Pulsatillae Radix

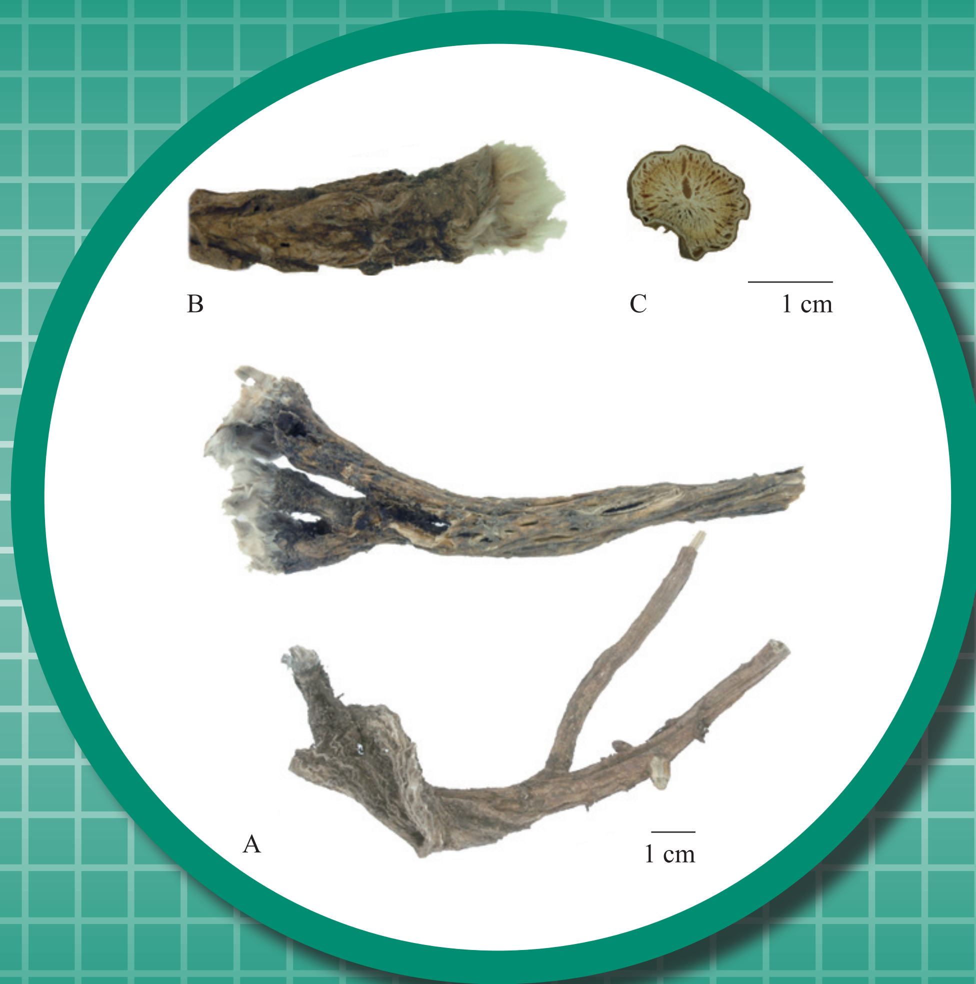


Figure 1 A photograph of Pulsatillae Radix

A. Pulsatillae Radix B. Magnified root stock with white-pubescent

C. Magnified transverse section of the root

1. NAMES

Official Name: Pulsatillae Radix

Chinese Name: 白頭翁

Chinese Phonetic Name: Baitouweng

2. SOURCE

Pulsatillae Radix is the dried root of *Pulsatilla chinensis* (Bge.) Regel (Ranunculaceae). The root is collected in spring and autumn, soil removed, then dried under the sun to obtain Pulsatillae Radix.

3. DESCRIPTION

Subcylindrical or conical, slightly twisted, 6-20 cm long, 5-20 mm in diameter. Externally yellowish-brown, brown to dark brown, with irregular and longitudinal wrinkles or furrows; bark easily exfoliated, the exposed wood yellow, some with reticulate fissures and cracks, usually with decayed and dented holes near the root stock. Root stock slightly swollen, white-tomentose-pubescent, some showing sheath-like remnant bases of petiole. Texture hard; fracture yellowish-white to pale yellowish-brown in the bark, pale yellow in the wood. Odour slight; taste slightly bitter and astringent (Fig. 1).

