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Based on UHPLC-Q-Orbitrap HRMS, the Effect of Chemical Composition before and after Fermentation of Gastrodia Tuder Halimasch Powder Was Compared

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Article

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Abstract: Objective: To compare the effects of fermentation on the chemical composition of Gastrodia Tuder Halimasch Powder, and to provide a basis for the processing, processing and clinical application of this medicinal material. Methods: UHPLC-Q-Orbitrap HRMS was used to identify compound structure by comparing excimer ions, characteristic fragment ions and reference substances, and referring to relevant literature and database search. The main differential components were screened by orthogonal partial least squares-discriminant analysis (OPLS-DA), and the differential components were quantitatively studied by high performance liquid chromatography, and the differences in the types and contents of chemical components before and after fermentation were compared. Results: A total of 77 compounds were identified or preliminarily derived after fermentation, including 21 terpenes, 18 organic acids, 9 flavonoids, 7 nucleosides, 6 amides, 4 amino acids, 2 pyrrolidone derivatives, 2 sterols, 2 alkaloids and 6 others. Through high performance liquid chromatography, it was found that the content of daidzein genistein and ergosterol was significantly increased after fermentation, and a new component ergosterol was produced. Compared with Gastrodia Tuder Halimasch Powder before fermentation, the content of genistin and other components was significantly reduced, and there may be a process of genistin conversion to genistein. Conclusion: The fermentation process will have a certain influence on the types and contents of the chemical components of Gastrodia Tuder Halimasch Powder, and ergosterol, genistein and daidzein may be the main pharmacodynamic components, which can provide a useful reference for the clinical rational application, quality control and mechanism of action of Gastrodia Tuder Halimasch Powder.

Keywords: gastrodia tuder halimasch powder; ultra high performance liquid chromatography tandem quadrupole electrostatic field orbitrap high resolution mass spectrometry; stoichiometry; fermentation processing

1. Introduction

Gastrodia Tuder Halimasch Powder is a member of the family Armillaria mellea (vane. ex. Fr.) Quel. The dried bacteria powder obtained by liquid fermentation and culture, separated and extracted, was included in the 2002 (Chemical Landmark Upgrading National Standard 13 Volumes) [1]. Honey ring bacteria are rich in chemical components, including terpenes, sugars, nucleosides, sterols and other components [2].Modern pharmacological studies have shown that aqueous extracts and ethanol extracts of mycelium of Mycomycetes have the effect of improving insomnia [3]; Ethyl

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acetate extract of Honeycilla is able to inhibit inflammatory mediators [4]; Melania polysaccharides are resistant to Alzheimer's disease and hypoglycemia [5,6]; Sesquiterpene aromatic esters in Honey Ring are antidepressant [7]; Melleolide-like compounds in honey ring bacteria have anti-liver cancer and anti-Aspergillus effects [8,9].

After reviewing the literature, soy glycogen can improve ischemic brain injury [10], Cerebral edema [11], Vascular endothelial dysfunction [12,13] and anti-epileptic [14];Genistein has anti-Aβ neurotoxicity [15], atherosclerosis [16], Regulates blood sugar [17] and lipids [18];Ergosterol against Alzheimer's disease [19], diabetes [20], Hepatic steatosis [21] and to neuroprotection [22];Clinically used for neuroprotection with Gastrodia Tuder Halimasch Powder [23], Treatment of tension headaches [24], Lower blood sugar [25] and other effects. Daidzein, genistein and ergosterol may be the main pharmacodynamic components of Gastrodia Tuder Halimasch Powder.

At present, there are few reports on the effect of Gastrodia Tuder Halimasch Powder on its chemical composition before and after fermentation, and the chemical components of traditional Chinese medicines are of great significance for elucidating the mechanism of action of traditional Chinese medicines and guiding the safety of clinical medication, which restricts the clinical rational application of Gastrodia Tuder Halimasch Powder to a certain extent [26]。 Ultra-performance liquid chromatography-quadrupole / electrostatic field orbital well high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) It is mostly used in the analysis of complex compounds in traditional Chinese medicine, and has the characteristics of high resolution, high sensitivity and strong qualitative ability [27]。 Therefore, the chemical components of Gastrodia Tuder Halimasch Powder before and after fermentation were analyzed based on UHPLC-Q-ORBITRAP HRMS, and the main chemical components were identified according to the chromatographic peak retention time (tR), secondary fragment ions and reference comparison, combined with relevant literature reports.;Orthogonal partial least squares-discriminant analysis (OPLS-DA) was used to screen the main differentiating components.;The HPLC method was used to determine the multi-component content in Gastrodia Tuder Halimasch Powder, and then PCA treatment was carried out to group the Gastrodia Tuder Halimasch Powder before and after fermentation, and the SPSS 26.0 software was used to analyze the LSD variance of the data in pairs;According to this, the Origin 8.0 drawing software was used to draw a significant difference marker histogram to compare the difference in the content of components before and after fermentation of Gastrodia Tuder Halimasch Powder. The fermentation-based method adopted by Gastrodia Tuder Halimasch Powder is of great significance to enhance the clinical efficacy, and provides a reference for exploring the clinical rational application and quality control research of Gastrodia Tuder Halimasch Powder.

2. Results and Discussion

2.1. Chemical composition analysis of Gastrodia Tuder Halimasch Powder

The ion flow (TIC) of Armillaria gastrodia powder before and after fermentation under positive and negative ion modes is shown in Figure 1. Xcalibur 4.5 was used to process the original mass spectrometry data, and chemical formulas were obtained by accurate relative molecular mass and excimer ion peaks of compounds. 77 components were preliminarily qualitatively analyzed by using MS/MS spectra, characteristic fragment ions and other fragment ions, reference reports and database analysis. It included 21 terpenoids, 18 organic acids, 9 flavonoids, 7 nucleosides, 6 amides, 4 amino acids, 2 pyrrolidone derivatives, 2 steroids, 2 alkaloids and 6 other classes, as shown in Table 1.

