Distinct Changes of Metabolic Profile and Sensory Quality With Different Varieties of Chrysanthemum (Juhua) Tea by LC-MS Based Metabolomics and Electronic Tongue

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Keywords: Chrysanthemum (Juhua); Tea; Metabolomics; Sensory Quality; LC-MS,32; Electronic Tongue

Article

Distinct Changes of Metabolic Profile and Sensory Quality with Different Varieties of *Chrysanthemum* (Juhua) Tea by LC-MS Based Metabolomics and Electronic Tongue[1]

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Abstract: ***Chrysanthemum*** tea, a typical health tea with the same origin as medicine and food, is famous for its unique health benefits and flavor. To clarify the influence of metabolites of different varieties of *chrysanthemum* tea on the formation of taste and quality differences, nontargeted metabolomics combined with electronic tongue analysis were used to characterize the correlation between metabolites profiles and taste characteristics of different quality *chrysanthemum* tea. Thirteen metabolites were identified as the key metabolites of the sensory quality difference between Huangju and Jinsi Huangju tea. Kaempferol, luteolin, genistein, and some quinic acid derivatives were correlated with the 'astringent' and taste attributes. In contrast, l-(-)-3 phenyllactic acid and L-malic acid were found to be the 'bitterness' and 'umami' in *chrysanthemum* tea. KEGG pathway enrichment analysis showed that the flavonoid and flavonol biosynthesis pathway had important effects on the sensory quality of *chrysanthemum* tea.

Keywords: *Chrysanthemum*(Juhua); tea; metabolomics; sensory quality; LC-MS; electronic tongue

Introduction

Dietary herbal teas, defined as water-based immersion or decoction preparation with herbal ingredients, have been used in healthcare and as a healthy diet[1]. As a traditional medicine and food homologous plant, *Chrysanthemum morifolium Ramat.*(Juhua) has been used for over 3000 years as a herbal tea-based drink, the third largest drink after tea and coffee[2]. Notably, drinking *chrysanthemum* tea or beverages was thought to have similar preventive or therapeutic effects for these diseases [3]. Modern pharmacological studies have shown that flavonoids, anthocyanins, alkaloids, phenolic acids, and other phytochemicals in *chrysanthemum*, which have anti-microbial, anti-oxidation, anti-inflammation, anti-cancer, anti-obesity, nerve protection and other functions, provide a theoretical basis for the development of *chrysanthemum* tea and its deeply processed

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS); Mass Spectrometry (MS);High-Performance Liquid Chromatography (HPLC); False Discovery Rate (FDR); Principal Component Analysis (PCA); Kyoto Encyclopedia of Genes and Genomes (KEGG);Traditional Chinese medicine (TCM); Gas Chromatography-Mass Spectrography (GC-MS); Headspace-gas Chromatography-ion Mobility Spectrometry (HS-GC-IMS);Gas chromatography olfactometry (GC-O); Electron Spray Ionization (ESI);

products [4]. However, as a medicine and food homologous plant, the literature on *chrysanthemums* mainly focuses on the biological activity, vegetative propagation, and cultivation technology of medicinal *chrysanthemum* [5]. In contrast, there are few reports on edible *chrysanthemum*'s flavor and sensory quality as dietary herbal teas.

The taste and sensory quality of *chrysanthemum* (Juhua) tea are mainly determined by secondary metabolites, such as flavonols, anthocyanins, amino acids, alkaloids, and organic acids [6]. Generally, the variety, region, climate, soil type, and production process of *chrysanthemum* tea determine the sensory quality of *chrysanthemum* tea related to its chemical composition [7]. At present, many researchers have systematically analyzed the flavor components of edible *chrysanthemum* using GC-MS, HS-GC-IMS, GC-O, HPLC, sensory evaluation, and other methods [8–10]. However, numerous studies on the sensory quality and flavor substances of edible *chrysanthemum* have problems, such as a single method and lack of multi-omics research [11].

Metabolomics, as a method of omics, is the science of studying the species, quantity and changes of metabolites (endogenous metabolites) with molecular weights less than 1500 Da caused by the response of organisms to external stimuli, pathophysiological changes and gene mutations [12]. Through plant metabolomics, a variety of analysis platforms can be used to study the metabolites of different plant samples after physical or chemical treatment, and obtain different meanings, including geographical traceability, food processing, biological activity, etc [1,13]. In addition, as an intelligent instrument to simulate human taste, electronic tongue system has been reported to can quantitatively and qualitatively analyze the taste of different foods and Chinese herbs [14–16]. Thus, the combined analysis of metabolomics and food flavomics based on LC-MS/MS and electronic tongue is an excellent method to establish the relationship between *chrysanthemum* tea's chemical constituents and sensory quality.

The chemical composition, metabolites, and taste of *chrysanthemum* (Juhua) tea vary considerably depending on its cultivar and production region[17]. The objective of this study builds on previous studies. It aims to compare the metabolites and sensory qualities of five different varieties of *chrysanthemum* tea by using LC-MS-based nontargeted metabolomics combined with electronic tongue analysis, investigating key differential metabolites associated with sensory quality differences in *chrysanthemum* tea. Importantly, the implementation of this study offers a high-resolution marker for the quality evaluation of *chrysanthemum* tea.

2. Materials and methods

2.1. Chrysanthemum tea samples

The 5 varieties of *chrysanthemum* (Juhua) tea used in this study were collected from local producer, including 6 of 'JinshihuangJu'(J), 6 of 'HuangJu'(X), 6 of'HanbaiJu'(H), 6 of'BaoJu'(B), and 6 of'GongJu'(G) growing in the regions of Hunan, Hangzhou, Anhui province. Detailed information on the *chrysanthemum* tea samples is provided in Figure 1.

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Fig. 1 Appearance and origin of five different species of chrysanthemum
Note: (J) JinshihuangJu; (X) HuangJu; (H) HanbaiJu; (B) Boju; (G) Gongju; (F) Diagram of the source of five different varieties of *chrysanthemum* tea

Figure 1. Appearance and origin of five different species of chrysanthemum Notes: (J) JinshihuangJu; (X) HuangJu; (H) HanbaiJu; (B) Boju; (G) Gongju; (F) Diagram of the source of five different varieties of *chrysanthemum* tea.

