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High-performance liquid chromatographic analysis of bioactive triterpenes in *Perilla frutescens*

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Abstract

Perilla frutescens (L.) Britt. (Lamiaceae), a famous traditional Chinese medicine, has been used for the treatment of various diseases. To evaluate the quality of *P. frutescens*, a simple, rapid and accurate high-performance liquid chromatography (HPLC) method was developed for the assessment of three bioactive triterpene acids: tormentic acid (TA), oleanolic acid (OA) and ursolic acid (UA). The HPLC system used an Spherisob octadecylsilyl silica (ODS) column with acetonitrile and aqueous H_3PO_4 as the mobile phase and detection at 206 nm. The method was precise with relative standard deviations for these three constituents that ranged between 0.6–1.5% (intraday) and 0.7–2.6% (interday). The content of these three phytochemicals in the leaves of *P. frutescens* growing at eight different locations of China was determined to establish the effectiveness of the method.

Keywords: Analysis; *Perilla frutescens*; Lamiaceae; HPLC, Tormentic acid; Oleanolic acid; Ursolic acid

1. 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, tumor, cough, bacterial and fungal infections, allergy, intoxication and some intestinal disorders [1–4]. As a follow-up to our previous analytical and antifungal investigation of the essential oil of *P. frutescens* [5], the three main bioactive constituents were characterized spectroscopically as tormentic acid (TA), oleanolic acid (OA) and ursolic acid (UA) (Fig. 1). TA was ascertained to be an anti-inflammatory and DNA polymerase-inhibitory phytochemical [6] while OA and UA were both reported to have the anti-inflammatory activity [7,8]. Furthermore, OA possesses hepatoprotective [9] and anti-ulcer activities [10], and UA exerts its anti-tumor action through enhancing the production of both nitric oxide and tumor necrosis factor-α [11]. Needless to say, a sufficiently high content of the bioactive phytochemicals is crucial for therapeutic effect.

Like most plant extracts composed of complex phytochemical constituents, proper method(s) is (are) particularly desired for the quality control of

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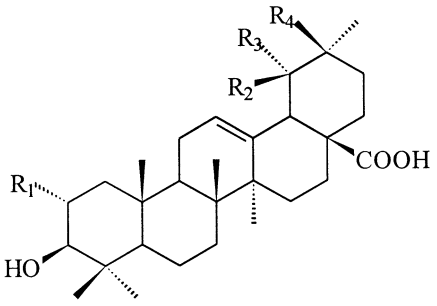

TA: R_1=R_3=OH, R_2=CH_3, R_4=H

OA: R_1= R_2= R_3=H, R_4= CH_3

UA: R_1= R_3= R_4=H, R_2= CH_3

Fig. 1. The chemical structures of three triterpenes.

the extract of the title plant as well as that of pharmaceutical and/or nutraceutical products made therefrom. As no method is available to date for the co-quantification of these three bioactive triterpene acids, we developed an accurate high-performance liquid chromatography (HPLC) method for the quality assessment of the leaves of *P. frutescens*. The contents of these three constituents in the leaves of the plant collected from eight geographically different areas of China has been determined to demonstrate the robustness of the established method.

2. Experimental

2.1. Plant material

A total of eight sets of plant materials of *P. frutescens*, purchased from Materia Medica Company in Nanjing, was harvested in early September 2000 from Jiangsu (A), Sichuan (B), Shaanxi (C), Shanxi (D), Yunnan (E), Guangdong (F), Hubei (G), Shandong (H) provinces of China, respectively. All materials were sorted and identified by Associate Professor L.X. Zhang with voucher specimens (NJU-070801–NJU-070808) deposited in the Herbarium of Nanjing University, Nanjing 210093, China.

2.2. Reagents

TA, OA and UA were isolated from the ethanol extract of the dried leaves of *P. frutescens*. Acetonitrile (HPLC grade) was purchased from Fisher (Fisherchemicals, USA). Ultra-pure distilled water was used. All organic solvents used in the study were of analytical grade.

