UHPLC-Q-TOF-MS/MS-oriented characteristic components dataset and multivariate statistical techniques for the holistic quality control of Usnea

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The holistic quality evaluation of Traditional Chinese Medicine (TCM) is confronted with significant challenges due to its extreme chemical complexity. In this study, a sensitive strategy based on ultra-high-performance liquid chromatography-triple/time-of-flight mass spectrometry (UHPLC-Q-TOF-MS/MS) and chemometric analysis was established and validated for the qualitative and semi-quantitative analyses of characteristic components in Usnea. First, three mass spectrometry fragmentation patterns of phenolic acid standards were studied and summarized. Then, an extract of this herb was analyzed by the full-scan MS spectra and identified by extracted ion chromatography (XIC). Based on the abovementioned methods, a total of 38 compounds (8 dibenzofurans, 11 didepsides, 13 depsidones, and 6 mono-substituted phenyl rings) were identified. Subsequently, the qualities of Usnea samples from different regions were evaluated by the semi-quantitative analysis based on their relative peak areas. Furthermore, principal component analysis (PCA) was performed to compare the Usnea herbs and to find possible diagnostic chemical components. This novel and powerful strategy could provide a potential approach for the holistic quality control of TCM.

1. Introduction

Usnea belongs to the Usnea genus and originates from the dried lichen body of Usnea longissima Ach. It is mainly distributed in Sichuan, Yunnan, Zhejiang, and Shanxi provinces in China. For hundreds of years, this plant has been used in folk medicine to protect the liver, clear the heat, and remove the toxins as is recorded in several Materia Medica books such as “Compendium of Materia Medica” and “The Dictionary of Medicinal Plant”. Although it also exhibits antioxidant, antineoplastic, anti-allergic, antimicrobial, analgesic, antiinflammatory, antitumor, and antiviral properties in clinic, it is mainly known for its anti-inflammator y anti-microbial properties in the treatment of cough and rheumatism. Simultaneously, it is often consumed as tea or as a healthy drink.

Phytochemical studies demonstrate that the members of Usnea genus contain phenolic acids, steroids, triterpenoids, and polysaccharides. Among them, the types of phenolic acids found in the lichens of Usnea genus mainly include dibenzofurans, didepsides, depsidones, and mono-substituted phenyls. Recently, phenolic acids were believed to be the main bioactive constituents in the lichens of Usnea genus due to their various activities such as anti-microbial, anti-cancer, and anti-inflammatory. However, according to the reports, only a few chemical ingredients have been isolated from Usnea. In our previous study, a large number of phenolic acids were detected in Usnea by MS, and most of them were at low levels. It is well-known that Traditional Chinese Medicine (TCM) is commonly believed to operate due to synergistic effects of all of the major and minor components in the medicines. Moreover, many factors such as different origins can affect the quality of TCM. Therefore, it is critically important to explore a more scientific, systematic, and strongly operated evaluation method that can help maintain the inherent qualities of TCMs so as to ensure their safety, efficacy, and batch-to-batch consistency. At present, as is observed in the recent studies, only a few quantitative markers have been selected for the quality evaluation of Usnea, which severely restricts its in-depth study and clinical application. Although a HPLC method has been developed for the analysis of usnic acid in this plant, this method mainly focuses on the quantitative determination of single components and also cannot reflect the intrinsic qualities of Usnea. Therefore, the in-depth studying of a qualitative and semi-quantitative...
analytical strategy on the basis of the active ingredients in *Usnea* is of great significance for its pharmacodynamics and comprehensive quality control.

Recently, HPLC coupled with Q-TOF-MS has been used in ingredient analysis and metabolite detections. Among the multifarious LC platforms, UHPLC holds advantages over HPLC because of its higher speed, higher detection sensitivity, increased specificity, and enhanced repeatability. In addition, Q-TOF-MS provides accurate precursor and product ion information with a mass error of less than 5 ppm, which authentically increases the characterization and dependability of the compound. The recent decade has witnessed an increasingly vital role of high-resolution mass spectrometry (HRMS) in complex chemical profiling and highly sensitive quantitative analysis of medicinal herbs. The HRMS system can provide the acquisition of full-scan MS spectra and fragment ion spectral datasets for compounds by virtue of on-line and off-line techniques, enabling the overall consideration of MS and MS/MS spectra for the identification of both target and non-target compounds. In previous studies, it was challenging and error-prone to monitor the relatively small signals from the complex chemical background in full-scan mass chromatograms. To detect as many metabolites as possible, a novel approach combining multiple mass defect filter (MMDF) and dynamic background subtraction (DBS) dependent on-line data acquisition is used to identify the chemical composition that has explicit mass defect values. On the other hand, the effective multiple data-mining techniques involved in extracted ion chromatography (XIC), mass defect filtering (MDF), product ion filtering (PIF), and neutral loss filtering (NLF) are employed to further identify and analyze the compounds. Because of the combined use of the above-described techniques, the detection and identification of herbal components becomes easier and faster than before. Furthermore, multivariate statistical analysis methods such as PCA are performed to provide more information about the chemical differences in TCMs. These approaches can be used to process tremendous datasets of TCM samples by showing a two-dimensional space of the variable metabolic profile.