2.2. Sensory Evaluation for chrysanthemum tea samples

The sensory quality of 5 *chrysanthemum* tea samples (J, X, H, B, and G) was evaluated by a sensory evaluator (3 males and 7 females, 18−21 years of age) from the Hunan University of Chinese Medicine according to a standardized method (GB/T 23776-2018). All subjects signed a written informed consent form to participate in this study.*Chrysanthemum* tea samples were assessed for various sensory attributes on a 5-point scale based on taste intensity, represented on a scale of 0 to 5, where 0 means none and 5 means very high intensity [18]. Briefly, the different varieties of *chrysanthemum* tea were weighed (2.00±0.05) g, brewed with 150 mL boiling water for 30 min, and then filtered out with gauze to prepare samples to be tested. Each sensory participant scored each *chrysanthemum* tea sample's taste for five taste characteristics (bitterness, astringency, umami sweetness, and aroma). Participants did not eat any food except water before participating in the sensory evaluation, and sensory panels did not communicate with each other during the whole sensory evaluation. Furthermore, the data of sensory evaluation used one-way analysis of variance (ANOVA) with IBM SPSS Statistics 25.0 software (IBM, Chicago, IL, USA).

2.3. Electronic tongue analysis for chrysanthemum tea samples

Taste attributes of the five different varieties of *chrysanthemum* tea were determined using TS-sa402b electronic tongue sysem (INSENT Inc., Japan) . The (2.00 ± 0.05) g *chrysanthemum* tea sample was accurately weighed and brewed with 150ml boiling water for 30 min. Then the tea broth was filtered through 3 layers of gauze to obtain the sample to be tested. The sample was then cooled to room temperature before being detected by the TS-sa402b electronic tongue system. In this study, each sample was cycled 4 times, and the average of the three times after the first cycle was analyzed.

2.4. HPLC Analysis for chrysanthemum tea samples

All *chrysanthemum* teas samples were ground separately into 100 mesh size fine powder. A 25 ± 0.01 mg sample of each *chrysanthemum* tea was extracted using ultrasonic extraction (Power:300W, Frequency: 45kHz) (SB-5200DTD; Scientz, China) with 25 mL of ultrapure water at room temperature for 40 min. After cooling, the weight was determined, and ultrapure water was used to replenish the lost weight. Subsequently, the sample was shaken and filtered to obtain the filtrate to be measured. The supernatants were collected and centrifuged for HPLC analysis.

The contents of chlorogenic acid, luteolin, and 3, 5-O-dicaffeyl quinin acid in *chrysanthemum* tea samples were analyzed using a high-performance liquid chromatography (HPLC) analytical method, following the Pharmacopoeia of the People's Republic of China. The HPLC system had pumps and an autosampler (Agilent 1260 Infinity II Prime liquid chromatography system, Agilent Technologies, Inc., Palo Alto, CA, USA). HPLC column (250 × 4.6 mm，5 μm particle size, Welch Technologies, China) was used. An auto-injector injected 10 μL of the test solution into the HPLC system, and the flow rate was 1.0 mL/min. The mobile phase consisted of mobile phase A [H2O containing 0.05% (v/v) phosphoric acid] and mobile phase B [0.1% (v/v) acetonitrile]. Additionally, the gradient elution was as follows: 0-11 min, the gradient of phase B increased from 10% to 18%, 11-30 min, the gradient of phase B increased to 20%, 30-40 min, the gradient of phase B was maintained at 20% for 10 min, 40-45 min, the gradient of phase B continued to rise to 95%, 45-60 min, and the gradient of phase B was maintained at 90% for 15 min. Furthermore, samples (10 μL) were eluted at 0.8-1.0 mL/min, and the column oven was kept at 30 °C.

2.5. LC-MS/MS-Based untargeted metabolomics analysis

The untargeted metabolomics analysis of *chrysanthemum* tea samples was performed using an UHPLC (1290 Infinity LC, Agilent Technologies, Japan) equipped with a binary pump and C18 column (2.1 mm × 100 mm, i.d., 1.8 μm, Agilent) operated at 40 ℃. Mobile phase A consisted of 25 mmolL ammonium acetate and 0.5% formic acid in water, and mobile phase B was methanol. Additionally, the gradient elution was as follows:0-0.5 min, 5 % B; then B changed to 100 % linearly from 0.5 to 10 min; 10-12.0 min, B was maintained at 100 %; From 12.0 to 12.1 min, B changed linearly from 100 % to 5 %; 12.1-16 min, B was maintained at 5 %. The sample was placed in an automatic sampler at 4 ℃ during the analysis. The separated components were then detected with a quadrupole time-of-flight (AB Sciex TripleTOF 6600, Shanghai Applied Protein Technology Co., Ltd., China). To avoid the effects of instrument fluctuations, a random sequence was used to analyze samples. QC samples are inserted into the sample queue to monitor and evaluate the stability and reliability of data.

The ESI source parameters were set as follows: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600 ℃, IonSpray Voltage Floating (ISVF)±5500 V. In MS-only acquisition, the instrument was set to acquire over the m/z range 60-1000 Da, and the accumulation time for the TOF MS scan was set at 0.20 s/spectra. In auto MS/MS acquisition, the instrument was set to acquire over the m/z range 25-1000 Da, and the accumulation time for the product ion scan was set at 0.05 s/spectra. Moreso, the product ion scan was acquired using information-dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with±15 eV; declustering potential (DP), 60 V (+) and −60 V (−); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.

2.6. Metabolomics data acquisition and analysis

The TIC diagram in the ESI positive and negative modes for the five different varieties of *chrysanthemum* tea is shown in Figure S1. The raw MS data were converted to MzXML files using ProteoWizard MSConvert before importing into freely available XCMS ^{plus} software (Sciex, USA). For peak picking, the following parameters were used: centWave m/z = 10 ppm, peak width = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, minfrac = 0.5 were used. The R-package CAMERA (Collection of Algorithms for MEtabolite pRofile Annotation) was used for annotating isotopes and adducts. In the extracted ion features, only the variables having more than 50% of the nonzero measurement values in at least one group were kept. Compound identification of metabolites was performed by comparing accuracy m/z value (<10 ppm) and MS/MS spectra with an in-house database established with available authentic standards.

