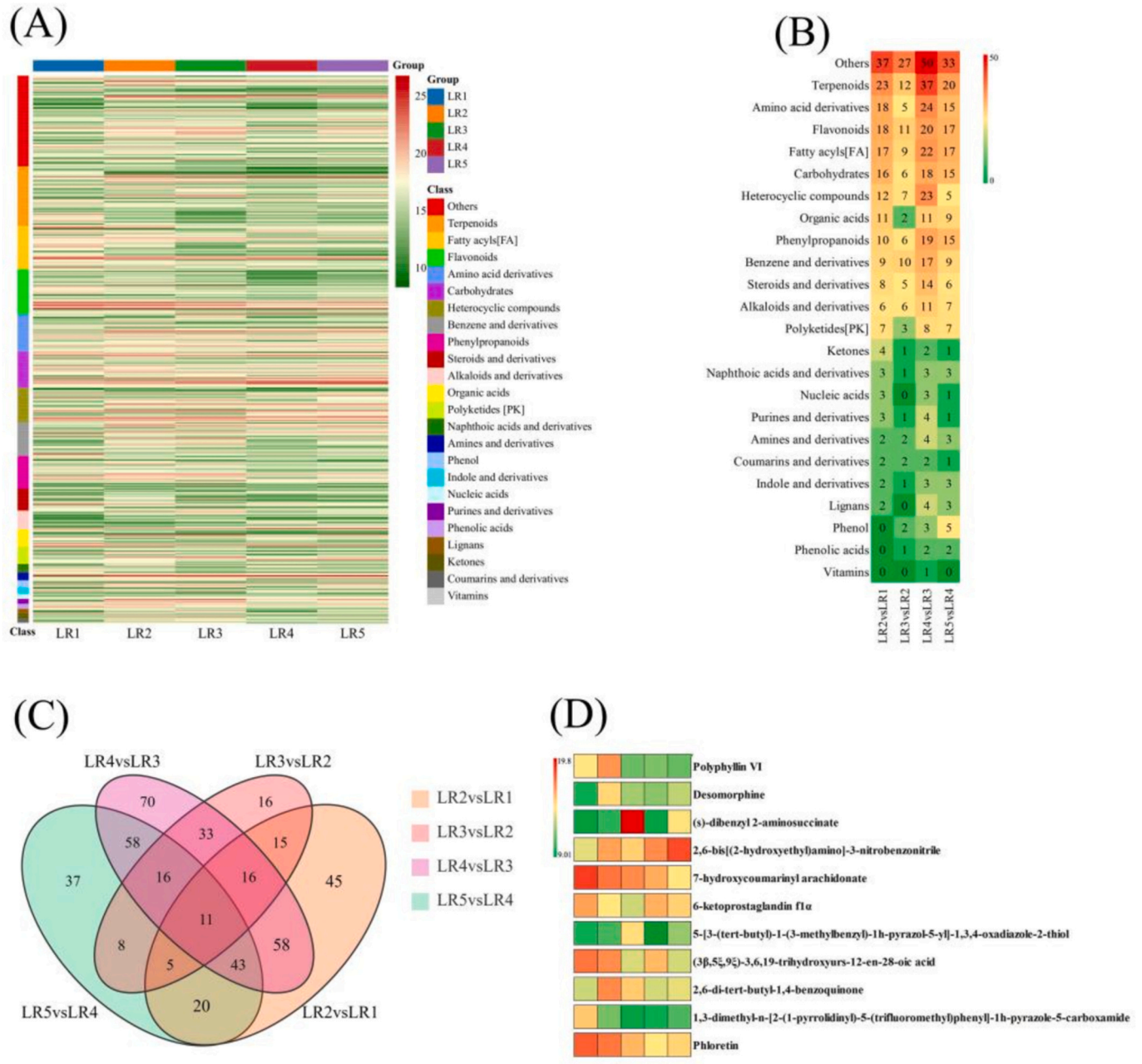

Fig. 4. Differential metabolites at 5 different maturity stages.
(A): Hierarchical cluster analysis of metabolites identified from LR *B. ramiflora*; (B): Number of differential metabolites; (C): Venn plot reveals 451 common differential accumulated metabolites; (D): 11 shared differential metabolites and their variation.The color from green (low) to red (high) indicates the level of each metabolite. The *Z*-score represents the deviation from the mean by standard deviation units. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Sugars, acids, and basic taste qualities of LR pulp at five mature stages.