2.3. Apparatus

Melting point was determined on a Boetius micromelting point apparatus, and was uncorrected. All NMR spectra were taken on a Bruker DRX500 spectrometer in $CDCl_3$ and C_5D_5N with ^1H and ^{13}C nuclei observed at 500 and 125 MHz, respectively. The chemical shifts were expressed in ppm (δ) relative to an internal standard TMS. Silica GF_{254} for TLC and silica gel (200–300 mesh) for CC were produced by Qingdao Marine Chemical Company, China.

2.4. Extraction and isolation

The air-dried leaves of *P. frutescens* (5 kg) were extracted thrice with ethanol (40 l, 24 h each time). Removal of the solvent from the extract under reduced pressure gave a semi-solid gum (150 g) which was suspended in water and partitioned successively with petroleum ether, $CHCl_3$ and *n*-BuOH to afford eventually the petroleum ether (35 g), $CHCl_3$ (30 g), *n*-BuOH (45 g) and water fractions (25 g). The $CHCl_3$ fraction was chromatographed on a silica gel column (600 g) eluted with petroleum ether–acetone mixtures of increasing polarity to yield TA, OA and UA, respectively.

All compounds were identified by ^1H-, and ^{13}C-NMR, and these spectroscopic data were compared with the data reported in the literatures [12–14].

2.5. High-performance liquid chromatography system

HPLC was performed on a Waters 600E intelligent pump system equipped with a Waters 966 photodiode array detector with the detection wavelength set at 206 nm. Satisfactory separation

was obtained with reversed-phase column (Spherisob ODS, 5 μm, 25 cm × 4.6 mm i.d.) eluted at a rate of 0.5 ml/min with a solvent of A-B (A, acetonitrile; B, 1.25% H_3PO_4 aqueous; A:B = 86:14, v/v).

2.6. Preparation of standard solution

To prepare standard solutions, an accurately weighed amount of the TA, OA and UA standards (3.1, 3.4 and 5.2 mg, respectively) were dissolved in methanol (10 ml) for analysis. The standard solutions (2, 4, 6, 8, 10 μl, respectively) were injected and run for calibration curves. Calibration graphs were plotted subsequently for linear regression analysis of the peak area with concentration.

2.7. Preparation of sample solution

The eight sets of the air-dried leave of *P. frutescens* (5 g) collected in different areas were separately pulverized and refluxed thrice with methanol (25, 20 and 20 ml, each 1 h) at 80 °C. The extracts with each set were combined and filtered while hot. The filtrate was concentrated to a tarry lump, which was dissolved in 10 ml of methanol. The afforded solution was filtered through a 0.45 μm syringe filter prior to HPLC.

2.8. Preparation of recovery studies

To tested plant leaves (5 g), appropriate amounts of TA, OA and UA were added to approximately the double contents of those acids in treated materials (Table 2). The follow-up extractions and HPLC analyses were accomplished in the same manner as detailed above. The recovery was determined as follows:

$$\text{Recovery } (\%) = (A - B)/C \times 100\%$$

where, A is the amount of detections above, B is the amount of sample without added standards, C is the added amount of the standards.

Table 1
Intra- and inter-day precision for determination of TA, OA and UA

Standard	Concentration (μg/ml)	R.S.D.	
		Intraday ($n = 6$)	Interday ($n = 6$)
TA	310	1.5	2.8
	620	1.2	2.4
OA	340	1.3	1.8
	680	0.5	0.9
UA	520	0.8	1.7
	1040	0.6	1.0

3. Results and discussion

The detection wavelength was chosen at 206 nm because the three triterpenes have better absorption and sensitivity at this wavelength. A chromatogram is showed in Fig. 2 which illustrates the separation of the three phytochemicals in this system.

Calibration graphs were constructed in the range 4.2–20.6 μg/ml for TA, 2.4–24.0 μg/ml for OA, and 2.5–62.5 μg/ml for UA. The regression equations of these curves and their coefficients of determination (R^2) were calculated as follows: TA, $y = 7.0480\text{E}+05x - 3.8080\text{E}+03$, 0.9997; OA, $y = 1.4921\text{E}+06x - 1.0977\text{E}+04$, 0.9998; UA, $y = 1.2951\text{E}+06x - 1.4734\text{E}+04$, 0.9998. The method showed a linear relationship between peak areas and concentrations over this range for all three compounds. A signal five times higher than the noise was regarded as the detection limit. Accordingly the detection limits of these constituents were: 0.2, 0.1, 0.1 μg/ml for TA, OA and UA, respectively.