4. IDENTIFICATION

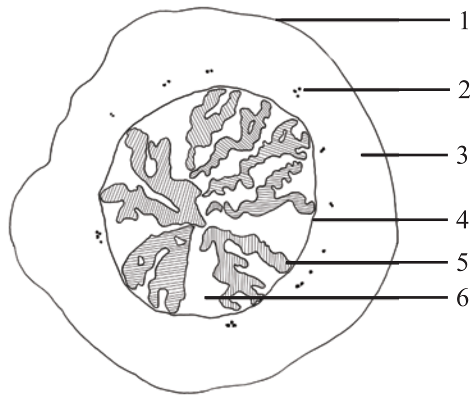
4.1 Microscopic Identification *(Appendix III)*

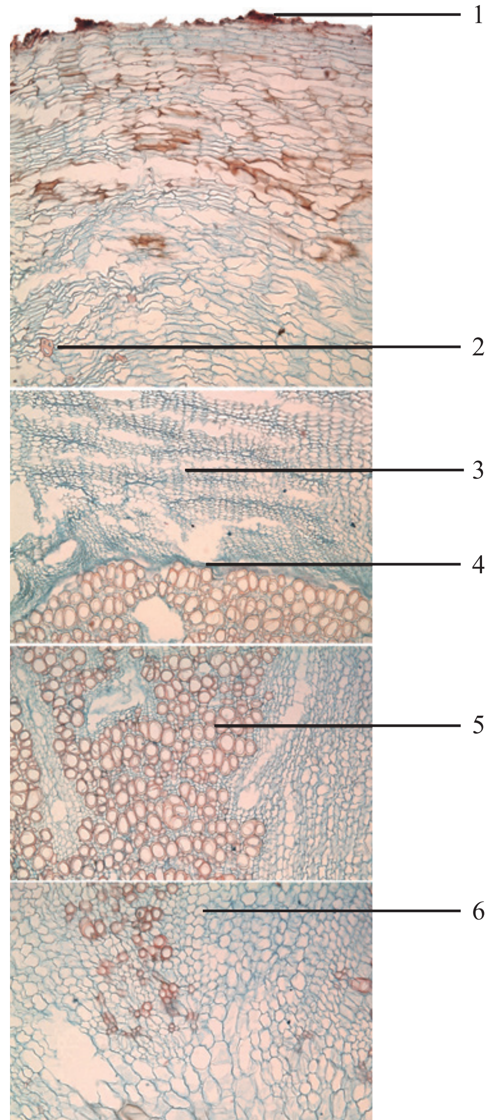
Transverse section

Cortex usually fallen off. Phloem broad, outer layers of cells of phloem brown, walls suberized. Phloem fibres single or several in groups. Cambium distinct. Vessels single or several in groups; ray wide, up to 20 rows of cells (Fig. 2).

Powder

Colour greyish-brown. Vessels bordered-pitted, reticulate and spiral, 10-60 μm in diameter. Phloem fibres fusiform or spindle-shaped, 100-300 μm long, 16-35 μm in diameter, walls lignified. Non-glandular hairs unicellular, 9-21 μm in diameter, base slightly inflated, walls mostly lignified, some with spiral or double-spiral striations (Fig. 3).