The aim of this study was to develop a novel analytical strategy for the comprehensive quality control of *Usnea*. First, the fragmentation patterns of three reference substances were studied and summarized. Then, a sensitive method based on on-line data acquisition combined with comprehensive data processing techniques was established for the identification of phenolic acids in *Usnea*. Next, a semi-quantitative analysis of *Usnea* samples based on the relative peak areas of the 38 identified compounds was performed in 25 batches of *Usnea* samples collected from various regions. Meanwhile, PCA was conducted to disclose the possible characteristic components resulting in quality differences in the *Usnea* samples. It is believed that this novel strategy could be valuable for the quality control of *Usnea* and other TCMs.

### 2. Materials and methods

#### 2.1. Chemicals and materials

Usnic acid was purchased from Shanghai Sunny Bio-technology Co., Ltd. Diffractaic acid was obtained from Shanghai Yuanye Bio-technology Co., Ltd. Evernic acid was purchased from Shanghai Bohu Bio-technology Co., Ltd. The purities of the abovementioned ingredients were more than 98% according to HPLC analysis. HPLC-grade methanol (Tedia, USA), formic acid (Fisher Scientific, USA), diethylamine (Dikma Technologies Inc., USA), and ammonium acetate (Fisher Scientific, USA) were used in the mobile phase. Redistilled water was prepared in our own lab using the Milli-Q water purification system (Millipore, ELIX100, USA).

#### 2.2. Plant materials and sample preparation

Twenty-five batches of *Usnea* samples (Table 1) were acquired from different regions in China. The *Usnea* samples, identified by Prof. Lianhuai Li from Department of Pharmacognosy in Hebei Medical University, were deposited in the College of Pharmacy Laboratory of Hebei Medical University, Shijiazhuang, China.

Dried *Usnea* samples were crushed into fine powder so that they could be passed through 60 mesh screens. An amount of 0.1 g of *Usnea* powder was extracted with 25 mL of 100% alcohol using an ultrasonic bath for 20 min. After the supernatant was filtrated through 0.22 μm syringe filters, the extract was concentrated to dryness under reduced pressure. The abovementioned ingredients were more than 98% according to HPLC analysis. HPLC-grade methanol (Tedia, USA), formic acid (Fisher Scientific, USA), diethylamine (Dikma Technologies Inc., USA), and ammonium acetate (Fisher Scientific, USA) were used in the mobile phase. Redistilled water was prepared in our own lab using the Milli-Q water purification system (Millipore, ELIX100, USA).

#### Table 1 Information on the 25 batches of *Usnea* samples

| Sample | Source                        | Collection time | Sample | Source                        | Collecting time |
|--------|-------------------------------|-----------------|--------|-------------------------------|-----------------|
| S1     | Zigong, Sichuan (AHBZ)        | 2015.04         | S13    | Fanxiong, Yunnan (AHBZ)       | 2015.04         |
| S2     | Zigong, Sichuan (AHBZ)        | 2015.04         | S14    | Fanxiong, Yunnan (AHBZ)       | 2015.04         |
| S3     | Zigong, Sichuan (AHBZ)        | 2015.04         | S15    | Fanxiong, Yunnan (AHBZ)       | 2014.04         |
| S4     | Zigong, Sichuan (AHBZ)        | 2015.05         | S16    | Fanxiong, Yunnan (AHBZ)       | 2015.05         |
| S5     | Zigong, Sichuan (AHBZ)        | 2015.07         | S17    | Zigong, Sichuan (HBAG)        | 2015.09         |
| S6     | Zigong, Sichuan (AHBZ)        | 2015.09         | S18    | Zigong, Sichuan (HBAG)        | 2015.09         |
| S7     | Zigong, Sichuan (AHBZ)        | 2015.09         | S19    | Zigong, Sichuan (HBAG)        | 2015.09         |
| S8     | Jinhua, Zhejiang (AHBZ)       | 2015.04         | S20    | Jinhua, Zhejiang (HBAG)       | 2015.09         |
| S9     | Jinhua, Zhejiang (AHBZ)       | 2015.04         | S21    | Jinhua, Zhejiang (HBAG)       | 2015.09         |
| S10    | Jinhua, Zhejiang (AHBZ)       | 2015.04         | S22    | Jinhua, Zhejiang (HBAG)       | 2015.09         |
| S11    | Jinhua, Zhejiang (AHBZ)       | 2015.05         | S23    | Fanxiong, Yunnan (HBAG)       | 2015.09         |
| S12    | Jinhua, Zhejiang (AHBZ)       | 2015.05         | S24    | Fanxiong, Yunnan (HBAG)       | 2015.09         |
| S25    | Fanxiong, Yunnan (HBAG)       | 2015.09         |        |                               |                 |
methanol in an ultrasonic bath (40 kHz, 500 w) for 30 min. An additional quantity of 100% methanol was added to compensate for the lost weight. Then, the extracted solution was centrifuged for 10 min at 150 000 rpm and filtered through a 0.2 μm GHP filter (Mfd. by Waters Corporation, USA). Afterwards, the supernatant was transferred to a clean polypropylene tube. An aliquot of 5 μL from each sample was injected into the UHPLC-Q-TOF-MS/MS system for further analysis.

