Research Article

Identification of Secondary Metabolites in *Flammulina velutipes* by UPLC-Q-Exactive-Orbitrap MS

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*Flammulina velutipes* is the fourth largest edible fungus in China with high nutritional value. In this paper, ultrahigh-performance liquid chromatography tandem hybrid quadrupole-Orbitrap mass spectrometry (UPLC-Q-Exactive-Orbitrap MS) was used to identify the secondary metabolites of *F. velutipes*. The metabolites were identified by comparing the retention time, accurate molecular weight, and MS² data with standard databases of mzVault and mzCloud (compound: 17,000+) and BGI high-resolution accurate mass plant metabolome database (plant metabolite: 2500+). Finally, 26 secondary metabolites were preliminarily identified, including flavonoids, phenylpropanoids, organic acids, and steroids.

1. Introduction

*Flammulina velutipes* is also known as golden needle mushroom and winter mushroom with high nutritional value and medicinal value. According to “Analysis of the National Statistical Survey Results of Edible Fungi in 2019,” *F. velutipes* is the fourth largest edible fungus in China with an output of 2.589,600 tons in 2019. *F. velutipes* contains a variety of nutrients, including proteins, carbohydrates, mineral elements, vitamins, and crude fibers [1]. *F. velutipes* contains eighteen amino acids, including eight essential amino acids, of which lysine content is 1.09%. It has been proved that lysine and its derivatives can promote children’s growth and development and enhance memory. Therefore, *F. velutipes* is also known as “Zengzhi mushroom” [2, 3]. It can not only be used as functional food but also has great potential in the development of medical and health products [4]. *F. velutipes* contains many active components, including polysaccharides, proteins, terpenoids, phenolic acids, and flavonoids [4–10]. Ishikawa et al. isolated and identified sesquiterpenoids enokipodins A-D with the cyathane skeleton from *F. velutipes* [7, 8]. Five flavonoids were isolated and identified from *F. velutipes* by Hu et al. [10], named epicatechin, phyllarin, apigenin, kaempferol, and formononetin. *F. velutipes* has many pharmacological effects, such as antitumor [4], regulating immunity [4, 11], improving memory [5], antibacterial [8], antioxidation [12, 13], protecting the kidney [12], protecting the liver [14], neuroprotection [15], regulating intestinal flora [16], and improving constipation [17].

Ultrahigh-performance liquid chromatography tandem hybrid quadrupole-Orbitrap mass spectrometry (UPLC-Q-Exactive-Orbitrap MS) is a new type of liquid chromatography-mass spectrometry developed in recent years; it is also one of the techniques commonly used in metabolomics with the characteristics of high resolution, good quality and precision, and strong qualitative and quantitative abilities. It is used for the qualitative analysis of Chinese medicinal materials and can realize the rapid identification of various components [18]. At present,
there are few systematic studies on the secondary metabolites of *F. velutipes*. Therefore, in this paper, the secondary metabolites of *F. velutipes* were investigated to provide a reference for research on the chemical composition of *F. velutipes*.

### 2. Materials and Methods

#### 2.1. Materials

Fruiting bodies of *F. velutipes* were obtained from Henan Longfeng Industrial Co., Ltd. The specimens (no. 2020-09-09) were saved at the National Research and Development Center of Edible Fungi Processing Technology, Henan University.

#### 2.2. Reagent

d$_3$-Leucine, $^{13}$C$_5$-phenylalanine, d$_3$-tryptophan, and $^{13}$C$_3$-progesterone were used as the internal standard. Both methanol (A454-4) and acetonitrile (A996-4) were of mass spectral grade, which were purchased from Thermo Fisher Scientific, USA. Ammonium formate (17843-250G) was obtained from Honeywell Fluka, USA. Formic acid (50144-50 mL) was obtained from DIMKA, USA.

#### 2.3. Preparation of the Sample

Dried fruiting bodies of *F. velutipes* were crushed by using the grinding machine. 200 g of *F. velutipes* powder was immersed with 50% ethanol (2000 mL) for 2 times at room temperature, each time for 3 days. The filtrate was lyophilized to obtain 87.2 g extract. The filtrate was lyophilized to obtain 87.2 g extract. Then the sample was managed according to Yang et al. [19].