2.2. Analysis of main components of Gastrodia Tuder Halimasch Powder

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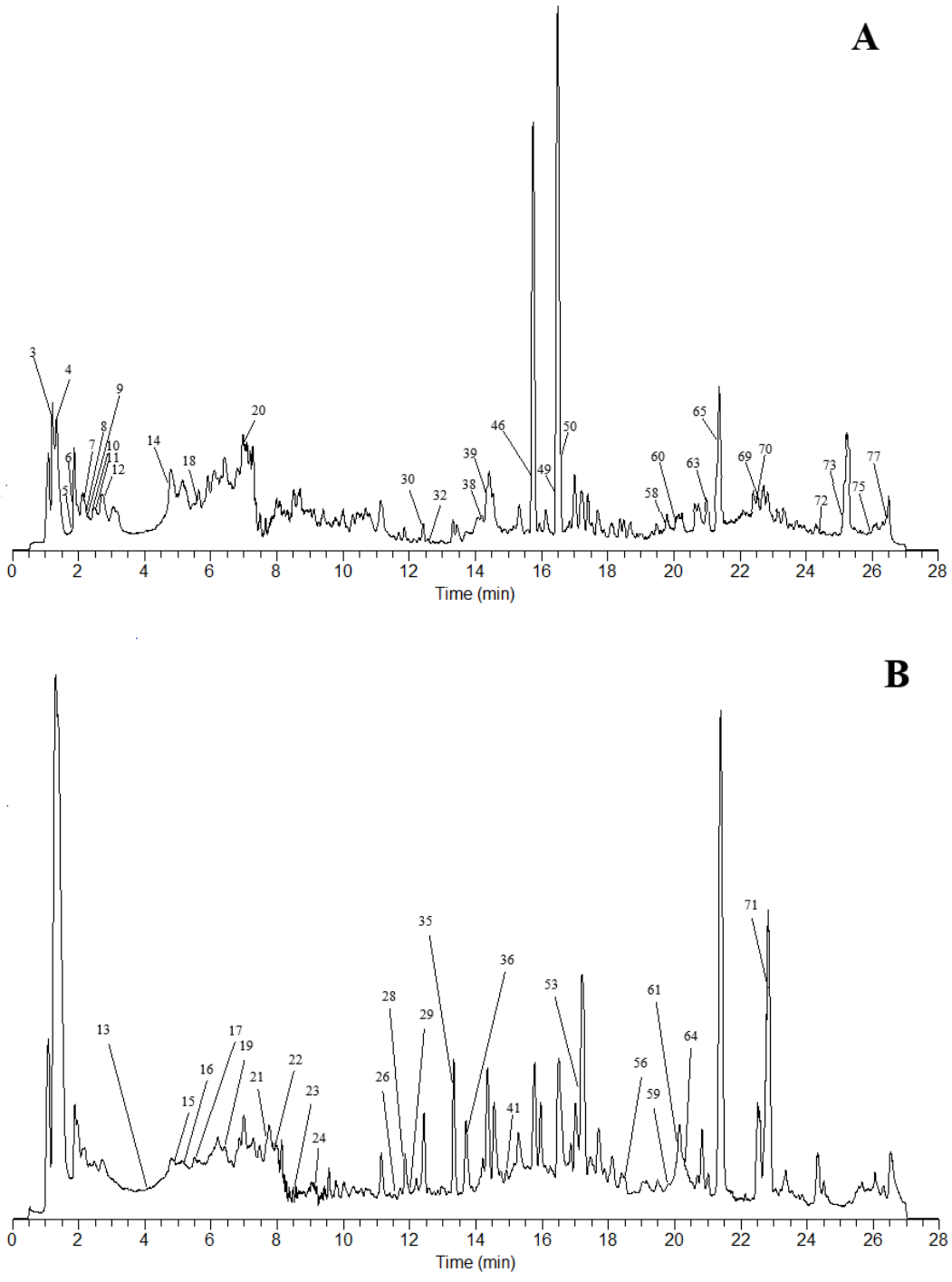

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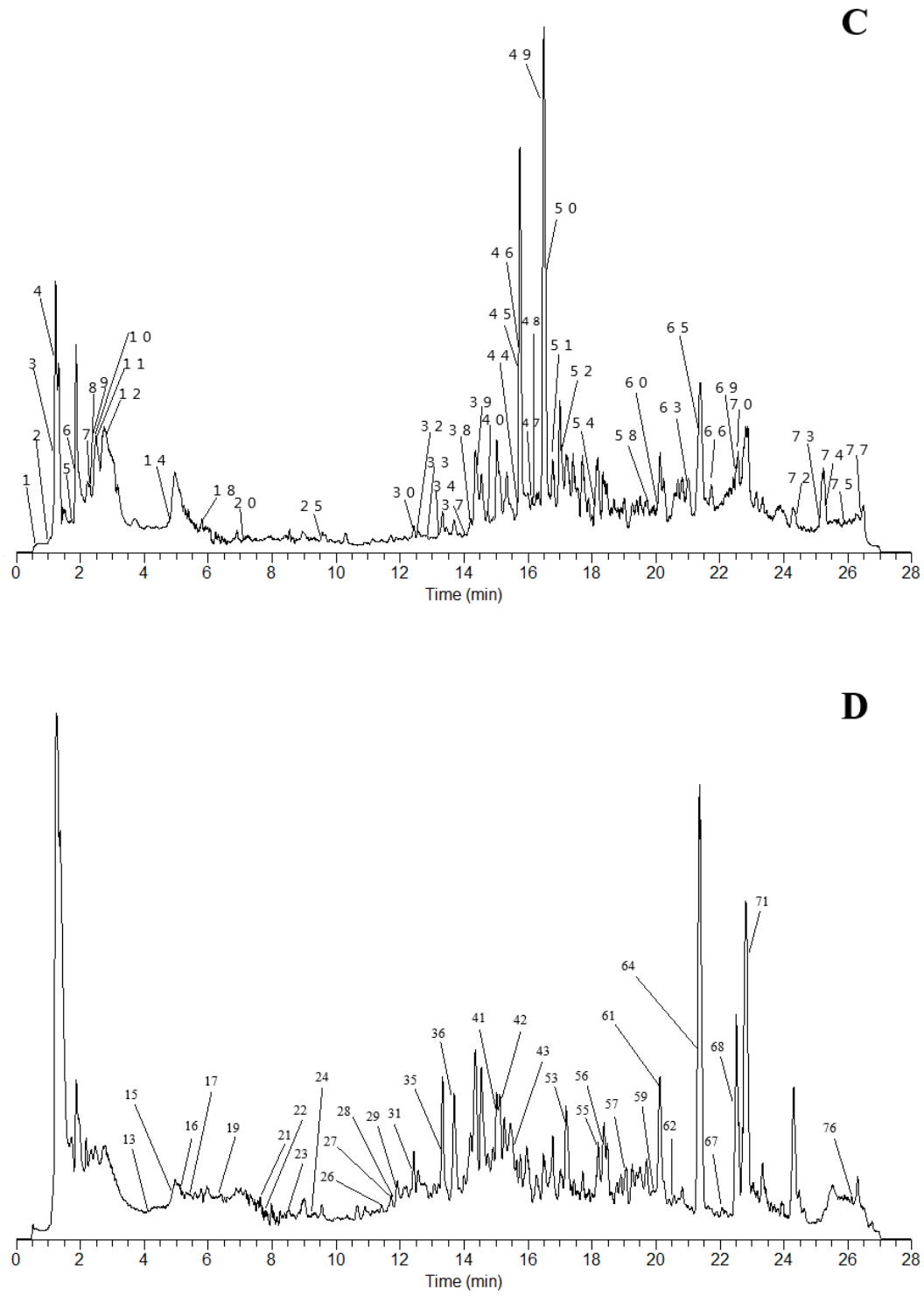

Figure 1. TIC diagram of Gastrodia Tuder Halimasch Powder under positive ion mode (A) and negative I on mode (B) before fermentation and positive ion mode (C) and negative ion mode (D) after fermentation.

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Table 1. UHPLC-Q-Exactive Orbitrap HRMS information of chemical components of Gastrodia Tuder Halimasch Powder before and after fermentation.

Numbering	Class	Compound	Retention Time	Molecular Formula	Mode	Measured Value	Calculate The Value	Error/×10⁻⁶	Fragment Ions	After Fermentation	Before Fermentation
1	Other	Benzaldehyde	0.54	C_7H_6O	[M+H]	107.04893	107.04914	-1.96	79.05432	+	-
2	Amides	Phenacetin	1.03	$C_{10}H_{13}NO_2$	[M+H]	180.10207	180.10191	0.89	138.09169	+	-
3	Alkaloids	Choline	1.29	$C_5H_{13}NO$	[M+H]	104.10693	104.10699	-0.58	60.08082, 58.06518	+	+
4	Nucleosides	Cytidine*	1.38	$C_9H_{13}N_3O_5$	[M+H]	244.09282	244.09279	0.12	112.05049	+	+
5	Nucleosides	Uridine*	1.81	$C_9H_{12}N_2O_6$	[M+H]	245.0768	245.07681	-0.04	113.03452	+	+
6	Organic acids	Nicotinic acid	1.86	$C_6H_5NO_2$	[M+H]	124.03936	124.0393	0.48	80.04947, 96.04435, 78.03384	+	+
7	Amino acids	L-Tyrosine	2.22	$C_9H_{11}NO_3$	[M+H]	182.08092	182.08117	-1.37	165.05461, 136.07568	+	+
8	Nucleosides	Adenosine*	2.34	$C_{10}H_{13}N_5O_4$	[M+H]	268.10406	268.10403	0.11	136.06178, 119.03522	+	+
9	Pyrrolidone derivatives	Piracetam	2.39	$C_6H_{10}N_2O_2$	[M+H]	143.08144	143.0815	-0.42	98.05994, 126.05515, 70.06517	+	+
10	Nucleosides	Guanosine*	2.45	$C_{10}H_{13}N_5O_5$	[M+H]	284.09894	284.09894	0	152.0567	+	+
11	Nucleosides	Guanine*	2.57	$C_5H_5N_5O$	[M+H]	152.05667	152.05669	-0.13	135.03012, 110.03482, 109.05079, 128.04536	+	+
12	Nucleosides	Uracil*	2.8	$C_4H_4N_2O_2$	[M+H]	113.03458	113.03455	0.27	96.00798	+	+
13	Organic acids	Methylsuccinic acid	4.1	$C_5H_8O_4$	[M-H]	131.035	131.03498	0.15	87.0451	+	+
14	Nucleosides	Thymine	4.84	$C_5H_6N_2O_2$	[M+H]	127.05011	127.0502	-0.71	110.03175	+	+
15	Amino acids	D-Phenylalanine	4.98	$C_9H_{11}NO_2$	[M	164.07172	164.0717	0.12	164.07172	+	+