Growing Stage	Sugars				Acids		Sugar-acid ratio
	Soluble sugar (%)	Sucrose	L-sorbose	D-(+)-glucose	Titratable acid (%)	Citric acid	
LR1	53.26 ± 6.41c	16.87 ± 0.33c	18.41 ± 3.63b	17.98 ± 2.52b	21.32 ± 0.11a	21.32 ± 0.11a	2.5 ± 0.3c
LR2	60.61 ± 1.61bc	18.89 ± 0.9b	21.55 ± 0.39ab	20.17 ± 0.35ab	21.35 ± 0.49a	21.35 ± 0.49a	2.84 ± 0.02bc
LR3	66.22 ± 0.4ab	21.38 ± 0.08a	22.83 ± 0.11ab	22 ± 0.21a	21.94 ± 0.05a	21.94 ± 0.05a	3.02 ± 0.02b
LR4	70.45 ± 0.57a	22.73 ± 0.28a	24.65 ± 0.13a	23.08 ± 0.18a	19.24 ± 0.02b	19.24 ± 0.02b	3.66 ± 0.03a
LR5	71.52 ± 0.61a	22.94 ± 0.35a	25.25 ± 0.08a	23.34 ± 0.24a	19.55 ± 0.7b	19.55 ± 0.7b	3.67 ± 0.16a

Note: Sugar-acid ratio is soluble sugar divided by titratable acid. Different letters on the number meant significant differences between growing stages ($p < 0.05$).

important period for the maturation of 'LR' *B. ramiflora*.

3.4. Amino acids dynamic changes analysis

Amino acids are one of the important components of fruit nutrients and one of the important flavor components of fruit (Aghdam et al., 2020; Zhu et al., 2021). 42 amino acids and derivatives were identified during the ripening period of *B. ramiflora*, the major amino acids being *L*-phenylalanine, L-tyrosine, L-tryptophan and Dl-arginine, which showed no significant changes until 73 day, and during the period of LR4 vs LR3 Dl-arginine (FC 1.7313, VIP 1.0650, P 0.0028) and l-tryptophan (FC 0.1037, VIP 1.0557, P 0.0044) increased significantly, and *L*-phenylalanine (FC 0.4395, VIP 1.0177, P 0.0984) decreased significantly. The insignificant pre-accumulation of amino acids and the significant changes at LR4 vs LR3 indicate that amino acids have an important role in the critical fruit ripening period and affect the flavor formation of *B. ramiflora*.

3.5. Flavonoids dynamic changes analysis

Flavonoids are a group of plant metabolites that provide health benefits through cell signaling pathways and antioxidant effects (Panghal et al., 2021; Tanwar et al., 2023). A total of 40 flavonoids were detected during the ripening process of the *B. ramiflora*, with rhusflavanone, quercitrin, procyanidin B1, and (+)-Catechin hydrate predominating, and as can be seen in Table S1, Quercitrin was the highest, with a significant decrease at LR2 vs LR1 (FC 0.2155, VIP 1.0031, P 0.1272) with a significant decrease followed by no significant change; rhusflavanone increased significantly during LR3 vs LR2 (FC 5.7429, VIP 0.0511, P 0.0004) and LR4vsLR3 (FC 2.4826, VIP 1.1590, P 0.0002; and procyanidin B1 did not change significantly, explaining the insignificant change in flesh color during ripening of 'LR' *B. ramiflora* (Fig. 1); (+)-Catechin hydrate decreased significantly during LR4vsLR3 (FC 0.2268, VIP 1.1433, P 0.0659).

3.6. Terpenoid dynamic changes analysis

Terpenoids are one of the main volatile substances in small berry fruits, widely distributed in the biological world of a class of natural products, with high antioxidant activity, can delay aging, enhance immunity (Li et al., 2014; Xu et al., 2019). A total of 34 Terpenoids were detected during the ripening process of *B. ramiflora*, among which atractyloside a, atractylenolide II and lupeol were the major ones. Atractylenolide II decreasing significantly (88.7653, VIP 1.1640, P 0.0161), lupeol increased significantly during LR2 vs LR1 (FC 0.3023, VIP 0.8920, P 0.0070), and LR4 vs LR3 (FC 2.3529, VIP 1.0097, P 0.0065), showing a trend of increasing and then decreasing. Increasing and then decreasing trend. The active synthesis or catabolism of terpenoids affects the flavor changes of *B. ramiflora*.

