Asian Journal of Chemistry Vol. 21, No. 4 (2009), 2739-2744

Determination of Triterpenoids in *Perilla frutescens* by High Performance Liquid Chromatography Based on Matrix Solid Phase Dispersion

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A new high performance liquid chromatography method based on matrix solid phase dispersion for the simultaneous determination of the major triterpenoids in *Perilla frutescens* (Chinese medicinal herb) was developed. The plant samples and silica gel (1:4) were homogenized in a mortar and then packed into a cartridge. The triterpenoids fraction was eluted from the cartridge with dichloromethane -acetone (85:15). The solvent were volatilized and the residue was dissolved with methanol. The triterpenoids was separated on a ZORBAX Stable Bound (4.6 mm × 100 mm, 1.8 μm) C_{18} column by gradient elution with acetonitrile and water as the mobile phase and detected with evaporative light scattering detection. This method provides good reproducibility and sensitivity for the quantification of bioactive triterpene acids *i.e.*, tormentic acid, oleanolic acid and ursolic acid, respectively. The relative standard derivation of overall intra-day variations were less than 1.8 %, and inter-day variations were less than 2.3 %. The standard recoveries (at 3 different concentrations of markers: 0.1, 0.5 and 2.0 mg) were ranged from 97-102 %.

Key Words: Triterpenoids, HPLC, Matrix solid phase dispersion, Evaporative light scattering detection, *Perilla frutescens*.

INTRODUCTION

The leaves of *Perilla frutescens* (L.) Britt. (Lamiaceae), which is a traditional Chinese medicinal herb, have been used in China for centuries to treat various diseases including depression, anxiety, tumour, cough, bacterial and fungal infections, allergy, intoxication and some intestinal disorders[1-3]. Various chemical and pharmacological studies have demonstrated that the major biologically active ingredients present in *Perilla frutescens* are tormentic acid (TA), oleanolic acid (OA) and ursolic acid (UA)[3,4]. Therefore, both quality and quantity controls of the major active triterpenoids in this herb have always been an important issue to ensure its effective and safe clinical usefulness[5]. However, most of the triterpenoids in *Perilla frutescens* (Fig. 1) are non-chromophoric, which make the use of direct UV detection without pre-or post-column derivatization has low sensitivity with a very low wavelength at 205 nm[5].

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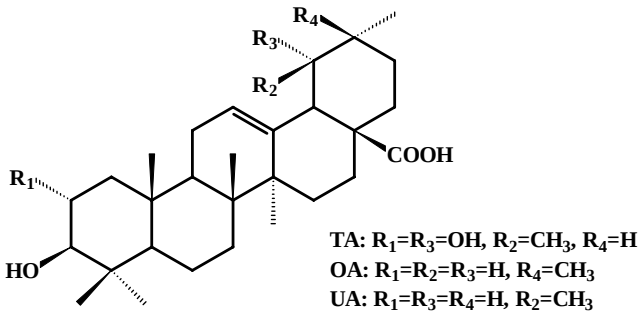

Fig. 1. Structures of triterpenoids in *Perilla frutescens*

Recently, publications on the use of HPLC coupled with evaporative light scattering detection (ELSD) have markedly increased and the published results demonstrated that ELSD is an excellent detection method for the analysis of non-chromophoric compounds[6-10]. Matrix solid-phase dispersion (MSPD) has also been successfully applied for the isolation of target molecules from biological matrices[11-14]. The procedure can considerably reduce the sample size and the solvent consumption. Therefore, in this paper, a simple and sensitive direct HPLC analytical method using MSPD and ELSD for the simultaneous determination of the major biologically active triterpenoids in *Perilla frutescens* was developed. This technique had been applied to the analysis of triterpenoids in different *Perilla frutescens* samples with good results.

EXPERIMENTAL

The samples analyzed are leaves of *P. frutescens* was harvested in early September 2007 and collected in Sichuan, Shanxi, Yunnan, Shandong provinces of P.R. China. All of the samples were identified by Prof. Xi-Wen Li, Kunming Institue of Botany, Chinese Academy of Sciences. For each samples, at least 0.5 kg of herbal samples were dried at room temperature for constant weight and pulverized to 200 mesh.

Chemicals, apparatus, and chromatographic conditions: The tormentic acid (TA), oleanolic acid (OA) and ursolic acid (UA) (Purity ≥ 98 %) were all purchased from Sigma (St. Louis MO, USA). The HPLC analysis was performed on a Waters 2695 Alliance separation system with an ELSD 2000 and a Nitrox nitrogen generator (Waters Corporation, Milford., MA 01757, USA). A ZORBAX Stable Bound column (4.6 mm × 100 mm, 1.8 μm) (Agilent Technologies Inc, Santa Clara., CA 95051, USA) was utilized. The global silica gel used in analytical experiment was 400 mesh purchased from Merck company (Germany).

