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Specific targeted quantification combined with non-targeted metabolite profiling for quality evaluation of *Gastrodia elata* tubers from different geographical origins and cultivars

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ABSTRACT

Gastrodia elata tuber (GET) has been widely used as a famous herbal medicine in China and other East Asian countries. In this work, we developed a comprehensive strategy integrating targeted and non-targeted analyses for quality evaluation and discrimination of GET from different geographical origins and cultivars. Firstly, 43 batches of GET samples of five cultivars from three regions in China were efficiently quantified by a "single standard to determine multi-components" (SSDMC) method. Six marker compounds were simultaneously determined within 11 min using gastrodin as the internal standard. It showed that samples from different regions and cultivars could not be differentiated by the contents of six marker compounds. Secondly, a non-targeted metabolite profiling analysis was performed by ultrahigh-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS). Samples from different geographical origins and cultivars were clearly discriminated by principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). 147 discriminant ions contributing to the group separation were selected from 1194 aligned variables. Furthermore, based on the relative intensities of discriminant ions, support vector machines (SVM) was employed to predict the geographical origins of GET. The obtained SVM model showed excellent prediction performance with an average prediction accuracy of 100%. These results demonstrated that the UHPLC-QTOF/MS-based non-targeted metabolite profiling analysis, as a vital supplement to targeted analysis, can be used to discriminate the geographical origins and cultivars of GET.

1. Introduction

In regular quality evaluation of herbal medicines (HMs), a specifically targeted method is commonly applied, i.e. marker compounds are subjectively or sometimes a little arbitrarily selected and followed by qualitative and quantitative analyses. This quality evaluation mode is widely adopted in international pharmacopoeial monographs. As a result, the quality of tested sample is easily to determine as "qualified" or "unqualified" when the analytical data is directly compared with the presetting acceptance criteria. However, this is merely one side of the coin. Even for a qualified HM, a variety of quality-related parameters, e.g. harvesting, processing, geographical origin and cultivar are still puzzling the quality managers, since under these circumstances targeted analysis approach is not always feasible in assessing subtle difference in quality [1,2]. Furthermore, the chemical differences in a certain HM arising from geographical origins or cultivars may result in different pharmacological effects. Therefore, the comprehensive chemical composition analysis and the sample classification are of great importance for the quality evaluation of HMs. As an important complement to targeted analysis focusing on the selected marker compounds, non-targeted metabolite profiling analysis involving a broader scope of metabolites has been successfully applied to differentiate the geographical origins and cultivars of HMs and foods [3–8].

Gastrodia elata Blume is a saprophytic perennial plant belonging to Orchidaceae family and indigenous to the East Asia. *Gastrodia elata* tuber (GET), with the Chinese name of Tianma, is a famous HM used in China, Korea and Japan for the treatment of headaches, epilepsy, dizziness, rheumatism, neuralgia, paralysis, hypertension and other neuralgic disorders [9–13]. In recent years, GET has also been popularly regarded as a sub-material for food and food ingredients [14,15]. Phytochemical studies of GET have revealed the presence of phenolic compounds and their

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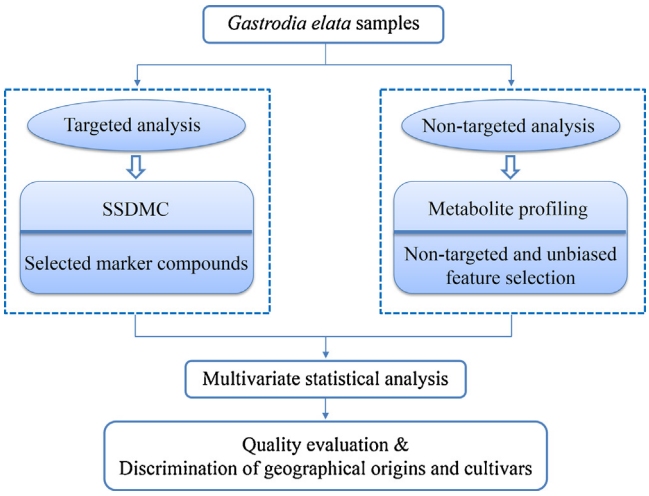

Fig. 1. A schematic illustration of the strategy based on targeted and non-targeted analyses for quality evaluation of GET.

glycosides, polysaccharide, organic acids and nitrogenous compounds [16–19]. Among these compounds, phenolic compounds, e.g. *p*-hydroxybenzyl alcohol (HA), gastrodin (GA) and parishins compounds have received more attentions because of their pharmacological features [9,20–23]. In Chinese Pharmacopoeia (2015 edition) [24], the sum of the content of GA and HA in GET is officially set as not less than 0.25 percent, while minimum 0.20 per cent of GA is required by European Pharmacopoeia [25].