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16	Organic acids	3,4-Dihydroxyphenylacetic acid	5.23	$C_8H_8O_4$	[M-H]-	167.03506	167.03498	0.48	123.04531, 122.03760	+	+
17	Amino acids	3-Hydroxy-3-methylglutaricacid	5.41	$C_6H_{10}O_5$	[M-H]-	161.0455	161.04555	-0.31	57.03456, 59.01381, 99.04536,	+	+
18	Organic acids	Pantothenic acid	5.79	$C_9H_{17}NO_5$	[M+H]+	220.11797	220.11795	0.09	202.10672, 184.09637	+	+
19	Organic acids	3-Hydroxy-3-methylbutanoic acid	6.43	$C_5H_{10}O_3$	[M-H]-	117.05579	117.05572	0.60	71.05026, 115.03962, 99.04516	+	+
20	Pyrrolidone derivatives	Levetiracetam	7.05	$C_8H_{14}N_2O_2$	[M+H]+	171.11284	171.1128	0.23	126.09127, 89.07092, 72.08067	+	+
21	Other	Salicylic acid	7.62	$C_7H_6O_3$	[M-H]-	137.02437	137.02442	-0.36	93.03452	+	+
22	Other	2-Isopropylmalic acid	7.9	$C_7H_{12}O_5$	[M-H]-	175.06126	175.0612	0.34	115.04008, 85.06586, 113.06087	+	+
23	Organic acids	Terephthalic acid	8.58	$C_8H_6O_4$	[M-H]-	165.01938	165.0193	0.48	121.0296	+	+
24	Organic acids	benzoic acid	9.12	$C_7H_6O_2$	[M-H]-	121.02943	121.0295	-0.58	93.03461	+	+
25	Organic acids	5-Hydroxyindole-3-acetic acid	9.63	$C_{10}H_9NO_3$	[M+H]+	192.06543	192.06552	-0.47	146.06000, 147.06816	+	-
26	Flavonoids	Genistin*	11.53	$C_{21}H_{20}O_{10}$	[M-H]-	431.09836	431.09837	-0.02	269.04517	+	+
27	Flavonoids	Hispidulin	11.89	$C_{16}H_{12}O_6$	[M-H]-	299.0563	299.05611	0.64	300.05914, 284.03268, 285.03671	+	-
28	Flavonoids	Kaempferide	11.93	$C_{16}H_{12}O_6$	[M-H]-	299.05658	299.05611	0.64	284.03271, 285.03638	+	+
29	Flavonoids	Naringenin	12.05	$C_{15}H_{12}O_5$	[M-H]-	271.06149	271.0612	1.07	151.00380, 119.05028, 107.01356	+	+
30	Flavonoids	Daidzein*	12.4	$C_{15}H_{10}O_4$	[M+H]+	255.06519	255.06519	0	227.07022, 199.07542, 137.02338	+	+
31	Flavonoids	Fisetin	12.49	$C_{15}H_{10}O_6$	[M	285.04059	285.04046	0.46	135.00888, 256.03601	+	-

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32	Flavonoids	4′,7-Dihydroxyflavanone	12.6	$C_{15}H_{12}O_4$	[M+H]-	257.08096	257.08084	0.47	137.02336, 91.05408, 81.03345	+	+
33	Steroids	Estriol	12.98	$C_{18}H_{24}O_3$	[M+H]-	289.17941	289.17982	-1.42	159.08067	+	-
34	Amino acids	Gabapentin	13.24	$C_9H_{17}NO_2$	[M+H]-	172.13322	172.13321	0.06	154.12212	+	-
35	Flavonoids	Genistein*	13.35	$C_{15}H_{10}O_5$	[M-H]-	269.0455	269.04555	-0.19	241.05162, 240.04305, 225.05518, 213.05608, 197.06015	+	+
36	Organic acids	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	13.66	$C_{18}H_{34}O_5$	[M-H]-	329.23343	329.23335	0.24	171.10269, 139.11282, 127.11284, 125.09731	+	+
37	Terpenes	Armillarinin	14.15	$C_{24}H_{29}O_7Cl$	[M+H]-	465.16751	465.16746	0.11	199.01511	+	-
38	Terpenes	Soyasaponin I	14.32	$C_{48}H_{78}O_{18}$	[M+H]-	943.52606	943.52609	-0.03	441.37283, 423.36234, 599.39441, 797.46826	+	+
39	Other	2-Amino-1,3,4-octadecanetriol	14.47	$C_{18}H_{39}NO_3$	[M+H]-	318.30029	318.30027	0.06	300.28946	+	+
40	Terpenes	Armillarilin	14.81	$C_{24}H_{30}O_7$	[M+H]-	431.20618	431.20643	-0.58	165.05458	+	-
41	Organic acids	Ethyl myristate	14.98	$C_{16}H_{32}O_2$	[M-H]-	255.23286	255.23295	-0.35	255.06647, 69.03460	+	+
42	Terpenes	10β,13α-dihydroxymelleolide	15.17	$C_{23}H_{28}O_8$	[M-H]-	431.17108	431.17114	-0.14	167.0356	+	-
43	Terpenes	4′-methoxy-8-hydroxymelledonal	15.49	$C_{24}H_{30}O_9$	[M-H]-	461.18134	461.18171	-0.8	181.05078	+	-
44	Organic acids	7-(2-aminophenyl)heptanoic acid	15.64	$C_{13}H_{19}NO_2$	[M+H]-	222.14914	222.14886	1.26	106.06501	+	-
45	Flavonoids	ar-Turmerone	15.75	$C_{15}H_{20}O$	[M+H]-	217.15852	217.15869	-0.78	119.14818, 91.05412	+	-
46	Terpenes	Armillarin	15.78	$C_{24}H_{30}O_6$	[M+H]-	415.21155	415.21152	0.07	165.05457	+	+
47	Terpenes	Dehydroeburicoic acid	16.13	$C_{31}H_{48}O_3$	[M	469.36761	469.36762	-0.02	451.35693	+	-

