Non-targeted metabolomic analysis of lotus leaf and lotus seed wine

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Abstract. Non-targeted metabolomics was used to analyze the active substances in Lotus Leaf and Lotus Seed Liquor to study their types and distribution characteristics, and to screen characteristic markers used to distinguish Lotus Leaf and Lotus Seed Liquor. The results showed that through analysis and screening, a total of 198 metabolically active substances were identified in the wine samples. A total of 74 differential metabolites were found in lotus leaf lotus seed wine and ordinary liquor base wine, among which matrine, eucalyptol and isoferulic acid The expression difference in lotus leaf and lotus seed wine is obvious, and it can be used as an identification marker of lotus leaf and lotus seed wine.

Keywords: Non-targeted metabolomics；Lotus leaf and lotus seed wine；marker.

1. Introduction

Lotus leaf is a common aquatic plant widely used in traditional Chinese medicine and food preparation. Lotus leaf is rich in nutrients and has the functions of clearing away heat and detoxification, lowering blood lipids, antioxidant, protecting liver and kidneys, regulating gastrointestinal function, etc. These functions make lotus leaf a popular natural medicinal and food ingredient. Lotus seeds are precious plant seeds that are often used as Chinese herbal medicine. They are rich in nutrients and help to strengthen physical strength and improve immunity. At the same time, lotus seeds have the effects of benefiting the spleen and stomach, clearing away heat and moisturizing the lungs, nourishing the kidneys, and beautifying the skin [1, 2]. Lotus leaves and lotus seeds are rich in minerals, vitamins, antioxidants and other nutrients. They are precious natural plants that have the same source of medicine and food [3]. Lotus leaf and lotus seed wine is a health-care wine that uses lotus seeds and lotus leaves as auxiliary materials and is fermented using traditional brewing methods. It has the effects of reassuring and nourishing the mind, replenishing the kidneys and strengthening essence [4-6]. The production process of lotus leaf and lotus seed wine includes soaking or fermenting lotus leaves and lotus seeds with base wine such as white wine or rice wine. The nutrients of lotus seeds can penetrate into the base wine, giving the wine a rich taste and nutritional value. At present, the research on lotus leaf and lotus seed wine mainly focuses on the fermentation process, and there are few research reports on its metabolite analysis.

Untargeted metabolomics is a method that can simultaneously detect and analyze all small molecule metabolites. It plays an important role in the research of lotus leaf and lotus seed wine and can help analyze its intrinsic metabolic characteristics. In addition, non-targeted metabolomics also has wide applications in fields such as biomedicine and nutritional health [7, 8]. Researchers can use metabolomics analysis methods to screen out differential metabolites, discover specific compounds, and study their functions and mechanisms of action in depth. In addition,

metabolomics can also be combined with bioinformatics to reveal the complex network of metabolism by analyzing differential metabolic pathways and predicting the biological mechanisms of metabolite formation. This analytical method can provide in-depth understanding for food research and promote knowledge of sample composition, quality and functionality [9-11]. In recent years, non-targeted metabolomics can accurately distinguish foods of different qualities by comprehensively analyzing and comparing the metabolite composition of samples. It can reveal significant differences in metabolites between different samples, thereby enabling effective variety identification and variety difference analysis. This allows untargeted metabolomics to support quality and flavor control of food products [12, 13]. Wang [14] et al. used a non-targeted metabolomics method to analyze the composition differences of the main brewing sorghum in the liquor-producing areas of southern Sichuan, and a total of 975 metabolites were identified.

In this study, samples of lotus leaf lotus seed wine and corresponding base wine produced by liquor companies in Shandong Province were selected, and samples were extracted. Non-targeted metabolomics methods were used to analyze the types and contents of metabolites in lotus leaf lotus seed wine and base wine. Conduct systematic research on the changing patterns. The research focuses on screening and analyzing differential metabolites to understand the composition and efficacy of the characteristic markers of lotus leaf and lotus seed wine. This study provides a valuable theoretical basis for the identification and quality assessment of lotus leaf and lotus seed wine.

2. Materials and methods

2.1 Main instruments and equipment

Vanquish UHPLC ultra-high pressure liquid chromatograph, Q Exactive Focus high-resolution mass spectrometer, Heraeus Fresco17 centrifuge, Thermo Scientific Company of the United States; BSA124S-CW balance, Sartorius; JXFSTPRP-24 grinder, Shanghai Jingxin Technology Co., Ltd.; Mingche D24 UV pure water meter, Merck Millipore; YM-080S ultrasonic meter, Shenzhen Fangao Microelectronics Co., Ltd.; ACQUITY UPLC BEH C18 1.7 μm 2.1*100 mm chromatographic column, Waters, etc.

