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Comprehensive analysis of 61 characteristic constituents from Siraitiae fructus using ultrahigh-pressure liquid chromatography with time-of-flight mass spectrometry

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ABSTRACT

A systematic method was established for the qualitative and quantitative analysis of characteristic components such as triterpenoids and flavonols in Siraitiae fructus, a famous antidiabetic traditional Chinese medicine (TCM). For qualitative analysis, 61 characteristic components were identified using the ultrahigh performance liquid chromatography coupled with photo-diode array and quadrupole/time-of-flight mass spectrometry (UPLC-PDA-QTOF-MS/MS) based on a multiple product ions filtering (mPIF) strategy, of which 22 compounds were characterized for the first time from Siraitiae fructus. For quantitative detection, a relative quantitation assay using an extract ion chromatogram (EIC) of the full scan MS experiment was validated and employed to assess the quantity of the 61 identified compounds in 40 batches Siraitiae fructus samples from different sources. Additionally, the principal component analysis (PCA) indicated that 40 samples could be clustered into four groups and the cultivated variety was an important factor for sample classification. The methods used in present study might be also valuable for simultaneous investigation of multiple components from Siraitiae fructus for the purpose of holistic quality control, phytochemistry and metabolic studies.

1. Introduction

Siraitiae fructus (*Luo-Han-Guo*) belongs to the family of Cucurbitaceae, and is primarily cultivated in Guangxi Province of China. Siraitiae fructus is a well-known traditional Chinese medicine (TCM) which is considered to have potential immuno-regulating, anti-oxidation, anti-cancer, anti-obesity and anti-asthmatic effects [1,2]. The major chemical constituents of Siraitiae fructus included triterpenoids, flavonols, amino acids, nucleosides and polysaccharides; triterpenoids and flavonols were considered as the effective components [2–5]. It was difficult to isolate or identify the many triterpenoids with high polarity and/or weak chromophore groups in Siraitiae fructus by the conventional phytochemistry techniques. So far, only 30 major triterpenoids and 3 flavonols have been identified and isolated from Siraitiae fructus [3]. Although it was reported that the effects of Siraitiae fructus highly reply on the triterpenoids and flavonols at trace levels, the isolation of the trace level constituents was much more difficult that of the enriched ones [6,7]. In addition, as most of the unknown triterpenoids and flavonols were often available in trace amounts from Siraitiae fructus. Thus, it is extremely necessary to comprehensively profile the triterpenoids and flavonols, especially their trace components.

In previous studies, chemical profiles of Siraitiae fructus were evaluated by thin layer chromatography (TLC) [8], high-performance liquid chromatography with ultraviolet-visible detection (HPLC-UV) [9] and UPLC with quadrupole time-of-flight tandem mass spectrometry (UPLC-QTOF-MS) [2]. However, these methods were either time consuming or incapable of detecting trace level components from Siraitiae fructus. Additionally, only 20 triterpenoids were described in the above studies because of the limitation of standards for triterpene saponins [2,8,9]. In this study, a simple, rapid and sensitive UPLC-UV-QTOF-MS/MS method was developed by multiple product ions filtering (mPIF) strategy, which could simultaneously analyze 57 triterpenoids and 4 flavonols from Siraitiae fructus in a short time.

It was well accepted that the efficacy of TCM was based on the synergistic effects of their multi-components on multi-targets [10]. Therefore, quantification of the multi-components was necessary for comprehensive quality control of TCMs. Currently, only a limited number of compounds were studied because there were very few usable standards of reference compounds in TCMs. Nowadays, relative quantification was becoming an expedient strategy

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for comparative studies of multi-components in complex matrices of TCMs, which could avoid expensive and time-consuming separation for the standard substances [11]. In this study, based on the strategy of relative quantification, UPLC-QTOF-MS/MS was developed to simultaneously quantify 57 triterpenoids and 4 flavonols in 40 samples of Siraitiae fructus using the extracted ion chromatography (EIC) mode. Furthermore, chemometrics was also employed to analyze the complex quantitative data from different Siraitiae fructus samples.

In summary, the objective of this study was to propose a strategy for comprehensive quality control of TCMs. In this paper, Siraitiae fructus was selected as an example to illustrate the feasibility of the proposed strategy. Siraitiae fructus samples were first prepared and subjected to UPLC-UV-QTOF-MS/MS. Then the triterpenoids and flavonols from the Siraitiae fructus samples were identified or tentatively presumed. Furthermore, to determine the relative concentrations of the identified/presumed components, the mass response of the each compound was obtained by the EIC of UPLC-QTOF-MS. Finally, chemometrics was used to classify Siraitiae fructus samples based on the obtained quantitative data. The present strategy might be employed to simultaneously investigate multi-components including the main and trace compounds for comprehensive quality control of TCMs.