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48	Terpenes	10α,13α-dihydroxyarmillaridin	16.22	$C_{24}H_{29}O_8Cl$	[M+H]+	481.1629	481.16237	1.10	199.01569	+	-
49	Other	Piptamine	16.5	$C_{23}H_{41}N$	[M+H]+	332.33102	332.33118	-0.48	240.26851, 91.05411	+	+
50	Terpenes	Armillaribin	16.66	$C_{24}H_{28}O_5$	[M+H]+	397.20105	397.20095	0.25	232.14107, 215.14299, 187.14807, 185.13257, 171.11671, 165.05460, 131.08546	+	+
51	Terpenes	Armillaricin	16.85	$C_{24}H_{27}O_5Cl$	[M+H]+	431.16205	431.16198	0.16	215.14302, 199.01566, 187.14810, 171.11707	+	-
52	Terpenes	Melleolide	17.18	$C_{23}H_{28}O_6$	[M+H]+	401.19601	401.19587	0.35	233.15363	+	-
53	Terpenes	coriolic acid	17.22	$C_{18}H_{32}O_3$	[M-H]-	295.22781	295.22787	-0.2	277.21704	+	+
54	Terpenes	Armillaridin	18.19	$C_{24}H_{29}O_6Cl$	[M+H]+	449.17239	449.17254	-0.33	233.15359, 199.01558	+	-
55	Terpenes	4′-methoxyarmillasin	18.33	$C_{23}H_{30}O_5$	[M-H]-	385.20212	385.20205	0.18	181.05067	+	-
56	Terpenes	4′-demethoxyarmillaribin	18.48	$C_{23}H_{26}O_5$	[M-H]-	381.17075	381.17075	0	167.03499	+	+
57	Terpenes	Armillaridine	19.05	$C_{24}H_{28}O_6$	[M-H]-	411.18137	411.18131	0.15	165.05545	+	-
58	Amides	Linoleamide	19.86	$C_{18}H_{33}NO$	[M+H]+	280.26379	280.26349	1.07	263.23645, 245.22620	+	+
59	Terpenes	Oleanolic acid	19.96	$C_{30}H_{48}O_3$	[M-H]-	455.35297	455.35307	-0.22	456.35941	+	+
60	Other	D-Sphingosine	20.04	$C_{18}H_{37}NO_2$	[M+H]+	300.28998	300.28971	0.90	282.27841, 283.26282	+	+
61	Terpenes	Ursolic Acid*	20.29	$C_{30}H_{48}O_3$	[M-H]-	455.35263	455.35307	-0.97	456.35699	+	+
62	Terpenes	1-dehydroxyarmilyeverninate	20.59	$C_{24}H_{32}O_5$	[M-H]-	399.21762	399.21769	-0.18	181.05075	+	-
63	Amides	Hexadecanamide	20.94	$C_{16}H_{33}NO$	[M	256.26349	256.26349	0	74.06010, 69.06995, 57.06992, 55.05431	+	+

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64	Organic acids	Linoleic acid	21.37	C18H32O3	[M+H]+	279.23291	279.23295	-0.14	261.22281	+	+
65	Amides	Oleamide	21.43	$C_{18}H_{35}NO$	[M+H]+	282.27917	282.27914	0.11	265.25272	+	+
66	Steroids	Ergosterol*	21.77	$C_{28}H_{44}O$	[M+H]+	397.34583	397.34649	-1.66	379.33572	+	-
67	Terpenes	4′-methoxy-4-dehydroxyarmillasin	22.15	$C_{23}H_{30}O_4$	[M-H]-	369.20715	369.20713	0.05	181.0507	+	-
68	Organic acids	Palmitic acid	22.45	$C_{16}H_{32}O_2$	[M-H]-	255.23296	255.23295	0.04	237.22198	+	-
69	Amides	N-octodecanoylsphinganine	22.51	$C_{36}H_{73}NO_3$	[M+H]+	568.56604	568.56632	-0.49	302.30414, 284.29504	+	+
70	Organic acids	Palmitoleic Acid	22.73	$C_{16}H_{30}O_2$	[M+H]+	255.23184	255.23186	-0.08	237.22128	+	+
71	Organic acids	Oleic acid	22.87	$C_{18}H_{34}O_2$	[M-H]-	281.24857	281.2486	-0.11	264.34039	+	+
72	Organic acids	Dioctyl phthalate	24.44	$C_{24}H_{38}O_4$	[M+H]+	391.28439	391.28429	0.26	149.02319, 71.08543, 167.03371	+	+
73	Amides	Erucamide	25.11	$C_{22}H_{43}NO$	[M+H]+	338.34198	338.34174	0.71	321.31503	+	+
74	Organic acids	Linolenic acid ethyl ester	25.31	$C_{20}H_{34}O_2$	[M+H]+	307.26337	307.26316	0.68	123.11671	+	-
75	Organic acids	Ethyl oleate	25.93	$C_{20}H_{38}O_2$	[M+H]+	311.29498	311.29446	1.67	265.25317, 247.24187, 163.14861,	+	+
76	Terpenes	2′,5-epoxy-4-dehydroxyarmillaridiene	26.28	$C_{24}H_{26}O_5$	[M-H]-	393.17172	393.17075	2.47	181.05064	+	-
77	Alkaloids	Betaine	26.48	$C_5H_{11}NO_2$	[M+H]+	118.08617	118.08626	-0.76	58.06517, 59.07298	+	+

Note: * Compounds identified by comparison of reference.

2.2.1. Identification of terpenoids

According to MS information, terpenoids were the most identified compounds. A total of 21 kinds of terpenoids were detected under positive and negative ion modes. Compounds 46, 50, 51, 52, 53, 54 are taken as examples. Compound 46 (t_R=15.78 min) gives the molecular ion peak m/z 415.21155

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$[M+H]^+$ in the positive ion mode, and its molecular formula is inferred to be $C_{24}H_{30}O_6$ The fragment ion 165.05457 $[M+H-C_{15}H_{21}0_2-OH]^+$ combined with reference identified it as armillarin[28]. Compound 50 (t_R=16.66 min) gives the molecular ion peak m/z 397.20105 $[M+H]^+$ in the positive ion mode, and its molecular formula is inferred to be $C_{24}H_{28}O_5$. Fragment ions 232.14107 $[M+H-C_9H_9O_3]^+$, 215.14299 $[M+H-C_9H_9O_3-OH]^+$, 187.14807 $[M+H-C_9H_9O_3-CO]^+$, 185.13257 $[M+H-C_9H_9O_3-CO-H_2]^+$, 171.11671 $[M+H-C_9H_9O_3-CO-H_2-CH_4]^+$, 165.05460 $[M+H-C_{15}H_{20}O_2]^+$ and 131.08546 $[M+H-CO-C_4H_8]^+$ were identified as armillaribin by reference[29]. Compound 51 (t_R=16.85 min) gives the molecular ion peak m/z 431.16205 $[M+H]^+$ in the positive ion mode, and its molecular formula is inferred to be $C_{24}H_{27}O_5Cl$. Fragment ion 215.14302 $[M+H-C_9H_8Cl0_3-OH]^+$, 199.01566 $[M+H-C_{15}H_{20}O_2]^+$, 187.14810 $[M+H-C_9H_8Cl0_3-CO]^+$ and 171.11707 $[M+H-C_{15}H_{20}O_2-CO]^+$,It was identified as armillaricin by reference[30]. Compound 52 (t_R=17.18 min) gives the molecular ion peak m/z m/z 401.19601 $[M+H]^+$ in the positive ion mode, and its molecular formula is inferred to be $C_{23}H_{28}O_6$, fragment ion 233.15363 $[M+H-C_8H_8O_4]^+$, It was identified as melleolide by reference[29]. Compound 53 (t_R =17.22 min) gives the molecular ion peak m/z 295.22781 in the positive ion mode, and its molecular formula is inferred to be $C_{18}H_{32}O_3$ and fragment ion 277.21704 $[M-H-H_2O]^+$, which is identified as coriolic acid by reference[31]. Compound 54 (t_R=18.19 min) gives a molecular ion peak m/z 449.17240 $[M+H]^+$ in the positive ion mode, and its molecular formula is inferred to be $C_{24}H_{29}O_6Cl$. The fragment ions m/z 233.15359 $[M+H-C_9H_9O_4Cl]^+$ and 199.01558 $[M+H-C_{15}H_{21}0_2-OH]^+$ were identified as armillaridin by reference [28].