After normalizing to total peak intensity, the processed data were analyzed using an R package (ropls), where it was subjected to multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). The 7-fold cross-validation and response permutation testing was used to evaluate the robustness of

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the model. Furthermore, the variable importance in the projection (VIP) value of each variable in the OPLS-DA model was calculated to indicate its contribution to the classification. In this study, metabolites with VIP values > 1.0 was further applied to Student's T-test (*P*-value < 0.05) at the univariate level to measure the significance of each metabolite.

The multiple comparisons of the five varieties of *chrysanthemum* tea groups were calculated by one-way analysis of variance (ANOVA) with Duncan's test for Statistics 25.0 software (IBM, Chicago, IL, USA). A $P < 0.05$ was considered statistically significant. Prism 8.0 software (GraphPad, San Diego, CA, USA) was used for drawing.

2.7. Bioinformatics analysis

The difference of metabolites was statistically significant among the varieties of *chrysanthemum* tea (the VIP value >1 in the OPLS-DA model and *P* value < 0.05) were screened for bioinformatics analysis, including hierarchical clustering analysis, correlation analysis, and pathway analysis. The hierarchical clustering analysis was also done using TBtools software (TBtools, Guangzhou, China). Moreso, the differentially expressed metabolites were matched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database by the KEGG Automatic Annotation Server (KAAS, website: https://www.genom e.jp/tools /kaas/). $P < 0.05$ in Fisher's exact test was considered statistically significant.

3. Results & Discussion

3.1. Sensory quality of the five different varieties of chrysanthemum tea

The difference in sensory attributes of the five different varieties of *chrysanthemum* tea was detected by electronic tongue. As shown in Table 1A, the astringency, bitterness, and umami taste of the five different varieties of *chrysanthemum* tea were significantly higher than the tasteless point ($P < 0.05$). Therefore, the astringency, bitterness, and umami indexes could be used as effective taste indexes for the five *chrysanthemum* tea varieties.

As a unique scented tea health drink, most *chrysanthemum* is consumed as a tea. However, *chrysanthemum* tea has astringency, which cannot meet consumers' oral pleasure, resulting in the low market recognition of simple chrysanthemum tea products [15]. It is worth noting that astringency is an important sensory property of *chrysanthemum* tea, with hydrolyzed and concentrated tannins responsible for this property[19]. Moreso, astringency, one of the most complex oral sensations, is an important essential affecting the flavor quality of food, tea, and other beverages[20]. The astringency index of the Huangju sample (X) was significantly higher than that of the other three species except for the Boju sample (B) ($P < 0.05$). Meanwhile, the richness of the Huangju sample (X) was significantly higher than that of the other four varieties of *chrysanthemum* tea ($P < 0.05$). Notably, this dry, wrinkled taste occurs when drinking tea or other foods containing polyphenols.

In addition, the taste (including bitterness, astringency, umami, and sweetness） and aroma profiles of the five different varieties of *chrysanthemum* tea were quantified using a sensory evaluator consisting of ten trained individuals (Table 1B). The results showed no significant difference in umami taste among the five different varieties of *chrysanthemum* tea ($P < 0.05$). At the same time, the astringency, bitterness, and aroma of'HuangJu'(X) were significantly ($P < 0.05$) higher than that of the other four species of chrysanthemum. Additionally, the results of astringency and bitterness were consistent with the results of taste characteristic value analysis by the electronic tongue. As polyphenols and other astringent substances interact with salivary proteins, resulting in protein precipitation, lubrication in the mouth is reduced, resulting in an astringent feeling. However, the content of polyphenols in different chrysanthemum tea varieties may affect the astringency of *chrysanthemum* tea. Therefore, further main active compounds analysis of the five different varieties of *chrysanthemum* tea was carried out.

Table 1A. Determination of taste characteristics of five different varieties of *chrysanthemum* tea by electronic tongue.

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Taste characteristics	*Chrysanthemum* varieties				
	J	X	H	B	G
Sourness	-25.02±0.00^{d}	-21.36±0.09^{a}	-24.06±0.09^{c}	-21.38±0.08^{a}	-23.36±0.05^{b}
Bitterness	11.11±0.00^{b}	7.36±0.01^{e}	11.34±0.01^{a}	9.33±0.01^{d}	9.63±0.02^{c}
Astringency	13.60±0.00^{d}	15.79±0.07^{b}	14.89±0.05^{c}	16.79±0.04^{a}	14.96±0.03^{c}
Aftertaset-b	1.89±0.00^{a}	0.58±0.03^{e}	0.90±0.05^{c}	1.03±0.05^{b}	0.69±0.02^{d}
Aftertaset-a	2.79±0.00^{c}	3.36±0.02^{a}	2.03±0.06^{e}	3.00±0.07^{b}	2.60±0.02^{d}
Umami	11.88±0.00^{a}	11.19±0.02^{b}	9.55±0.02^{e}	10.25±0.01^{d}	10.30±0.02^{c}
Richness	2.17±0.00^{c}	3.19±0.06^{a}	1.54±0.06^{e}	2.33±0.09^{b}	1.87±0.01^{d}
Saltiness	-7.32±0.00^{b}	-2.97±0.03^{a}	-13.25±0.01^{e}	-7.74±0.01^{c}	-9.82±0.06^{d}

Standard error of means (n = 3), $^{a-d}$ Means within the same row with different superscript differ significantly (P <0 .05).

Table 1B. Traditional sensory evaluation of five different varieties of *chrysanthemum* tea.