#### 2.4. Chromatographic Conditions

The type of column was C18 Hypersil GOLD aQ (100 mm $\times$ 2.4 μm). The mobile phases were 0.1% formic acid-water (liquid A) and 0.1% formic acid-acetonitrile (liquid B) with the elution gradient of 0–2 min 5% B; 2–22 min 5%–95% B; 22–27 min 95% B; 27.1–30 min 5% B. 0.3 mL/min, 40°C, and 5 μL were used as the flow rate, column temperature, and injection volume, respectively.

#### 2.5. Mass Spectrometry Conditions

Ultraprecision liquid chromatography (Waters 2D UPLC, USA) tandem Q-Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, USA) was used to separate and detect the metabolites. The mass spectrometry parameters were set according to Yang et al. [19]. In brief, 150–1500 and 70,000 were used as the mass range and MS resolution, respectively. 35,000 was used as MS$^2$ resolution. The fragmentation energy was 20, 40, and 60 eV. Sheath gas flow rate and aux gas flow rate were 40 and 10, respectively. Spray voltage (KV) of the positive ion mode and negative ion mode was 3.80 and 3.20, respectively. Ion capillary temperature and aux gas heater temperature were 320°C and 350°C, respectively.

#### 2.6. Data Analysis

BGI high-resolution accurate mass plant metabolome database (plant metabolite: 2500$^+$), mzCloud database (compound: 17000$^+$), and mzVault database were used to identify the metabolites.

### 3. Results

#### 3.1. Total Ion Chromatogram

The total ion current chromatogram of *F. velutipes* is shown in Figure 1.

#### 3.2. Results of Metabolites’ Identification

The metabolites of *F. velutipes* were analyzed by UPLC-Q-Exactive-Orbitrap MS, the structural identification of compounds in *F. velutipes* was based on the retention time, MS data, and MS$^2$ data compared with the BGI high-resolution accurate mass plant metabolome database (plant metabolite: 2500$^+$), mzCloud database (compound: 17000$^+$), and mzVault database. The identified metabolites were classified into three grades (level 1, level 2, and level 3) according to the comparison results. The credibility sequence is as follows: level 1 > level 2 > level 3. The detailed results are shown in Table 1. 26 compounds were preliminarily identified in *F. velutipes*, including 3 phenylpropanoids, 7 flavonoids, 1 steroid, and 15 organic acids.

#### 3.2.1. Structural Analysis of Flavonoids

Flavonoids are easily deprotonated in the negative ion mode to produce ion [M – H]$^–$. Some flavonoids are protonated in the positive ion mode to produce ion [M + H]$^+$.

Methylated flavonoids are prone to losing methyl to obtain ion [M – H–CH$_3$]$^–$ or [M + H–CH$_3$]$^+$.

Flavone O-glycoside mainly lost the sugar group by glycosidic bond fracturing. The parent nucleus of flavonoids is prone to RDA cracking and lost the CO group [20, 21].

Compound 1 had a weak quasi-molecular ion in the positive ion mode, the ion of [M + H]$^+$ was m/z 593, and then it continuously lost rhamnosyl and glucosyl groups to obtain two fragment ions m/z 447 [M + H-rhamnosyl]$^+$ and m/z 285 [M + H-rhamnosyl-glucosyl]$^+$. The ion [M + H-rhamnosyl-glucosyl]$^−$ further lost methyl to obtain m/z 270 and then further lost the CO group to produce m/z 242 [M + H-rhamnosyl-glucosyl-CH$_3$-CO]$^−$. It was speculated that compound 1 was linarin. Possible MS fragmentation pathway of linarin is shown in Figure 2. The cracking process of compound 3 is similar to that of compound 1 with fragment ions of m/z 447 [M + H]$^+$, m/z 285 [M + H-glucosyl]$^−$, m/z 270 [M + H-glucosyl-CH$_3$]$^−$, and m/z 242 [M + H-glucosyl-CH$_3$-CO]$^−$. It was speculated that compound 3 may be glycitin.

