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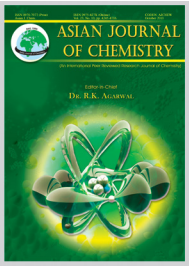

Simultaneous Determination of Four Phenolic Acids in *Terminalia* by HPLC

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A HPLC method is established for the simultaneous determination of four phenolic acids (gallic acid, vanillic acid, caffeic acid and *p*-coumaric acid) in the extract of *Terminalia*. XDB-C_{18} (4.6 × 150 mm i.d., 5 μm) column was used, the chromatography was carried out with a linear gradient program. The mobile phase was A: methanol and B: 1 % ethylic acid at flow rate of 1.0 mL min^{-1}, the detection wavelength was 280 nm and column temperature was 25 ºC. The linear ranges were 80-800 mg/L for gallic acid, 2.5-250 mg/L for vanillic acid, 2.0-200 mg/L for caffeic acid and 1.0-100 mg/L for *p*-coumaric acid. The average recoveries (n=5) were 99.40 % with relative standard deviation (RSD) of 1.84 % for gallic acid, 98.5 % with RSD of 2.15 % for vanillic acid, 98.7 % with RSD of 1.90 % for caffeic acid and 99.3 % with RSD of 2.00 % for *p*-coumaric acid. These four phenolic acids were separated successfully from each other.

Key Words: *Terminalia*, Gallic acid, Vanillic acid, Caffeic acid, *p*-Coumaric acid, Phenolic acids, HPLC.

INTRODUCTION

Terminalia is a plant belonging to *Terminalia mantaly Tricolor* of *Combretaceae*, which is widely distributed in the west of Yunnan[1]. It is a well-known crude drug and a key herb ingredient in many important multi-herb remedies in traditional Chinese medicine (TCM) and it has been used for hundreds of years in China and other countries[2]. It was reported that *Terminalia* contains a large amount of phenolic compounds and tannins and both of them have a strong antioxidative activities[3,4].

Pharmacological studies revealed that phenolic acids were the main active components showed that these naturally occurring phenolic acids had various pharmacological properties and could be used to act as antibacterial, antiinflammatory, antioxidant, anticancer[5]. They also could effectively prevent the cerebrovascular and cardiovascular diseases[6-9]. Gallic acid, vanillic acid, caffeic acid and *p*-coumaric acid are the main active components of *Terminalia*[10-13]. Thus it is essential and meaningful to establish an analytical method for phenolic acids of *Terminalia*. In order to utilize this crude drug more reasonably and scientifically, we select these four phenolic acids as standard compounds to evaluate the quality of the *Terminalia*. In this paper, we have established a simple and effective analytical method for simultaneous determination of four phenolic acids in the extract of *Terminalia* and in the meantime satisfying results were obtained.

EXPERIMENTAL

Gallic acid, vanillic acid, caffeic acid and *p*-coumaric acid (used as reference substance, the purity higher then 99.0 %) were purchased from National Institute of China for the Control of Pharmaceutical and Biological Products (NICPBP, Bejing, China). *Terminalia* seeds was purchased from Lincang of Yunnan and Burma, which was ground into powder and then sieved (60 mesh). Methanol (HPLC-grade) was from Tedia (Fairfield, OH). Acetic acid and ethanol (analytical-grade). AB-8 macroporous adsorption resin (Chemical Plant of Nankai University). All aqueous solution were prepared with purified water (Wahaha, Hangzhou, China).

Agilent 1200 series high performance liquid chromatograph system (Aglient Technologies, USA), equipped with diode array detector (G1315A), quaternary pump (G1311A), automatic sampler (SIL-10AP), UV variable-wavelength detector (1314A-UV) and column oven (CTO-10ASVP). The analysis column was a XDB-C_{18} column (150 mm × 4.6 mm, i.d., 5 μm) maintained at 25 ºC. The mobile phase consisted of methanol (A) and 1 % acetic acid (B). A linear gradient of mobile phase A was applied during the analysis. The gradient profile was as follows: 0-10 min (5 % A), 10-25 min (10 % A), 25-26 min (50 % A), 26-35 min (80 % A), 35-35.1 min (100 % A). The flow rate of mobile phase was 1.0 mL min^{-1}. The detection occurred at 280 nm. The injection volume was 20 μL.