Each value is expressed as mean ± SD (n =10). $^{a–c}$ Different letters within a column indicate a significant

Sensory indicators	*Chrysanthemum* varieties				
	J	X	H	B	G
Bitterness	2.90±0.72^{b}	4.13±0.53^{a}	2.70±0.60^{bc}	3.13±0.67^{b}	2.23±0.65^{c}
Astringency	2.47±0.69^{b}	3.37±0.95^{a}	2.33±0.50^{b}	2.50±0.45^{b}	2.27±0.68^{b}
Umami	1.93±0.75^{a}	1.47±0.85^{a}	2.06±0.93^{a}	1.57±0.39^{a}	2.10±0.75^{a}
Sweetness	1.60±0.54^{ab}	1.07±0.14^{c}	1.77±0.57^{a}	1.23±0.42^{bc}	1.93±0.66^{a}
Aroma	2.17±0.57^{c}	3.43±0.83^{a}	3.07±0.72^{ab}	2.23±0.72^{c}	2.43±0.97^{bc}

difference (P < 0.05). The taste strength of each sample was evaluated using a standard scale (0 no taste; 1 to 2, slightly strong; 3 to 4 strong; 5, very strong).

3.2. Comparison of the contents of main active compounds

Chlorogenic acid, luteolin, isochlorogenic acid, and other phenolic acids are the major components responsible for the health benefits of *chrysanthemum* tea [21]. Phenolic acids are responsible for *chrysanthemum* tea's distinctive color and taste, and the bioactive components contribute to its antibacterial, antiviral, antioxidation, antihypertension, and hypolipidemic activities[2]. In this study, there were apparent differences in phenolic acid content among five varieties of chrysanthemum. As shown in Table S1, among the five different varieties of *chrysanthemum* tea, the highest content of phenolic acids was Huangju (H), which was significantly higher than the other four kinds of *chrysanthemum* tea. In contrast, the lowest content of phenolic

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acids was Boju (B), which was significantly lower than that of the other four *chrysanthemum* tea (P<0.05). The data from sensory analysis have confirmed that phenolic acids were positively correlated with astringent taste [22]. Notably, the astringent taste produced by drinking *chrysanthemum* tea is caused by the polyphenol-protein complex reaction [23]. Huangju (H) had the highest content of three phenolic acids and astringent sensation, indicating that major bioactive substances in five varieties of *chrysanthemum* tea showed a highly comparable curve with the sensory quality data. In fact, due to these differences in variety and origin, Huangju (H) may be quite different from other types of *chrysanthemum* tea in terms of chemical compounds and sensory characteristics. Hence, follow-up nontargeted metabolomics analysis were conducted to provide in-depth information regarding the relationship between characteristic metabolites and sensory qualities of *chrysanthemum* tea by identifying metabolites in five different varieties.

3.3. Nontargeted metabolomics analysis

Nontargeted metabonomics combined with multivariate analysis was applied to investigate the differences of metabolites in five varieties of *chrysanthemum* tea and to identify critical metabolites responsible for metabolomics variation caused by different varieties of *chrysanthemum* tea. The typical total ion current chromatogram for each *chrysanthemum* tea sample is shown in Figure S1. This study identified metabolites in *chrysanthemum* tea samples according to the in-house database (Shanghai Applied Protein Technology)[24]. After pre-treatment and data normalization, 1105 and 670 metabolites were identified from the total ion chromatogram of UPLC-QTOF-MS in positive and negative ion modes. According to their Chemical Taxonomy, all metabolites (identified by combining positive and negative ions) were classified and performed on the attribution information. The proportion of the number of various metabolites is shown in Figure 2, including 473 lipids and lipid-like molecules (25.918%), 361 phenylpropanoids and polyketides (19.781%), 184 organoheterocyclic compounds (10.082%), 173 benzenoids (9.479%), 148 organic oxygen compounds (8.11%), 109 organic acids and derivatives (5.973%), 40 alkaloids and derivatives (2.192 %), 32 nucleosides and analogs (1.753%), 26 lignans, neolignans and related compounds (1.425%), 21 organic nitrogen compounds (1.151%), 1 hydrocarbon derivatives (0.055%), and 257 other undefined compounds (14.082%).

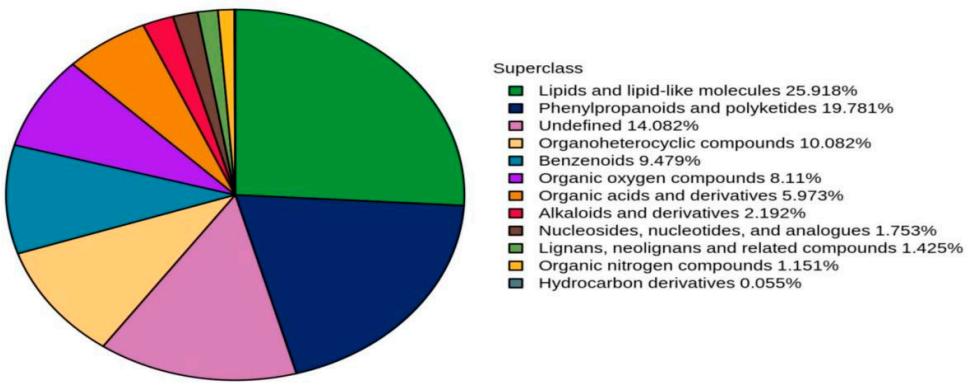

Fig.2: The proportion of the number of various metabolites in chrysanthemum samples

Notes: Different color blocks represent different chemical classification belonging items, and the percentage represents the chemical classification belonging items, The number of metabolites as a percentage of all identified metabolites.Metabolites that have no chemical classification are defined as undefined.

Figure 2. The proportion of the number of various metabolites in *chrysanthemum* tea samples
Notes: Different color blocks represent different chemical classification belonging items, and the percentage represents the chemical classification belonging items. The number of metabolites as a percentage of all identified metabolites. Metabolites that have no chemical classification are defined as undefined.

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The principal component analysis (PCA), partial least squares discrimination Analysis (PLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) methods have been used to identify combinations of metabolites accounting for the most variance, and to visualize sample cluster trends in tea[25]. All metabolites were subjected to multivariate analysis using SIMCA-P 14.1 multivariate statistical software. As an unsupervised data analysis method, the PCA can reflect variability between and within sample groups. As shown in Figure 3A, when using all of the data on metabolite ion features of five *chrysanthemum* tea samples, the QCs were clustered together on the PCA score plots, which revealed that the data variability was small. It is noteworthy that the X ('HuangJu') and J ('JinshihuangJu') *chrysanthemum* tea samples were similar in PCA but were separated in PLS-DA (Figure 3A,B). Therefore, to obtain a higher level of population separation and better understand the differences between different varieties of *chrysanthemum* tea, the OPLS-DA was used for classification and to confirm the separation between the'JinshihuangJu'(J) and'HuangJu'(X) tea samples in terms of the various significant parameters. Based on OPLS-DA, the separation trends between J and X samples show more obvious variations (Figure 3C), and the cross-validation with 200 permutation tests indicated that this OPLS-DA model was reliable, with intercepts of R^2 and Q^2 being 0.5555 and -0.6665, respectively (Figure 3D).

