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# Profiling of phenolic constituents in *Polygonum multiflorum* Thunb. by combination of ultra-high-pressure liquid chromatography with linear ion trap-Orbitrap mass spectrometry

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ABSTRACT

A simple and effective method was developed for characterization of phenolic constituents in the roots of *Polygonum multiflorum* by combination of ultra-high-pressure liquid chromatography with linear ion trap-Orbitrap tandem mass spectrometry (UHPLC–LTQ-Orbitrap). Stilbenes, anthraquinones, tannins and naphthalenes were differentiated by diagnostic fragment ions with accurate mass measurements and characteristic fragmentation pathways. Based on the proposed strategy, fifty-nine constituents were characterized or tentatively identified, of which twenty-two constituents were the first to be reported in *P. multiflorum* and twelve compounds were characterized as potential new compounds. The identification and structure elucidation of these chemicals provided essential data for further phytochemical studies and quality control of *P. multiflorum*. The results also demonstrated that our novel method can be extended to screen and characterize other phenolic constituents and their metabolites in botanical extracts.



## 1. Introduction

*Polygonum multiflorum* Thunb. (Polygonaceae) (PM), well known as Heshouwu in China and Fo-ti in North America [1], is one of the most popular Chinese traditional medicinal herbs and is officially listed in the Chinese Pharmacopoeia. Pharmacological studies and clinical practice have demonstrated that the roots of PM possess various biological activities, including anti-tumor, antibacterial, hemostatic, spasmolytic, analgesics, immunological properties and antiaging activities [2]. Phenolic constituents such as anthraquinones, stilbenes and tannins are thought to be the major active components.

Chromatographic methods have been used for qualitative analysis of major chemical constituents in PM. T Yi et al. [3] developed an HPLC–DAD–MS method for qualitative analysis of the major constituents in PM and successfully identified nine major compounds. Liang et al. [4] characterized eleven major constituents in PM by means of LC–QTOF-MS. Less effort has been dedicated to further characterize minor and novel phenolic components. The usual way to elucidate these minor components was by extracting, isolating and purifying into adequate amounts for MNR tests; however, these time-consuming and expensive methods are not suitable for isolation and purification of sufficient amounts of minor or unstable constituents for further structural identification. Therefore, development of new strategy for rapid and effective analysis and discovery of new minor constituents from medicinal herbs is of great significance.

Liquid chromatography coupled with mass spectrometry (LC/MS) shows unique advantages in analyzing unknown target from botanical extracts, however the limitation of MS-based methods is instrument-dependant. Among all existing LC/MS techniques, ultra-high-pressure liquid chromatography (UHPLC) is a very powerful tool for fast and efficient separation of complex chemicals mixtures [5]. High-resolution tandem mass spectrometry, on the other hand, can provide abundant information for structural elucidation of a wide range of compounds. Recently, the combination of Orbitrap technology with a linear ion trap has been shown to enable fast, sensitive and reliable detection and identification of small molecules regardless of relative ion abundance [6,7]. This hybrid LTQ-Orbitrap analysis platform can easily test $MS^5$ of fragment ions in collision-induced dissociation (CID) mode and provide high mass accuracy measurements for precursor and product ions with fast scan speeds ($\leq$1 ms per scan in full scan). Furthermore, external calibration can be used to obtain high mass accuracy (<3 ppm) of all measured spectra resulting in a simplified experimental protocol.

The present study was aimed at developing a simple and rapid UHPLC–LTQ-Orbitrap-$MS^n$ method to analyze phenolic components in the roots of PM. Several strategies including diagnostic fragment ions screening and high-resolution measurement

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was employed to distinguish aromatic compounds. The whole chromatogram was less than 35 min and over 100 peaks were detected by the UHPLC separation. In addition, fifty-nine phenolic compounds were identified or tentatively characterized, of which twenty-two constituents were the first to be reported in PM and twelve compounds were characterized as potential new compounds. Besides, direct infusion of stilbene and anthraquinones were conducted to propose a comprehensive fragment pattern rule of them. The results of the present investigations demonstrated the ability of UHPLC coupled with LTQ-Orbitrap MS to rapidly and sensitively detect and identify minor or novel small molecules from crude herbal extracts.

## 2. Experimental

### 2.1. Reagents and chemicals

HPLC-grade acetonitrile, methanol and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Water (18.2 MΩ) was from a Milli-Q water system (Millipore, Bedford, MA, USA). The roots of *P. multiflorum* thumb, was collected at Deqing county, Guangdong Province, China and authenticated by Professor Zhihai Huang in our laboratory. The standards of emodin, physcion, alo-emodin, chrysophanol, catechin, epicatechin and 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). The rest compounds (emodin-8-*O*-β-D-glucoside, physcion-8-*O*-β-D-glucoside, and gallic acid) were isolated and identified from PM in our laboratory, and their chemical structures were elucidated by means of $^{1}H$ and $^{13}C$ NMR with more than 95% purity.