Compound 2 was deprotonated in the negative ion mode to produce ion m/z 285 [M – H]$^–$ and then underwent RDA cracking to obtain two fragment ions m/z 133 [M – H–C$_7$H$_4$O$_4$]$^−$ and m/z 151 [M – H–C$_8$H$_9$O$_2$]$^−$. It was speculated that compound 2 may be luteolin. Possible MS fragmentation pathway of luteolin is shown in Figure 3. The cracking process of compound 4 is similar to that of compound 2 with fragment ions of m/z 269 [M – H]$^–$, m/z 117 [M – H–C$_7$H$_4$O$_4$]$^−$, and m/z 151 [M – H–C$_8$H$_9$O$_2$]$^−$. It was speculated that compound 4 may be apigenin.

Compound 5 was protonated in the positive ion mode to produce the ion m/z 301 [M + H]$^+$, then lost methyl to obtain m/z 286, and further lost the CO group to obtain m/z 258 [M + H–CH$_3$-CO]$^+$. It was speculated that compound 5 may be diosmetin.
Both compounds 6 and 7 contain a methoxy group, the quasi-molecular ion was m/z 299 [M − H] and m/z 283 [M − H]², respectively, and then they lost the methyl unit to produce the ion [M − H-CH₃]⁻ of m/z 284 and m/z 268, respectively. It was speculated that compounds 6 and 7 were hispidulin and acacetin, respectively.

3.2.2. Structural Analysis of Phenylpropanoids.

Compound 8 was protonated in the positive ion mode to produce ion m/z 193 [M + H]⁺ and then produced two fragment ions m/z 165 and m/z 137; they may be [M + H–CO]⁺ and [M + H–2CO]⁺; the cracking process of compound 8 is consistent with that of coumarins [22]. It was speculated that compound 8 may be 5,7-dihydroxy-4-methylcoumarin.

Compounds 9 and 10 had the same quasi-molecular ion m/z 515 [M − H]⁻, and both had characteristic fragment ions m/z 191 [quininic acid-H]⁻ and m/z 173 [quininic acid-H-H₂O]⁻. It was speculated that they were chlorogenic acids. The replacement position of caffeic acid can be determined according to the strength of the fragment ions [18]. Combined with the retention time, it was speculated that compounds 9 and 10 may be 5,7-dihydroxy-4-methylcoumarin.

3.2.3. Structural Analysis of Steroids.

Compound 11 was protonated in the positive ion mode to obtain the ion m/z 387 [M + H]⁺ and then continuously lost the H₂O group to obtain fragment ions m/z 369 [M + H–H₂O]⁺ and 351 [M + H–2H₂O]⁺ [23]; combined with the retention time and accurate molecular weight, it was speculated that compound 11 may be bufalin.

3.2.4. Structural Analysis of Organic Acids.

Organic acids generally respond in the negative ion mode to produce ion [M − H]⁻. The organic acids in F. velutipes were mostly fatty acids. They were prone to break apart and lose groups such as (CH₂)ₙ and COOH [24]. In this paper, organic acids in F. velutipes mainly produce fragments that lose H₂O and CO₂. The structural analysis of some organic acid compounds is as follows.

Compound 12 responded in the negative ion mode to produce ion m/z 133 [M − H]⁻, then lost the group H₂O to produce ion m/z 115 [M − H-H₂O]⁻, and further lost the group CO₂ to produce ion m/z 71[M – H – H₂O – CO₂]⁻. Combined with the retention time, accurate molecular weight, and the data of [25], it was speculated that compound 12 may be DL-malic acid. The structural analysis of other organic acids is similar to that of compound 12.