In China, *G. elata* is mainly planted in the southwestern and central regions [15]. During the long history of cultivation, the species of cultivated *G. elata* are agronomically divided into six cultivars based on the colors of flower and scape [26], of which Wutianma (*G. elata* Bl. *f. glauca* S. Chow), Hongtianma (*G. elata* Bl. *f. elata* ex S. Chou et S. C. Chen), Lvtianma (*G. elata* Bl. *f. viridis* Makino) and Huangtianma (*G. elata* Bl. *f. flavida* S. Chow) are the four prevalent cultivars. Besides, the hybrid variety of Wutianma × Hongtianma is also cultivated in some areas [27]. Although many efforts have been devoted to quality evaluation of GET [27–30], further exploring works are still desired owing to the following reasons: (1) apart from GA, parishins were unfortunately neglected in those quantitative investigations; (2) the developed HPLC fingerprint analysis was not comprehensive since very limited constituents could be detected by ultraviolet (UV) detector; (3) the quality variation resulting from geographical origin and cultivar was far from being considered.

In this study, quality evaluation and precise discrimination of GETs from different geographical origins and cultivars was performed to deliver the strategy based on both targeted and non-targeted analyses. The schematic flow is illustrated in Fig. 1. For the purpose of quality evaluation, a single standard to determine multi-components (SSDMC) method based on HPLC was developed and fully validated, to simultaneously determine six marker phenolic compounds including GA, HA, parishin E (PE), parishin B (PB), parishin C (PC) and parishin (PA), using GA as internal standard. For the purpose of precise discrimination, a non-targeted UHPLC-QTOF/MS-based metabolite profiling approach was employed to analyze the intendedly collected GET samples. The data obtained by targeted and non-targeted analyses were both subjected to multivariate statistical analysis, including unsupervised principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA), in order to improve the interpretability of initial results. Support vector machines (SVM) was also used for classification and prediction.

2. Materials and methods

2.1. Chemicals and reagents

Standard compounds of GA, HA and PA were purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China), Aladdin-reagent Co., Ltd. (Shanghai, China) and Shanghai Pure-One Biotechnology Co., Ltd. (Shanghai, China), respectively. PB, PC and PE were purchased from Chengdu Biopurify Phytochemicals Co., Ltd. (Chengdu, China). Their structures are shown in Fig. 2. 4-Hydroxybenzoic acid (Chengdu Biopurify Phytochemicals Co., Ltd.) was used as the internal standard for MS analysis. Acetonitrile and formic acid of HPLC grade were purchased from ROE (Newark, New Castle, DE, USA), and HPLC-grade methanol was purchased from Jiangsu Hanbon Sci. & Tech Co., Ltd. (Nanjing, China). Deionized water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Other reagents and chemicals were of analytical grade.

2.2. Plant materials

Forty-three batches of freshly collected GET samples of different cultivars, including Hongtianma, Wutianma, Lvtianma, Huangtianma and Wutianma × Hongtianma were provided by the local farmers or manufactures from Hubei, Sichuan and Yunnan provinces of China in December 2014 (Table 1). In our laboratory, upon arrival, each samples were washed clean immediately, put into an electric steamer (Midea Group, Shunde, China) and heated for 10 min to replicate the steaming process used by farmers. Then samples were spread out, dried at 40 °C and ground into fine powder. All the procedures were strictly controlled to minimize the uncertain parameters during sample collection, storage and preprocessment.

2.3. Sample preparation and standard solution

Accurately weighed GET powders (500 mg) were extracted by ultrasonication for 30 min at 100 Hz with 50 mL of 50% methanol. Then the extracted solution was adjusted to the initial weight by adding 50% methanol as needed and was filtered. The filtrate was centrifuged for 10 min at 13000 rpm, and the supernatants were used as the sample solution for HPLC quantitative analysis. For MS analysis, the sample solution was diluted 10 times with 50% methanol and 1.12 μg/mL of 4-hydroxybenzoic acid was used as the internal standard.

The standard stock solutions for SSDMC were prepared by dissolving the six reference standards in 50% methanol to a final concentration of 0.3165 mg/mL for GA, 0.0995 mg/mL for HA, 0.6480 mg/mL for PA, 0.2618 mg/mL for PB, 0.1102 mg/mL for PC and 0.4070 mg/mL for PE. Then the stock solutions were diluted with 50% methanol to a series of appropriate concentrations for the construction of calibration curves.

2.4. SSDMC-based targeted analysis

2.4.1. Apparatus and chromatographic conditions

Analyses were primarily performed on an Agilent 1260 HPLC System (Agilent Technologies, Palo Alto CA, USA) comprising a diode-array detector. Additionally, a Waters 2995 HPLC System (Waters Corp, Milford, MA, USA) consisting of a photodiode array detector was used for the ruggedness test. Both of the two instruments were equipped with a quaternary solvent delivery system, an on-line degasser, an auto-sampler and a column temperature controller. The separation was carried out on an Agilent Zorbax SB-C_{18} column (4.6 mm i.d. × 50 mm, 1.8 μm, Agilent Technologies, USA). For the ruggedness test, another two columns was