2.3. Preparation of standard solutions

Reference standards were accurately weighed and then dissolved in methanol to yield standard stock solutions for usnic acid, evernic acid, and diﬀractic acid at concentrations of 0.400 g L⁻¹, 0.408 g L⁻¹, and 0.392 g L⁻¹, respectively. Then, each stock solution was mixed with methanol to prepare a ﬁnal mixed standard solution. The working solutions were stored at 4 °C for further analysis.

2.4. UPLC-Triple TOF-MS conditions

UHPLC-Q-TOF-MS/MS analysis was conducted on the Shimadzu UHPLC system (Kyoto, Japan) coupled with the triple TOF™ 5600Plus MS/MS system (AB SCIEX, CA, USA). The chromatographic separation was accomplished on a Kinetex C₁₈ (100 mm × 3.0 mm, 2.6 μm) column with a Security Guard UPLC C₁₈ column (4.0 mm × 3.0 mm, 2.6 μm) (Phenomenex, Torrance, CA, USA), and the column oven was maintained at 25 °C. The mobile phase consisted of water containing 0.1% formic acid as well as 4 mM L⁻¹ ammonium acetate (A) and methanol (B) with a ﬂow rate of 0.4 mL min⁻¹. A binary gradient elution was employed, and the consecutive program was as follows: 5–75% B from 0 to 3 min, 75–85% B from 3 to 15 min, 85–95% B from 15 to 20 min, and 95–98% B from 20 to 25 min; later, the column was returned to its starting conditions for 30 min for column balance.

The mass spectrometer with a Duo-Spray™ source was operated in the negative ion electrospray mode, and the parameters of the MS/MS detector were as follows: ion spray voltage: −4500 kV; turbo spray temperature: 550 °C; curtain gas: 25 psi; nebulizing gas (GAS1): 55 psi and TIS gas (GAS2): 55 psi; and declustering potential (DP): −60 V. For the full MS-IDA (information-dependent acquisition)-8MS/MS analysis, the eight most intense fragment ions of each analyte that exceeded 100 cps counts were selected for the survey scan, and the MS/MS experiments were run in the scan range from m/z 100 to m/z 1000 with 250 m of accumulation time for full MS and 100 m of accumulation time for MS/MS experiments. The collision energy (CE) was set at −30 eV, and the collision energy spread (CES) was 15 eV in the MS/MS experiments. The IDA criteria were given to ions that matched the mass defect window to obtain the MS/MS spectra. Simultaneously, real-time MMDF and DBS were used to fulﬁll the IDA criteria. To get convincing results, an automated calibration delivery system (CDS) was used to automatically calibrate MS and MS/MS with every 6 samples in our study.

The UHPLC-Q-TOF-MS data of the extracted samples were analysed using the PeakView® Software (AB SCIEX, CA, USA). The XIC manager tool in Master View® (AB Sciex, CA, USA) provided quasi-molecular weights, mass errors and isotope patterns. The possible compounds, their elemental compositions and chemical formulae could be calculated by screening the ChemSpider database. The predicted formula with errors less than ±5 ppm was searched against the compounds reported for Usnea to obtain tentative identiﬁcation.

2.5. Analytical procedures

The chemical compound identiﬁcation strategy was based on a Triple TOF instrument with DBS-dependent on-line data acquisition and multiple post data-mining tools. The analytic strategy is presented in detail as follows: ﬁrst, the mass spectrometry cracking behaviors of the reference substances were studied, and the fragmentation patterns were summarized. Second, a full mass scan was performed, and accurate MS/MS data sets were obtained using an eﬀective MMDF and DBS-dependent data acquisition method. The application of DBS could intelligently promote the ability of identiﬁcation of drug-related MS/MS ions from the background and matrix-related MS/MS ions. Therefore, the lower-level of target compounds could be clearly captured. Third, post-acquisition data mining was obtained by using various data mining tools including XIC, MDF, PIF, and NLF. Finally, the compounds were identiﬁed on the basis of accurate mass measurements and previously investigated fragmentation regulations of reference substances.