2. Experimental

2.1. Materials and samples

Deionized water was prepared from distilled water through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetonitrile and formic acid were of HPLC grade from Merck (Darmstadt, Germany). Other reagents and chemicals were of analytical grade. 16 standards of grosvenorine I (**1**), 7-*O*-mogroside V (**2**), 11-*O*-mogroside V (**13**), isomogroside V (**15**), mogroside V (**16**), siamenoside I (**20**), mogroside IVA (**21**), kaempferol (**25**), mogroside $IIIA_2$ (**28**), mogroside $IIIA_1$ (**37**), mogroside IIA_2 (**39**), mogroside IIA_1 (**41**), mogroside IE_1 (**42**), mogroside IA_1 (**45**), mogrol (**55**) and 11-*O*-mogrol (**60**) were isolated from Siraitiae fructus in our laboratory and were identified by chemical and spectroscopic analysis. The purity of each compound was > 98%.

Forty batches of Siraitiae fructus samples (S**1**–**40**) were collected. Detailed information of these samples was summarized in Table 1. The samples were authenticated as the fruits of *Siraitia grosvenorii* Swingle by Prof. Xiaobo Li from Shanghai Jiao Tong University. Voucher specimens were deposited at the School of Pharmacy, Shanghai Jiao Tong University (Shanghai, China).

2.2. Sample preparation

The dry sample powder was sieved through a 40 mesh stainless-steel sieve before extraction. 1.0 g of each dry sample powder was weighed and put into 250 mL conical flask, and 150 mL of 90% methanol-water (v/v) was added to each conical flask. All the mixtures were placed into an ultrasonic bath (200 W) for 40 min at room temperature, and then the same solvent was added to compensate for the weight lost during the extraction. After centrifugation (13,000 rpm, 10 min), the supernatant was collected and filtered through a 0.22 μm nylon membrane before analysis.

2.3. Preparation of standard solutions

Individual stock solution of 16 standards was prepared by dissolving the references in 90% methanol-water (v/v). A mixed solution containing all of the 16 references was prepared for the qualitative analysis.

For the relative quantitation assay, each structural class had a representative reference which has high level of content, easier accessibility and better stability in Siraitiae fructus. Each compound in different structural class was monitored semi-quantitatively and quantified as the corresponding selected reference. In this study, the identified 61 compounds were divided into six structural classes including mogrol, 11-*O*-mogrol, mogroside, 7-*O*-mogroside, 11-*O*-mogroside and grosvenorine. Mogrol, 11-*O*-mogrol, mogroside $IIIA_2$, 7-*O*-mogroside V,11-*O*-mogroside V and grosvenorine I were selected as the representative references of mogrol, 11-*O*-mogrol, mogroside, 7-*O*-mogroside 11-*O*-mogroside and grosvenorine, respectively. These references were prepared by dissolving 1.0 mg of the standard in 25 mL of 90% methanol-water (v/v) for the relative quantitation assay. Working standard mixtures (20–4000 ng mL^{-1}) were prepared by diluting each primary stock solution with 90% methanol-water (v/v). All of the standard solutions were stored at 4 °C until further use.

2.4. Chromatography

UPLC was performed using a Waters ACQUITY UPLC system (Waters, Milford, MA, USA), equipped with a binary solvent delivery system, an autosampler, and a photodiode array (PDA) detector. An Acquity BEH C18 column (2.1 mm × 100 mm, 1.7 μm) maintained at 50 °C was used with an injection volume of 2 μL. Mobile phase A was 0.1% formic acid/water solution (1/1000, v/v), and mobile phase B was acetonitrile solution. The flow rate was 0.4 mL min^{-1}. The linear gradient conditions were: 95–80% A (0–1.5 min), 80–70% A (1.5–7.0 min), 70–35% A (7.0–9.5 min), 35–0% A (9.5–11.0 min), 0% A (11.0–12.5 min). The wavelength of on-line PDA detector was set from 200 nm to 400 nm.

2.5. Mass spectrometry

Mass spectrometry was carried out using a Waters Synapt mass spectrometer (Waters) equipped with an electrospray ionization source (ESI) in negative mode with scan range of *m/z* 50–1500 Da. The instrument was calibrated with sodium formate. The mass accuracy and reproducibility were maintained using a LockSpray mode and the $[M-H]^-$ ion of leucine–encephalin (*m/z* 554.2615 Da) at 10 μL min^{-1} (the concentration of 100 pg mL^{-1}). A dwell time of 0.2 s was employed with an inter-acquisition delay of 0.02 s. Nitrogen was used as nebulization and desolvation gas, and argon was used as the collision gas. The desolvation gas flow rate was set to 600 L h^{-1} at temperature of 350 °C. The source temperature was held at 115 °C. In the full scan, the capillary voltage and cone voltage were set to 2800 V and 40 V, respectively. The quasi-molecular ions $[M-H]^-$ as precursor ions and subjected to MS/MS analyses using collision energy between 55 eV and 70 eV. The accurate mass and elemental composition for the precursor ions and fragment ions were analyzed using the MassLynx V4.1 software (Waters).

2.6. Method validation

2.6.1. Calibration curves, limit of detection (LOD) and limit of quantification (LOQ)

Stock solutions containing 6 representative references for the relative quantitation assay were prepared and diluted to appropriate concentrations. The calibration curves were obtained by plotting the peak areas *versus* the corresponding concentrations of each representative analyte. The LODs and LOQs were determined using diluted standard solution containing the 6 representative references with the signal-to-noise ratios (S/N) of 3 and 10, respectively.