3.7. Vitamins dynamic changes analysis

Vitamins are the main nutrients of many plant fruits and have many biological activities, which have high value in industrial and pharmaceutical applications (Aghdam et al., 2020; Özyürek et al., 2007). A total of 3 Vitamins were detected during the ripening process of *B. ramiflora*, including trolox, pantothenic acid and dl-Thioctic acid. as can be seen from Table 1, trolox increased significantly during LR2 vs LR1 (FC 77.2089, VIP 0.7467, P 0.1561), LR4 vs LR3 (FC 0.0136, VIP 0.2642, P 0.0211), showing a trend of increasing and then decreasing; pantothenic acid increased significantly during LR2vsLR1 (FC 2.6991, VIP 0.0186, P 0.0724) and did not show any significant change in the remaining periods; dl-Thioctic acid decreased significantly during LR2vsLR1 (FC 0.5874, VIP 0.4845, P 0.0953) and decreased significantly during LR4vsLR3 (FC 0.0796, VIP 1.0790, P 0.0181), showing a continuous decrease.

3.8. KEGG enrichment analysis of DMs

The metabolites of different growth stages of 'LR' *B. ramiflora* were comprehensively resolved by the KEGG database, and a total of 63 differential metabolites were annotated to 27 pathways, respectively, of which 18 pathways were duplicated and enriched. The enrichment pathways were dominated by amino acid and sugar metabolism, of which there were nine amino acid metabolisms, including aminoacyl-tRNA biosynthesis, beta-Alanine metabolism, biosynthesis of amino acids, glutathione metabolism, glycine, serine and threonine metabolism, phenylalanine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, tryptophan metabolism and valine, leucine and isoleucine biosynthesis. There were overlapping metabolic pathways such as aminoacyl-tRNA biosynthesis, glycine, serine and threonine metabolism. 7 differential metabolites were annotated to the amino acid metabolic pathways, including l-tryptophan, 2-isopropylmalic acid, spermine, citric acid, dehydroascorbic acid, *n*-acetyl-*l*-phenylalanine, and tryptamin, with l- tryptophan was the most active and was annotated to 5 metabolic pathways. The sugar metabolism has two pathways, pyruvate metabolism and citrate cycle (TCA cycle), including two differential metabolites citric acid and 2-isopropylmalic acid. a large number of metabolites are involved in amino acid metabolism synthesis, which greatly improves the nutritional value and taste of the *B. ramiflora* during ripening. The involvement of citric acid and 2-isopropylmalic acid in both sugar and amino acid metabolic pathways suggests that these two differential metabolites are important for the flavor of *B. ramiflora*.

LR2 vs LR1 had 4 differential metabolites annotated to 4 pathways (Fig. S2A); LR3 vs LR2 had 4 differential metabolites annotated to 4 pathways (Fig. S2B); LR4 vs LR3 had the most differential metabolites enriched, with 48 differential metabolites annotated to 21 pathways (Fig. S2C). In terms of enrichment factors, the metabolites were mainly enriched in TCA cycle and plant hormone signal transduction metabolic pathways, and two differential metabolites were annotated to citric acid and jasmonic 2 differential metabolites, citric acid and jasmonic acid, were annotated. A large number of differential metabolites were enriched in the LR4 vs LR3 period, indicating that this period is a critical period for flavor changes during the ripening process of *B. ramiflora*, and the differential metabolites of the major pathways affect the key changes in the flavor of *B. ramiflora*; a total of 22 differential metabolites were annotated to 16 pathways in the LR5 vs LR4 period (Fig. S2D), and from the enrichment factors, the metabolites mainly enriched in the plant hormone signal transduction metabolic pathway (PHTM). Metabolites were mainly enriched in plant hormone signal transduction and linoleic acid metabolism pathways, including 3 differential metabolites of jasmonic acid, γ-linolenic acid, and linoleic acid. Plant hormone signal transduction pathway and differential metabolism pathway were mainly enriched in the plant hormone signal transduction pathway and the differential metabolism pathway. Signal transduction pathway and the differential metabolite jasmonic acid were repeatedly enriched at LR4 vs LR3 and LR5 vs LR4, suggesting that they affect the fruit quality of 'LR' *B. ramiflora* at the late ripening stage and have an important influence on the fruit flavor changes of *B. ramiflora*.