4. Discussion and Conclusion

Most of the compounds in F. velutipes have good biological activities. Hu et al. [15] investigated neuroprotective effects of six compounds from F. velutipes on H₂O₂-induced oxidative damage in PC12 cells, including arbutin, epicatechin, phillyrin, apigenin, kaempferol, and formononetin, and the results revealed that all components except apigenin mediate the apoptosis of PC12 cells via the endogenous pathway. In this paper, 7 flavonoids were identified by UPLC-Q-Exactive-Orbitrap MS, including linarin, luteolin, glycitin, api-genin, diosmetin, hispidulin, and acacetin. These flavonoids have many pharmacological effects such as anticancer, anti-inflammatory, and antioxidation. Luteolin has been showing numerous therapeutic activities such as anticancer, anti-inflammatory, antioxidation, and antimicrobial [26]. Apigenin has the cytostatic and cytotoxic effects on various cancer cells, prevents atherogenesis, hypertension, cardiac hypertrophy, ischemia/reperfusion-induced heart injury,
| Number | RT (min) | Formula | Adducts | Measured values (m/z) | Theoretical value (Da) | Error (ppm) | MS² | Identification level | Name | Compound class |
|--------|---------|---------|---------|------------------------|------------------------|-------------|------|----------------------|------|-------------------|
| 1      | 8.41    | C_{28}H_{32}O_{14} | [M + H]⁺ | 593.18524              | 593.18514              | 0.17        |      | 593 [M + H]⁺, 447 [M + H-rhamnosyl]⁺, 285 [M + H-rhamnosyl-glycosyl]⁺, 270 [M + H-rhamnosyl-glycosyl-CH₃]⁺, 242 [M + H-rhamnosyl-glycosyl-CH₃-CO]⁺ | Level 1 | Linarin | Flavonoids |
| 2      | 8.509   | C_{13}H_{12}O_{6} | [M - H]⁻ | 285.03983              | 285.04053              | -2.44       |      | 285 [M - H]⁻, 151 [M - H-C₇H₄O₄]⁻, 133 [M - H-C₆H₅O₂]⁻ | Level 2 | Luteolin | Flavonoids |
| 3      | 8.804   | C_{22}H₂₂O₁₀ | [M + H]⁺ | 447.12946              | 447.12992              | -1.03       |      | 447 [M + H]⁺, 285 [M + H-glycosyl]⁺, 270 [M + H-glycosyl-CH₃]⁺, 242 [M + H-glycosyl-CH₃-CO]⁺ | Level 2 | Glycitin | Flavonoids |
| 4      | 9.46    | C_{15}H₁₀O₅ | [M - H]⁻ | 269.04501              | 269.04573              | -2.66       |      | 269 [M - H]⁻, 151 [M - H-C₆H₅O₄]⁻, 117 [M - H-C₅H₄O₄]⁻ | Level 1 | Apigenin | Flavonoids |
| 5      | 9.759   | C_{16}H₁₂O₆ | [M + H]⁺ | 301.07047              | 301.07061              | -0.46       |      | 301 [M + H]⁺, 286 [M + H-CH₃]⁺, 258 [M + H-CH₃-CO]⁺ | Level 2 | Diosmetin | Flavonoids |
| 6      | 9.764   | C_{16}H₁₂O₆ | [M - H]⁻ | 299.05548              | 299.0562               | -2.41       |      | 299 [M - H]⁻, 284 [M - H-CH₃]⁻ | Level 1 | Hesperidin | Flavonoids |
| 7      | 11.787  | C_{16}H₁₂O₅ | [M - H]⁻ | 283.06076              | 283.06135              | -2.08       |      | 283 [M - H]⁻, 268 [M - H-CH₃]⁻ | Level 1 | Acacetin | Flavonoids |
| 8      | 5.14    | C_{10}H₈O₄ | [M + H]⁺ | 193.04933              | 193.04927              | 0.29        |      | 193 [M + H]⁺, 165 [M + H-CO]⁺, 137 [M + H-2CO]⁺ | Level 2 | 5,7-Dihydroxy-4-methylcoumarin | Phenylpropanoids |
| 9      | 6.712   | C_{23}H₂₄O₁₂ | [M - H]⁻ | 515.11823              | 515.1195               | -2.47       |      | 515 [M - H]⁻, 353, 335, 191 [quininic acid-H]⁻, 179, 173 [quininic acid-H₂O]⁻, 155, 135 | Level 1 | Isochlorogenic acid B | Phenylpropanoids |
| 10     | 7.221   | C_{23}H₂₄O₁₂ | [M - H]⁻ | 515.11835              | 515.11962              | -2.47       |      | 515 [M - H]⁻, 353, 191 [quininic acid-H]⁻, 179, 173 [quininic acid-H₂O]⁻, 135, 93 | Level 1 | Isochlorogenic acid C | Phenylpropanoids |
| 11     | 14.001  | C_{21}H₂₃O₄ | [M + H]⁺ | 387.2532               | 387.25326              | -0.15       |      | 387 [M + H]⁺, 369 [M + H-H₂O]⁺, 351 [M + H-2H₂O]⁺, 341, 143, 131, 105, 91 | Level 2 | Bufalin | Steroids |
