Rhizoma Anemarrhenae

Figure 1 A photograph of Rhizoma Anemarrhenae (A: Maozhimu B: Guangzhimu)

1. NAMES

Official Name: Rhizoma Anemarrhenae

Chinese Name: 知母

Chinese Phonetic Name: Zhimu

2. SOURCE

Rhizoma Anemarrhenae is the dried rhizome of *Anemarrhena asphodeloides* Bge. (Liliaceae). The rhizome is collected in spring or autumn, remnants of the aerial stem and fibrous roots removed, then dried under the sun (to produce 'Maozhimu') or alternatively, the cork is peeled off when the rhizome is still fresh, then dried under the sun (to produce 'Guangzhimu') to obtain Rhizoma Anemarrhenae.

3. DESCRIPTION

Maozhimu: Cork intact. Slat-shaped, slightly curved, somewhat compressed, occasionally branched, 3-15 cm long, 8-20 mm in diameter, with pale yellowish stem and leaf scars. Externally yellowish-brown to brown, the upper part exhibiting a concave groove and closely arranged annular nodes with dense yellowish-brown remains of leaf bases arising upward laterally; the lower part is raised and somewhat shrivelled, exhibiting depressions or protruding dotted root scars. Texture hard, easily broken, fracture yellowish-white. Odour slight; taste slightly sweetish, bitterish and viscous on chewing (Fig. 1A).

Guangzhimu: Outer cork removed, 5-15 mm in diameter. Externally yellowish-white, with twisted grooves, sometimes with leaf scars and root scars (Fig. 1B).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Phelloderm or cork mostly remains in Maozhimu, absent (due to removal) in Guangzhimu, consists of several layers of polygonal cork cells and over 10 layers of sub-rectangular cork cells, slightly lignified. Leaf-trace vascular bundles scattered in suberized and non-suberized cortex occasionally. Mucilage cells numerous, containing raphides of calcium oxalate. Endodermis invisible. Stele broad, root-trace vascular bundles tangentially arranged along the pericycle; collateral or amphivasal vascular bundles small and scattered. Some parenchyma cells are mucilaginous and contain raphides of calcium oxalate (Fig. 2).

Powder

Colour pale yellow. Raphides of calcium oxalate easily observed, in bundles or scattered, 20-110 μm long. Cork cells yellowish-brown, varying in shape in surface view, anticlinal walls thin, several cells appearing overlapped when viewed from the top view. Vessels bordered-pitted or spiral, 6-25 μm in diameter. Fibres very slender, 6-15 μm in diameter (Fig. 3).

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

Standard solution

Sarsasapogenin standard solution

Weigh 2.0 mg of sarsasapogenin CRS (Fig. 4) and dissolve in 1 mL of dichloromethane.

Developing solvent system

Prepare a mixture of ethyl acetate and cyclohexane (1:4, v/v).

Spray reagent

Mix cautiously 25 mL of sulphuric acid (20%, v/v) into 25 mL of ice-cold glacial acetic acid. Add 2.5 mL of *p*-anisaldehyde. Add further 50 mL of sulphuric acid (20%, v/v).

Test solution

Weigh 0.5 g of the powdered sample and place it in a 50-mL conical flask, then add 25 mL of absolute ethanol. Shake the mixture for about 15 h. Sonicate (220 W) the mixture for 40 min. Filter and transfer 10 mL of filtrate to a 50-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Add 11 mL of 1.1 M hydrochloric acid. Reflux the solution for 2 h. Cool in an ice bath. Add dropwise about 1 mL of sodium hydroxide solution (40%, w/v) with shaking. Transfer the solution to a separating funnel. Extract for three times each with 50 mL of dichloromethane. Combine the extracts. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 4 mL of dichloromethane.