Fig. 5 provides a comprehensive resolution of the relevant KEGG pathways based on the predominance of sugar metabolism and amino acid biosynthesis pathways, explaining the mechanism of the changes in the flavor of the *B. ramiflora*. All metabolites enriched by the KEGG pathway and their peak changes in each period are indicated by colored boxes. During fruit ripening, changes in sugar and acid are usually influenced by changes in fruit flavor, and no major sugar metabolism differentiators were found in the enriched pathways, suggesting that the substances affecting the flavor changes of *B. ramiflora* are mainly organic acids. The major organic acid citric acid is active in the TCA cycle pathway and was accumulating from LR1 to LR3 periods, with a significant decrease at LR4. Interestingly, the differential metabolite spermine on the downstream metabolic pathway glutathione

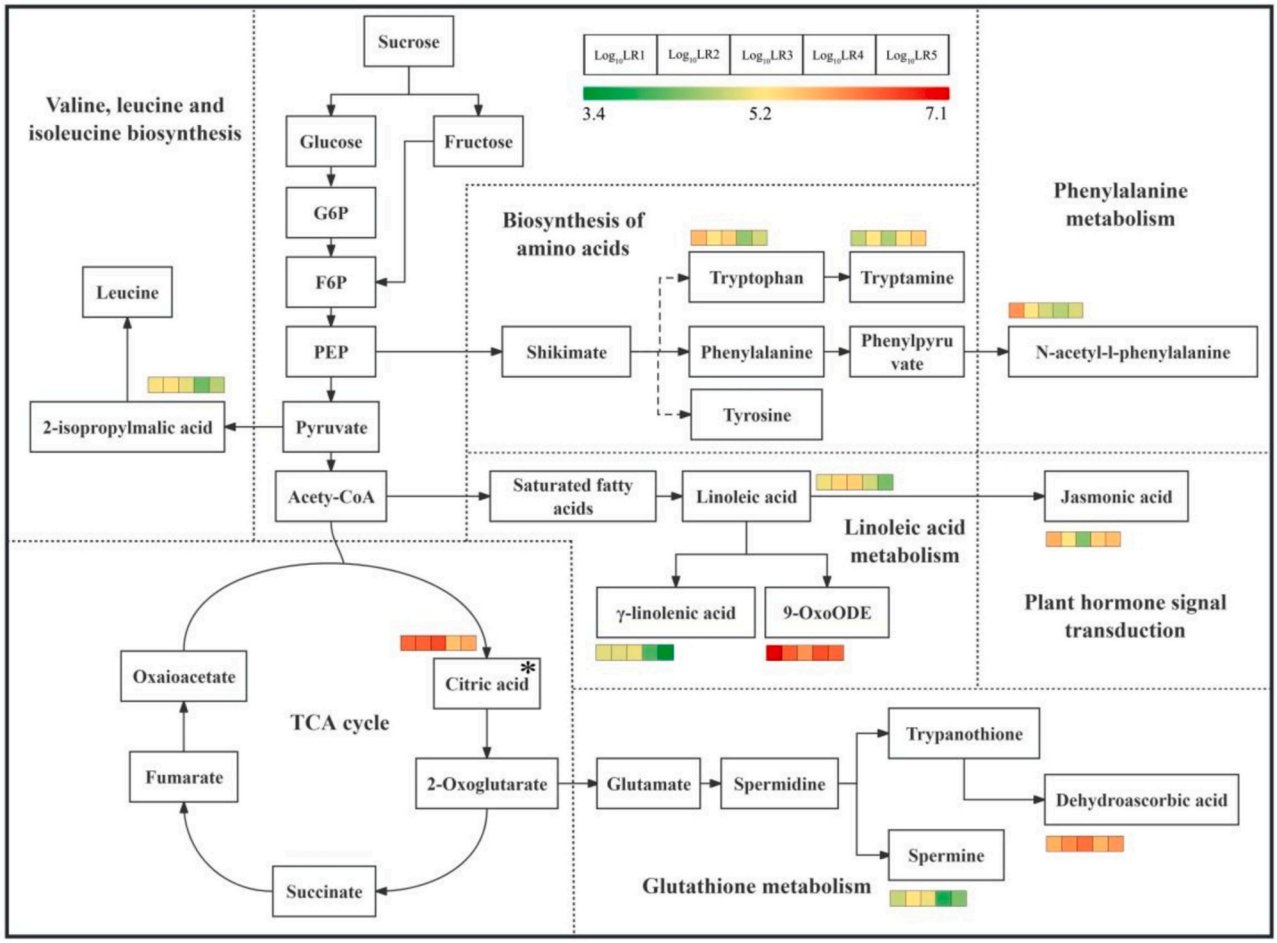

Fig. 5. KEGG map of key DMs in LR *B. ramiflora.* This map is constructed based on the KEGG pathway and literary references. Colored boxes in front of each metabolite indicate log_{10}LR1, log_{10}LR2,log_{10}LR3,log_{10}LR4and log_{10}LR5 values according to the color scale. * represents the taste biomarker.

metabolism showed a significant correlation with the trend of citric acid content in the five periods (R^2 = 0.9068, $y = -5.49 + 0.66\times$), and dehydroascorbic acid was significantly increased from LR3 to LR4. increased, probably influenced by the effect from citric acid, suggesting that changes in the content of citric acid, an upstream differential metabolite, play an active role in the ripening process of 'LR' *B. ramiflora* and have a key role in the flavor changes of *B. ramiflora*. Tryptophan, which enters the metabolic pathway of biosynthesis of amino acids via shikimate, decreased significantly from LR3 to LR4, and led to a significant increase in tryptamine, a downstream substance, from LR3 to LR4, suggesting that they are actively involved in the synthesis and catabolism of amino acids in 'LR' *B. ramiflora n*-acetyl-*l*-phenylalanine, which enters the metabolic pathway of phenylalanine metabolism via phenylpyruvate, showed a significant decrease from LR1 to LR3, which may be influenced by the upstream biosynthesis of amino acids metabolic pathway. Linoleic acid and γ-linolenic acids and 9-OxoODE are differential