Production area discrimination of *Ophiopogon Japonicus* using ^{1}H-NMR fingerprints and stoichiometric method

Abstract: Objective: Compared the nuclear magnetic resonance (NMR) fingerprint spectra of *Ophiopogon Japonicus* from three planted regions in Sichuan province and used multiple stoichiometric methods to distinguish these medicinal material's production areas. **Methods:** The ^{1}H-NMR data of three *Ophiopogon Japonicus*-producing areas in Sichuan province were obtained. Three stoichiometric methods---similarity analysis, clustering analysis, and principal component analysis (PCA) were used to analyze these 1H-NMR characteristics. Results: There were significant differences in *Ophiopagon japonicus* from the three production areas. Samples in distinct regions of the same large production area could also observe a slight divergence. The similarity analysis, clustering analysis, and PCA analysis results consistently indicated that NMR spectroscopy combined with chemical measurement analysis could identify the production areas of medicinal material. **Conclusion:** This study established a method of determining the *Ophiopogon Japonicus*'s district based on the 1H-NMR fingerprint map and stoichiometric methods. The technique was relatively simple and could serve as a basis for identifying the production areas of *Ophiopogon Japonicus*, providing a reference for developing and utilization of *Ophiopogon Japonicus*.

Keywords: *Ophiopogon Japonicus (*Linn.f.) Ker-Gawl.; ^{1}H-NMR fingerprints; identification of production areas; stoichiometric methods;

1 INTRODUCTION

Ophiopogon Japonicus(Figure 1)is a dried root of *Ophiopogon Japonicus* (Linn. f.) Ker-Gawl. in the Liliaceae family[1]. It is first recorded as the top grade in the "Shennong Materia Medica Classic." As a commonly used Traditional Chinese Medicine, it reinforces quenching thirst, moisturizes the lungs, relieves cough, and nourishes the stomach[2–4]. *Ophiopogon Japonicus* is widely distributed in China. Its main production areas are in Sichuan and Zhejiang provinces, respectively known as "Chuanmaidong" and "Zhemaidong." Among them, the most significant output and market circulation is the former. "Chuanmaidong" is mainly produced in the Minjiang River Basin in Sichuan province, especially in the Santai *County* of Mianyang city [5]. It is also cultivated in other areas of Sichuan, such as Dazhou and Guang'an City, *et al.*

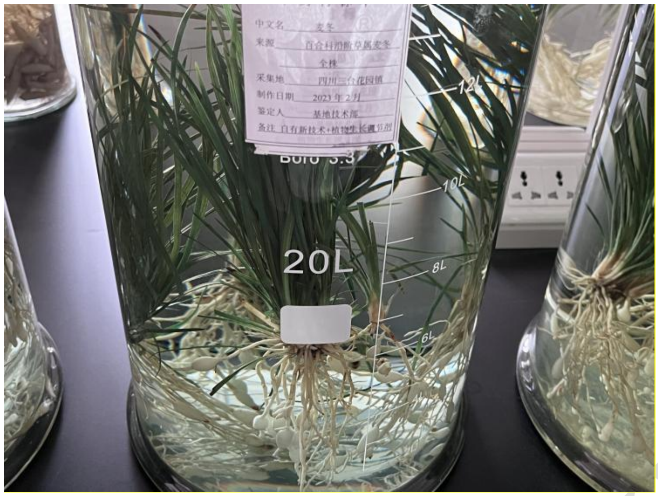

Figure 1*Ophiopogon Japonicus*specimen

Nuclear magnetic resonance (NMR) spectroscopy is an analytical method to study the absorption of radio frequency radiation by atomic nuclei in a strong magnetic field to obtain information about the molecular structure of compounds [6]. The chemical shift, number of resonance peaks, relative intensity, and other parameters of protons obtained by ^{1}H-NMR can be used to determine the structure of related chemical components in Traditional Chinese Medicine. Under standard extraction and separation methods, there is an accurate correspondence between ^{1}H-NMR spectra and plant varieties, which has the characteristics of singularity, comprehensiveness, reproducibility, and quantification [7-8]. Therefore, this technique has particular applicability in analyzing and identifying Traditional Chinese Medicine. With the development of technology, ^{1}H-NMR fingerprint technology has become increasingly mature in studying the effective ingredients of medicine herbs to identify and control the quality of Traditional Chinese Medicine internationally[9-10].