2.2.2. Identification of organic acid compounds

According to MS information, a total of 18 organic acid compounds were detected, taking compounds 18, 64 and 68 as examples. Compound 18 (t_R=5.79 min) gives the molecular ion peak m/z 220.11797 in the positive ion mode, and its molecular formula is inferred to be $C_9H_{17}NO_5$, fragment ion 202.10672 $[M+H-H_2O]^+$ and 184.09637 $[M+H-2H_2O]^+$. It was identified as Pantothenic acid by reference[32]. Compound 64 (t_R=21.37min) was given a molecular ion peak m/z 279.23291 in the negative ion mode, and its molecular formula was inferred to be $C_{18}H_{32}O_3$ and fragment ion 261.22281 $[M-H-H_2O]^-$, which was identified as linoleic acid by reference[33]. Compound 68 (t_R=22.45min) gives a molecular ion peak m/z 255.23296 in the negative ion mode, and its molecular formula is inferred to be $C_{16}H_{32}O_2$ and fragment ion 237.22198 $[M-H-H_2O]^-$, which is identified as palmitic acid by reference[34].

2.2.3. Identification of flavonoids

According to MS information, a total of 9 flavonoids were detected, taking compounds 26, 30 and 35 as examples. Compound 26 (t_R=11.53min) was given a molecular ion peak m/z 431.09836 in the negative ion mode, and its molecular formula was inferred to be $C_{21}H_{20}O_{10}$ and fragment ion 269.04517 $[M-H-C_6O_5H_{10}]^-$, which was identified as genistin by comparison with reference materials[35]. Compound 30 (t_R=12.4 min) gives a molecular ion peak m/z 255.0652 in the positive ion mode, and its molecular formula is inferred to be $C_{15}H_{10}O_4$. Fragment ions227.07022$[M+H-CO]^+$, 199.07542 $[M+H-2CO]^+$ and 137.02338 $[M+H-H2O-C_8H_4]^+$ were identified as daidzein by comparison with reference [36]. Compound 35 (t_R=13.35min) gives a molecular ion peak m/z 269.04547 in the negative ion mode, and its molecular formula is inferred to be $C_{15}H_{10}O_5$. Fragment ion 241.05162 $[M-H-CO]^-$, 240.04305 $[M-H-CHO]^-$, 225.05518 $[M-H-CO_2]^-$, 213.05608 $[M-H-2CO]^-$ and 197.06015$[M-H-CO_2-CO]^-$, According to the literature[37] and the comparison of control products, it was identified as genistein.

2.2.4. Identification of nucleoside compounds and amide compounds

According to MS information, 7 nucleoside compounds and 6 amide compounds were detected. Compounds 8, 12, 58, 65 and 73 were taken as examples. Compound 8 (t_R =2.34 min) gives a molecular ion peak m/z 268.10406 in the positive ion mode, and its molecular formula is inferred to be $C_{10}H_{13}N_5O_4$, fragment ion 136.06178 $[M+H-C_5H_9O_4]^+$ and 119.03522 $[M+H-C_5H_9O_4-NH_3]^+$. It was

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identified as adenosine by comparison with reference[38]. Compound 12 (t_R=2.8min) was given a molecular ion peak m/z 113.03458 in the positive ion mode, and its molecular formula was inferred to be $C_4H_4N_2O_2$ and fragment ion 96.00798 $[M+H-NH_3]^+$, which was identified as uracil by comparison with reference materials[39]. Compound 58 (t_R=19.86min) gives a molecular ion peak m/z 280.2638 in the positive ion mode, and its molecular formula is inferred to be $C_{18}H_{33}NO$, fragment ions 263.23645 $[M+H-NH_3]^+$ and 245.22620 $[M+H-NH_3-H_2O]^+$. According to literature[34],it was identified as linoleamide. Compound 65 (t_R =21.43min) gives a molecular ion peak m/z 282.2792 in the positive ion mode, and its molecular formula is inferred to be $C_{18}H_{35}NO$ and fragment ion 265.25272 $[M+H-NH_3]^+$. According to the literature[40], it was identified as oleic acid amide. Compound 73 (t_R =25.11min) gives a molecular ion peak m/z 338.342 in the positive ion mode, and its molecular formula is inferred to be $C_{22}H_{43}NO$ and fragment ion 321.31503 $[M+H-NH_3]^+$. Combined with literature[40], it is identified as erucic amide.

2.2.5. Identification of amino acid compounds and pyrrolidone derivatives

According to MS information, a total of 4 amino acid compounds and 2 pyrrolidone derivatives were detected, taking compounds 7, 9 and 20 as examples. Compound 7 (t_R = 2.22min) gives a molecular ion peak m/z 182.08092 in the positive ion mode, and its molecular formula is inferred to be $C_9H_{11}NO_3$, fragment ion 165.05461$[M+H-NH_3]^+$ and 136.07568 $[M+H-COOH_2]^+$. It was identified as L-tyrosine[41]. Compound 9 (t_R=2.39min) gives the molecular ion peak m/z 143.08144 in the positive ion mode, and its molecular formula is inferred to be $C_6H_{10}N_2O_2$. The fragment ions 126.05515 $[M+H-NH_3]^+$, 98.05994 $[M+H-NH_3-CO]^+$ and 70.06517 $[M+H-NH3-2CO]^+$, combined with references[42], were identified as Piracetam. Compound 20 (t_R=7.05min) gave a molecular ion peak m/z 171.11284 in the positive ion mode, and its molecular formula was inferred to be $C_8H_{14}N_2O_2$ and fragment ion 126.09127 $[M+H-NH_3-CO]^+$. Combined with literature[42], it was identified as Levetiracetam.

2.2.6. Identification of steroids and alkaloids

According to MS information, a total of 2 sterols and 2 alkaloids were detected, taking compounds 66 and 77 as examples. Compound 66 (t_R = 21.77 min) was given a molecular ion peak m/z397.34583 in the positive ion mode, and its molecular formula was inferred to be $C_{28}H_{44}O$ and fragment ion 379.33572 $[M+H-CH_2COOH]$. Combined with the reference[43] and the comparison, it was identified as ergosterol. Compound 77 (t_R=26.48min) was given a molecular ion peak m/z118.08617 in the positive ion mode, and its molecular formula was inferred to be $C_5H_{11}NO_2$, fragment ions 59.07298 $[M+H-CH_2COOH]$ and 58.06517 $[M+H-CH_3COOH]$. It was identified as betaine[44].

2.2.7. Other classes

According to MS information, a total of 6 other classes were detected, including 2 sphingolipids, 1 aromatic aldehyde, phenolic, aromatic amine and acid compounds, taking compound 21 as an example. Compound 21 (t_R =7.62min) gave a molecular ion peak m/z 137.02437 in the negative ion mode, and its molecular formula was inferred to be $C_7H_6O_3$ and fragment ion 93.03452 $[M-H-CO_2]$. Combined with literature[45], it was identified as salicylic acid.

2.2.8. Orthogonal Partial least squares-Discriminant Analysis (OPLS-DA)

SIMCA 14.1 software was used, based on PCA, and OPLS-DA modeling analysis was shown in Figure 2. The VIP value diagram was used to determine the main difference components of the powder before and after fermentation. The greater the VIP value, the greater the weight value and the stronger the ability to distinguish samples, as shown in Figure 3. Results The R^2X (cum) = 0.947, Q^2 (cum) = 0.998 of the OPLS-DA models of the pre-fermentation and post-fermentation Gastrodia Tuder Halimasch Powder. It is proved that the OPLS-DA model is good and can explain the component difference generally. Projecting importance as a variable(VIP) value >1 shows the difference components: armillarin, Genistein, Linoleic acid, armillaridin, 4′,7-Dihydroxyflavanone, 10α,13α-dihydroxyarmillaridin, adenosine, 4′-methoxyarmillasin, 7-(2-aminophenyl)heptanoic acid,

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daidzein, 2′,5-epoxy-4-dehydroxyarmillaridiene, choline, uracil, 3-Hydroxy-3-methylbutanoic acid, phenacetin, ergosterol, 2-Amino-1,3,4-octadecanetriol, guanine, L-Tyrosine, soyasaponin I, armillaricin, genistin, palmitoleic Acid, nicotinic acid, melleolide, Guanosine, armillaridine, armillaribin，however, due to the limitation of chromatographic conditions, a multi-component determination method was established for the different components of Gastrodia Tuder Halimasch Powder before and after fermentation.