2.6. Semi-quantitative data and chemometric analysis

To evaluate the qualities of Usnea samples from different regions, a semi-quantitative analysis based on relative peak areas was introduced. PCA was applied to holistically observe the general clustering and trends among all samples using the SPSS 21.0 software (IBM SPSS, USA). The principal component (PC) score plots of the ﬁrst 2 PCs were generated from the results of PCA.

3. Results and discussion

3.1. Optimization of LC-MS/MS conditions

With the purpose of achieving an eﬃcient and rapid analysis, a high pressure chromatographic column packed with 2.6 μm porous particles was applied in UPLC analysis. Diﬀerent gradient elutions, ﬂow rates (0.3, 0.4, and 0.5 mL min⁻¹) and column temperatures (20, 25, and 30 °C) were optimized to achieve a higher eﬀect of chromatography and mass spectrometry. Meanwhile, to enhance the signal response, mobile phase modiﬁers such as formic acid and ammonium acetate were also evaluated. A mobile phase containing 0.1% formic acid and 4 mM L⁻¹ ammonium acetate improved the chromatographic behavior, reduced the peak tailing and facilitated ionization. The mobile phase at 25 °C with a ﬂow rate of 0.4 mL min⁻¹ was ﬁnally selected for the shorter analysis. Thus, all the detected compounds could be well separated within 25 min. As shown in Fig. 1, the representative XIC spectra of standards and samples were obtained under the abovementioned optimized conditions.
Furthermore, the mass spectrometric conditions were optimized in positive and negative ion modes. The negative ion mode was selected because the ionization of phenolic acids was more efficient when compared with the positive ion mode. The parameter CE was optimized to get the richest relative abundance by appropriate fragmentation and molecular intensity. Collision-induced dissociation (CID) combined with a CE of \(-30\) eV and CES of 15 eV was performed to obtain more fragmentation information for the elucidation of inferred compounds.

Fig. 1 The XIC and MS/MS spectra of samples.
3.2. Establishment of qualitative analysis strategy

All Usnea samples were detected using the optimized HRMS methods. To rapidly identify the target and non-target compounds in the Usnea extracts, a powerful integrated analytical strategy using on-line data acquisition combined with multiple data processing techniques including database searching, reference standard comparison, TOF-MS information, and MS/MS data analysis was first employed for the...
unambiguous identification of constituents in the *Usnea* plant. The structures of the compounds having reference standards were deduced based on the retention times and HRMS data. At the same time, their cracking patterns were probed and summarized. On the contrary, the unknown constituents were deduced based on the high-accuracy \([M - H]^-\) precursor ion in the negative ion mode using the XIC Manager software. The next step was to search for a possible compound in the
Fig. 2 Chemical structures of 38 compounds identified in *Usnea longissima* Ach.
# Table 2
Characterization of phenolic compounds in *Usnea* by UHPLC-Q-TOF-MS/MS