As the diversity of producing areas, the unstable quality of medicinal materials seriously affects the *Ophiopogon Japonicus* pharmacological effect. Although many researches have shown no significant differences in the types of ingredients and main pharmacological effects of "Chuanmaidong" from different production areas, the difference in composition content is substantial[11]. At present, the identification of production areas for *Ophiopogon Japonicus* is mainly based on ultraviolet spectroscopy[12], infrared spectroscopy[13], and High-performance chromatography (HPLC) fingerprint[14].To comprehensively reflect the regional differences, this study constructed a practical and feasible method for determining this medicine herb's production areas by combining ^{1}H-NMR and various stoichiometric analysis methods. This method provides a theoretical basis for selecting high-quality production areas of *Ophiopogon Japonicus*.

2 MATERIALS AND METHODS

2.1 Instruments and reagents

FA2004 Electronic Balance (Ningbo Yinzhou Huafeng Instrument Factory); DHG-9053A electric drying oven (Shanghai Yiheng Scientific Instrument Co., Ltd.); Chinese herbal medicine grinder (Shanghai Dianjiu Traditional Chinese Medicine Machinery Manufacturing Co., Ltd.); B-220 constant temperature water bath pot (Shanghai Yarong Biochemical Instrument Factory); SHB - III circulating water multi-purpose vacuum pump (Zhengzhou Changcheng Science and Technology Industry and Trade Co., Ltd.); Bruker Avance 600 MHz nuclear magnetic resonance spectrometer (Brooke, Germany).

DMSO-d_6 (Energy Chemical Co., Ltd), Methanol (Chengdu Kelong Chemical Co., Ltd.).All reagents are analytically pure.

2.2 Materials

"Chuanmaidong" were collected from Mianyang City, Dazhou City, and Guang'an City in Sichuan Province. These materials were identified as dry root tubers of the plant *Ophiopogon Japonicus* (Linn. f.) Ker-Gawl by Professor Hongbo Jiang from Sichuan College of Traditional Chinese Medicine. The voucher was stored in the School of Lifescience and Engineering, Southwest University of Science and Technology. These samples (Table 1) were dried at 60 ℃ for 24 hours and stored in the dark at 4 ℃ for future use.

Table 1 Source information of *Ophiopogon Japonicus* samples

Sample number	Regional of city	Sample location	
		Longitude (East)	Latitude (North)
S1~S3	Santai *County* of Mianyang	104.941972	31.265016
S4~S6	Jiangyou city of Mianyang	105.10559	31.91277
S7~S9	Anzhou district of Mianyang	104.51463	31.47339
S10~S12	Qu *County* of Dazhou	106.967004	30.772231
S13~S15	Wusheng *County* of Guang'an	106.114281	30.499548

2.3 Methods

2.3.1 Samples preparation and ^{1}H-NMR determination

Took 3.0g of crushed and sieved *Ophiopogon Japonicus* powder through Pharmacopoeia No. 4 sieve, added 50ml of methanol, and refluxed in a water bath for 2 hours. After cooling at room temperature, the methanol was added to the original weight. Took 1ml filtered extraction solution and dried it at 60 ℃ under reduced pressure for 2 hours. Then, Add 0.8ml DMSO-d6 to dissolve the sample and transfer it to a 600M nuclear magnetic tube for detection.

2.3.2 Similarity analysis

Sample similarity analysis based on angle cosine method: Each fingerprint is treated as an N-dimensional space vector (x_1, x_2, x_3,..., x_n) composed of numerical values of peak areas corresponding to a set of peaks. If X=X, there are two vectors, X and Y, simultaneously. The angle cosine value between them is calculated using the following formula.The cos θ A value close to 1 indicates a higher similarity.

$$\cos\theta = \frac{\sum_{i=1}^{n} X_i Y_i}{\sqrt{\sum_{i=1}^{n} X_i^2} \cdot \sqrt{\sum_{i=1}^{n} Y_i^2}}$$

Import the ^{1}H-NMR spectrum into the software MestReNova (version 6.1.1-6384, Mestrelab Research S.L.). Use δ 0.02 integral segment to perform segmented integration from 0 to 11.00. The residual water peak and residual dimethyl sulfoxide peak are not integrated. The integration matrix is imported into Excel and software IBM SPSS Statistics 20 (version 20.0.0.0, IBM Corp.) for similarity evaluation.