Differential metabolites of J and X samples were found by the OPLS-DA and variable importance in projection (VIP >1, P<0.01) and | log2 (fold change) | values > 1.5 was used for screening. In both positive- and negative-ion modes, 143 VIP metabolites responsible for metabolic changes between J and X samples were screened out, including 40 flavonoids and flavone glycosides, 31 acids, 22 ketones, 8 esters, 7 amino acids, 7 glycosides, 6 alkaloids, 6 alcohols, and 16 other metabolites. (Table S2).

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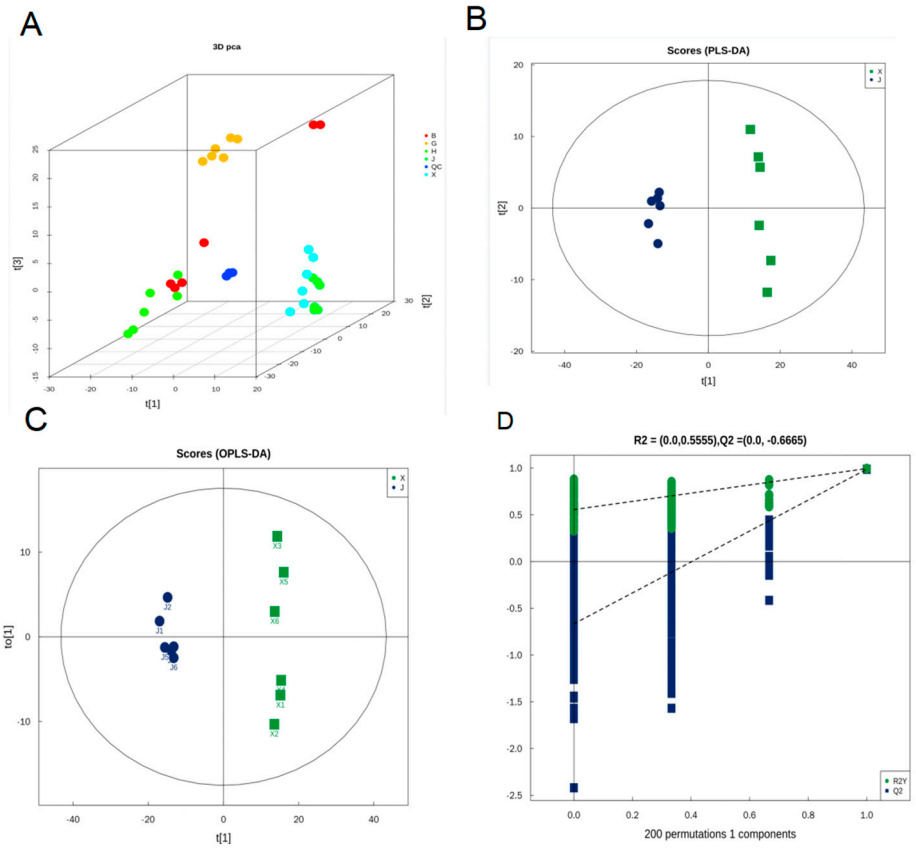

Fig.3 Multivariate analysis of *chrysanthemum* tea samples. (A) The 3D PCA of five different species of chrysanthemum. **(B)** The PLS-DA plot (X VS J), R^2X=0.751, R^2Y=0.994, Q^2=0.979. **(C)** The OPLS-DA score plot(X VS J), R^2X = 0.753, R^2Y = 0.994, Q^2 =0.987. **(D)** Permutation plot of OPLS-DA, R^2 =(0.0,0.5555), Q^2 =(0.0,-0.6665).
Notes: (J) JinshihuangJu; (X) HuangJu; (H) HanbaiJu; (B) Boju; (G) Gongju

Figure 3. Multivariate analysis of *chrysanthemum* tea samples. (A) The 3D PCA of five different species of chrysanthemum. (B) The PLS-DA plot (X VS J), R2X=0.751, R2Y=0.994，Q2=0.979. (C) The OPLS-DA score plot (X VS J), R2X = 0.753, R2Y = 0.994, Q2 =0.987. (D) Permutation plot of OPLS-DA, R2 = (0.0,0.5555), Q2 = (0.0, -0.6665). Notes: (J) JinshihuangJu; (X) HuangJu; (H) HanbaiJu; (B) Boju; (G) Gongju.

A multiple analysis was applied to visualize the difference of these critical metabolites between the'JingshihuangJu'(J) and'HuangJu'(X) samples (Figure 4). The x-coordinate represents the log2 FC value of the differential metabolite, and each row represents a critical metabolite. The red and green bar charts correspond to differential metabolites up and down, visually showing the changes in the multiple metabolic differences identified as significant.