| 12     | 1.006   | C_{4}H₄O₅ | [M - H]⁻ | 133.01421              | 133.01437              | -1.22       |      | 133 [M - H]⁻, 115 [M - H-H₂O]⁻, 71 [M - H-H₂O-CO₂]⁻ | Level 2 | DL-malic acid | Organic acids |
| 13     | 1.086   | C_{5}H₅O₃ | [M - H]⁻ | 147.03015              | 147.03022              | -0.48       |      | 147 [M - H]⁻, 129 [M - H-H₂O]⁻, 103 [M - H-CO₂]⁻, 101, 87, 85, 57 | Level 2 | D-α-Hydroxyglutaric acid | Organic acids |
| 14     | 1.093   | C_{6}H₄N₂O₂ | [M - H]⁻ | 122.02499              | 122.02503              | -0.33       |      | 122 [M - H]⁻, 94 [M - H-CO]⁻, 91 [M - H-CO₂]⁻, 111 [M - H-CO₂-H₂O]⁻, 87 | Level 1 | Picolinic acid | Organic acids |
| 15     | 1.095   | C₆H₄O₂ | [M - H]⁻ | 191.01964              | 191.01986              | -1.17       |      | 191 [M - H]⁻, 129 [M - H-CO₂-H₂O]⁻, 111 [M - H-CO₂-H₂O]⁻, 87 | Level 1 | Citric acid | Organic acids |
| 16     | 1.141   | C₆H₄O₂ | [M - H]⁻ | 117.01933              | 117.01931              | 0.21        |      | 117 [M - H]⁻, 99 [M - H-H₂O]⁻, 73 [M - H-H₂O-CO₂]⁻ | Level 1 | Succinic acid | Organic acids |
| 17     | 1.161   | C₆H₄O₂ | [M - H]⁻ | 115.00361              | 115.00362              | -0.10       |      | 115 [M - H]⁻, 71 [M - H-CO₂]⁻ | Level 1 | Fumaric acid | Organic acids |
| 18     | 4.225   | C₇H₁₂O₄ | [M - H]⁻ | 159.0661               | 159.06625              | -0.95       |      | 159 [M - H]⁻, 115 [M - H-CO₂]⁻, 97 [M - H-CO₂-H₂O]⁻ | Level 2 | 3-Methyladipic acid | Organic acids |
| 19     | 5.199   | C₇H₁₄O₄ | [M - H]⁻ | 185.08171              | 185.0819              | -1.03       |      | 185 [M - H]⁻, 141 [M - H-CO₂]⁻, 123 [M - H-CO₂-H₂O]⁻ | Level 2 | 1-(Carboxymethyl)cyclohexanecarboxylic acid | Organic acids |
| Number | RT (min) | Formula | Adducts | Measured values (m/z) | Theoretical value (Da) | Error (ppm) | MS² | Identification level | Name | Compound class |
|--------|---------|---------|---------|----------------------|-----------------------|-------------|-----|---------------------|------|---------------------|
| 20     | 7.109   | C₉H₁₆O₄ | [M – H]⁻ | 187.09744            | 187.09764             | −1.05       | 187 [M – H]⁻, 169 [M – H-H₂O]⁻, 125 [M – H-H₂O-CO₂]⁻, 97 [M – H-H₂O-CO₂-CO]⁻ | Level 1 | Azelaic acid | Organic acids |
| 21     | 8.302   | C₁₀H₁₈O₄ | [M – H]⁻ | 201.11302            | 201.11329             | −1.35       | 201 [M – H]⁻, 183 [M – H-H₂O]⁻, 139 [M – H-H₂O-CO₂]⁻ | Level 2 | 3-tert-Butyladipic acid | Organic acids |
| 22     | 12.434  | C₁₄H₂₆O₄ | [M – H]⁻ | 257.17542            | 257.17584             | −1.62       | 257 [M – H]⁻, 239 [M – H-H₂O]⁻, 195 [M – H-H₂O-CO₂]⁻ | Level 2 | Tetradecanedioic acid | Organic acids |
| 23     | 16.105  | C₁₄H₂₆O₃ | [M – H]⁻ | 243.1965             | 243.19673             | −0.96       | 243 [M – H]⁻, 197 [M – H-HCOOH]⁻ | Level 2 | 2-Hydroxymyristic acid | Organic acids |
| 24     | 18.147  | C₁₆H₃₂O₃ | [M – H]⁻ | 271.22733            | 271.22782             | −1.80       | 271 [M – H]⁻, 225 [M – H-HCOOH]⁻ | Level 2 | 16-Hydroxyhexadecanoic acid | Organic acids |
| 25     | 18.531  | C₁₆H₃₀O₂ | [M + H]⁺ | 255.23093            | 255.2311              | −0.66       | 255 [M + H]⁺, 237 [M + H-H₂O]⁺, 219 [M + H-H₂O-CO₂]⁺, 149, 135, 121, 97, 83, 81, 69 | Level 2 | Palmitoleic acid | Organic acids |
| 26     | 18.934  | C₁₈H₃₂O₂ | [M – H]⁻ | 279.23245            | 279.23298             | −1.91       | 279 [M – H]⁻, 261 [M – H-H₂O]⁻, 59 | Level 2 | Linoleic acid | Organic acids |
and autoimmune myocarditis, protects the chemical- and ischemia/reperfusion-induced liver injury, inhibits asthma, bleomycin-induced pulmonary fibrosis, abnormal behavior, and oxygen and glucose deprivation/reperfusion-induced neural cell apoptosis, and improves pancreatitis, type 2 diabetes and its complications, osteoporosis, and collagen-induced arthritis [27]. Acacetin has neuroprotective, cardioprotective, anticancer, anti-inflammatory, antidiabetic, and antimicrobial activities [28]. Hispidulin has diverse pharmacological effects such as anticancer, anti-