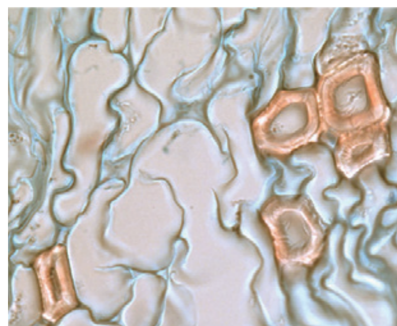


Figure 2 Microscopic features of transverse section of Pulsatillae Radix

A. Sketch　B. Section illustration　C. Fibres

1. Phloem cell walls suberized　2. Phloem fibres　3. Phloem　4. Cambium
5. Xylem　6. Rays

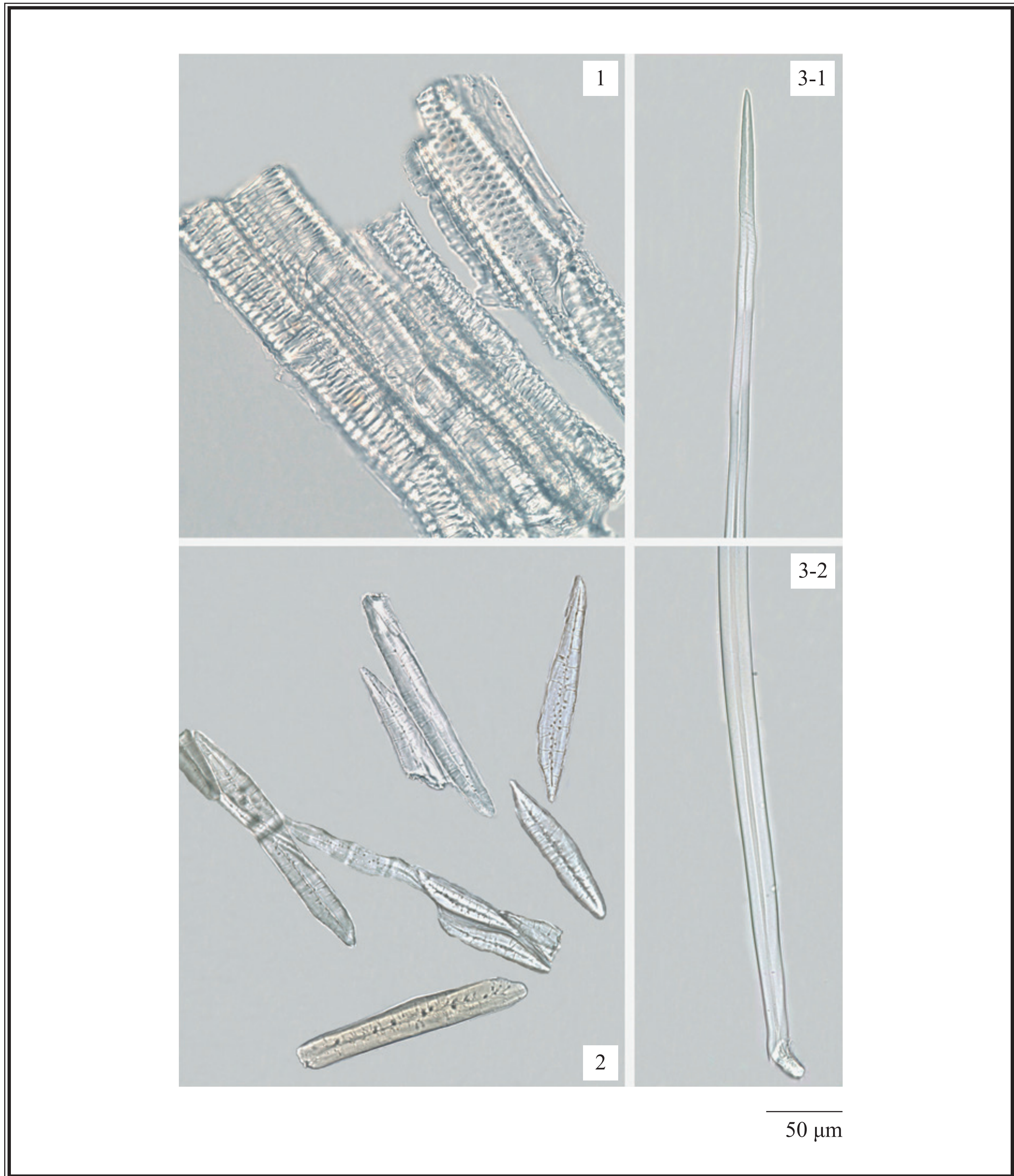


Figure 3 Microscopic features of powder of Pulsatillae Radix (under the light microscope)

1. Vessels 2. Phloem fibres 3. Non-glandular hair (3-1 upper part, 3-2 lower part)

4.2 Thin-Layer Chromatographic Identification *[Appendix IV(A)]*

Standard solution

Anemoside B4 standard solution

Weigh 1.0 mg of anemoside B4 CRS (Fig. 4) and dissolve in 1 mL of ethanol.

Developing solvent system

Prepare a mixture of *n*-butanol, water and glacial acetic acid (6:2:1, v/v).

Spray reagent

Add slowly 10 mL of sulphuric acid to 90 mL of ethanol.

Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL conical flask, then add 10 mL of ethanol. Sonicate (270 W) the mixture for 10 min. Filter the mixture.

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately anemoside B4 standard solution (3 μL) and the test solution (2 μL) to the plate. Before the development, add the developing solvent to one of the troughs of the chamber and place the HPTLC plate in the other trough. Cover the chamber with a lid and let equilibrate for about 15 min. Carefully tilt the chamber to allow sufficient solvent to pass from the trough containing the solvent to the other containing the HPTLC plate for development. Develop over a path of about 5 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Spray the plate evenly with the spray reagent and heat at about 105ºC until the spots or bands become visible (about 5-10 min). Examine the plate under visible light. Calculate the R_f value by using the equation as indicated in Appendix IV (A).