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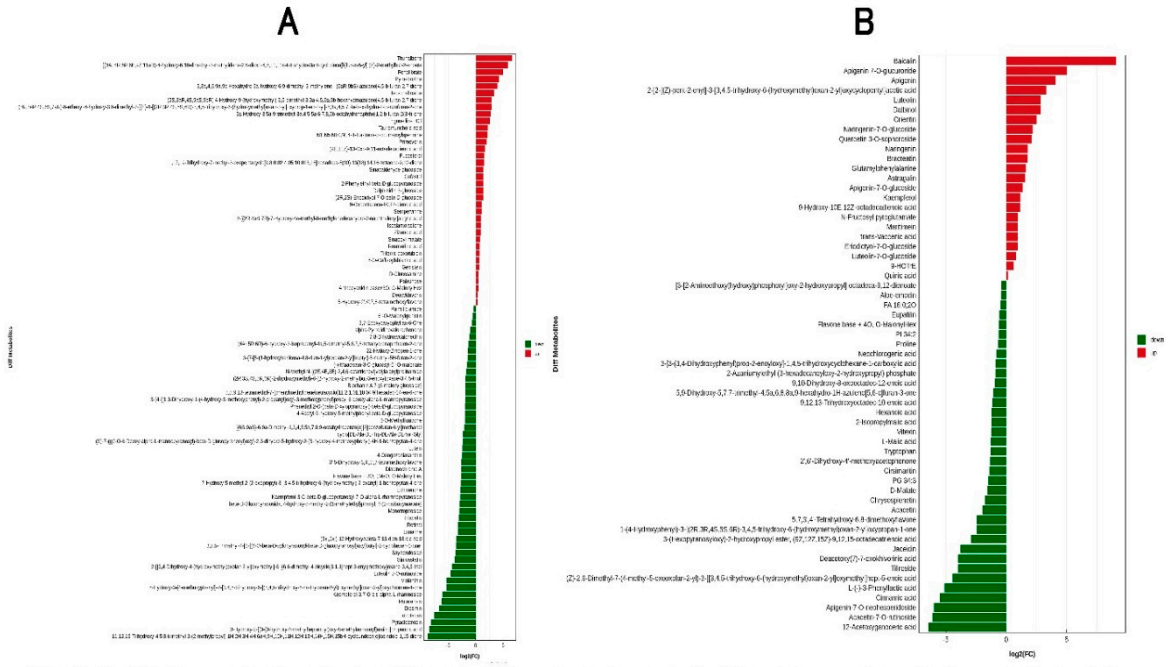

Fig 4 Multiple analysis of significant difference in metabolite expression between X and J samples((VIP>1, P< 0.01). (A) In positive-ion modes. **(B)** In negative-ion modes. **Notes:** the x-coordinate represents the log2 FC value of the differential metabolite, that is, the logarithm value of the differential multiple of the differential metabolite is taken as the base 2.The ordinate axis represents significant differential metabolites. The red indicates up-regulated differential metabolites and green indicates down-regulated differential metabolites.

Figure 4. Multiple analysis of significant difference in metabolite expression between X and J samples (VIP >1, *P* < 0.01). (A) In positive-ion modes. (B) In negative-ion modes. Notes: the x-coordinate represents the log2 FC value of the differential metabolite, that is, the logarithm value of the differential multiple of the differential metabolite is taken as the base 2, the ordinate axis represents significant differential metabolites. The red indicates up-regulated differential metabolites and green indicates down-regulated differential metabolites.

Polyphenols are phytonutrients, the most abundant content in chrysanthemum tea, containing flavonoids, phenolic acids, lignans, and stilbenes [3]. Isochlorogenic acid C, luteolin, apigenin-7-glucoside, chlorogenic acid, apigenin, and cryptochlorogenic acid play an important role in distinguishing different chrysanthemum varieties [11]. According to Figure 4, the most abundant markers metabolites in J and X samples are flavonoids and flavone glycosides. For example, the abundance of thunalbene, isoschaftoside, delphinidin 3-glucoside, primeverin, genistein, astragalin, bracteatin, maritimein, apigenin, kaempferol, luteolin, naringenin-7-O-glucoside, apigenin-7-O-glucoside, apigenin 7-O-glucuronide, naringenin, orientin, luteolin-7-O-glucoside, baicalinEriodictyol-7-O-glucoside, quercetin 3-O-sophoroside was up-regulation, while the content of kaempferol-3,7-O-bis-alpha-L-rhamnoside, rutarensin, violanthin, luteolin 7-O-rutinoside, 3',5-dneohesperidoside, chrysosplenetin, acacetin-7-O-rutinoside, jaceidin, 5,7,3',4'-tetrahydroxy-6,8-dimethoxyflavone,vitexin, cirsimaritin, and eupatilin were down-regulated. In fact, flavonoids and flavonoid glycosides play a central role in all aspects of plant life, particularly in the interactions between the plant and the environment, and determine taste and biological activity [13]. Flavonoid glycoside is an important astringent compound in *chrysanthemum* teas, with a velvety taste and oral coating sensation[16]. For instance, luteolin and apigenin had the highest contribution to the difference between these *chrysanthemum* teas as indicated by high VIP values, which were responsible for tea infusion's bitter and astringent taste [26]. In this study, the data of the untargeted metabolomics analysis showed a correlation with the taste index of the electronic tongue analysis. Moreso, the abundance of luteolin and apigenin in X (‘HuangJu’) samples was significantly higher

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than that in J ('JinshihuangJu') samples (Figure 4, $P<0.01$). Thus, the degradation of flavonols and flavonoids in different *chrysanthemum* varieties may play a crucial role in forming chrysanthemum tea flavor. In practice, *chrysanthemum* tea contains an extraordinarily high level of flavonoids that contribute to tea health benefits and flavor characteristics[27]. However, many flavonoids and xenoflavones have bitter and astringent tastes that are undesirable to consumers and hinder their use as bioactive substances in food [28]. Therefore, improving the bioavailability of *chrysanthemum* tea by modifying its flavonoids without affecting its sensory quality will be one of the directions of in-depth, comprehensive research in the future.

3.4. Identifying the core metabolites

Since different metabolites coordinate their biological functions, the KEGG pathway-based analysis would be helpful to further understand their biological function[29]. A KEGG analysis was conducted to correlate the core metabolites identified between X and J Samples (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/). The KEGG pathway enrichment analysis is based on the KEGG pathway as the unit and the metabolic pathways involved in this species or closely related species as the background. Fisher's Exact Test was used to analyze and calculate the significance level of metabolite enrichment in each pathway to identify the metabolic and signal transduction pathways that are significantly affected. Additionally, the KEGG enrichment pathway map between X and J samples is shown in Figure 5A. Most of the identified metabolites were mainly related to flavone and flavonol biosynthesis, flavonoid biosynthesis, isflavonoid biosynthesis, and other pathways identified by the KEGG enrichment analysis ($P < 0.05$). The important secondary metabolites, flavones, and flavonols were also detected in *chrysanthemum* of all the cultivars.