TABLE-1
CALIBRATION EQUATIONS OF THE FOUR PHENOLIC ACIDS

Compound	Retention time (min)	Calibration equation (r)	Linear range (mg/L)
Gallic acid	4.008	y = 6.4693x + 11.025 (0.9995)	80-800
Vanillic acid	17.558	y = 4.5576x + 7.9008 (0.9995)	2.5-250
Caffeic acid	18.380	y = 2.5309x - 0.8339 (0.9996)	2.0-200
p-Coumaric acid	22.006	y = 7.5224x + 4.3575 (0.9995)	1.0-100

AS10200AD ultrasound cleaning bath (China Tianjin Autoscience instrument Co. Ltd.) with 330 cm × 270 cm × 290 cm internal dimensions, a volume of 10.0 L, 40/60 kHz and 4 transducers was used in the experiment, operating at a frequency of 60 kHz with input power of 320 W. The extraction vessel was put at the center of the bath in the process of the experiment. The temperature was controlled and maintained at (35 ± 1) ºC by circulating external water from a thermostated water bath. EYELAN-1100 Rotary Eaporators (Shanghai EYELAN Instrument Co. Ltd.). AB-8 macroporous adsorption resin column (40 × 10 mm), AR224CN electronic balance (OHAUS Instrument Co. Ltd.).

Standard solution preparation: The standard stock solutions of gallic acid (1.0 mg mL^{-1}), vanillic acid (1.0 mg mL^{-1}), caffeic acid (1.0 mg mL^{-1}) and *p*-coumaric acid (1.0 mg mL^{-1}) were prepared in methanol and stored away from light at 4 ºC. A mixed working solution of gallic acid (0.8 mg mL^{-1}), vanillic acid (0.5 mg mL^{-1}), caffeic acid (0.4 mg mL^{-1}) and *p*-coumaric acid (0.2 mg mL^{-1}) were prepared by dilution of the stock solution with methanol. The solution was filtered through 0.45 μm organic millipore membrane filters.

Sample solution preparation: 5 g of *Terminalia* seeds powder was accurately weighed and placed in ultrasound bath and sonicated at room temperature for 1 h and this extraction process was repeated two times under the same conditions. The extraction solution was filtered and the filtrate was condensed at 50 ºC by decompression. Next, the residual solution of the supernatant was lyophilized to form the ethanol extract of *Terminalia* powder; then the ethanol extract was taken by weighing and was separation and purification by AB-8 macroporous adsorption resin and got the pure polyphenols extract of *Terminalia,* which was taken by weighing (0.3 g) and dissolved with methanol in a 10 mL measuring flask. The solution was filtered through 0.45 μm organic millipore membrane filters. The filtrate was used for HPLC analysis.

RESULTS AND DISCUSSION

Optimization of chromatographic condition: The diode array detector (DAD) was used to conduct a full spectrum scan of sample. It was found that phenolic acids compounds had strong ultraviolet absorption at 220 nm and 280 nm, the absorption of four phenolic acids at 280 nm is more sensitive than that at 220 nm and a lot of substances have the absorption at 220 nm, so 280 nm was chosen as detector wavelength. The mobile phases with different composition were tested, methanol(A) -1 % acetic acid (B) solution was found to have the optimum isolation effect for four phenolic acids. The gradient elution was used and Gradient profile was as follows: 0-10 min (5 % A), 10-25 min (10 % A), 25-26 min (50 % A), 26-35 min (80 % A), 35-35.1 min (100 % A).