![Figure 2: Possible MS fragmentation pathway of linarin.](image)

![Figure 3: Possible MS fragmentation pathway of luteolin.](image)

![Figure 4: MS² spectrum of compound 9.](image)
inflammatory, antifungal, antiplatelet, anticonvulsant, and antiosteoporotic [29]. Linarin could suppress glioma through inhibition of NF-κB/p65 and upregulating p53 expression in vitro and in vivo [30]. Glycitin has effects of alleviating lipopolysaccharide-induced acute lung injury via inhibiting NF-κB and MAPK pathway activation in mice [31]. Diosmetin has anti-inflammatory effects on IL-4- and LPS-induced macrophage activation and the atopic dermatitis model [32]. Therefore, it is beneficial to develop flavonoids in F. velutipes.

One steroid (bufalin) was identified in F. velutipes in this paper. Bufalin is one of the main pharmacological and toxicological components of Venenum Bufonis and many traditional Chinese medicine preparations [33]. Currently, there is no report of bufalin in F. velutipes. Whether F. velutipes contains bufalin needs more research to determine.

Chen et al. [25] investigated chemical compositions in the stipe and pileus of F. filiformis by UPLC-Q/TOF-MS, 130 compounds were identified, including 33 amino acids and derivatives, 34 nucleotides and derivatives, 37 organic acids and lipids, 9 carbohydrate alcohols, 8 alkaloids, and 9 other compounds, and most of them were primary metabolites. Han et al. [34] investigated chemical compositions of F. velutipes, 11 compounds were isolated and identified, including arabinitol, ergosterol, cis-9-tricosene, uracil, nicotinamide, xanthine, glycerol, adenosine, trehalose, mannitol, and tyrosine, and most of them were primary metabolites. In this paper, 26 secondary metabolites were preliminarily identified by UPLC-Q-Exactive-Orbitrap MS in F. velutipes from Henan province, including 3 phenylpropanoids, 7 flavonoids, 1 steroid, and 15 organic acids. It provides a reference for the future separation of chemical components of F. velutipes.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

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Figure 5: MS² spectrum of compound 10.
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