| No. | Molecular formula | [M – H]⁻ m/z | Error (ppm) | tᵣ (min) | MS/MS fragments | Identification |
|-----|------------------|---------------|-------------|----------|-----------------|---------------|
| 1   | C₁₈H₁₆O₈         | 359.0772      | −1.0        | 6.306    | 359.0781, 344.0547, 275.0561, 257.0459, 229.0507 | (S)-8-Acetyl-7-hydroxy-1,3-dimethoxy-9α-methyl-9-oxo-9β-dihydrodiolbenzo[b,d]furan-4-carboxylic acid |
| 2   | C₁₈H₁₄O₇         | 341.0667      | −0.5        | 6.316    | 341.0638, 326.0407, 311.0191, 257.0459, 229.0507 | (R)-Benzo[b]furo[3',2':3,4]benzo[1,2-d]furan-1,6(2H,5bH)-dione, 7-acetyl-5,8-dihydroxy-4,5b-dimethyl Dimethyl 3,4-dihydroxy-9-methoxy-1,7-dimethylbenzo[b,d]furan-2,6-dicarboxylate |
| 3   | C₁₉H₁₈O₈         | 373.0928      | −0.2        | 8.386    | 373.0928, 358.0662, 341.0659, 326.0426, 311.0197, 298.0471 | Placodiolic |
| 4   | C₁₈H₂₀O₈         | 375.1085      | −1.1        | 8.960    | 375.1085, 343.0818, 315.0854, 259.0601, 231.0653 | Usnic acid |
| 5   | C₁₈H₁₆O₇         | 343.0823      | −0.6        | 12.446   | 343.0815, 328.0584, 313.0345, 299.0904, 259.0598, 231.0652 | Isohydrousic acid |
| 6   | C₁₈H₁₆O₇         | 345.0980      | −0.9        | 12.437   | 345.0905, 330.0643, 315.0433, 261.0624, 233.1527 | Porphyric acid |
| 7   | C₁₉H₁₆O₇         | 313.0354      | −0.5        | 12.446   | 313.0344, 269.0486, 257.0454, 241.0478, 229.0483, 213.0184 | |
| 8   | C₁₉H₁₈O₇         | 357.0980      | −0.1        | 15.694   | 357.0987, 342.0751, 314.0434, 286.0478, 273.0763, 245.0817 | 1,1-O-(3,7-Dihydroxy-9-methoxy-8,9b-dimethyl-1-oxo-1,9b-dihydrodibenzo[b,d]furan-2,6-diyl) bis(ethan-1-one) |
| 9   | C₁₉H₁₄O₇         | 317.0667      | −0.8        | 5.558    | 317.0663, 255.0862, 167.0350, 149.0239, 123.0449, 105.0347 | Lecanoric acid |
| 10  | C₂₀H₂₂O₈         | 389.1242      | −0.6        | 5.620    | 389.2910, 343.0633, 313.1075, 283.0964, 225.0749, 193.0303, 151.0758 | 2-Acetyl-3,5,6-trimethoxyphenyl 2,3-dimethoxybenzoate |
| 11  | C₂₀H₂₂O₇         | 375.0722      | −1.4        | 6.002    | 375.1000, 357.0557, 343.0816, 329.2327, 301.0750, 167.0335 | Vermicularin |
| 12  | C₂₇H₃₆O₇         | 471.2388      | 0.6         | 7.121    | 471.2986, 425.2879 | |
| 13  | C₁₈H₁₆O₇         | 359.0772      | −1.0        | 7.997    | 359.0765, 331.0617, 167.0353, 149.0252 | Planaria acid |
| 14  | C₁₈H₁₆O₇         | 331.0823      | −1.1        | 8.095    | 331.0827, 167.0353, 149.0246, 123.0453, 105.0350 | Hemamnoll |
| 15  | C₁₉H₁₄O₇         | 333.0616      | 4.3         | 8.117    | 333.0878, 167.0345, 149.0240, 123.0449, 105.0342 | Evernic acid |
| 16  | C₂₀H₂₂O₇         | 373.1293      | −1.3        | 9.709    | 373.1297, 329.1392, 297.1133, 282.0900, 267.0667, 238.0997, 209.0820, 165.0926, 135.0814 | Dipractiasic acid |
| 17  | C₁₈H₁₆O₈         | 373.0929      | −0.2        | 9.709    | 373.0927, 329.1392, 297.1133, 282.0900, 267.0667, 238.0997, 209.0820, 165.0926, 135.0814 | 4-Acetoxy-3-methoxybenzo-phenyl 2-hydroxy-4-methoxy-6-methylbenzoate |
| 18  | C₁₈H₁₄O₆         | 301.0718      | −0.5        | 12.461   | 301.0688, 286.0481, 271.0237, 258.0505, 232.0558 | 3-Benzoyloxy-4,5-dimethoxybenzoic acid |
| 19  | C₁₉H₂₀O₇         | 359.1131      | −1.4        | 13.013   | 359.1141, 181.0501, 163.0396, 137.0605 | Barbitic acid |
| 20  | C₁₉H₁₂O₁₀        | 387.0358      | −2.4        | 4.591    | 387.0355, 369.0211, 343.0457, 341.0324, 325.0343, 297.0405, 269.0450 | Norperistatic acid |
| 21  | C₂₀H₁₈O₉         | 401.0878      | −0.7        | 4.775    | 401.0876, 357.0625, 333.2269, 313.0708 | Cetaric acid |
| No. | Molecular formula | [M – H]^- m/z | Error (ppm) | t_R (min) | MS/MS fragments | Identification |
|-----|------------------|---------------|-------------|-----------|----------------|---------------|
| 22  | C_{20}H_{18}O_{9} | 401.0878      | -0.7        | 5.341     | 401.0867, 383.2700, 355.0763, 269.0818 | 1-Hydroxy-11-(hydroxymethyl)-4,10-dimethoxy-5,8-dimethyl-7H-benzo[6,7][1,4]dioxepino[2,3-e]isobenzofuran-3,7(1H)-dione |
| 23  | C_{19}H_{14}O_{8} | 357.0616      | -0.8        | 5.549     | 357.0604, 342.0455, 314.0420, 313.0805, 286.0458 | Psoromic acid |
| 24  | C_{19}H_{14}O_{8} | 371.0772      | -0.8        | 6.633     | 371.0745, 356.0547, 341.0308, 313.0383, 285.0407 | Methyl psoromate |
| 25  | C_{19}H_{14}O_{8} | 373.0565      | -0.9        | 7.054     | 373.0564, 358.0329, 355.0455, 327.0518, 271.0222 | Menegazziaic acid |
| 26  | C_{19}H_{14}O_{8} | 357.0616      | -2.6        | 8.091     | 357.0632, 313.0708, 270.0498, 227.0690 | Virensic acid |
| 27  | C_{20}H_{16}O_{8} | 397.1657      | -1.9        | 9.329     | 397.1649, 355.1528, 326.0805, 313.1025 | Norlobaridone |
| 28  | C_{20}H_{16}O_{12}| 471.0569      | -1.4        | 9.713     | 471.0597, 427.0293, 383.0380 | Pumaroprotocetraric acid |
| 29  | C_{20}H_{16}O_{8} | 469.1504      | 0.4         | 9.784     | 469.1374, 425.1444, 381.1532, 323.2563 | Glomellonic acid |
| 30  | C_{20}H_{16}O_{8} | 469.1504      | 0.4         | 10.281    | 469.1873, 451.1872, 425.1962, 381.2097 | Oxoobaric acid |
| 31  | C_{20}H_{16}O_{8} | 469.1504      | -1.2        | 10.424    | 469.1862, 425.1917, 381.2074, 310.1284 | Physodic acid |
| 32  | C_{20}H_{16}O_{8} | 455.1711      | -0.2        | 13.083    | 455.1719, 437.1562, 411.1817, 357.044, 367.1892, 352.1664 | Lobaric acid |
| 33  | C_{8}H_{10}O_{4}  | 167.0350      | 0.5         | 5.556     | 167.0357, 149.0251, 123.0461 | Orsellinic acid |
| 34  | C_{11}H_{14}O_{4} | 223.0612      | 0.3         | 7.041     | 223.0611, 205.0491, 181.0499, 179.0705, 163.0399, 119.0498 | 3-Butyryl-beta-resorcylic acid |
| 35  | C_{8}H_{10}O_{4}  | 181.0506      | 1.9         | 7.492     | 181.0485, 163.0520, 137.0592, 122.0357 | Everninic acid |
| 36  | C_{8}H_{10}O_{4}  | 167.0350      | 0.5         | 8.107     | 167.0337, 149.0202, 123.0435, 105.0330 | 2,6-Dihydroxy-4-methylbenzoic acid |
| 37  | C_{11}H_{14}O_{4} | 209.0820      | 0.9         | 9.608     | 209.0611, 180.0603, 164.9451, 134.9485, 121.0296 | Everninate ethyl |
| 38  | C_{10}H_{12}O_{4} | 195.0663      | 0.5         | 13.373    | 195.0292, 177.0184, 165.0555, 151.0397, 133.0284, 123.0456 | 2,4-Dimethoxy-6-methylbenzoic acid |
established *Usnea* characteristic components data set and then, the candidate structures were tentatively deduced from MS/MS spectrogram, the fragmentation patterns of reference standards, and the data of the reported literatures. As known, it is difficult to differentiate isomers because they have the same molecular formula. A certain degree of difference was presented in their retention times and chemical structures with lower energies. The minimized energy value and steric hindrance were used to account for the stability of the molecule. The minimized energy values were provided by the program ChemOffice 14.0 (CambridgeSoft, USA).