Under the optimum conditions mentioned above, the chromatogram of four reference substances is shown in Fig. 1(A). The typical chromatograms of *Terminalia* extracts are shown in Fig. 1(B). The chromatograms of the four phenolic acids showed that these four phenolic acids were separated successfully from each other. All compounds displayed good linearity ($R^2 > 0.999$) in the given concentration range. The peaks of analytes were identified by two means: (i) by comparing the retention times of the peaks with those of the reference compounds eluted under the same conditions and (ii) by spiking the sample with stock standard solutions of four phenolic acids.

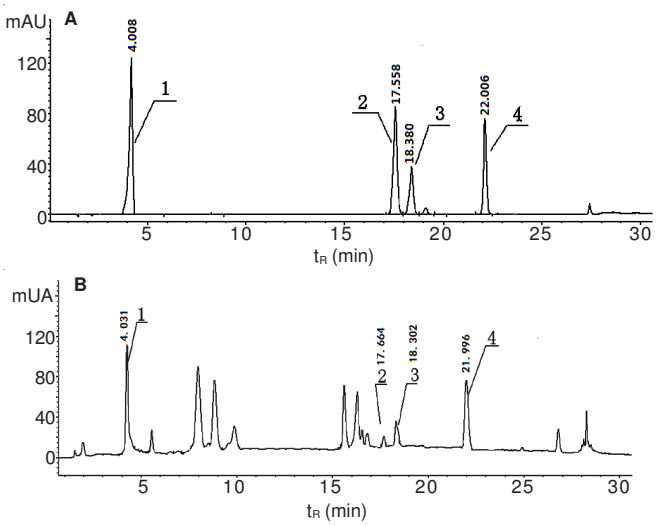

Fig. 1. HPLC chromatograms of reference substances (A)and sample (B); Peaks: 1. Gallic acid 2. Vanillic acid 3. Caffeic acid 4. *p*-Coumaric acid

Method validation

Linear relation: Five reference solution were taken accurately respectively and then added into a 50 mL measuring flask and dissolved with methanol. The mixture of reference solution were diluted to volume 1.0, 1.6, 2.5, 5.0, 10 mL ,which were respectively transferred to five 10 mL volumetric flask each and methanol was added to volume. 20 μL of the above solutions were accurately injected for analysis. Chromatogram of the standard compounds was seen in Fig. 1. The retention time of gallic acid, vanillic acid, caffeic acid and *p*-coumaric acid were 4.008 min, 17.558 min, 18.380 min, 22.006 min, respectively. The linearity for each compound was established by plotting the peak area(y) *versus* concentration(x). Linear regression analysis was performed by the external standard method. The results were shown in Table-1. All compounds displayed good linearity ($R^2 > 0.999$) in the given concentration range.

Precision, repeatability, stability and recovery: The precision was determined on the same stock solution selected from previous prepared, 20 μL of which was taken for analysis for 5 times. The relative standard deviation (RSD, %) of peak areas of four phenolic acids were 1.6, 1.5, 1.8 and 1.4 % respectively, which showed a good precision (Table-2).

TABLE-2
RESULT OF PRECISION TEST

Compound	Gallic acid	Vanillic acid	Caffeic acid	*p*-Coumaric acid
Peak area	2378	441	1351	856
	2458	448	1363	872
	2353	443	1373	863
	2374	446	1395	856
	2356	450	1367	889
RSD (%)	1.6	1.5	1.8	1.4

In order to test the repeatability, 5 portions of the pure polyphenols with the same amount were prepared for the testing solutions. The testing solutions were analyzed under the above chromatographic conditions. It was found that the RSD of peak areas of four phenolic acids were 2.7, 2.8, 2.6 and 2.6 %, respectively. The above data showed that the analytical method had a good repeatability.