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate and a freshly prepared developing solvent system as described above. Apply separately sarsasapogenin standard solution (2 μL) and the test solution (4 μL) to the plate. Develop over a path of about 8 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, then heat at about 110°C until the spots or bands become visible (about 10 min). Examine the plate under visible light. Calculate the R_f value by using the equation as indicated in Appendix IV(A).

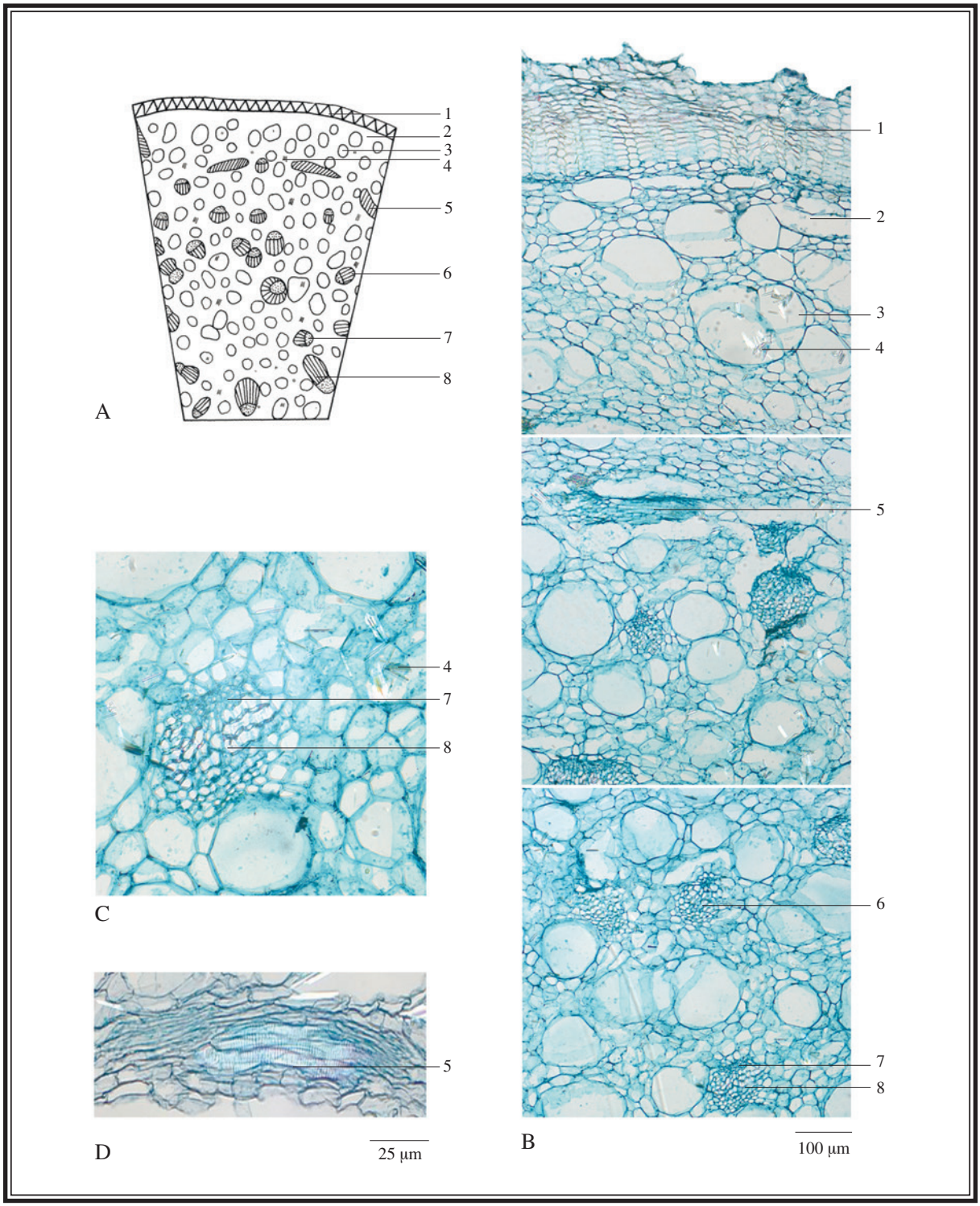


Figure 2 Microscopic features of transverse section of Rhizoma Anemarrhenae

A. Sketch B. Section illustration C. Vascular bundles
D. Root-trace vascular bundle

1. Suberized cortex 2. Cortex 3. Mucilage cells 4. Raphides of calcium oxalate
5. Root-trace vascular bundles 6. Vascular bundles 7. Phloem 8. Xylem

