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Determination of three monosaccharides in *Dendrobium denneanum* and *Dendrobium officinale* by UPLC-QQQ-MS

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Abstract
An ultra-high performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-QQQ-MS) method was established to simultaneously determine the content of three monosaccharides in *Dendrobium denneanum* and *D. officinale*, and construct a system with monosaccharide components as an indicator to evaluate the quality of *D. denneanum* and *D. officinale* from different sources. The results show that the three monosaccharide components have a good linear relationship within a certain concentration range, the correlation coefficients are all greater than 0.999, the stability, precision, and repeatability RSD are all less than 4.0%, and the sample recovery rate is 99.62%~102.31%, with RSD between 1.49% and 2.67%. Both *D. denneanum* and *D. officinale* polysaccharides contain three monosaccharide components: glucose, mannose, and galactose, but there are certain differences in content. The UPLC-QQQ-MS method established in this study is fast, accurate, efficient, and reproducible. It can provide a reference for the quality evaluation of *D. denneanum* and *D. officinale*, and provide technical support for the further development and utilization of its resources.

Keywords: *Dendrobium denneanum*; *D. officinale*; UPLC-QQQ-MS; monosaccharide

1. Introduction
Dendrobium is a rare Chinese traditional medicine with a long history of application. It is called the first of the nine great immortals in China by Taoists. It has the effects of nourishing yin, clearing heat, nourishing body fluid and nourishing stomach [1]. In recent years, Zhejiang, Yunnan, Guangdong, Guangxi, Hunan, Anhui and other provinces have actively developed the *Dendrobium* industry, and the *Dendrobium* industry has become a hot spot in China's traditional medicine industry [2]. According to the 19th volume of Flora of China, southern Sichuan (Changning, Emei Mountain, and Leshan) is one of the authentic producing areas of *Dendrobium*. At present, Xiema Township, Jiajiang County, under the jurisdiction of Leshan City, is the main producing area of Dendrobium. The main cultivated varieties include *Dendrobium denneanum*, *D. officinale*, etc. [3, 4]. However, *Dendrobium* has different plant sources [5]. Existing research, including chemical composition and ocular activity research, is mainly focused on *D. officinale*, *D. nobile* and other species listed in pharmacopoeia [6-11]. There are few researches on *D. denneanum*, so it is impossible to carry out effective quality evaluation. The establishment of exclusive quality standards has severely restricted the expansion and strengthening of the *Dendrobium* industry. Polysaccharides are one of the main active ingredients of natural medicines, and the analysis of the composition of polysaccharides is of great significance to explain the mechanism of action of polysaccharides in organisms [12, 13]. However, it is rare to compare of the composition of polysaccharides in *D. denneanum* and *D. officinale*. Therefore, *D. denneanum* produced form Leshan city was chosen as the research object in this study, and *D. officinale*, a species was used included in the Chinese Pharmacopoeia as the reference, using pre-column derivatization combined with ultra-high performance liquid chromatography-electrospray ionization triple quadrupole mass spectrometry (UPLC-QQQ-MS) technology to compare and analyze the monosaccharide composition of the same two kinds of *Dendrobium* polysaccharides after hydrolysis, and explore the composition and structural characteristics of different kinds of *Dendrobium* polysaccharides from the monosaccharide composition and content, and provide for the formulation of subsequent quality standards and the study of the relationship between spectrum efficiency reference.

2. Materials and methods
2.1. Apparatus
Agilent Technologies 1290 Infinity ultra-high performance liquid chromatograph in tandem

with Agilent 6460 triple quadruple mass spectrometer (Agilent, USA); 1/100,000 electronic balance (Sartorius, USA); Eppendorf 5810R high-speed centrifuge (Eppendorf, Germany).

2.2. Materials and reagents

A total of 6 batches of *D. denneanum* and *D. officinale* samples were collected on-site in their main production areas. It was identified as the dried stems of *D. denneanum* Kerr and *D. officinale* Kimura *et al*. Migo.