2.2 Main reagents

Chromatographic grade methanol and acetonitrile, CNW Technologies; chromatographic grade formic acid, SIGMA; L-2-chlorophenylalanine (purity ≥98%), Shanghai Hengbai Biotechnology Co., Ltd.

2.3 Experimental methods

2.3.1 Test sample

Lotus leaf and lotus seed wine and the corresponding liquor base liquor come from liquor companies in Shandong Province.

2.3.2 Sample extraction method

Vortex for 30 s, centrifuge the sample at 4°C and 12000 rpm for 15 min; take 300 μL of the supernatant in an EP tube and add 1000 μL of extraction solution (methanol: water = 4:1, internal standard concentration is 10 μg/mL); Vortex for 30 s, ultrasonicate in an ice-water bath for 5 min; let stand at -40°C for 1 h, then centrifuge the sample at 4°C and 12000 rpm for 15 min; the supernatant is filtered through a 0.22 μm filter; 50 μL of each liquor sample is mixed into a quality control Samples; samples are stored at -80°C.

2.3.3 Sample data preprocessing

The research object of the experiment was lotus leaf and lotus seed wine samples, which were divided into two groups on average: 1 (base wine) and 2 (lotus leaf and lotus seed wine). The

corresponding biological replicates in each group were 3 cases. Metabolomic analysis was performed. A total of 6 samples.

2.3.4 Chromatography and mass spectrometry conditions

Chromatographic conditions: The chromatographic column used was a UPLC BEH C18 column purchased from Waters (1.7 μm * 2.1 * 100 mm). The injection volume is 5 μL; the flow rate is 0.5 mL/min; the mobile phase composition is: 0.1% formic acid aqueous solution (A) - 0.1% formic acid acetonitrile solution (B); elution gradient: 0~11 min, 85%~15% B; 11~12 min, 25%~75% B; 12~14 min, 2%~98% B; 14~14.1 min, 85%~15% B; 14.1~15 min, 85%~15% B; 15~ 16 min, 85%~15% B.

Mass spectrometry conditions: Q Exactive Focus mass spectrometer was used to collect primary and secondary mass spectrometry data of the sample. After the samples were separated by the Vanquish UHPLC system, they were analyzed by mass spectrometry and detected using electrospray ionization mode. The electrospray and mass spectrometry setting parameters are as follows: spray voltage (+): 4.0 kV, electrospray voltage (-): 3.6 kV; heating temperature 400 °C; sheath gas flow rate 45 Arb; auxiliary gas flow rate 15 Arb; collision energy 15-30- 45 eV; resolution Full MS) 70000; resolution (MS2) 17500.

2.3.5 Data processing

After the original data is converted into format, XCMS software is used for subsequent data processing. Combined with the Chinese medicine non-target metabolomics database built by Shanghai Baiqu Biomedical Technology Co., Ltd. and the corresponding matching method, metabolite identification was performed on the peaks containing MSMS data. Through this method, we can determine the compound information and fragmentation rules of the substances in the peak, and further understand the composition and characteristics of the metabolites in the sample.

Data analysis includes principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA), and differential metabolite screening [13]. From the variable weight values (VIP) obtained by the OPLS-DA model, we can identify metabolites that play a key role in sample classification and further study their functions and mechanisms of action in biological processes. The high and low VIP values can guide us to identify and interpret important metabolic changes, thereby gaining insight into the biological differences between samples. In this study, the differential metabolite screening criteria were set as follows: VIP obtained by the OPLS-DA model was > 1 and P < 0.05.

2.3.6 Statistical analysis

The processed data is subjected to logarithmic (LOG) conversion and centralization (CTR) formatting in SIMCA (V16.0.2) software, and then automatic modeling analysis is performed to complete principal component analysis [15]. The results were analyzed using the statistical method of orthogonal partial least squares-discriminant analysis (OPLS-DA), and the effective differential metabolites were clustered and displayed.

3. Results and analysis

3.1 Principal Component Analysis (PCA)

The PCA scores are shown in Figure 1. From the results of the PCA score chart, it can be observed that all samples are within the 95% confidence interval. The differences between the groups are obvious, there is no overlap and crossover, and the differences within the groups are also small, which shows that the differences between the samples The biological repeatability is good, and it can reflect the metabolic differences between samples as a whole, which shows that the PCA

analysis model is reliable and shows that there are differences in metabolites between lotus leaf lotus seed wine and base wine samples.