### 2.2. Sample preparation

Powdered root (5 g) was refluxing extracted in 250 mL of 70% aqueous methanol at 80 °C for 2.5 h, filtered through a 0.22 μm membrane filter and then analyzed directly by LC/MS.

### 2.3. LC system

LC analyses were performed on a Thermo Accela UHPLC system (Thermo fisher Scientific, San Joes, CA, USA) consisting of a quaternary pump, a diode-array detector (DAD), an autosampler, and a column compartment. Samples were separated on a Hypersil Gold C18 column (2.1 mm × 100 mm, 1.8 μm) at room temperature. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) and the elution gradient was set as follows: 5% A (0 min), 15% A (6 min), 15% A (12 min), 38% A (25 min), 70% A (30 min) and 90% A (35 min). The mobile phase flow rate was 300 μL/min. The on-line UV spectra were recorded in the range of 200–400 nm.

### 2.4. Mass spectrometry

For LC/ESI-$MS^n$ experiments, a Thermo-fisher LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was connected to the UHPLC instrument via an ESI interface. Samples were analyzed in positive and negative ion modes, separately. The tune methods were developed using a multi-objective optimization experiment, similar to those described for ESI-MS [8]. Accurate mass analyses were calibrated according to the manufacturer's guidelines using a standard solution mix of sodium dodecyl sulfate, sodium taurocholate, the tetrapeptide MRFA acetate salt and Ultramark. Centroided mass spectra were acquired in the mass range of $m/z$ 150–1500. In the full scan mode, resolution of the Orbitrap mass analyzer was set as 30,000 (FWHM as defined at $m/z$ 400). Data-dependent $MS^n$ scanning was performed to minimize total analysis time as it can trigger fragmentation spectra of target ions and prevent repetition by dynamic exclusion settings. In the data-dependent scan, the FT resolution was 15,000 for $m/z$ 400 FWHM. The collision energy for CID was adjusted to 35% of maximum, and the isolation width of precursor ions was $m/z$ 2.0. A syringe pump was used for the direct infusion of standard solutions (about 10 μg/mL in methanol) in negative mode and flow rate was set at 8 μL/min. Capillary temperature was set at 275 °C, sheath gas 5 arbitrary units and no auxiliary gas was needed for direct infusion. All other parameters were identical to those in LC–$MS^n$ experiments.

Considering the possible elemental composition of potential components existing in PM, the types and amount of expected atoms were set as follows: carbons ≤40, hydrogens ≤200, oxygens ≤20, nitrogens ≤5. The accuracy error threshold was fixed at 5 ppm.

## 3. Results and discussion

### 3.1. Chromatographic separation of phenolic constituents

The LC separation method was optimized firstly. An UHPLC system with a small 1.8 μm particle size column showed more powerful separation ability with a higher peak resolution and the total analysis time was less than 35 min (Fig. 1), which were approximately double times faster than that for a conventional column packed with 5.0 μm particles. A 2.1 mm × 100 mm column was selected for gradient elution as a compromise between an acceptable generated column dead time ($t_0$) and a maximum plate count ($N$) since $N$ is directly proportional to $t_0$ in the limit of high speed when the small particles were used [9]. The mobile phase compositions were screened and it was found that acetonitrile and 0.1% aqueous formic acid were the most suitable eluting solvent system. We optimized and adjusted the elution gradient to make a balance between the good separation efficiency and sufficient peak widths to meet the requirements of the high resolution acquisitions (as the longer the acquisition time, the higher accurate the mass determination and the more $MS^n$ sequences provided by Orbitrap). As a result, the chromatograms presented in this study showed broader peak widths (approximately 15 s) than some fast UHPLC studies where gradient time are usually below 10 min, and thus sufficient data points were acquired when high resolution survey scan and $MS^n$ scan involved. This optimized LC method was found acceptable and adequate for further $MS^n$ analysis.

### 3.2. Performance of LTQ-Orbitrap

The instrumentation employed in this study was an ion trap MS coupled with an Orbitrap FT mass analyzer. The FT scan consists of several resolution power settings (7500, 15,000, 30,000, 60,000 and 100,000) which allowed high accurate measurements of the targeted ions. The resolution power of Orbitrap is correlated with acquisition time such that the longer the acquisition time, the higher the mass resolution. In our preliminary experiments, a resolution power of 30,000 in Orbitrap needed an acquisition time of 0.4 s and provided less than 3 ppm mass error when the ion mass range is between 150 and 1500. When the LTQ-Orbitrap was coupled with UHPLC, fast scan or acquisition rates were required so as to provide sufficient data points for minor and narrow peaks. Fig. 2 illustrated that a circle time of approximate 1.2 s was needed to acquire mass spectra with both a full scan at resolution power of 30,000 and a data-dependent scan mode at that of 15,000. It means that at least 6 scan points per peak can be acquired to obtained adequate MS and $MS^n$ information for structural elucidation. Thus the resolution power was set as 30,000 for the full scan mode and 15,000 for data-dependent scan.