metabolites active in the metabolic pathway of linoleic acid metabolism, and linoleic acid located in the upstream directly affects the downstream γ-linolenic acids differential metabolite changes (R^2 = 0.8948, $y = -3.73 + 1.6\times$), and 9-OxoODE showed stable high levels in five periods, indicating its stable involvement in the synthesis and catabolism of metabolites during the ripening of *B. ramiflora*. Plant hormone signal transduction inside the metabolic pathway of jasmonic acid is located downstream of linoleic acid, and it was found to be stable during the jasmonic acid is located downstream of linoleic acid and showed a significant increase from LR3 to LR4, which may be caused by the passage of linoleic acid. 2-isopropylmalic acid, which enters the valine, leucine and isoleucine biosynthesis pathway through pyruvate, showed a significant decrease from LR3 to LR4, indicating that it is actively involved in the metabolic changes during fruit ripening in *B. ramiflora*.

3.9. Potential taste biomarker

Among the 11 shared differential metabolites (Fig. 4D), the major sugars and organic acids were not found and were not enriched by the KEGG pathway, suggesting that the key taste markers for ripening of *B. ramiflora* were not among the shared differential metabolites. LR4vsLR3 was the critical period for *B. ramiflora* ripening, in which the major sugars showed non-significant changes, while the major organic acid, Citric acid, showed a significant decrease, while in KEGG pathway enrichment, we found that the significant change of Citric acid was significantly correlated with the downstream substance spermine (R^2 = 0.9068, $y = -5.49 + 0.66$x). We hypothesized that citric acid is a key taste differentiator in the ripening of 'LR' *B. ramiflora*. The peak areas of citric acid and spermine were transformed by log_{10} before correlation analysis. As shown in Fig. 6A, citric acid was negatively correlated with sugar-acid ratio (R^2 = 0.9982) during the critical period of fruit ripening in LR3, LR4 and LR5, and citric acid, which is the main component of titratable acid, was positively correlated with titratable acid. As shown in Fig. 6B, spermine was negatively correlated with sugar-acid ratio (R^2 = 0.9841),and positively correlated with titratable acid (R^2 = 0.9991). Therefore, citric acid may be a key flavor marker for 'LR' *B. ramiflora*.