Stability experiments were also done. The testing solution was analyzed for 5 times under the above chromatographic conditions with interval of 2 h. The RSD of each compound was calculated as follows: gallic acid 2.1 %, vanillic acid 2.4 %, caffeic acid 2.5 %, *p*-coumaric acid 2.1 %. The results showed that the contents of these four ingredients were stable in 8 h.

The recovery experiments were carried out to evaluate the accuracy of the method. Five portions of the pure polyphenols with the same weight (about 0.3 g) were taken for analysis. 1 mL of standard sample solution with concentration of 0.8 mg mL^{-1} for gallic acid, 0.5 mg mL^{-1} for vanillic acid, 0.4 mg mL^{-1} for caffeic acid and 0.2 mg mL^{-1} for *p*-coumaric acid were respectively added into every sample. Five sample solutions were prepared according to analytical procedure. Take each of them for analysis and the average recovery rate were 99.4.0, 98.5, 98.7 and 99.3 %, with the RSD being 1.84, 2.15, 1.90 and 2.0 %, respectively, which indicated the good reliability of the methodology (Table-3).

Sample determination: Samples from different origins were weighed precisely and prepared for the testing solution according to the analytical procedure. And 20 μL of the filtrate was injected for analysis. The content of each component was calculated according to the corresponding calibration equation. The content of phenolic acids of two samples from different areas were determined and the results were summarized in Table-4. Chromatograms were shown in Fig. 1(B).

TABLE-4
CONTENTS OF FOUR PHENOLIC ACIDS IN DIFFERENT *TERMINALIA* SAMPLES (mg/g) (n=3)

Samples	Gallic acid	Vanillic acid	Caffeic acid	*p*-Coumaric acid
Terminalia (Lincang of Yunnan)	1.22	0.13	0.78	1.11
Terminalia (Burma)	2.22	0.11	0.76	0.94

Conclusion

The developed HPLC method for simultaneous determination of four phenolic acids in *Terminalia*, which was characterized by sufficient accuracy, precision and reproducibility, as well as sensitivity and selectivity and also offers a short time of analysis of four phenolic acids. The differences were seen by comparing the contents of these four ingredients of different origins. The contents of gallic acid in Burma are much higher than those in Lincang of Yunnan province and the contents of the other ingredients in these two origins did not have that much differences. In conclusion, the developed method can be used for the assay of four phenolic acids in *Terminalia*.

TABLE-3
RESULTS OF SAMPLE RECOVERY RATE (n=5)

Compound	Samples (g)	Back-ground (mg)	Added (mg)	Found (mg)	Recovery (%)	Average (%)	RSD (%)
Gallic acid	0.3004	3.6649	0.8	4.4363	99.4	99.4	1.84
	0.3001	3.6612	0.8	4.3008	96.4		
	0.3008	3.6698	0.8	4.4547	99.7		
	0.3003	3.6637	0.8	4.4798	100.4		
	0.3011	3.6734	0.8	4.5286	101.2		
Vanillic acid	0.3004	0.3905	0.5	0.8875	99.7	98.5	2.15
	0.3001	0.3901	0.5	0.8928	100.3		
	0.3008	0.3910	0.5	0.8479	95.2		
	0.3003	0.3904	0.5	0.8665	97.3		
	0.3011	0.3914	0.5	0.8862	99.4		
Caffeic acid	0.3004	2.3431	0.4	2.6883	98.0	98.7	1.9
	0.3001	2.3408	0.4	2.7243	99.4		
	0.3008	2.3462	0.4	2.6948	98.1		
	0.3003	2.3423	0.4	2.6486	96.6		
	0.3011	2.3486	0.4	2.7929	101.6		
p-Coumaric acid	0.3004	3.3344	0.2	3.4941	98.9	99.3	2.0
	0.3001	3.3311	0.2	3.4264	97.0		
	0.3008	3.3389	0.2	3.5038	99.0		
	0.3003	3.3333	0.2	3.4805	98.5		
	0.3011	3.3422	0.2	3.6255	102.4		

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