D-mannose, D-glucose, D-galactose, formic acid, 1-phenyl-3-methyl-5-pyrazolone (PMP) were purchased from Simga, USA; acetonitrile of chromatographic grade and ethanol of reagent grade were purchased from Merck, Germany; ultrapure water was prepared with Milli-Q™ purification system from Millipore, USA.

2.3. Chromatography and mass spectrometry conditions

Column Waters BEH C18 (1.7μm, 2.1 × 100 mm) and guard column Van Guard™ (BEH, C18, 1.7μm, 2.1 × 5 mm); mobile phase: water (containing 0.01% formic acid) (A)-acetonitrile (B), gradient elution (0~2 min, 5% B; 2~25 min, 5%~25% B); column temperature 30^0, flow rate 0.2 mL·min-1, injection volume 1μL.

Multiple reaction monitoring (MRM) detection was adopted for quantitative analysis in ionization mode ESI^+; ionization temperature (TEMP) was 550^0; GaTP1 was 379.2kPa (55 psi), Gas2 was 379.2kPa (55 psi); curtain gas was s 241.3kPa (35 psi). The detection ion pair, Declustering voltage (DP) and collision voltage (CE) were optimized at the same time. The optimized mass spectrometry detection condition parameters of the three target components were shown in Table 1

Table 1: The parameters of optimized mass spectrometry conditions in the determination of three components

Components	tR/min	Molecular weight	MRM Parameters			
			Precursor ions	Product ions	Impact voltage (V)	Declustering voltage (V)
D-Mannose	6.85	180.16	511.22	175.10/217.10	30	175
D-Glucose	9.96	180.16	511.22	175.10/217.10	35	170
D-Galactose	11.34	180.16	511.22	175.10/217.10	25	170

2.4. Preparation of the reference solution

The three monosaccharide reference substances of D-galactose, D-mannose, and D-glucose (1.0 mg each) were accurately weighed and placed in 1 mL volumetric flasks. The volume was made up with ultrapure water to prepare 1.0 $g \cdot L^{-1}$ reference substance stock solution. Each stock solution (0.1 mL) was accurately pipetted, and distilled with ultrapure water (1.0 mL) to prepare monosaccharide mixed standard solution with a final concentration of 100 $mg \cdot L^{-1}$ for later use.

2.5. Preparation of test solution

2.5.1. Extraction and hydrolysis of Dendrobium polysaccharide

About 0.1 g of *D. denneanum* and *D. officinale* sample powder (passed through the No. 3 sieve) was accurately weighed, placed in a Soxhlet extractor, added with 80% ethanol (50 mL), heated and refluxed for extraction for 4 hours, and the ethanol solution was discarded. The ethanol of the dregs was evaporated to dryness, the filter paper cylinder was disassembled and placed in a beaker, added with 100 mL water, decocted for 1 hour, stirred constantly, let it cool, added with water to make up to about 100 mL, mixed well, centrifuged, and set aside for later use.

The above polysaccharide solution (1 mL) was aspirated, placed in a 10 mL ampoule, and added with 3.0 $mol \cdot L^{-1}$ hydrochloric acid solution (0.5 mL), sealed, mixed, and hydrolyzed at 110^0 for 1 h. After allowing to cool, the solution was adjusted to a neutral pH value by 3.0 $mol \cdot L^{-1}$ sodium hydroxide solution, and the volume was fixed for use.

2.5.2. PMP derivatization of reference substance and Dendrobium sample

The three monosaccharide mixed standard solutions were serially diluted to a mass concentration of 0.01~1000 $\mu g \cdot L^{-1}$. The mixed standard solution (400μL), PMP solution (400μL) and 0.3mol/L sodium hydroxide solution (400μL) were shaken and mixed thoroughly, and then placed in an oven at 70 °C for 100 min. After the reaction, 0.3mol/L hydrochloric acid (500μL) was added to the above solution. After mixing, the solution was added with 2 mL chloroform solution. After shaking and mixing thoroughly, the solution was stood for 5-10 minutes. After the upper liquid was repeatedly washed with chloroform for 2~3 times, it was passed through a 0.22μm microporous membrane for later use.

