SUPPLEMENTARY MATERIAL

HPLC fingerprint analysis of polysaccharides from different accessions of *Polygonatum odoratum*

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ABSTRACT

Polysaccharide was one of the considered major active ingredient in *Polygonatum odoratum* which was crucial for its quality evaluation. In this study, High performance liquid chromatography (HPLC) combined with chemometrics methods were performed to assess the quality of *P. odoratum* polysaccharide (POP) harvested from different locations. The methodology validation and similarity evaluation results showed that the analysis method was able to meet the requirement of fingerprint analysis, and 10 batches of POPs had a high degree of similarity based on the similarity values were greater than 0.960. The results of hierarchical cluster analysis (HCA) showed that different regions POPs could be classified by clustering analysis based on their nuances. The results of principal component analysis (PCA) showed that the mannose (58.13%~78.18%) and glucuronic acid (2.36%~11.72%) could be selected as herb markers for the quality control of *P. odoratum*. In conclusion, a more quantitative quality control method was established, and could be applied to the identification and quality control of different *P. odoratum* and their products.

Keywords: *Polygonatum odoratum*; Polysaccharide; Fingerprint analysis; High performance liquid chromatography

3. Experimental

3.1 Plant material

Ten batches of the root of *P. odoratum* were collected from different regions of China. Samples were collected by randomized sampling techniques. Professor Yuguang Zheng (College of Pharmacy, Hebei University of Chinese Medicine, China) did the authentication of species. The root of *P. odoratum* was long cylindrical, slightly flat, light yellow-brown on the surface, translucent, with longitudinal wrinkles and slightly raised links, with white dot-like fibrous root marks and disc-like stem marks. Microscopic observation revealed that the epidermal cells in the cross-section were oblate or oblong, with a slightly thicker outer wall and keratinized. There are many mucous cells scattered in the parenchyma, containing calcium oxalate needle crystal bundles. These were consistent with the Pharmacopeia of P. R. China (Chp 2020) description.

Ten batches of the root of *P. odoratum* were kept in the herbarium, and accession numbers B2019102401 (*P. odoratum*-1, Huake Ecological Agriculture Development Co., Ltd., Changsha, Hunan), B2019102402 (*P. odoratum*-2 Hebei Meiwei Pharmaceutical Co., Ltd., Yiyang, Hunan), B2019102403 (*P. odoratum*-3, Beijing Tongrentang (Anguo) Chinese Medicine Decoction Co., Ltd., Shaoyang, Hunan), B2019102404 (*P. odoratum*-4, MinxianHetai Chinese Medicine Co., Ltd., Shaoyang, Hunan), B2019102405 (*P. odoratum*-5, Hangzhou Longmo Biological Technology Co., Ltd., Shaoyang, Hunan), B2019102406 (*P. odoratum*-6, Chengdu Benzhen Biomedical Technology Co., Ltd., Shaoyang, Hunan), B2019102407 (*P. odoratum*-7, Guangzhou Gulefu Food Co., Ltd., Panyu, Guangdong), B2019102408 (*P. odoratum*-8, Tongfu Ginseng Products Co., Ltd., Shaoyang, Hunan), B2019102409 (*P. odoratum*-9, Guangdong Fengchun Pharmaceutical Co., Ltd., Shaoyang, Hunan) and B2019102410 (*P. odoratum*-10, AnguoYaoyuan Trading Company, Yiyang, Hunan)were obtained from Herbarium, College of Pharmacy, Hebei University of Chinese Medicine.

3.2 Experimental reagents and materials

Glucose (Glc), galactose (Gal), mannose (Man), arabinose (Ara), xylose (Xyl), fucose (Fuc), rhamnose (Rha), glucuronic acid (GlcA), and galacturonic acid (GalA) were purchased from Sigma-Aldrich Co. (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP) and Trifluoroacetic acid (TFA) were obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). HPLC-grade acetonitrile was purchased from America Tedia Co. The water for HPLC

analysis was purified by a Milli-Q water purification system (Merck & Co Inc., USA). All the other reagents were of analytical-reagent grade or HPLC-grade.