2.3.3 Cluster analysis

Cluster analysis uses "similarity" to measure the degree of familiarity between samples and classify them accordingly. Firstly, each sample in the set is individually defined as a class, and then the distance or similarity measure between the samples is defined. The samples with high similarity were classified into one category, and those with low similarity were classified into different classes after the clustering analysis [15]. In this experiment, the ^{1}H-NMR data of 15 batches of "Chuanmaidong" samples were used as variables, and the integration matrix was imported into the software IBM SPSS Statistics 20 for clustering analysis. The Squared Euclidean distance coefficient was used as a measure, and the between-groups linkage method was used for clustering.

2.3.4 Principal Component Analysis

Principal Component Analysis (PCA) transforms a set of variables that may correlate with a group of linearly unrelated variables through orthogonal transformation. The resulting set of variables is called principal components [16]. This experiment uses SIMCA (version 14.1, Umetrics) software to plot the ^{1}H-NMR spectra of samples as variables and the Principal Component Vector as the coordinate axis, reflecting the differences between different categories.

3 RESULTS AND DISCUSSION

3.1 Similarity analysis

Figure 2 shows the NMR chromatogram of the sample S2. According to the traditional angle cosine method, Similarity analysis of the integral matrixshowed high similarity in ^{1}H-NMR spectra of *Ophiopogon Japonicus* from the production areas of Mianyang City (Santai *County*, Jiangyou City, Anzhou District) (Table 2). However, Mianyang's similarity significantly differs from the production areas of Dazhou and Guang'an. It indicates that similarity analysis can be used to trace the source of production areas, which is of great significance for ensuring the stability of product quality.