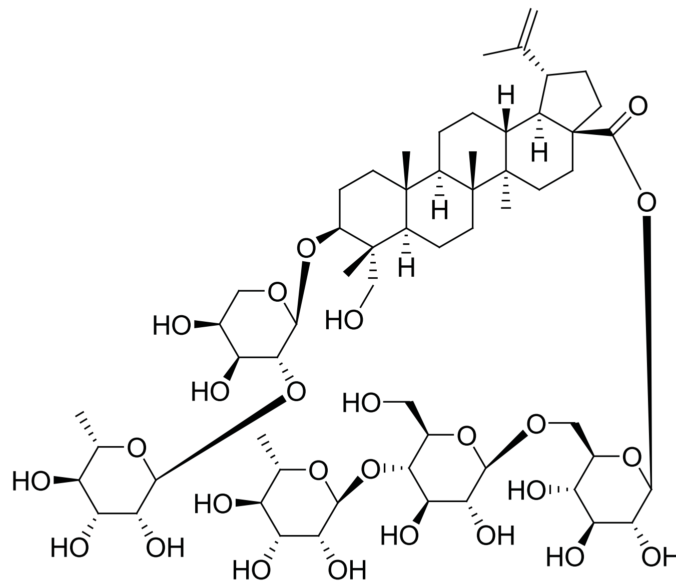


Figure 4 Chemical structure of anemoside B4

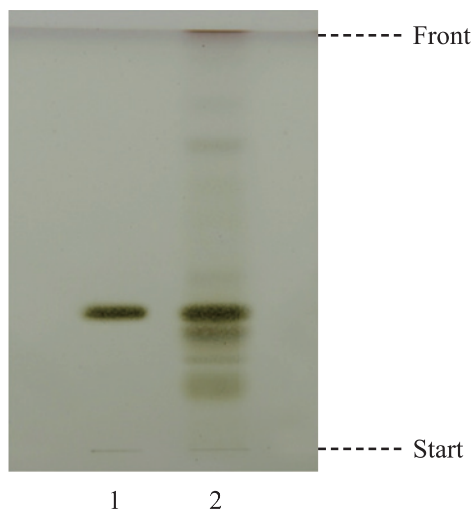


Figure 5 A reference HPTLC chromatogram of Pulsatillae Radix extract observed under visible light after staining

1. Anemoside B4 standard solution 2. Test solution

For positive identification, the sample must give spot or band with chromatographic characteristics, including the colour and the R_f value, corresponding to that of anemoside B4 (Fig. 5).

4.3 High-Performance Liquid Chromatographic Fingerprinting *(Appendix XII)*

Standard solution

Anemoside B4 standard solution for fingerprinting, Std-FP (500 mg/L)

Weigh 5.0 mg of anemoside B4 CRS and dissolve in 10 mL of methanol.

Test solution

Weigh 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 10 mL of methanol. Sonicate (270 W) the mixture for 30 min. Centrifuge at about 5000 × *g* for 5 min. Filter and transfer the filtrate to a 25-mL volumetric flask. Repeat the extraction for one more time. Combine the filtrates and make up to the mark with methanol. Filter through a 0.45-µm RC filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (201 nm) and a Hydrophilic Interaction Chromatography (HILIC) column (4.6 × 250 mm), 5 µm particle size. The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 30	10 → 20	90 → 80	linear gradient
30 – 45	20 → 10	80 → 90	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of anemoside B4 Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of anemoside B4 should not be more than 5.0%; the RSD of the retention time of anemoside B4 peak should not be more than 2.0%; the column efficiency determined from anemoside B4 peak should not be less than 9000 theoretical plates.

The *R* value between peak 2 and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 6).

Procedure

Separately inject anemoside B4 Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention time of anemoside B4 peak in the chromatogram of anemoside B4 Std-FP and the retention times of the three characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify anemoside B4 peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of anemoside B4 Std-FP. The retention times of anemoside B4 peaks from the two chromatograms should not differ by more than 2.0%. Calculate the RRTs of the characteristic peaks by using the equation as indicated in Appendix XII.

The RRTs and acceptable ranges of the three characteristic peaks of Pulsatillae Radix extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the three characteristic peaks of Pulsatillae Radix extract

Peak No.	RRT	Acceptable Range
1	0.65	± 0.03
2 (marker, anemoside B4)	1.00	-
3	1.50	± 0.05