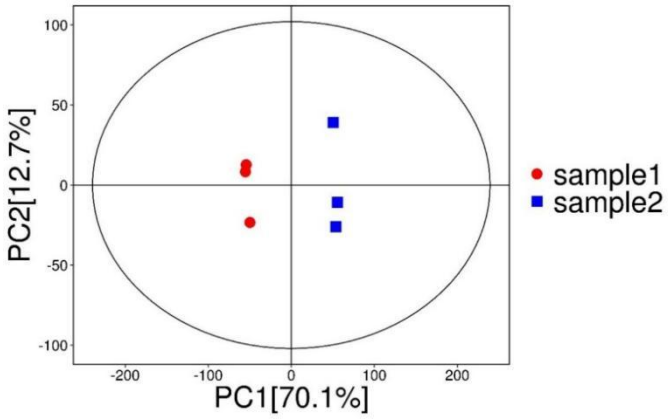

Fig. 1 Scatter plot of PCA model results of lotus leaf lotus seed wine and base wine

3.2 Orthogonal partial least squares discriminant analysis (OPLS-DA) and permutation verification

OPLS-DA analysis results showed that there were significant differences between the metabolome of lotus leaf lotus seed wine and base wine. The samples in the model are all within the confidence interval. Through multiple permutation tests and evaluating the R2 and Q2 values of the model, we verified the stability and reliability of the OPLS-DA model and ruled out the possibility of overfitting, thereby validating the lotus leaves. A reliable statistical analysis was conducted on the metabolic differences between lotus seed wine and base wine [16, 17].

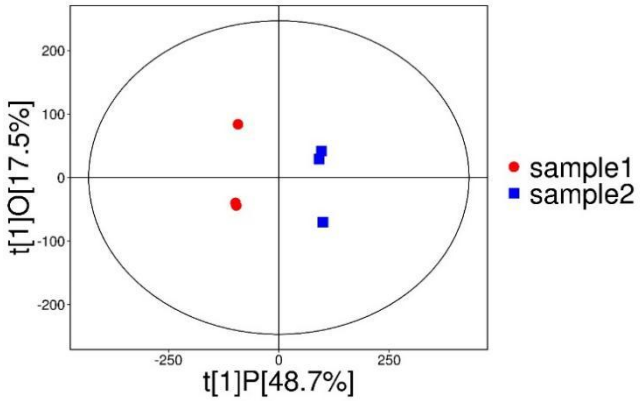

Fig. 2 OPLS-DA model scores of lotus leaf lotus seed wine and base wine

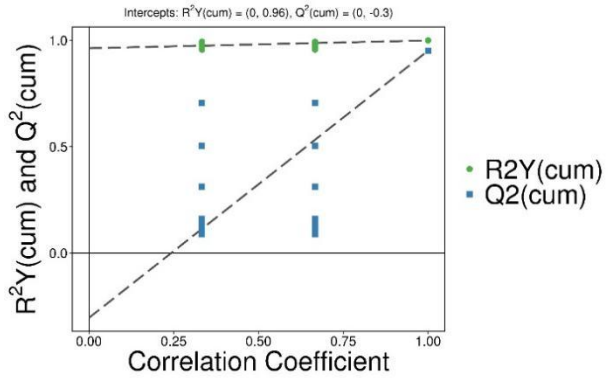

Fig. 3 Permutation test results of the OPLS-DA model of lotus leaf lotus seed wine and base wine

3.3 Analysis of differences in metabolites between lotus leaf and lotus seed wine and base wine

Through screening analysis, it was found that there were significant differences between the metabolites of lotus leaf lotus seed wine and base wine, and classification statistics and difference analysis were performed. A total of 198 metabolites were identified in the liquor samples in this

article. The metabolites were mainly divided into the following categories according to HMDB classification: 13 organic oxygen compounds, 9 organic acids and derivatives, 27 lipids and lipid molecules, phenylpropanoids and There are 22 polyketone compounds, 10 organic heterocyclic compounds, 8 benzene ring compounds, 3 organic nitrogen compounds, and 106 other metabolites. Lotus leaf and lotus seed wine is rich in organic oxygen compounds, which mainly include 13 sugars and sugar conjugates. The main sugar substances are sucrose and D-galactose. Lipids and lipid molecules mainly include 7 isopentenol lipids and 16 fatty acyls. Amino acid peptides mainly include L-glutamic acid, L-aspartic acid, L-isoleucine, and L-arginine.