3.3 Extraction of polysaccharides

The extraction of the polysaccharide of *P. odoratum* (POP) was performed according to the reported method with some modifications (Jing et al. 2018). Briefly, the root of *P. odoratum* was pulverized and sifted through and 40-mesh sieve for getting the powder. The powder (50 g) was immersed in ethanol (1:20, w/v) reflux extraction for 2 h to defat. The residues were dried and subsequently mixed with distilled water (1:30, w/v) in a round-bottomed flask. The solution was extracted in boiling water (100℃) and extracted 2 times (2 h each time). The extract was concentrated to one-fourth of its original volume under vacuum and then treated with ethanol (final concentration: 80%) for precipitation at 4℃ for 12 h. The precipitate was obtained by centrifugation (3000×g, 10 min, 4℃) and lyophilization. Finally, crude polysaccharides powder was coded asPOP-1 (Huake Ecological Agriculture Development Co., Ltd., Changsha, Hunan), POP-2 (Hebei Meiwei Pharmaceutical Co., Ltd., Yiyang, Hunan), POP-3 (Beijing Tongrentang (Anguo) Chinese Medicine Decoction Co., Ltd., Shaoyang, Hunan), POP-4 (MinxianHetai Chinese Medicine Co., Ltd., Shaoyang, Hunan), POP-5 (Hangzhou Longmo Biological Technology Co., Ltd., Shaoyang, Hunan), POP-6 (Chengdu Benzhen Biomedical Technology Co., Ltd., Shaoyang, Hunan), POP-7 (Guangzhou Gulefu Food Co., Ltd., Panyu, Guangdong), POP-8 (Tongfu Ginseng Products Co., Ltd., Shaoyang, Hunan), POP-9(Guangdong Fengchun Pharmaceutical Co., Ltd., Shaoyang, Hunan), POP-10 (AnguoYaoyuan Trading Company, Yiyang, Hunan), and the yield of POPs were calculated.

3.4 Validation of the method

The developed method was fully validated in terms of linearity and range, accuracy, precision, and stability. The calibration curve was established by calculation of the absorbance versus the concentration of each monosaccharide. The intra-day precision was evaluated by analyzing six replicate sample solutions within one day and the inter-day precision was examined in duplicates of sample solutions for three consecutive days. The relative standard deviation (RSD) was calculated as the measure of precision. Recovery was determined by analyzing the spiked samples. An aliquot of the sample (5.0 g) was extracted and analyzed according to the sample preparation procedure. A known amount of the standard (equivalent to 100% of the contents in the sample

solution) was added before the obtained solution was transferred to a 10-mL volumetric flask. Six replicates were used to calculate the recovery rate. The stability of the sample solution was evaluated by analyzing a newly prepared test solution at 0, 2, 4, 8, 12, and 24 h at room temperature (Long et al. 2016).

3.5 Analysis of monosaccharide composition

The monosaccharide composition analysis followed previous reports with some modifications (Sun and Zhou 2014). The reference standard solutions for each monosaccharide, including Man, Rha, GalA, GlcA, Glc, Gal, Xyl, Ara, and Fuc were dissolved in water at the concentration of 1.0 mg/mL. The derivatization of each monosaccharide was as follows: 1.0 mL of the reference standard solution were mixed with 0.4 mL of 0.3 mol/L sodium hydroxide and 0.4 mL of 0.5 mol/L methanolic solutions of PMP, then kept at 70℃ for 30 min. After cooling, the mixture was neutralized with 1 mL of 0.3 mol/L hydrochloric acid and diluted to 3 mL with distilled water. Then, 3 mL of chloroform was added and the organic phase was discarded after vigorous shaking and layering. The extraction was performed three times. Finally, the upper aqueous solution at the concentration of 0.5 mg/mL was passed through a 0.45 μm syringe filter for HPLC analysis. The linear relationship were Man (y=1045011405x-473531, R^2=0.9991), Rha (y=1112510301x-6708270, R^2=0.9990), GalA (y=826206937x-6325303, R^2=0.9997), GlcA (y=812777091x-5576823, R^2=0.9995), Glc (y=733967913x-5790716, R^2=0.9990), Gal (y=597067268x+5894322, R^2=0.9990), Xyl (y=792165256x+4464612, R^2=0.9996), Ara (y=1064274451x+1146610, R^2=0.9990), Fuc (y=1056099805x-19028636, R^2=0.9992).

20 mg of prepared polysaccharides was accurately weighed and hydrolyzed with 2 mol/L trifluoroacetic acid in a sealed tube at 120℃ for 4 h. After cooling, 2 mL of methanol was added to the hydrolyzates and the mixture was evaporated at 60℃ under vacuum to remove trifluoroacetic acid. The procedure was performed several times. The dried hydrolyzates were dissolved with 1 mL of water and derivatized as above of monosaccharides.