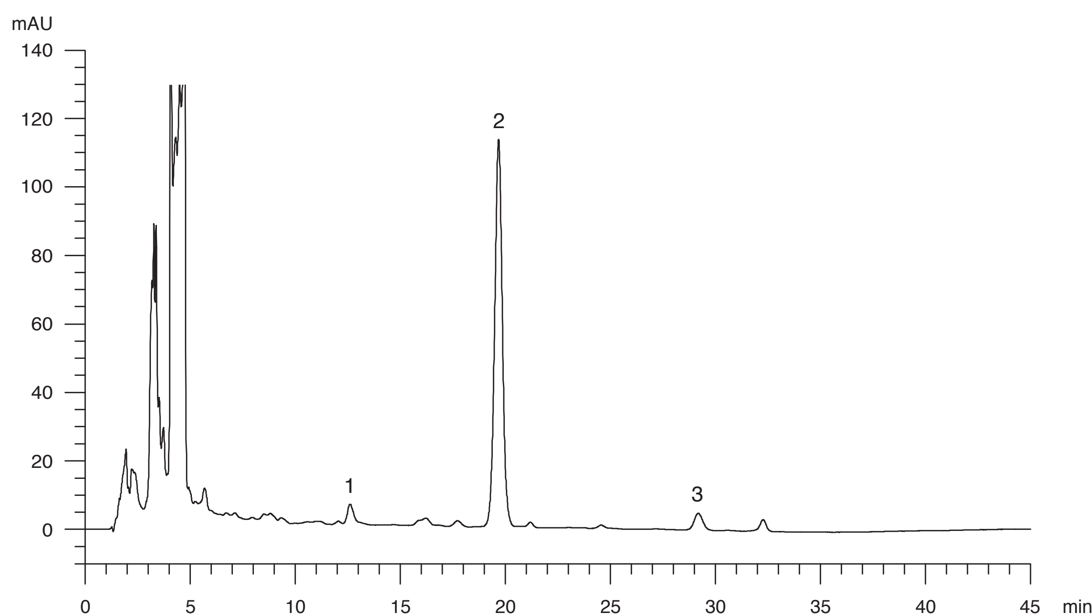


Figure 6 A reference fingerprint chromatogram of Pulsatillae Radix extract

For positive identification, the sample must give the above three characteristic peaks with RRTs falling within the acceptable range of the corresponding peaks in the reference fingerprint chromatogram (Fig. 6).

5. TESTS

5.1 **Heavy Metals** *(Appendix V)*: meet the requirements.

5.2 **Pesticide Residues** *(Appendix VI)*: meet the requirements.

5.3 **Mycotoxins** *(Appendix VII)*: meet the requirements.

5.4 **Sulphur Dioxide Residues** *(Appendix XVII)*: meet the requirements.

5.5 **Foreign Matter** *(Appendix VIII)*: not more than 3.0%.

5.6 **Ash** *(Appendix IX)*

Total ash: not more than 11.0%.
Acid-insoluble ash: not more than 5.0%.

5.7 **Water Content** *(Appendix X)*

Oven dried method: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 19.0%.
Ethanol-soluble extractives (cold extraction method): not less than 21.0%.

7. ASSAY

Carry out the method as directed in Appendix IV (B).

Standard solution
Anemoside B4 standard stock solution, Std-Stock (1000 mg/L)
Weigh accurately 5.0 mg of anemoside B4 CRS and dissolve in 5 mL of methanol.
Anemoside B4 standard solution for assay, Std-AS
Measure accurately the volume of the anemoside B4 Std-Stock, dilute with methanol to produce a series of solutions of 10, 200, 500, 800, 1000 mg/L for anemoside B4.

Test solution
Weigh accurately 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 10 mL of methanol. Sonicate (270 W) the mixture for 30 min. Centrifuge at about 5000 × *g* for 5 min. Filter and transfer the filtrate to a 25-mL volumetric flask. Repeat the extraction for one more time. Combine the filtrates and make up to the mark with methanol. Filter through a 0.45-µm RC filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (201 nm) and a Hydrophilic Interaction Chromatography (HILIC) column (4.6 × 250 mm), 5 µm particle size. The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 30	10 → 20	90 → 80	linear gradient
30 – 45	20 → 10	80 → 90	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of anemoside B4 Std-AS (500 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of anemoside B4 should not be more than 5.0%; the RSD of the retention time of anemoside B4 peak should not be more than 2.0%; the column efficiency determined from anemoside B4 peak should not be less than 9000 theoretical plates.

The *R* value between anemoside B4 peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curve

Inject a series of anemoside B4 Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of anemoside B4 against the corresponding concentrations of anemoside B4 Std-AS. Obtain the slope, y-intercept and the r^2 value from the 5-point calibration curve.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify anemoside B4 peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of anemoside B4 Std-AS. The retention times of anemoside B4 peaks from the two chromatograms should not differ by more than 5.0%. Measure the peak area and calculate the concentration (in milligram per litre) of anemoside B4 in the test solution, and calculate the percentage content of anemoside B4 in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 6.2% of anemoside B4 ($C_{59}H_{96}O_{26}$), calculated with reference to the dried substance.