Finally, a total of 74 differential metabolites were identified between the lotus leaf lotus seed wine and the base wine. The lotus leaf lotus seed wine had 25 markers (FC value > 1), and the base wine had 49 markers (FC value < 1). The screening found that there were significant differences in the expression of kurarinone, eudesmin and isoferulic acid in lotus leaf and lotus seed wine. These compounds can be used as identification markers of lotus leaf and lotus seed wine. By detecting and analyzing the content of these specific compounds, we can accurately identify and characterize lotus leaf lotus seed wine. These differentially expressed compounds may be related to the characteristics and quality of lotus leaf and lotus seed wine, and further research can help us understand their role and efficacy in lotus leaf and lotus seed wine.

Serial number	name	Mass-charge ratio（m/z）	Ionization mode	Chemical formula	FC
1	Kurarinone	439.21	[M+H]+	C26H30O6	31230.11
2	eudesmin	409.16	[M+Na]+	C22H26O6	2781.78
3	isoferulic acid	193.05	[M-H]-	C10H10O4	676.14
4	Notopterol	355.15	[M+H]+	C21H22O5	147.49
5	Ethyl cinnamate	177.09	[M+H]+	C11H12O2	33.09
6	Isogentisin	259.06	[M+H]+	C14H10O5	26.15
7	beta-Farnesene	205.19	[M+H]+	C15H24	21.08
8	7-Methoxy-4-methylcoumarin	191.07	[M+H]+	C11H10O3	14.86
9	3-Butylidenephthalide	189.09	[M+H]+	C12H12O2	12.91
10	trans-pterostilbene	257.11	[M+H]+	C16H16O3	10.43
11	(+)-alpha-Muurolene	205.19	[M+H]+	C15H24	9.09
12	Viburtinal	161.05	[M+H]+	C10H8O2	9.01
13	Dehydrovomifoliol	223.13	[M+H]+	C13H18O3	7.76
14	Ligustilide	191.10	[M+H]+	C12H14O2	5.40
15	Gamma-terpinene	137.13	[M+H]+	C10H16	5.13
16	2-Hydroxyacetophenone	137.05	[M+H]+	C8H8O2	4.05
17	3-(1-Hydroxymethyl-1-propenyl)pentanedioic acid	203.09	[M+H]+	C9H14O5	3.56
18	Cyclamate	178.05	[M-H]-	C6H13NO3S	2.51
19	(+)-Catechin hydrate	289.07	[M-H]-	C15H14O6.H2O	2.41
20	4-Hydroxystyrene	121.06	[M+H]+	C8H8O	2.21
21	Isosativan	287.12	[M+H]+	C17H18O4	2.03
22	Pogostone	225.11	[M+H]+	C12H16O4	1.88
23	Gentiatibetine	166.08	[M+H]+	C9H11NO2	1.55
24	2'-Hydroxyacetophenone	137.05	[M+H]+	C8H8O2	1.43
25	Cassiastearoptene	163.07	[M+H]+	C10H10O2	1.43

Table 1. Markers of lotus leaf and lotus seed wine

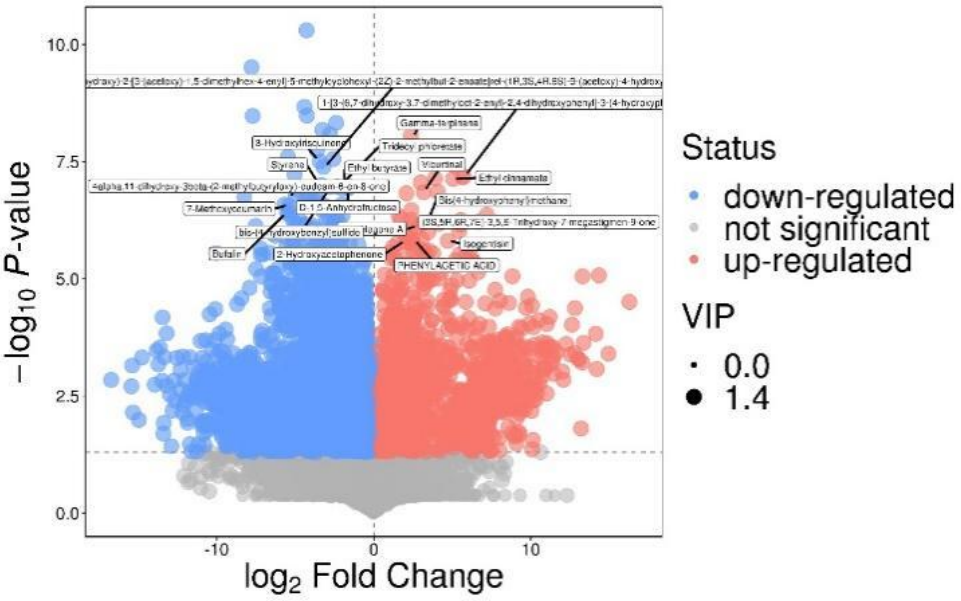

Fig. 4 Volcano plot of differential metabolite screening between lotus leaf lotus seed wine and base wine