The analysis of PMP derivatives was performed on an Agilent 1260 series HPLC system. A 20 μL of derivatives was injected onto a ZORBAX Eclipse XDB-C18 HPLC column (250 mm × 4.6 mm i.d., 5 μm) (Agilent, USA) operated at 30 ℃, and eluted with a mixture of 0.1 M phosphate buffer (pH 6.7) and acetonitrile (83:17, v/v) at a flow rate of 1.0 mL/min. The UV detection wavelength was set at 245 nm. The monosaccharides were identified by comparing the

retention time with those of the reference substances.

3.6 Standard fingerprint profiles and evaluation of similarity

HPLC data of all samples were submitted for analysis by professional software named Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version2004A, China Pharmacopoeia Committee, Beijing, China). High-resolution chromatographic data is submitted to professional software to build standard fingerprints and assess the similarity of hydrolysates based on matching data relative retention times and areas, which can reveal differences in polysaccharide samples from different fractions or species.

3.7 Statistical analysis

To further analyze the fingerprint characteristics of polysaccharides in different regions and analyze the main components that affect their identification, this study used software IBM SPSS (Version 21.0, SPSS Inc., Chicago, Ill., USA) to perform hierarchical cluster analysis (HCA) on the data matrix formed by the relative peak areas of monosaccharides in the fingerprints. and principal component analysis (PCA), to be able to distinguish the *P. odoratum* samples.

References

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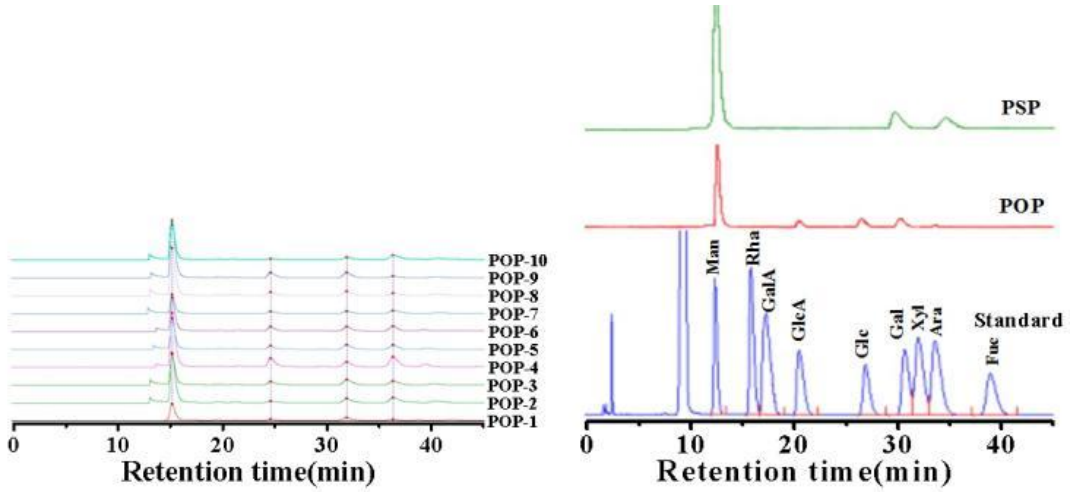

Figure S1. HPLC of 10 batches of POPs and PSP (a: 10 batches of POPs, b: POP, PSP and standard sample)

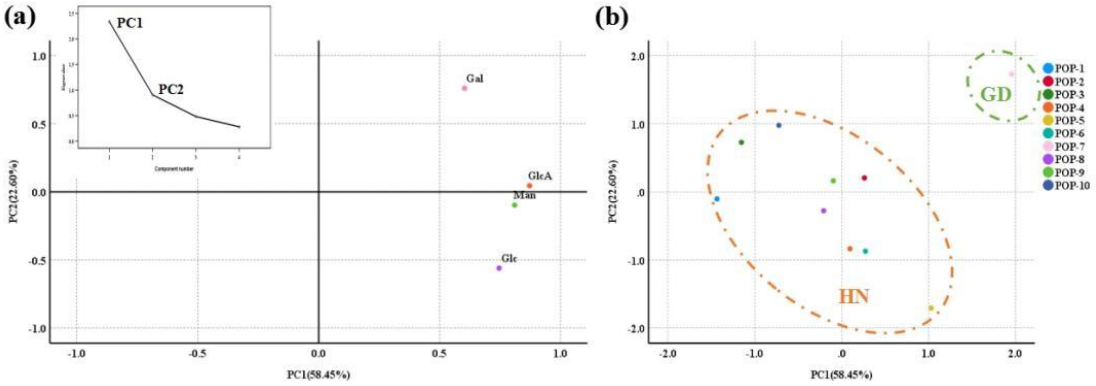

Figure S2. Loading plot (The inset was scree plot) (a) and plot of PCA scores (b)