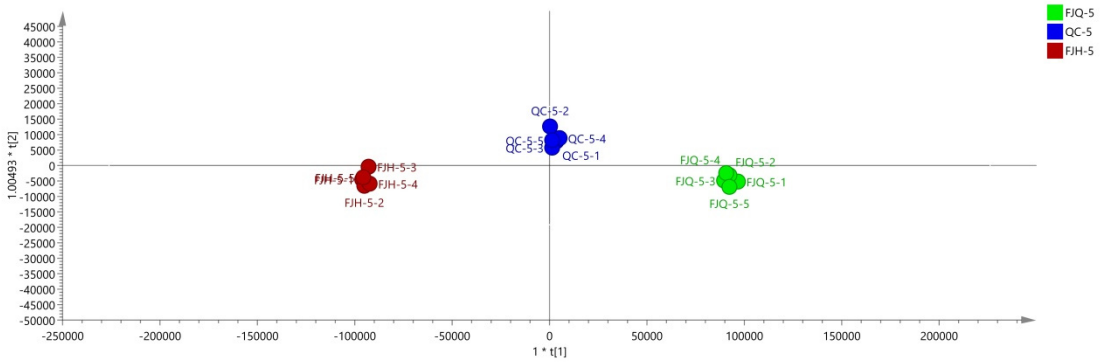

Figure 2. OPLS-DA analysis of Gastrodia Tuder Halimasch Powder (FJY-5) before fermentation and Gastrodia Tuder Halimasch Powder (FJH-5) after fermentation. Note: YL-5-1~5 and FJH-5-1~5 represent 5 parallel experiments.

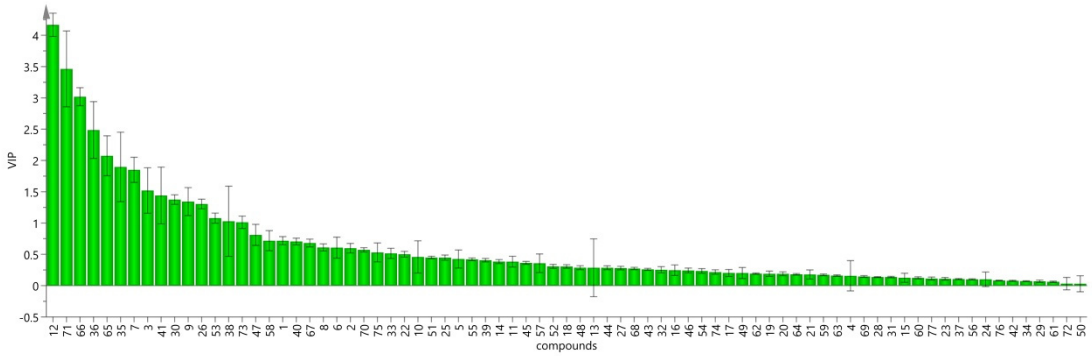

Figure 3. VIP value of Gastrodia Tuder Halimasch Powder before and after fermentation.

2.3. Establishment of a method for the determination of multi-component content of Gastrodia Tuder Halimasch Powder before and after fermentation

2.3.1. Chromatographic conditions

Shimadzu LC-20AD high performance liquid chromatogram (made in Shimadzu, Kyoto, Japan) was performed on Waters Symmetry C18 column (4.6 mm×250 mm, 5 μm) with detection wavelength of 270 nm and flow rate of 1.0 mL•min-1. The column temperature was 30 ℃. Gradient elution of methanol (A) -0.1% acetic acid aqueous solution (B) (0~5 min, 40% A; 5~15 min, 40~60% A; 15~30 min, 60~70% A; 30~35 min, 70%~100% A; 35-45 min, 100% A), the sample size was 5 μL. Figure 4.

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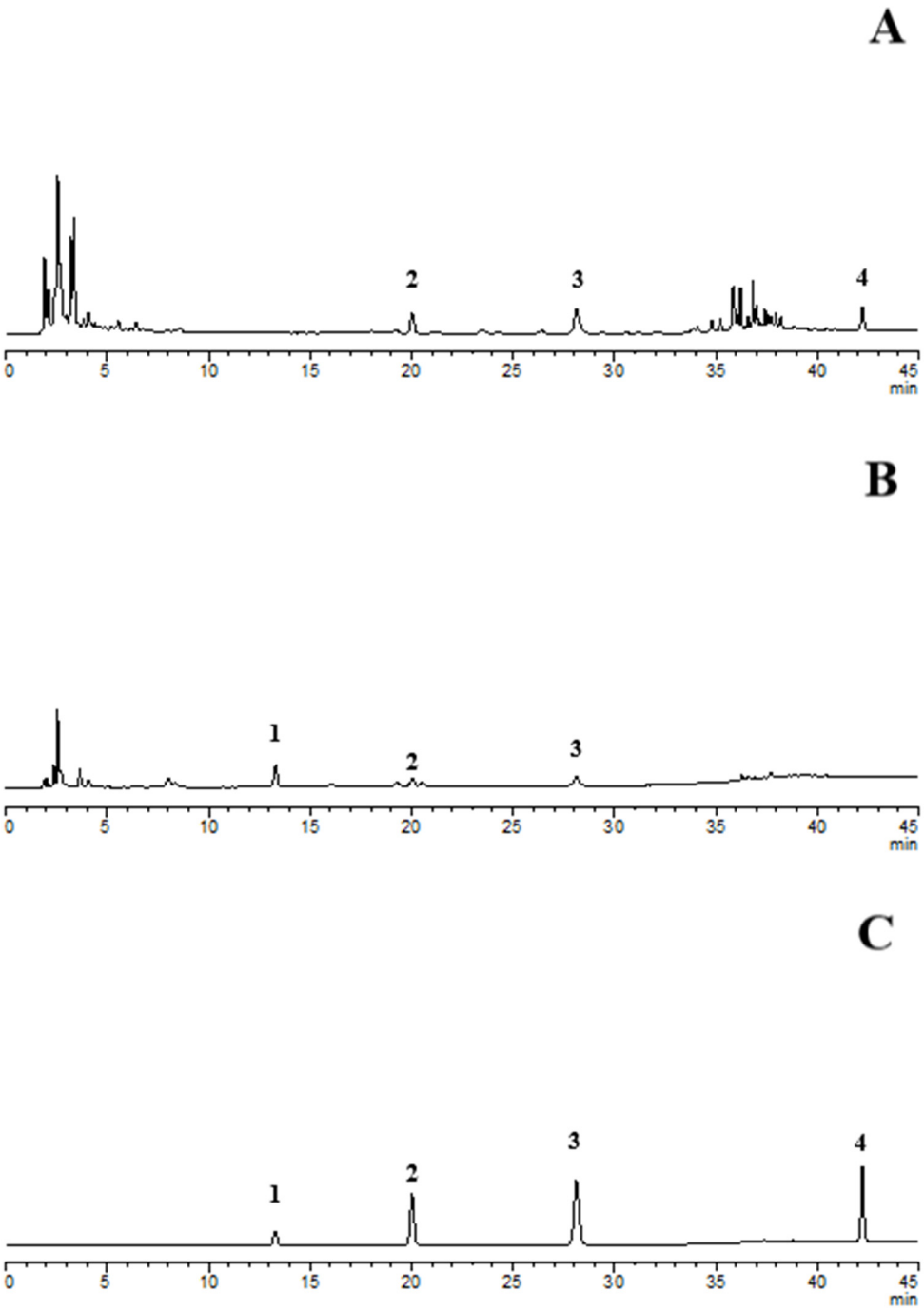

Figure 4. A. Gastrodia Tuder Halimasch Powder after fermentation, B. Gastrodia Tuder Halimasch Powder before fermentation, C-4 mixed label. Note: 1-genistin, 2-daidzein, 3-genistein, 4-ergosterol.