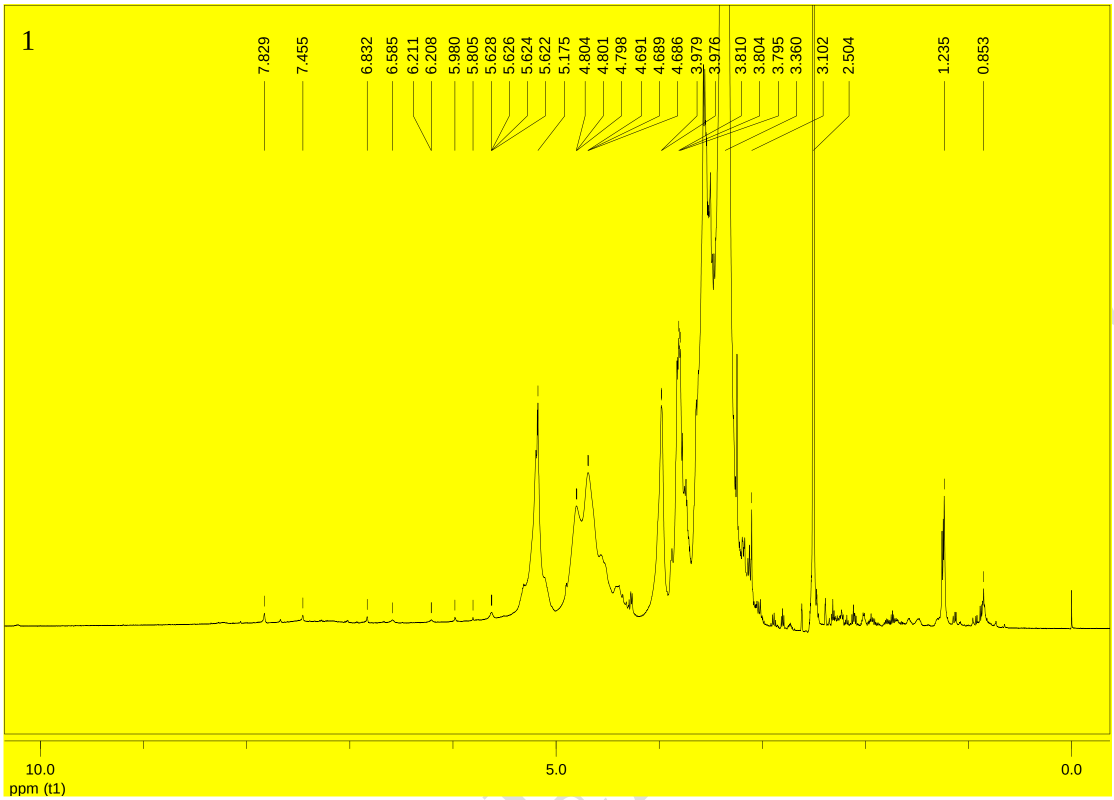

Figure 2 ^{1}H-NMR chromatogram of Sample 2

Table 2 The ^{1}H-NMR spectrum similarity table of samples

Sample source and number		Santai *County* of Mianyang			Jiangyou City of Mianyang			Anzhou district of Mianyang			Qu *County* of Dazhou			Wusheng *County* of Guang'an		
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Santai *County* of Mianyang	S1	1.000	0.995	0.999	0.987	0.982	0.990	0.992	0.988	0.991	0.956	0.956	0.956	0.975	0.975	0.974
	S2	0.995	1.000	0.997	0.986	0.983	0.987	0.993	0.988	0.992	0.938	0.938	0.938	0.960	0.960	0.959
	S3	0.999	0.997	1.000	0.988	0.983	0.990	0.994	0.989	0.993	0.951	0.951	0.951	0.970	0.970	0.969
Jiangyou city of Mianyang	S4	0.987	0.986	0.988	1.000	0.999	0.998	0.982	0.972	0.979	0.957	0.957	0.957	0.973	0.973	0.973
	S5	0.982	0.983	0.983	0.999	1.000	0.995	0.978	0.964	0.974	0.951	0.951	0.951	0.968	0.968	0.968
	S6	0.990	0.987	0.990	0.998	0.995	1.000	0.984	0.977	0.982	0.960	0.960	0.960	0.976	0.976	0.976
Anzhou district of Mianyang	S7	0.992	0.993	0.994	0.982	0.978	0.984	1.000	0.996	0.999	0.959	0.959	0.959	0.973	0.973	0.972
	S8	0.988	0.988	0.989	0.972	0.964	0.977	0.996	1.000	0.998	0.954	0.954	0.954	0.967	0.967	0.966
	S9	0.991	0.992	0.993	0.979	0.974	0.982	0.999	0.998	1.000	0.957	0.957	0.957	0.970	0.970	0.969
Qu *County* of Dazhou	S10	0.956	0.938	0.951	0.957	0.951	0.960	0.959	0.954	0.957	1.000	1.000	1.000	0.991	0.991	0.991
	S11	0.956	0.938	0.951	0.957	0.951	0.960	0.959	0.954	0.957	1.000	1.000	1.000	0.991	0.991	0.991
	S12	0.956	0.938	0.951	0.957	0.951	0.960	0.959	0.954	0.957	1.000	1.000	1.000	0.991	0.991	0.991
Wusheng *County* of Guang'an	S13	0.975	0.960	0.970	0.973	0.968	0.976	0.973	0.967	0.970	0.991	0.991	0.991	1.000	1.000	1.000
	S14	0.975	0.960	0.970	0.973	0.968	0.976	0.973	0.967	0.970	0.991	0.991	0.991	1.000	1.000	1.000
	S15	0.974	0.959	0.969	0.973	0.968	0.976	0.972	0.966	0.969	0.991	0.991	0.991	1.000	1.000	1.000

3.2 Cluster analysis

The cluster analysis (Figure 3) can classify the *Ophiopogon Japonicus* samples from the three major production areas on the ^{1}H-NMR spectra. The cluster tree graph shows a cross between the Mianyang region (Santai *County*, Jiangyou City, and Anzhou District), which cannot be classified. This phenomenon indicated that the chemical composition differences of *Ophiopogon Japonicus* in the above regions are relatively small. The clustering analysis results are consistent with the similarity analysis results.