### 3.3. Compounds identified in *Usnea*

Applying the abovementioned methods, a total of 38 components (8 dibenzofurans, 11 didepsides, 13 depsidones, and 6 mono-substituted phenyl rings) were identified in *Usnea* samples. Among them, three compounds (including usnic acid, diffractaic acid and evernic acid) were unambiguously characterized via comparison with chemical standards in terms of retention times and mass spectra. Their structures are shown in Fig. 2, and their detailed MS data are shown in Table 2.

#### 3.3.1. Dibenzofurans

The dibenzofurans exhibited specific and abundant \([M – H]^-\) precursor ions, and the negative ion MS/MS fragmentations of dibenzofurans were characterized by the Retro-Diels-Alder (RDA) cleavage of C1–12 and C2–3 bonds accompanied by the loss of characteristic fragments at \(m/z\) 84 Da \((C_4H_4O_2)\) and \(m/z\) 112 Da \((C_5H_4O_3)\). Multiple neutral loss pathways always occur in dibenzofurans. Taking usnic acid (5) as an example, the predominant quasi-molecular ion \([M – H]^-\) at \(m/z\) 343.0823 gave the formula \(C_{18}H_{16}O_7\), and the product fragments observed at \(m/z\) 259 (\([M – H – C_4H_4O_2]^-\)) and 231 (\([M – H – C_5H_4O_3]^-\)) corresponded to their RDA cleavage. Furthermore, distinctive product ions at \(m/z\) 328, 313, and 299 were detected in the spectrum of usnic acid due to the continuous loss of CH3 (15 Da) and CH2 (14 Da) units from the deprotonated molecule at \(m/z\) 343. Based on the above reference, 7 compounds were
broadly identified by comparison with usnic acid in terms of the fragmentation patterns. Compounds 1, 2, 6, and 8 demonstrated a distinctive C4H4O2 loss and C6H6O3 loss from the parent ion. Compound 4 readily produced a strong fragment ion [M-H-CH3O]− (m/z 343), which yielded the structure of incidental RDA cleavage and then generated the fragment ion at m/z 259 and 231. Thus, compound 4 was confirmed to be plagiocladic acid (Fig. 3). Moreover, compounds 3 and 7 presented neutral losses of CH3, CO, and CO2 molecules.