2.3.2. Preparation of reference solution

Take appropriate amount of genistin, daidzein, genistein and ergosterol reference products, weigh them accurately, add methanol to dissolve them, prepare the reference product mother liquor with mass concentrations of 0.1550, 0.6167, 0.4360 and 0.1200 mg•mL-1, and precisely absorb the appropriate amount of each reference product mother liquor. Mixed reference solution with methanol with mass concentrations of 5.3952, 17.9904, 19.3446 and 47.0368 ug•mL^{-1} was filtered by 0.22μm microporous filter membrane and then prepared for use.

2.3.3. Preparation of test product solution

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Take 0.5 g of Gastrodia Tuder Halimasch Powder before and after fermentation, add 5 mL methanol solution, ultrasonic for 1 h, take out, cool to room temperature, make up for weight loss, filter paper, filter through 0.22 μm microporous filter membrane, and then obtain.

2.3.4. Investigation of linear relationship

The mixture of reference solution 0.4, 0.5, 1, 3, 5, 7 μL under item 2.6.2 was precisely absorbed and determined according to the chromatographic conditions under item 2.6.1. The standard curve was drawn with the injection volume as the horizontal coordinate (X) and the peak area as the vertical coordinate (Y), as shown in Table 2.

Table 2. Regression equations, correlation coefficients and linear ranges of the four components.

Compound	Linear Equation	R^2	Linear Range/μg·mL^{-1}
Genistin	Y=3682.9592X-745.8583	0.9999	2.1581~37.7667
Daidzein	Y=3519.0934X-1460.4269	0.9999	7.1962~125.9328
Genistein	Y=5724.1012X-3375.2034	0.9999	7.7379~135.4125
Ergosterol	Y=1504.3237X-1830.0128	0.9999	18.8147~329.2574

2.3.5. Precision test

The same batch of test product solution was taken before and after fermentation, and the relative standard deviation (RSD) of genistin, dzeidin, genistein and ergosterol were calculated as 1.49%, 1.65%, 0.85% and 0.64%, respectively, according to the chromatographic conditions of 3.9.1. The accuracy of the instrument was good.

2.3.6. Stability test

The same batch of test solution was taken before and after fermentation, and the samples were injected at 0, 2, 4, 8, 12 and 24 h after preparation according to chromatographic conditions under item 3.9.1. The RSD of genistin, dzein, genistein and ergosterol peak area were 1.56%, 1.63%, 0.49% and 0.67%, respectively. The results showed that the test solution had good stability within 24 h.

2.3.7. Repeatability test

The same batch of Gastrodia Tuder Halimasch Powder was taken before and after fermentation, and 6 sample solutions were prepared in parallel according to the method in item 3.9.3. The average mass fraction of genistin, dzeidin, genistein and ergosterol was calculated as 0.04, 0.14, 0.16 and 0.43 mg•g-1, respectively, according to the chromatographic conditions in item 3.9.1. RSDS were 1.06%, 1.18%, 1.03% and 2.13%, respectively, indicating good reproducibility of the method.

3.3.8. Sample addition recovery test

The test Gastrodia Tuder Halimasch Powder with known component content before and after fermentation was finely weighed to about 0.25g, 6 parallel parts were added to the control product with the same content as the sample, and the test product solution was prepared according to the method under 3.9.2, and determined according to the chromatographic conditions under 3.9.1. The average recoveries of genistin, daidzein, genistein and ergosterol were 97.50%, 102.97%, 98.65% and 102.87%, and RSD were 0.39%, 0.97%, 0.51% and 0.85%, respectively. See Table 3.

Table 3. Recovery table of 4 components in Gastrodia Tuder Halimasch Powder samples before and after fermentation.

Compound	Weighing sample（g）	Sample content (mg/g)	Added content (mg/g)	Real measurement (mg/g)	Recovery（%）	Average recovery（%）	RSD value（%）
	0.2497	0.0084	0.0083	0.0165	0.9741	97.50%	0.39%

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Genistin	0.2503	0.0084	0.0083	0.0165	0.9700	102.97%	0.97%
	0.2501	0.0084	0.0083	0.0165	0.9753		
	0.2498	0.0084	0.0083	0.0165	0.9766		
	0.2498	0.0084	0.0083	0.0166	0.9892		
	0.2502	0.0084	0.0083	0.0164	0.9649		
Daidzein	0.2501	0.0341	0.0343	0.0692	1.0239		
	0.2503	0.0342	0.0343	0.0685	1.0008		
	0.2502	0.0341	0.0343	0.0696	1.0352		
	0.2501	0.0341	0.0343	0.0690	1.0164		
	0.2498	0.0341	0.0343	0.0701	1.0491		
	0.2501	0.0341	0.0343	0.0702	1.0527		
Genistein	0.2502	0.0387	0.0389	0.0766	0.9738	98.65%	0.51%
	0.2501	0.0387	0.0389	0.0773	0.9915		
	0.2502	0.0387	0.0389	0.0767	0.9747		
	0.2503	0.0388	0.0389	0.0772	0.9874		
	0.2502	0.0387	0.0389	0.0773	0.9919		
	0.2499	0.0387	0.0389	0.0776	0.9997		
Ergosterol	0.2503	0.1081	0.1081	0.2203	1.0384	102.87%	0.85%
	0.2501	0.1080	0.1081	0.2192	1.0286		
	0.2502	0.1080	0.1081	0.2157	0.9962		
	0.2501	0.1080	0.1081	0.2199	1.0348		
	0.2502	0.1080	0.1081	0.2210	1.0451		
	0.2503	0.1081	0.1081	0.2193	1.0290		

2.4. Sample Determination

Prepare Gastrodia Tuder Halimasch Powder before and after fermentation in parallel, 3 parts per batch, prepare the test solution according to the method in item 3.9.3, and determine according to the chromatographic conditions in item 3.9.1, as shown in Table 4. SPSS 26.0 software was used to average the contents of 4 components in each parallel sample, and then PCA was applied to each batch of data. The results showed that the Gastrodia Tuder Halimasch Powder before and after fermentation was divided into 3 groups, the first group before fermentation, the second group after fermentation, and the third group after fermentation, the eighth to the 10th group. SPSS 26.0 software was used to perform LSD variance analysis on the data, and Origin 8.0 was used to draw the histogram of significant difference markers. See Figure 5. The results showed that the contents of daidzein, genistein and ergosterol (not detected before fermentation) increased significantly after fermentation, and the contents of genistin were not detected after fermentation, which may be due to the possible transformation of chemical components during fermentation.

Table 4. Contents of 4 components in Gastrodia Tuder Halimasch Powder samples before and after fermentation.