The *D. denneanum* and *D. officinale* polysaccharide hydrolysate (400μL)under the item 2.5.1 was precisely pipetted, and derivatized in the same manner as above, the derivatized *Dendrobium* sample test solution was obtained.

3. Results

3.1. Linear relationship and sensitivity test

Each 1μL reference solution was used for sample determination under the conditions of chromatography-mass spectrometry, and the peak area of the reference substance and the mass concentration of the reference substance were used to draw a calibration curve to determine the regression equation, correlation coefficient and linear range. The signal-to-noise ratio (S/N) 3 was set as the detection limit (LOD = 3), the signal-to-noise ratio (S/N) 10 was set as the quantification limit (LOQ = 10). The results were shown in Table 2. The regression line correlation coefficients (R2) of the three monosaccharides all were more than 0.999, showing a good linear relationship.

Table 2: Linear range, regression equation and correlation coefficient of three monosaccharides

Monosaccharides	Regression equations	R^2	LOD (μg/mL)	LOQ (μg/mL)
D-Mannose	$y=1.2\times10^5+17.5$	0.9998	0.21	0.61
D-Glucose	$y=1.7\times10^5+20.4$	0.9999	0.35	0.97
D-Galactose	$y=1.3\times10^5+30.3$	0.9998	0.18	0.49

3.2. Precision test
The derivatization solution (1μL) of the mixed reference substance of a certain concentration was precisely pipetted, and injected 6 times under the chromatographic-mass spectrometry conditions of "1.3". By measuring the peak area of each reference substance, the RSD of the peak area of the three reference substances was calculated. The range was 1.66%~1.89%, indicating that the instrument has good precision.

3.3. Repeatability test
Six samples of TP1, each 0.1 g, were accurately weighed. The test solutions of 6 *Dendrobium* samples were prepared in parallel as described in "1.5", and then determined according to the conditions of chromatogram-mass spectrometry under "1.3". The RSD of the content of the three target components was calculated, which was 2.58%~3.67%, indicating the method has good repeatability.

3.4. Stability test
The test solution of the TP1 sample was determined at 0h, 2h, 4h, 8h, 12h, and 24h under the conditions of chromatography-mass spectrometry of item "1.3". The RSD of the peak areas of the three target components was calculated, and the range was 2.17%~2.82%, indicating that the test solution was stable within 24 hours.

3.5. Recovery test
Six aliquots of TP1 samples are accurately weighed, each 1.0 g, and a reference substance equivalent to the content of each component in the 1.0 g sample was added. The test solution was prepared according to the method under "1.5" and injected for determination. The recovery was calculated, and the average recovery rate of the four components was 99.62%~102.31% with RSD less than 2.67%, indicating that the recovery rate of the method was good.

Table 3: Results of the method validation

Monosaccharides	Precision	Stability	Repeatability	Recovery (*n*=3)	
	RSD (%) (*n*=6)			Average	RSD (%)
D-Mannose	1.66	2.82	3.26	99.62	2.67
D-Glucose	1.89	2.17	2.58	102.31	1.82
D-Galactose	1.76	2.78	3.67	100.36	1.49

3.6. Sample determination
The composition and content of the three monosaccharides in 6 batches of samples were determined by using the UPLC-QQQ-MS method. The results were shown in the Figure 1 and Table 4.