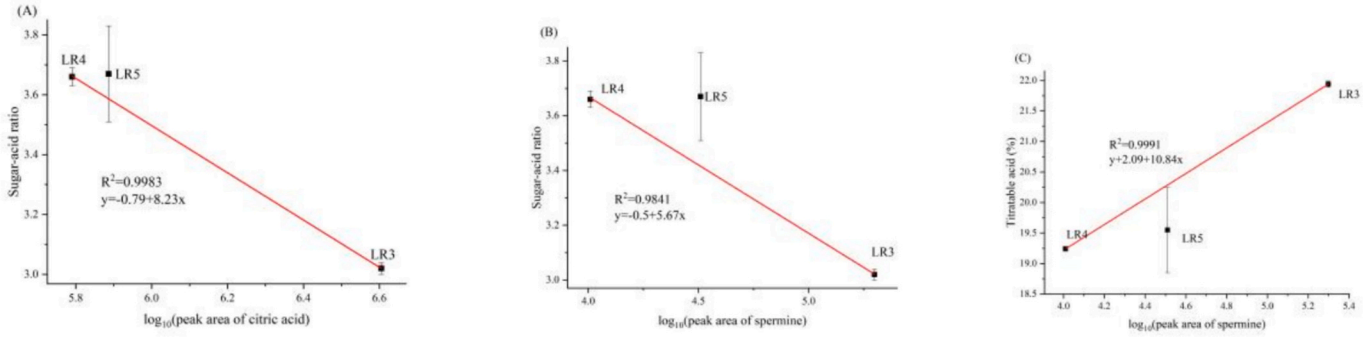

Fig. 6. Correlation between potential taste biomarker, sugar acid ratio and titratable acids in 'LR' *B. ramiflora.*

4. Conclusions

Fruit flavor is a key determinant of fruit quality as it significantly affects consumer perception and preference (Shu et al., 2023). As an under-exploited wild fruit tree, *B. ramiflora* shows different climatic periods in different growing environments, for example, *B. ramiflora* in Southeast Asia blossoms and bears fruit about 45 days earlier than in mainland China (J. Huang et al., 2024), different strains of *B. ramiflora* have different flavor variations (Chen et al., 2023). This has led to significant differences in the picking and storage periods of different strains of *B. ramiflora*, affecting the promotion of its cultivation as a commercial fruit. Previously, fruit ripening could be judged by estimating a variety of variables such as fruit firmness, soluble solids content (SS), dry matter content (DMC), and flesh color, but these factors varied between varieties and different orchard plantings (Burdon et al., 2014), inability to accurately determine fruit maturity differences affects fruit picking and storage (Favre et al., 2023). The study of the mechanism of fruit ripening and nutritional value of the *B. ramiflora* is of great significance as a guide for its commercial cultivation (Fyfe et al., 2023). The development of metabolomics technology can determine the critical period of fruit ripening and the changes in the content of related metabolites, which can help the commercialization of fruits and the extension of shelf life (Favre et al., 2022). Here, we analyzed metabolic differentiators of 'LR' *B. ramiflora* at different ripening stages by non-targeted metabolomics techniques, and identified a total of 530 metabolites, including sugars, organic acids, amino acids, flavonoids, terpenoids, and vitamins in a dynamic manner. Combined with the comprehensive analysis of metabolic pathways, we found that LR4vsLR3 is the key period of ripening of *B. ramiflora*, and the major sugars increased significantly and organic acids decreased significantly during this period, which determines the fruit quality of 'LR' *B. ramiflora*, and we explained from the field of metabolomics that the harvest standard of 'LR' *B. ramiflora* is 91-98d after the maturity degree of flower, and the fruits with the maturity degree of 98d after the flower are of good quality for fresh eating and high storability (Kong et al., 2024). The 'LR' *B. ramiflora* can be picked at LR4 for long-distance transportation and LR5 for short-distance transportation to ensure the best flavor and shelf life. The results of the study revealed the flavor changes of 'LR' *B. ramiflora* in different periods, which can help to upgrade the nutritional value and quality of *B. ramiflora* in the subsequent period, and also provide a theoretical basis for the commercial cultivation and harvesting of *B. ramiflora.*