3.3.2. Didepsides. A large number of didepsides were identified in the Usnea samples. Most of these compounds were substituted with phenyl salicylate, and they yielded salicylic acid ion A and phenolic moiety ion B by breaking the ester bonds of the eight-membered ring transition state rearrangements. Evernic acid and difractaica acid were used as examples to explore and verify the fragmentation patterns of these didepsides in detail. In the MS/MS spectra of the reference standard of evernic acid (14), minor product ion was detected at m/z 164, and the ion B at m/z 167 corresponded to the cleavage of the ester bond. Then, the distinctive fragment ions at m/z 149 and 123 were formed by the losses of H2O and CO2, respectively, from ion B (Fig. 4A). Another typical instance was the unknown compound physodic acid (16), which yielded identical product ions at m/z 329, 297, 282, 267, and 238 through consecutive losses of neutral molecules such as CO2, CH2O, CH3O, CH3, and CHO, respectively. At the same time, the fragment ions at m/z 209 and 165 were observed by breaking the ester bond (Fig. 4B). Accordingly, the results validated the abovementioned summarized fragmentation regularities. A comparison with the cracking rules of reference standards and literature data resulted in the compounds 9, 10, 11, 12, 13, 15, 17, 18, and 19 being unequivocally identified.

3.3.3. Depsidones. The depsidones from the Usnea samples were characterized based on the literature data. Similar to the structure of didepsides, the molecular structure of these compounds possessed a diphenyl ether bond between the A ring and B ring, which hindered the simple cracking of the ester bond. The decarboxylation reaction and dehydration reaction, especially the decarboxylation reaction, could occur when the B ring had the structure of salicylic acid. The continuous pyrolysis loss of H2O and CO mainly occurred when the carboxyl group of phenolic moieties entered the state of lactones. According to the aforementioned evidence, 13 depsidones were identified by the summarized fragmentation patterns combined with product ions observed in the MS/MS spectra. These summarized cleavage features assisted in the identification of the unknown compounds 20-32. Taking lobaric acid (32) as an example, the predominant quasi-molecular ion [M-H]− at m/z 455.1711 could be detected in the TOF-MS spectrum, giving the formula of C25H28O8. Further evidence was obtained from the negative MS/MS fragmentation of the characteristic ion at m/z 411 ([M-H-CO2]−) (Fig. 5A). The unknown compound physodic acid (31) underwent similar cleavage pathways as those of 32, corresponding to the quasi-molecular ion [M-H]− at m/z 469.1868 and the M-44 peak at m/z 425 ([M-H-CO2]−). In addition, compounds 21, 23, 24, 26, 27, 28, 29, and 30 showed similar MS data, thereby suggesting that these analytes were also depsidones. Another classic example was the unknown compound

![Fig. 5](Image)

The cleavage pathways of lobaric acid and menegazziaic acid.
(25), which displayed the \([M - H]^−\) precursor ion at \(m/z\) 373.0565 \(\text{C}_{18}\text{H}_{14}\text{O}_{9}\). It yielded abundant product ions at \(m/z\) 355 and \(m/z\) 327 corresponding to the continuous loss of \(\text{H}_2\text{O}\) and \(\text{CO}\) molecules, and this was in close agreement with the fragment pathway of depsidones (Fig. 5B). Consequently, compound 25 was identified as menegazziaic acid based on the abovementioned evidence. In a similar manner, compounds 20 and 22 exhibited similar fragmentation pathways to produce \([M-H-\text{H}_2\text{O}]^−\) and \([M-H-\text{H}_2\text{O}-\text{CO}]^−\) fragments, respectively, in the negative mode.

### 3.3.4. Mono-substituted phenyl rings

The mono-substituted phenyl rings displayed specific and abundant \([M - H]^−\) quasi-molecular ions, of which the MS/MS fragmentation featured successive losses of \(\text{CO}\) and \(\text{H}_2\text{O}\) as illustrated by the representative compound 33 shown in Fig. 6. The compound 33 \(\left(t_R = 5.56 \text{ min}\right)\) exhibited the \([M - H]^−\) precursor ion at \(m/z\) 167.0357. The typical product ions at \(m/z\) 149 \([\text{M-H-H}_2\text{O}]^{+}\), 123 \([\text{M-H-CO}_2]^{+}\), and 105 \([\text{M-H-CO}_2-\text{H}_2\text{O}]^{+}\) were observed in the HRMS data. Thus, the compound 33 was tentatively assigned as orsellinic acid after comparison with the data in the literature. Likewise, the compounds 34, 35, 36, 37, and 38 were tentatively characterized based on the HRMS information, database searching sets, differentiated retention behavior, and previous literatures.\(^{36}\) By comparing with the MS data and the results of previous studies, compounds 34, 35, 36, 37, and 38 were tentatively assigned as 3-butyryl-β-resorcylic acid, everninacid, 2,6-dihydroxy-4-methylbenzoic acid, respectively.