To assess the precision of the method, we injected standard solutions of TA, OA and UA, respectively, six times on the same day and over a 6-day period analysis. The coefficient variations of intraday and interday studies were both less than 3.0% (Table 1). The precision as well as reproducibility of this method was satisfactory. The results of the recoveries of TA, OA and UA ranged from 95.6 to 101.7%. The relative standard deviations

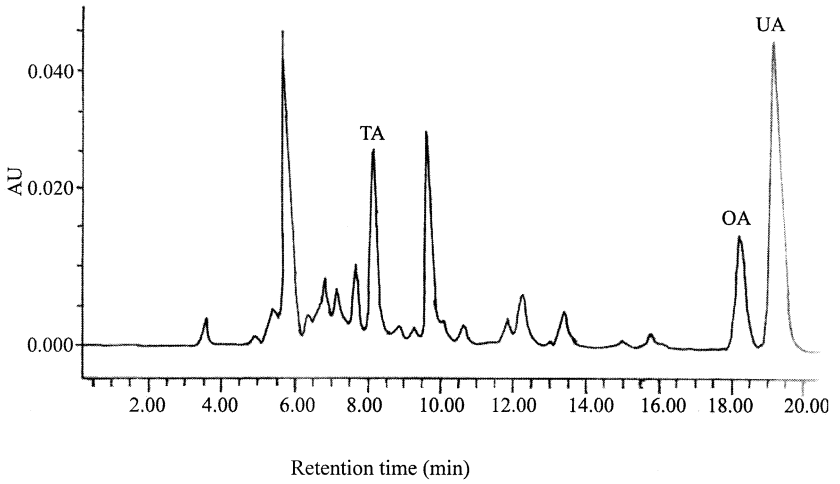

Fig. 2. Chromatogram of methanol extract of *P. frutecens*.

Table 2
Recovery of TA, OA and UA

Standard	Amount added (mg)	Recovery ($n = 5$)	
		Mean	R.S.D.
TA	4.81	98.1	3.1
OA	5.72	95.6	2.3
UA	35.15	101.7	1.8

(R.S.D.) of recoveries of three constituents ranged between 1.8 and 3.1% (Table 2).

When the sample solution was analyzed in the same manner, the peaks were identified by comparison of the retention time with those corresponding to authentic samples purified from *P. frutecens*. Regarding the extraction efficiency, three times work-up was sufficient since it allowed an over 98% extraction of the three constituents.

The content of the three constituents in leaves of *P. frutecens* growing in different locations was also analyzed demonstrating that all of these phytochemicals in the title plant are strikingly dependent of the locality (Fig. 3).

In conclusion, the newly established HPLC method is validated for the quantification of the main bioactive triterpenes TA, OA, and UA, and the quality control of the plant materials such as *P. frutecens* where the triterpene(s) is (are) the dominant phytochemical(s). This method is rapid, precise, reproducible, sample-saving, and maybe helpful for the quantitative analysis of phytochemicals analogous to the triterpenes.

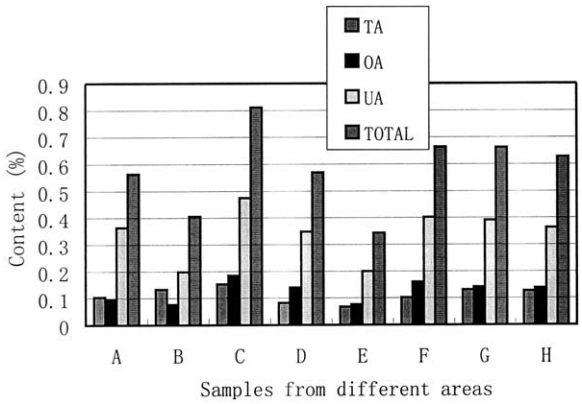

Fig. 3. The mean contents of three constituents in *P. frutescens* from eight different locations of China ($n = 5$).

Acknowledgements

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