3.4 Differential metabolite pathway analysis

The coordinated interaction between different metabolites enables organisms to have corresponding functions. By conducting KEGG pathway analysis on metabolites with significant differences screened out from metabolomics data, we can better understand the functions of organisms at the metabolome level. biological functions [18, 19]. Pathway analysis was performed using the screened KEGG IDs that shared significantly different metabolites, and a total of 15 metabolic pathways were obtained, including metabolism, biosynthesis of valine, leucine and isoleucine, valine, and leucine. and isoleucine degradation, glycine, serine and threonine metabolism, piperidine and pyridine alkaloid biosynthesis, fructose and mannose metabolism, metabolic pathways, glycerophospholipid metabolism, ABC transport, folate biosynthesis, niacin and nicotinamide metabolism, biosynthesis of ubiquinone and other terpenoid quinones, glutathione metabolism, cyanamic acid metabolism, and aminoacyl biosynthesis.

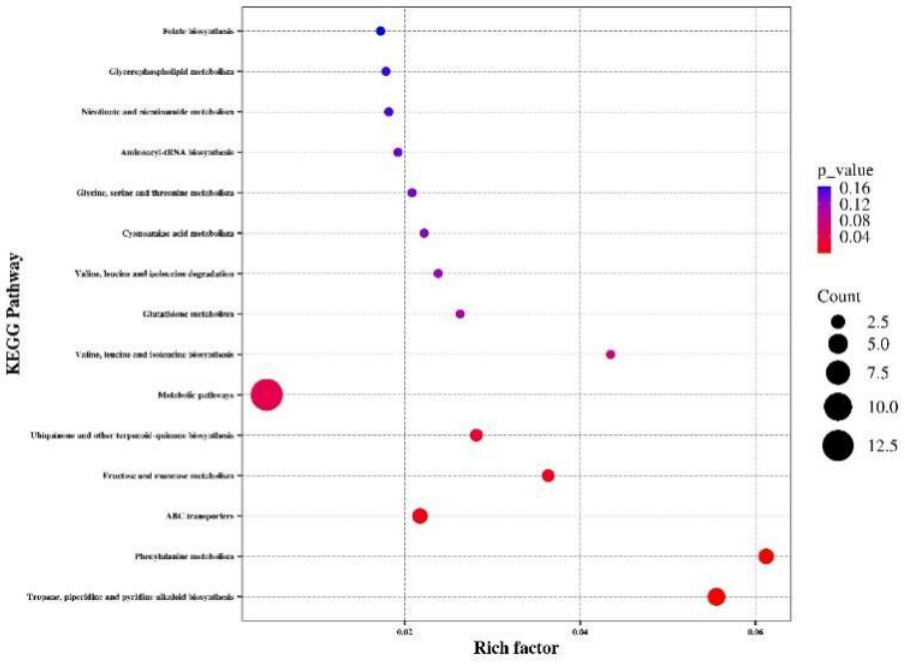

Fig. 5 KEGG classification diagram of differential metabolites between lotus leaf lotus seed wine and base wine

4. Summary

This study screened and analyzed the metabolites of lotus leaf lotus seed wine and base wine based on non-targeted metabolomics technology. After data processing and statistical analysis, it was found that matrine, eucalyptol and isoferulic acid were present in lotus leaf lotus seeds. The expression difference in the wine is obvious, which is significantly higher than the content in the base wine. These compounds have many nutritional and medicinal values. Among them, matrine has an inhibitory effect on endothelial cell proliferation and can protect cells from oxidative damage [20]; eucalyptol has anti-inflammatory, antibacterial, anti-inflammatory, antispasmodic and It has analgesic and other effects. It can also stimulate the discharge of respiratory secretions and help relieve the symptoms of respiratory diseases [21]; isoferulic acid is a phenolic acid compound with a wide range of pharmacological effects, can lower blood sugar, and has Antibacterial, anti-inflammatory, antioxidant, antiviral, and anti-tumor effects [22].

Through non-targeted metabolomics screening analysis and multivariate statistical analysis methods, significantly different metabolites in lotus leaf and lotus seed wine can be accurately identified, thus providing an in-depth theory for understanding the composition and characteristics of lotus leaf and lotus seed wine. Base. As a special liquor, the taste and aroma characteristics of lotus leaf and lotus seed wine are very important. Further research and verification are needed to deeply understand the mechanism of action and potential application value of these ingredients in lotus leaf and lotus seed wine, and to develop them. and applications provide substantial support.

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