### 3.4. Semi-quantitative analysis of Usnea samples

To overcome the serious restriction due to lack of reference standards, a feasible semi-quantitative approach on the single herb was developed to evaluate the differences in the qualities of Usnea samples. It was reported that semi-quantitative analysis was successfully used to assess the quality of different batches of the Niuhuang Shangqing pill;\(^{38}\) this analysis could compare the differences between the qualities of various batches. Thus, a semi-quantitative assay was introduced to appraise the similarities and differences in the qualities of the 25 batches of Usnea samples obtained from different sources. At the same time, the main chemical markers affecting the quality of this herb were checked out. In regard to semi-quantification of Usnea samples, peaks of 38 compositions in the 25 batches were identified and then, their absolute peak areas were recorded in succession. Meanwhile, sample no. 1 was selected as the reference sample based on the previous quantitative study by HPLC-ESI-MS/MS.\(^{22}\) After the absolute peak areas of 38 compounds in the other 24 samples were respectively divided by those in sample no. 1, the relative peak areas (RPAs) were obtained. The absolute peak areas and the relative peak areas of 38 compounds are presented in Tables S1 and S2,\(^{†}\) respectively.

To further evaluate the quality differences and find the possible characteristic components in Usnea samples, PCA was performed on the basis of the calculated RPAs, and the results exhibited remarkable group segregations and potentially significant components. The first two principle components (PC1 and PC2) with more than 75.2% of the total variance were extracted for analysis; among them, PC1 accounted for 56.9% of the variance and PC2 accounted for 18.3% of the variance. As seen in Table S3,\(^{†}\) PC1 included strong contributions from most compounds except the compounds 3, 8, 13, 21, 22, and 29; PC2 was directly correlated with the compounds 3 and 8. Consistent with the above-mentioned direct data, a graphical illustration is also presented in Fig. 7A. Compound 5 and other compounds such as 1, 2, 6, 7, 9, 11, 12, 18, 20, 24, 25, 27, 32, and 34 were located apart from the above-mentioned components.

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**Fig. 6** The cleavage pathway of orsellinic acid.

**Fig. 7** Loading plots (A) and score plots (B) for the compounds of Usnea longissima Ach. obtained from different origins.
results suggested that the compounds 1, 2, 6, 7, 9, 11, 12, 18, 20, 24, 25, 27, 32, and 34 might be the possible diagnostic chemical markers in Usnea samples like usnic acid (5). Pharmacological experiments have proved that usnic acid is a natural active ingredient in Usnea and a broad-spectrum anti-bacterial component. Thus, the consideration of the compounds together with usnic acid as quantitative indicators for further quality control of Usnea herbs is very meaningful.29,40

As can be seen clearly in Fig. 7B, all the samples were clearly clustered in three domains. Samples S1–S7 and S17–S19 were in circle A (from Sichuan province in China), samples S8–S12 and S20–S22 were in circle B (from Yunnan province in China), and samples S13–S16 and S23–S25 were in circle C (from Zhejiang province in China). The results showed that there was a quality difference in Usnea herbs obtained from different origins. Moreover, the circle D characterized by positive scores on PC1 was located apart from circle B, which suggested potential similarities between the qualities of Usnea samples obtained from the Yunnan province and Sichuan province. It was also plausible that the place of origin could have an impact on the quality of Usnea.

4. Conclusions

A novel analysis strategy based on UHPLC-Q-TOF-MS/MS integrated with multivariate statistical analysis methods was developed and used for the holistic quality control of Usnea herbs. The fragmentation patterns of phenolic acids in Usnea were summarized for the first time. A total of 38 phenolic compounds (8 dibenzoﬂurans, 11 didepsides, 13 depsidones, and 6 mono-substituted phenyl rings) were identiﬁed. Then, a semi-quantitative analysis was performed in Usnea samples, and PCA was conducted to evaluate the quality of the Usnea samples. The results showed that there were obvious differences in the qualities of the Usnea samples obtained from different regions, and the main factors inﬂuencing these qualities were picked out. The proposed method provided a solution for the comprehensive quality assessment of not only Usnea herbs but also other TCMs.

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Conflicts of interest

The authors declare no competing financial interest.

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