Sample	Genistin(mg/g)	Daidzein(mg/g)	Genistein(mg/g)	Ergosterol(mg/g)
Prefermentation sample	0.0336	0.0184	0.0188	/
After fermentation sample 1	/	0.1391	0.1617	0.4653
After fermentation sample 2	/	0.1364	0.1549	0.4318
After fermentation sample 3	/	0.1390	0.1576	0.4371
After fermentation sample 4	/	0.1116	0.1321	0.4392
After fermentation sample 5	/	0.1092	0.1348	0.4359
After fermentation sample 6	/	0.1487	0.1547	0.4368
After fermentation sample 7	/	0.1498	0.1259	0.4472
After fermentation sample 8	/	0.1938	0.1915	0.4248
After fermentation sample 9	/	0.1820	0.1803	0.4342

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After fermentation sample 10	/	0.2127	0.1942	0.4258

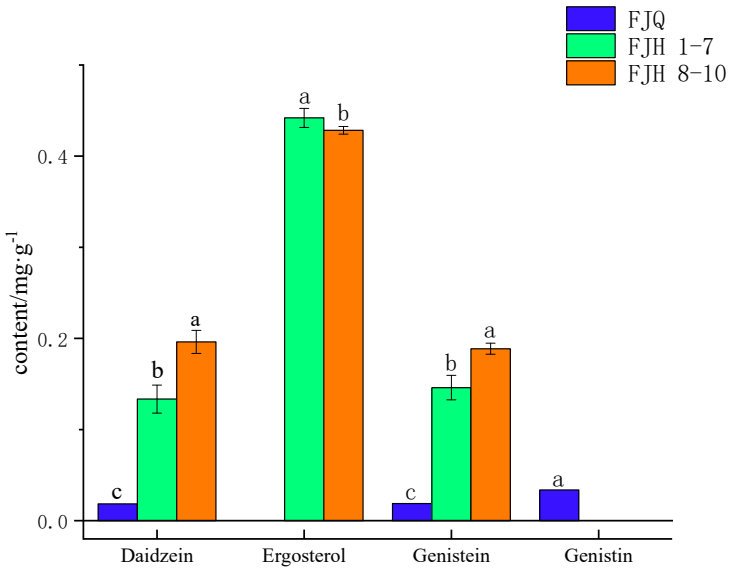

Figure 5. Difference of Gastrodia Tuder Halimasch Powder before and after fermentation.

3. Concluding Remarks

According to the analysis of components of Gastrodia Tuder Halimasch Powder before and after fermentation, due to the large number of chemical components, different compounds have different responses under different modes, so the positive and negative ion modes were respectively scanned, and combined with relevant data such as reference products, PubChem database and references, a total of 77 compounds were speculated and identified. It contains 21 terpenoids, 18 organic acids, 9 flavonoids, 7 nucleosides, 6 amides, 4 amino acids, 2 pyrrolidone derivatives, 2 sterols, 2 alkaloids and 6 other compounds. Combined with VIP>1, the main chemical components of Gastrodia Tuder Halimasch Powder before and after fermentation were further quantitatively analyzed, including genistin, daidzein, genistein and ergosterol. By PCA analysis, the Gastrodia Tuder Halimasch Powder before fermentation, 10 batches of different batches and the Gastrodia Tuder Halimasch Powder after fermentation by the manufacturer were divided into 3 groups, the raw materials were group 1, and the samples 1-7 after fermentation were group 2. After fermentation, samples 8~10 were the third group. Combined with SPSS 26.0 software and LSD analysis of variance, daidzein and genistein were significantly higher than those before fermentation, with daidzein content less than 0.02 mg•g^{-1} before fermentation and higher than 0.10 mg•g^{-1} after fermentation. The content of genistein was less than 0.02 mg•g^{-1} before fermentation and higher than 0.13 mg•g^{-1} after fermentation. After fermentation, no genistin was detected in Gastrodia Tuder Halimasch Powder, which may be converted to other compounds. Ergosterol was not detected in the Gastrodia Tuder Halimasch Powder before fermentation, which may be due to the formation of a new compound after fermentation. The ergosterol content was higher than 0.42 mg•g^{-1} after fermentation. This is of great significance for the clinical use of Gastrodia Tuder Halimasch Powder, and this study provides a theoretical basis for the subsequent research on the material basis of Gastrodia Tuder Halimasch Powder. Therefore, ergosterol, genistein and daidzein may be the active ingredients of Gastrodia Tuder Halimasch Powder.after fermentation, which can be used as quality control indicators for Gastrodia Tuder Halimasch Powder. after fermentation in the future, and provide reference for the quality control of Gastrodia Tuder Halimasch Powder. and the basic research of pharmacodynamic substances.

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4. Materials and Methods

4.1. Drugs and reagents

Before fermentation, all the Gastrodia Tuder Halimasch Powder were provided by Luoyang Wokang Pharmaceutical Co., LTD. After fermentation, there were 10 batches of Gastrodia Tuder Halimasch Powder; Luoyang Wokang Pharmaceutical Co., LTD. 7 batches, batch numbers are 220101, 220102, 220103, 220201, 220202, 220203, 220701. Jiangsu Shenhua Pharmaceutical Co., Ltd. 3 batches, batch numbers are 211108, 211109, 211110.

Genistin, genistein, uridine, adenosine reference products (China Institute for Identification of Pharmaceutical and Biological Products, batch number: 111709-200501, 111704-200501, 887-200202, 110879-200202, purity ≥98%);Ergosterol, ursolic acid, guanosine, uracil (Chengdu Pusi Biotechnology Co., LTD., batch number PS000668, PS000730, PS012671, PS010291, PS020117, purity ≥98%); Daidzein and cytidine reference products (Shanghai Yuanye Biotechnology Co., LTD., batch number B20227, B20073, purity ≥98%).

BSA224S-CW type 1/10,000 balance and BT25S type 1/100,000 balance (Sartorius Technology Instrument Co., LTD.); UPT-II-10T ultrapure water device (Chengdu Ultrapure Technology Co., LTD.); KQ-500DV type ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., LTD.), water is Watsons drinking water and laboratory self-made ultra-pure water, acetonitrile, formic acid are mass spectrometry pure, methanol, acetic acid are chromatographic pure, the rest of the reagents are analytical pure.

4.2. Preparation of Gandouling tablets and standard solutions

Precision weigh 0.5g Gastrodia Tuder Halimasch Powder into 100 mL volumetric bottle, add 10 mL 50% methanol, ultrasonic extraction for 1 h, cool, make up for weight loss, filter, and pass 0.22μm microporous filter membrane, take 1ml filtrate into 2ml centrifugal tube, put it into high-speed refrigerated centrifuge, then add 500ul methanol to redissolve. Then put it into a high-speed centrifuge at 12000 r•min^{-1} for 10 min, and take the supernatant to obtain the test product solution.

Accurately weigh the appropriate amount of each reference product, add 50% methanol to prepare the reference product reserve solution with a mass concentration of 1 mg•mL^{-1}, accurately absorb each reference product reserve solution, mix and dilute it into a mixed reference solution with a mass concentration of about 10 μg•mL^{-1} for HRMS analysis.

4.3. UHPLC conditions

Using the Ultimate 3000-Orbitrap Exploris 240 LMS (Thermo Fisher Scientific, USA), Hypersil GOLD column (100×2.1mm, 1.9μm), The flow rate was 0.3 mL•min-1, the column temperature was 35 ℃, and the mobile phase was gradient elution with acetonitrile (A) -0.1% formic acid aqueous solution (B) (0~1 min, 2% A; 1~8 min, 2~20% A; 8~14 min, 20~70% A; 14-22 min, 70-95% A; 22-24 min, 95% A; 24~24.5 min, 95%~2% A; 24.5~28 min, 2% A;) The sample size was 2 μL.

4.4. Mass spectrometry conditions

The scanning range of positive and negative ion detection modes is m/z 100~1200. The positive and negative ion spray voltages are 3.5kV and -3.0kV respectively. The sheath gas flow rate is 25 arb, the auxiliary gas flow rate is 10 arb, and the auxiliary temperature is 350 ℃. Ion transfer tube temperature 350 ℃.

4.5. Data processing

Compounds related to Gastrodia Tuder Halimasch Powder were collected by searching PubMed, TCMSP, CNKI and other databases, and the main chemical components were identified according to the chromatographic peak retention time (t_R), secondary fragment ions and reference materials, combined with relevant literature reports.

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Sample Availability: Samples of the compounds are available from the authors.

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