HPLC grade acetonitrile (mobile phase), methanol and chloroform (for sample preparation) were provided by Fisher Scientific Inc (Madison, WI 53711, USA). The ultrapure water used was obtained from a Milli-Q50 SP Water system (Millipore Inc, MA 01730 Bedford). The mobile phase used is a linear solvent gradient of A → B (A, acetonitrile; B, water), varying as follows: 0 min (80 % A + 20 % B) and

3.0 min (90 % A + 10 % B) at a flow-rate of 1.5 mL/min. The temperature for the detector drift tube was set at 85 ºC. The nitrogen (Kunming Cylinder Charging Plants, Kunming, P.R. China) flow was 2.50 SLPM (standard liters per min) with the pressure of a nebulizing gas of 5 bar. The sample injection volume is 20 µL.

Preparation of sample: A 0.1 g of finely powdered sample was placed in a glass mortar containing 0.4 g of silica gel (400 mesh), the mixture was gently blended with a pestle. Once the mixture was homogeneous, it was then transferred into the top of an 8 mm × 20 mm cartridge containing 0.2 g silica gel (400 mesh). The cartridge was eluted with 10 mL dichloromethane-acetone (85:15) to obtain the fraction containing triterpenoids. The triterpenoids fraction eluent was volatilized to dryness by nitrogen stream and the residue was dissolved precisely in 1.0 mL methanol. This methanol solution was filtered through a 0.45 µm syringe filter and ready for HPLC analysis.

Preparation of standard solution: To prepare standard solutions, an accurately weighed amount of TA, OA and UA which were dissolved in methanol for HPLC. Five concentrations were chosen, with the range 0.8-120 µg/mL, respectively. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentrations.

RESULTS AND DISCUSSION

Matrix solid-phase dispersion: Matrix solid-phase dispersion (MSPD) has been successfully applied for the isolation of target molecules from biological matrices. The mechanism of MSPD includes sample homogenization, cellular disruption, exhaustive extraction, fractionation and purification in a simple process. MSPD technology involves blending a small amount of matrix with an appropriate sorbent followed by washing and elution of compounds with a small volume of solvent. The procedure can considerably reduce the sample size and the solvent consumption.

Different parameters that affect MSPD extraction such as dispersing agent and eluent solvent were studied. The polar solid phase (silica gel, alumina and florisil) and non-polar solid phase (C_{18}, graphite carbon black) were tested for matrix dispersion. High recoveries (> 95 %) were obtained when use C_{18} (methanol-water system as eluent) and silica gel (dichloromethane-acetone system as eluent) as dispersing agent. The silica gel is cheaper than the C_{18}. Therefore, the silica gel (400 mesh) was selected as dispersing agent and the dichloromethane-acetone system was selected as eluent in this experiment.

For eluation the triterpenoids from the cartridge, the effect of different proportion of dichloromethane-acetone mixed used as eluent on the recoveries of triterpenoids were tested. The results showed when the acetone proportion less than 5 %, the recoveries for triterpenoids is low. However, with the increase of the eluent polarity, the recoveries of triterpenoids were gradually increased. When the acetone proportion reaches 15 %, the triterpenoids can be eluted from cartridge completely (recovery

> 95 %) with 10 ml of eluent used. The further increase of the acetone proportion can reduce the eluent volume. However, with the increase of acetone proportion, more interfering compounds (flavone and organic acid) can also be eluted from cartridge. Therefore, dichloromethane-acetone (85:15) was selected as eluent and 10 mL of eluent was recommended in this experiment.

Optimal of chromatographic separation: Optimal chromatographic condition was obtained after testing different mobile phase systems with two reversed-phase columns (C_8 and C_{18}). In the case of the C_8 column, the two major titerpenoids, OA and UA could not be resolved as a baseline separation. However, all analytes were resolved well with a baseline separation using the C_{18} column. Furthermore, among various mobile phases examined, the mobile phase used is a linear solvent gradient of A → B (A, acetonitrile; B, water) varying as follows: 0 min (80 % A + 20 % B) and 3.0 min (90 % A + 10 % B) at a flow-rate of 1.5 mL/min was found to be the best separation. Therefore, acetonitrile-water gradient elution was selected as mobile phase in this experience. To shorten the chromatographic separation time, a ZORBAX Stable Bound rapid analysis column (4.6 mm × 100 mm, 1.8 μm) was used in this experiment. With this rapid analysis column, all the 7 triterpenoids were separated completely within 3.0 min (Fig. 2). As compared to the previous reports[5,9,10,15-18], the present method is one of the most rapid method to separate triterpenoids.

Calibration graphs: Under the optimum conditions, the regression equations of 7 triterpenoids were established based on the standard samples injected and their peak areas. The residual standard deviations (σ) were plotted. The limits of detection are calculated by the ratio of signal to noise (S/N = 3). The results were shown in Table-1. The reproducibility of this method was also examined for 10 μg mL^{-1} of the 7 triterpenoids. The relative standard deviations (n=9) were shown in Table-1.