Key taste-differentiating metabolites in the fruit metabolome determine fruit flavor quality by affecting arom, sweetnes, acidi and bitterness(Colonges et al., 2022; Liu et al., 2024) and the mechanisms of the inheritance and domestication of these metabolites are important for the improving fruit quality and breeding. By looking for key flavor markers, it is possible to assess the relevance of variation at harvest to subsequent storage performance (Deng et al., 2023; Favre et al., 2023) In the present study, we found that the differential metabolite citric acid may be the key taste marker of 'LR' *B. ramiflora* through metabolic differential metabolite changes and kegg pathway analysis, which is inconsistent with the previous studies that the key taste differential metabolites of other strains of *B. ramiflora* are l-sorbose and 5-hydroxyindole-3-acetic acid (Yang et al., 2024). Sweetness is the main flavor of fleshy fruits, and the taste of the fruit depends largely on the right ratio of sugar to acid (Hussain et al., 2017; Topuz et al., 2005). We found that the major sugars did not change significantly during the critical period of ripening in 'LR' *B. ramiflora*, and the major organic acids declined significantly as the fruits ripened, whereas in the 'BR' *B. ramiflora*, the major sugar, L-sorbose, was the key taste differentiator in 'BR' *B. ramiflora.* Differences in sugar-acid ratios determine the major flavor variations of *B. ramiflora*, and we revealed the reasons for the flavor differences in different strains of *B. ramiflora* through variations in differential metabolites and key taste markers. In addition, changes in organic acids not only affect fruit growth and quality, but also regulate fruit senescence and storage. The key flavor sensation of 'LR' *B. ramiflora* is the variation of organic acid content, and this finding will be informative for future quality improvement of 'LR' *B. ramiflora*. With the increasing demand for personalized fruit flavor selection and exploration of the medicinal functions of wild plant fruits, research on wild fruit trees is receiving increasing attention (Ahmad et al., 2016; Kiprovski et al., 2021). Bioactive compounds such as tannins, flavonoids, alkaloids, fatty acids and polysaccharides present in the plant are of high medicinal value (Tiwari et al., 2023). *B. ramiflora* is a kind of wild fruit tree with high medicinal and health value, which has not been well developed and utilized due to different planting environments and big difference in taste of different strains. Metabolomics technology facilitates the development and utilization of medicinal plants through metabolite identification and differential analysis. We provided a staged interpretation of the metabolite changes in *B. ramiflora* at five periods by non-targeted metabolomics, and *B. ramiflora* of different maturity were selected for harvesting according to different medicinal needs. The identification of metabolic differentiators in different periods provides a useful reference for its further medicinal function development.

5. Conclusions

B. ramiflora is a highly exploitable tropical wild fruit tree, which is underutilized due to the differences in fruit quality among different strains, the uncertainty of the best picking period, and the difficulty of storage. In this study, five typical periods of fruit ripening of 'LR' *B. ramiflora* were analyzed by non-targeted metabolomics techniques. The results showed that a total of 451 differential metabolites were identified for the five periods. The ripening of 'LR' *B. ramiflora* started 73 days after flowering and reached the ripening criterion at 93 days. LR4 vs LR3 was the key period of ripening of *B. ramiflora*. There was no significant change in the major sugars in this period, while there was a significant decrease in the organic acids, increase or decrease in amino acids and flavonoids, and significant decrease in terpenoids and vitamins. The key changes in these metabolites were the main taste markers

of ripening of *B. ramiflora* fruits. Enrichment by KEGG pathway showed that significant changes in citric acid were significantly correlated with changes in the downstream substance spermine ($R^2 = 0.9068$, $y = -5.49 + 0.66\times$), while citric acid ($R^2 = 0.9982$) and spermine ($R^2 = 0.9841$) were negatively correlated with the sugar-acid ratio. Citric acid was the main component of titratable acid and spermine ($R^2 = 0.9991$) was positively correlated with titratable acid. We speculated that citric acid is a key taste marker for fruit ripening in 'LR' *B. ramiflora*. These findings provide new metabolic evidence for flavor changes during *B. ramiflora*. In the future, we can combine the genomics and transcriptomics technologies to further understand the interrelationships between gene expression, metabolite synthesis and catabolism, which will help to improve the quality of *B. ramiflora*, develop its medicinal value, and promote the commercial cultivation of *B. ramiflora*.

CRediT authorship contribution statement

Chongcheng Yang: Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Huachen Wang:** Writing – review & editing, Visualization, Supervision, Software, Resources, Project administration, Methodology, Investigation, Data curation. **Yang Zhang:** Writing – review & editing, Supervision, Resources, Project administration. **Jianjian Huang:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jie Chen:** Validation, Supervision, Project administration, Methodology, Data curation.

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

I have shared the link to my data at the Attach File step

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101894.

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