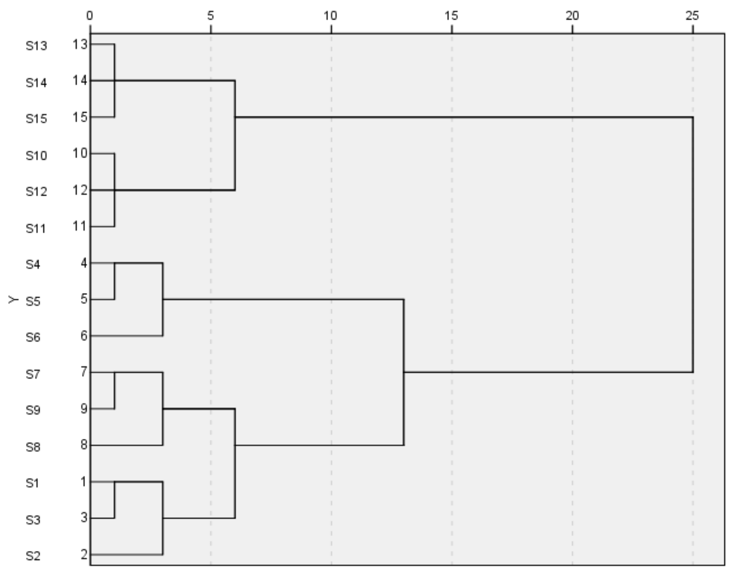

Figure3 Cluster analysis diagram

3.3 Principal Component Analysis (PCA)

Figure 4 shows that the PCA model can clearly distinguished the materials of *Ophiopogon Japonicus* from Dazhou, Guang'an, and Mianyang. However, the samples from small production areas in Mianyang cannot be clearly distinguish. By magnifying the scatter analysis of the principal components of the samples in the Mianyang area, it can be seen that there are slight differences in various small production areas in Mianyang. This analysis result is consistent with similarity and cluster analysis results.

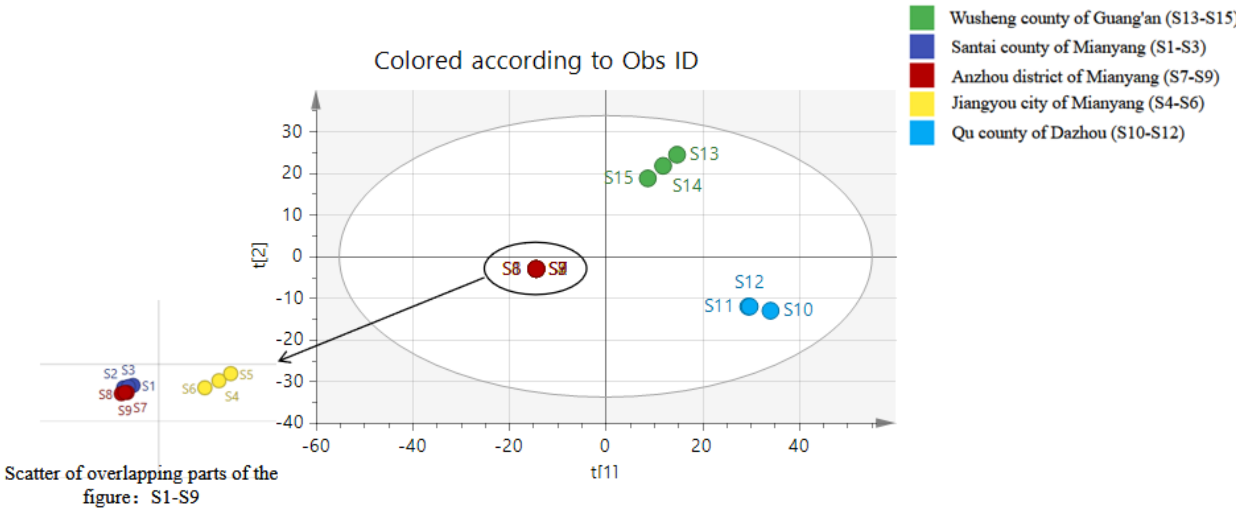

Figure 4 Principal Component Analysis scatter plot diagram

4 Conclusion

This study established ^{1}H-NMR spectra of "Chuanmaidong" from three production areas: Mianyang, Dazhou, and Guang'an. A comparative study was conducted using stoichiometry and combining IBM SPSS Statistics and SIMCA-P analysis software. The similarity of the spectra of *Ophiopogon Japonicus* from the same source was high, indicating that the chemical composition from the similar origin was consistent and the quality was stable. A quality control method for *Ophiopogon Japonicus* based on ^{1}H-NMR was established by rapid clustering identification of *Ophiopogon Japonicus* from different regions. This method has the advantages of accuracy, convenience, speed, and objectivity, making it suitable for the rapid identification of production areas of *Ophiopogon Japonicus* samples. Compared to the segmented integration comparison method reported in the literature [17-18], this method has higher reliability and practicality.

In summary, the nuclear magnetic fingerprint used in this study for identifying the production areas of *Ophiopogon Japonicus*provides a new approach to selecting high-quality production areas for *Ophiopogon Japonicus* of Sichuan province.

ACKNOWLEDGEMENT

This research was supported by the Undergraduate Innovation Fund Project of Southwest University of Science and Technology(CX23-046, CX23-017 amd JZ23-060).

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