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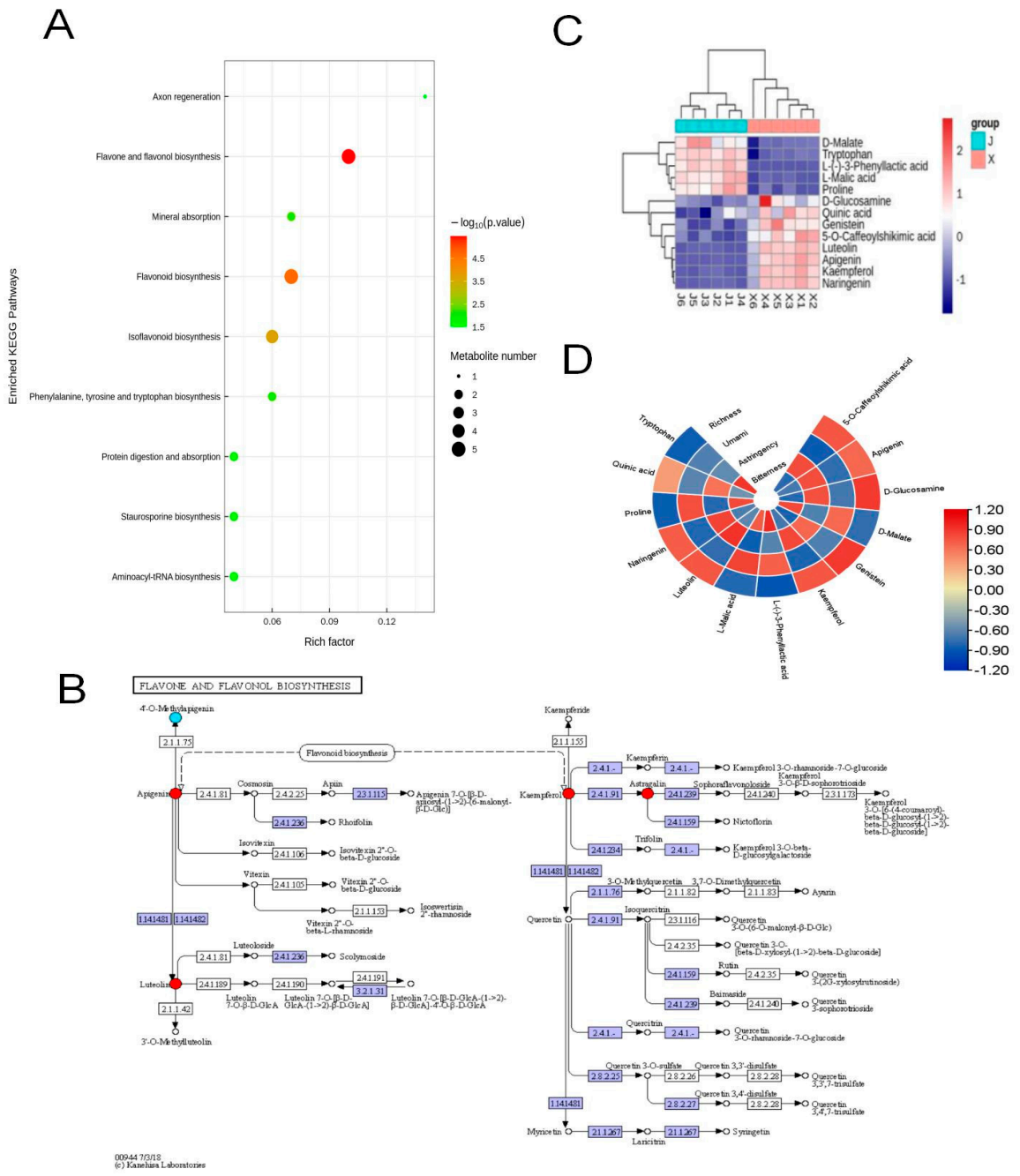

Fig. 5 Identifying the core metabolites . (A)The KEGG enrichment pathway bubble map between X and J samples, (B) The flavone and flavonol biosynthesis pathway, (C) Heatmap analysis of critical metabolites in theflavone and flavonol biosynthesis pathway. (D) Association between taste characteristics and metabolites data.

Note: (A) Each bubble in the figure represents a metabolic pathway (the top 20 with the highest significance are selected according to P value).The horizontal coordinate where the bubble is located and the bubble size represent the influence factor size of the path in the topology analysis, and the larger the size, the larger the influence factor.The vertical coordinate where the bubble is located and the bubble color represent the *P*-value of enrichment analysis (take the negative common logarithm, i.e. -log10 p-value); the darker the color, the smaller the *P*-value, the more significant the enrichment degree; the rich factor represents the proportion of the number of differential metabolites in this pathway in the number of annotated metabolites in this pathway. (B) The small circle nodes in the metabolic pathway diagram represent metabolites.The metabolites labeled in red are the significantly up-regulated differential metabolites detected in the experiment (VIP >1, $p < 0.05$, Fold change>1),while the metabolites labeled in blue are the significantly down-regulated differential metabolites detected experimentally (VIP >1, $p < 0.05$, Fold change>1).The depth of the color indicates the degree of downward adjustment.

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Figure 5. Identifying the core metabolites. (A)The KEGG enrichment pathway bubble map between X and J samples, (B) The flavone and flavonol biosynthesis pathway, (C) Heatmap analysis of critical metabolites in theflavone and flavonol biosynthesispathway. （D) Association between taste characteristics and metabolites data. Note: (A) Each bubble in the figure represents a metabolic pathway (the top 20 with the highest significance are selected according to P value). The horizontal coordinate where the bubble is located and the bubble size represent the influence factor size of the path in the topology analysis, and the larger the size, the larger the influence factor. The vertical coordinate where the bubble is located and the bubble color represent the *P*-value of enrichment analysis (take the negative common logarithm, i.e. -log10 p-value); the darker the color, the smaller the P-value, the more significant the enrichment degree; the rich factor represents the proportion of the number of differential metabolites in this pathway in the number of annotated metabolites in this pathway. (B) The small circle nodes in the metabolic pathway diagram represent metabolites, the metabolites labeled in red are the significantly up-regulated differential metabolites detected in the experiment (VIP >1, $p < 0.05$, Fold change>1), while the metabolites labeled in blue are the significantly down-regulated differential metabolites detected experimentally (VIP >1, $p < 0.05$, Fold change >1).The depth of the color indicates the degree of downward adjustment.