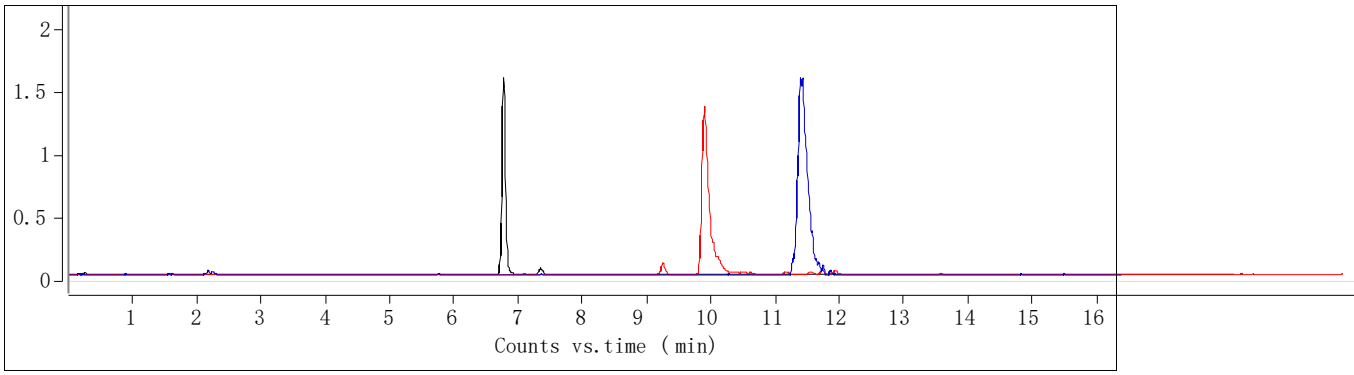

Fig 1: MRM chromatogram of the three monosaccharides in the test solutions

The results showed that both *D. denneanum* and *D. officinale* contained mannose, glucose and galactose. At the same time, among the monosaccharide components of *D. denneanum*and *D. officinale* polysaccharides, mannose has the highest content, with an average content of 267.8 mg/g, followed by glucose (175.3 mg/g) and galactose (31.9 mg/g).

Table 4: The content of three monosaccharides in *D. denneanum* and *D. officinale*

Species	No.	Collection location and time	Contents (mg/g)		
			D-mannose	D-glucose	D-galactose
D. officinale	TP1	Shitai County, Anhui Province, 2019	275.2 ± 6.9	145.2 ± 3.7	23.2 ± 0.6
	TP2	Qimen County, Anhui Province, 2019	338.6 ± 8.6	174.7 ± 4.4	16.3 ± 0.4
	TP3	Huoshan County, Anhui Province, 2018	362.7 ± 9.1	197.6 ± 4.8	32.7 ± 0.8
D. denneanum	DQ1	Jiajiang County, Sichuan Province, 2018	214.3 ± 5.3	163.2 ± 4.1	36.4 ± 0.9
	DQ2	Jiajiang County, Sichuan Province, 2017	193.5 ± 4.6	164.9 ± 4.2	35.2 ± 0.9
	DQ3	Jiajiang County, Sichuan Province, 2017	222.4 ± 5.6	206.2 ± 5.1	47.6 ± 1.2

4. Discussion
The pretreatment method of the test sample was referred to the item of *D. officinale* in the Chinese Pharmacopoeia, but the chromatographic conditions are slightly improved. The peaks of each monosaccharide derivative were well separated, which did not affect the determination of monosaccharides. The *Dendrobium* polysaccharides were Derivatization with PMP by pre-column, and then determined by UPLC-QQQ-MS. It had the advantages of simplicity, rapidity, sensitivity, accuracy, and good reproducibility, which provides a new reference for the qualitative identification of *D. officinale* and *D. denneanum* from different species and the quality evaluation of the corresponding medicinal materials.

The test results showed that, taking monosaccharides as the evaluation index, the content of mannose in *D. officinale* was higher, the content of galactose in *D. denneanum* was higher, and the content of glucose in the two *Dendrobium* species was basically the same. Although the content is different, the

samples of *D. officinale* and *D. denneanum* have detected three monosaccharides: mannose, glucose, and galactose. Therefore, two species from different provenances may be used instead. The established method of the present research uses the content of three monosaccharides in *D. denneanum* and *D. officinale* as indicators, which not only improves the accuracy of the analysis method, but also achieves the purpose of comparing the quality of *D. denneanum* and *D. officinale*. It has realized the association of the pharmacodynamic basic substances between *D. denneanum* and *D. officinale*, and provided strong support for the industrialization demonstration of traditional Chinese medicine such as cultivation technology, quality standards and new drug creation for the *Dendrobium* industry.

5. Acknowledgement

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6. Conflict of Interest

The author declares no conflict of interest.

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