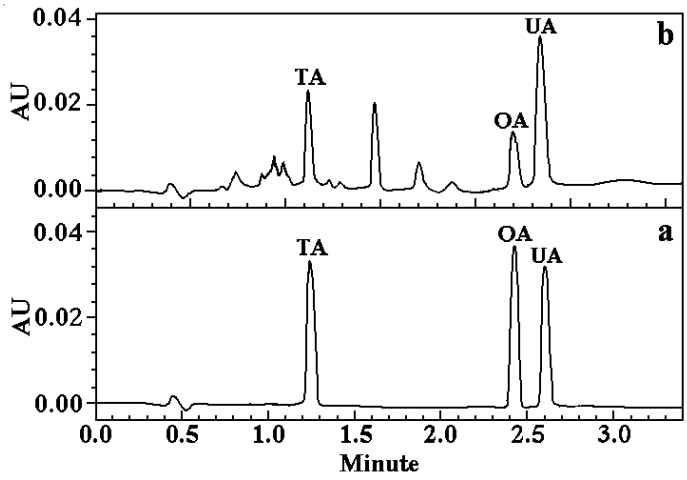

Fig. 2. Chromatogram of standard sample (a) and *Perilla frutescens* sample (b)

TABLE-1
REGRESSION EQUATION, COEFFICIENT AND DETECT LIMIT

Components	Regression equation C (μg mL^{-1})	Linearity range (μg mL^{-1})	Coefficient	σ % (n = 7)	Detect limits (μg mL^{-1})	RSD % (n = 9)
Tormentic acid	$A=1.31\times10^3$ C + 28.9	1.2~100	r = 0.9996	0.51	0.20	1.4
Oleanolic acid	$A=1.48\times10^3$ C + 33.4	1.4~120	r = 0.9994	0.48	0.25	1.2
Ursolic acid	$A=1.36\times10^3$ C - 45.8	1.6~110	r = 0.9992	0.71	0.25	1.5

Method recovery and precision: The recovery tests were carried out by adding TA, OA and UA to the samples (3 different concentrations of markers: 0.1, 0.5 and 2.0 mg). The sample was prepared as above "preparation of sample" procedure and injected for HPLC analysis to calculate the amount of the triterpenoids founded. The results shown that the recoveries (n = 5) were ranged from 97-102 %. This method is of high recovery.

The measurements of intra- and inter-day variability (determination of the same samples for 7 times) were utilized to determine the precision of the developed method. The results shown that the relative standard derivation of overall intra-day variations were less than 1.8 % and the relative standard derivation of inter-day variations were less than 2.3 %. This method is of high precision.

Analysis of pentacyclic triterpenoids in samples: This method was subsequently applied to simultaneous determination of the triterpenoids in different *Perilla frutescens* samples. The contents of triterpenoids are summarized in Table-2. A traditional solvent extraction and solid phase extraction (SE-SPE) was used as reference method. The results were shown in Table-3.

TABLE-2
DETERMINATION RESULTS (%) OF THE TRITERPENOIDS IN *Perilla frutescens* SAMPLE BY PRESENT METHOD

Components	Samples of different area *Perilla frutescens* (%)					RSD % (n = 5)	Recovery **%** (n = 5)
	Sichuan	Shaanxi	Shanxi	Yunnan	Shandong		
Tormentic acid	0.428	0.546	0.612	0.475	0.502	2.1	96-101
Oleanolic acid	0.152	0.174	0.162	0.181	0.147	2.3	97-103
Ursolic acid	0.134	0.148	0.151	0.162	0.129	2.0	96-102

TABLE-3
DETERMINATION RESULTS (%) OF THE TRITERPENOIDS IN *Perilla frutescens* BY SOLVENT EXTRACTION AND SOLID PHASE EXTRACTION METHOD

Components	Samples of different area *Perilla frutescens* (%)					RSD % (n = 5)	Recovery % (n = 5)
	Sichuan	Shaanxi	Shanxi	Yunnan	Shandong		
Tormentic acid	0.434	0.554	0.606	0.462	0.511	2.6	93-103
Oleanolic acid	0.158	0.168	0.155	0.187	0.142	2.8	95-105
Ursolic acid	0.142	0.153	0.147	0.155	0.136	2.4	96-104

Conclusion

In this manuscript, a ZORBAX Stable Bound C18 (4.6 × 10 mm, 1.8 μm) rapid analysis columns was used. The 7 triterpenoids can achieve baseline separation with 3.0 min on this column. Compared to the routine column, 80 % of separation time was saved. The matrix solid-phase dispersion (MSPD) was used as sample preparation method. MSPD combines both sample homogenization and extraction of the analyzed compounds in one step. It considerably reduced the sample size and the solvent consumption. The method precision and recovery are higher than that of traditional solvent extraction and solid phase extraction method. For the analysis of nonchromophoric compounds, the evaporative light scattering detection (ELSD) was used in this method. The nortriterpenoids can directly be detected without derivation. The sample preparation for this method is simple.

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(*Received*: 15 April 2008; *Accepted*: 15 January 2009) AJC-7115