The flavonoid biosynthesis pathway has been extensively investigated in different *chrysanthemum* species [30]. Flavonols belong to polyphenols, which mainly exist as glycosides in *chrysanthemum* tea, contributing to tea's bioactivities, bitterness, and astringency [31]. Considering the detection of flavonoids in *chrysanthemum* and previous studies on flavonoids biosynthesis pathway [32], a hypothesized that *chrysanthemum* biosynthesis pathway was detected for flavonoids and flavonols (Figure 5B). As shown in Figure 5B, this pathway includes the mutual synthesis and transformation of apigenin, luteolin, two flavonoid components (glycosides) and their derivatives, and the mutual synthesis and transformation of kaempferol, quercetin, myricetin 3 flavonol components and their derivatives. At the same time, apigenin and kaempferol can also be synthesized and transformed through the flavonoid biosynthesis pathway. To facilitate the observation of the expression of different metabolites annotated in the KEGG metabolic pathway, heat maps of the different metabolites in the flavone and flavonol biosynthesis pathways were plotted in Figure 5C. Furthermore, quinic acid, genistein, 5-O-caffeoylshikimic acid, luteolin, apigenin, kaempferol, and naringenin were found to be the top marker metabolites for X ('HuangJu') samples, which were significantly higher than that in J ('JingshihuangJu') samples; D-Malate, Tryptophan, L-(-)-3-Phenyllactic acid, L-Malic acid, and proline were found to be the top marker metabolites for J ('JinshihuangJu') samples, which it was significantly higher than that in X ('HuangJu') samples.

Numerous studies have shown that flavonols and flavones are key contributors to tea infusions' astringent and bitter tastes and can also significantly enhance the bitterness of caffeine [33,34]. Therefore, to statistically calculate the relationship between core metabolites compound and taste intensity, Spearman's correlation analysis coefficient was utilized in Figure 5D. There was a significant correlation between taste characteristics and some core metabolites. The main astringent contributors with tight correlation are kaempferol, luteolin, genistein, and some quinic acid derivatives. As the more common flavor characteristics of *chrysanthemum* tea, these key metabolites can form various flavonol glycosides with various sugar groups to bring an astringent and convergent taste to the tea[16]. In fact, astringency is a tactile sensation caused by the interaction of astringent substances (such as polyphenols) with salivary proteins, resulting in protein precipitation and decreased lubrication in the mouth. As important bitter and astringent compounds, quinic acid derivatives dissolve easily during tea brewing, thus enhancing acidity and affecting the taste of other polyphenols[17]. Furthermore, L-(-)-3 phenyllactic acid and L-malic acid were found to be bitter compounds in *chrysanthemum* tea. Interestingly, these compounds are also associated with the umami flavor of *chrysanthemum* tea. At the same time, bitterness and astringency are generally undesirable. Still, they are important for providing the complex sensory perceptions of *chrysanthemum* teas. All of these are essential tasting elements of a delicious drink. According to Table 1A and 1B, there was no significant difference in umami taste between J ('JinshihuangJu') samples and X ('HuangJu') samples ($P > 0.05$). In contrast, they obviously differed in bitterness and astringency ($P < 0.05$). Metabolic

pathway analysis (Figure 5A,B) showed significant differences in flavonoid metabolism levels between the two varieties of *chrysanthemum* tea, which may be the reason for the difference in taste quality between the two varieties. In fact, the taste of *chrysanthemum* tea is closely related to some core chemical constituents, shown in Figure 5D, and forms sensory qualities. Therefore, we hypothesized that these 13 core metabolites could be used as quick markers for the difference in taste between the two varieties of *chrysanthemum* tea. Importnatly, this study advances our understanding of metabolic changes and sensory quality formation in different varieties of *chrysanthemum* tea and these data provided a theoretical basis for the identification of chrysanthemum varieties and the control of flavor quality.

4. Conclusion

Chrysanthemum tea is rich in many secondary metabolites related to its sensory qualities. This study used a nonargeted metabolomic and sensory evaluation method based on UPLC-QTOF-MS and electronic tongue to investigate key differential metabolites associated with sensory quality differences among five *chrysanthemum* (Juhua) tea varieties. A total of 1775 metabolites were identified in five varieties of *Chrysanthemum* tea by using UPLC-Q-TOF/MS analysis. The PCA, PLS-DA, and OPLS-DA results indicated significant differences in metabolome between X ('HuangJu') samples and J ('JinshihuangJu') samples.

Of these metabolites, the content of 13 key metabolites (5-O-caffeoylshikimic acid, apigenin, D-glucosamine, D-malate, genistein, kaempferol, L-(-)-3-phenyllactic acid, L-malic acid, luteolin, naringenin, proline, quinic acid, tryptophan) could be used as quick markers for the difference in taste between the two varieties of *chrysanthemum* tea. Additionally, KEGG pathway enrichment analysis showed that there were significant differences in flavonoids metabolism levels between X ('HuangJu') and J ('JinshihuangJu') *chrysanthemum* tea samples, and the pathways involved in flavonoid metabolism had important effects on sensory quality of different *chrysanthemum* tea varieties. Notably, this study enriches our understanding of the relationship between metabolites and the sensory quality of *chrysanthemum* tea varieties. untargeted metabolomics combined with electronic tongue analysis based on LC-MS can be effectively used to evaluate the difference of sensory of different varieties *chrysanthemum* tea cultivars. Further studies are ongoing in the author's lab, focusing on the formation mechanism of the key flavor components and the functional components in *chrysanthemum* tea. We hope to report more about these advancements in the future.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

CRediT authorship contribution statement: Xing Tian: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Haodong Wang:** Formal analysis, Investigation, Writing - original draft.**Wei Wang:** Supervision, Writing - review & editing, Funding acquisition. **Hanwen Yuan:** Methodology, Conceptualization, Data curation. **Liang Chen**: Visualization, Data curation. **Yaoli Ouyang**: Funding acquisition.

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