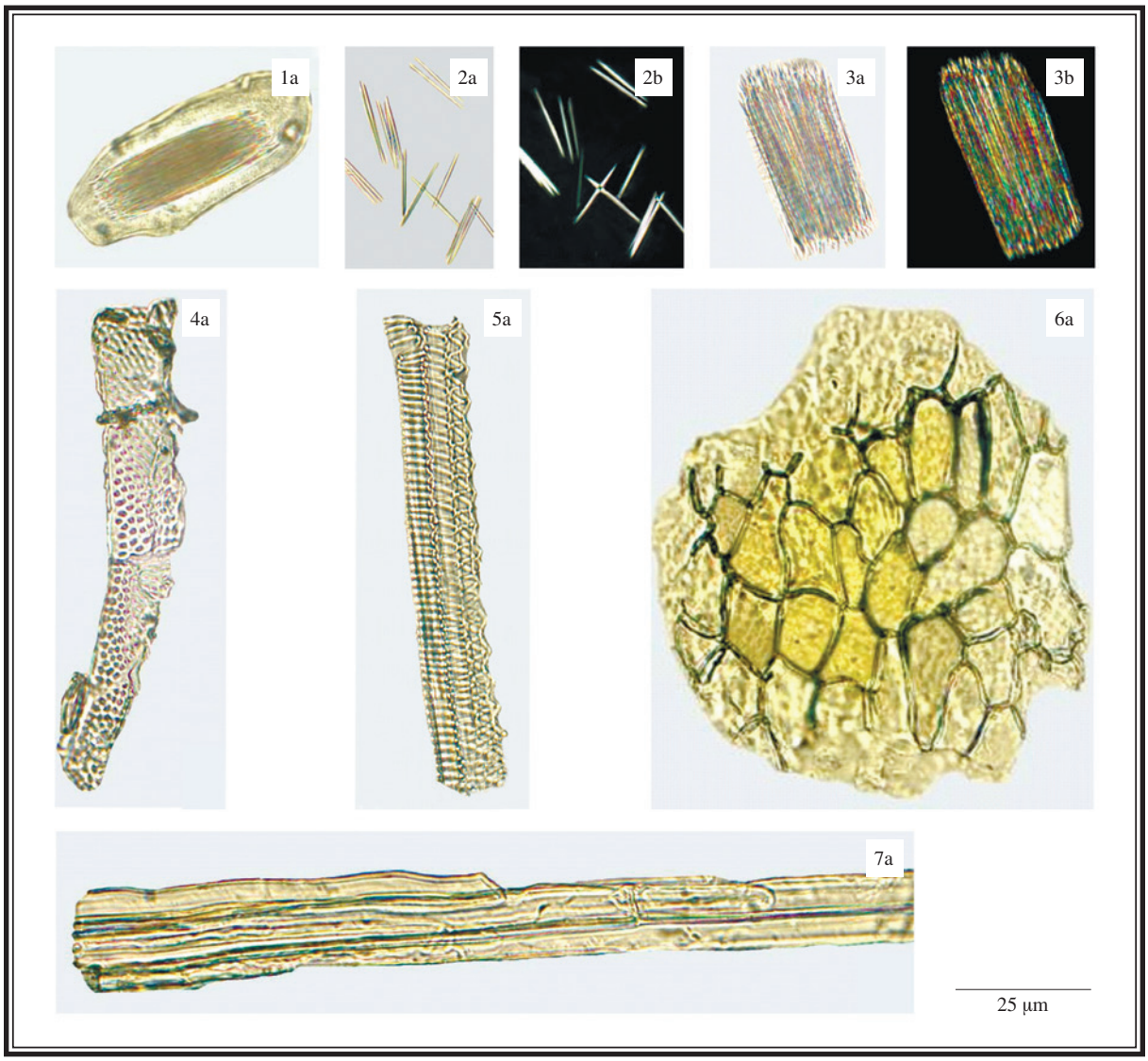


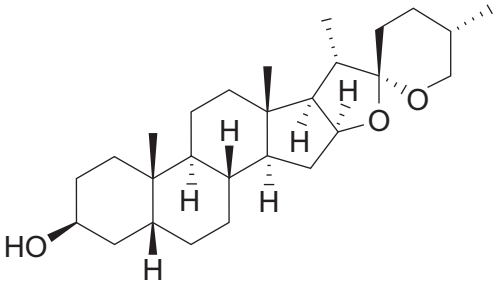
Figure 3 Microscopic features of powder of Rhizoma Anemarrhenae

1. Mucilage cells 2. Raphides of calcium oxalate (broken bundle)
3. Raphides of calcium oxalate (in bundles) 4. Bordered-pitted vessels
5. Spiral vessels 6. Cork cells 7. Fibres

a. Features under the light microscope b. Features under the polarized microscope

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 sarsasapogenin.

(i)



(ii)

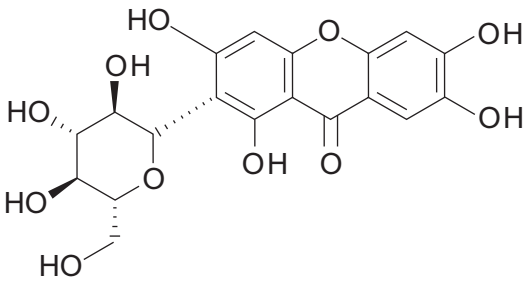


Figure 4 Chemical structures of (i) sarsasapogenin and (ii) mangiferin

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

Standard solution

Mangiferin standard solution for fingerprinting, Std-FP (30 mg/L)

Weigh 1.5 mg of mangiferin CRS (Fig. 4) and dissolve in 50 mL of methanol (70%).

Test solution

Weigh 0.5 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 40 mL of methanol (70%). Sonicate (220 W) the mixture for 30 min. Filter and transfer the filtrate to a 50-mL volumetric flask and make up to the mark with methanol (70%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (257 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 0.5 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	0.03% Phosphoric acid (%, v/v)	Methanol (%, v/v)	Elution
0–10	100→90	0→10	linear gradient
10–35	90→50	10→50	linear gradient
35–40	50→20	50→80	linear gradient
40–60	20	80	isocratic

System suitability requirements

Perform at least five replicate injections, each using 20 μL of mangiferin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of mangiferin should not be more than 3.0%; the RSD of the retention time of mangiferin peak should not be more than 2.0%; the column efficiency determined from mangiferin peak should not be less than 50000 theoretical plates.

The *R* value between peak 1 and peak 2 in the chromatogram of the test solution should not be less than 1.5 (Fig. 5).

Procedure

Separately inject mangiferin Std-FP and the test solution (20 μL each) into the HPLC system and record the chromatograms. Measure the retention time of mangiferin peak in the chromatogram of mangiferin Std-FP and the retention times of the three characteristic peaks (Fig. 5) in the chromatogram of the test solution. Identify mangiferin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of mangiferin Std-FP. The retention times of mangiferin 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 Rhizoma Anemarrhenae extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the three characteristic peaks of Rhizoma Anemarrhenae extract

Peak No.	RRT	Acceptable Range
1	0.86	±0.03
2 (marker, mangiferin)	1.00	-
3	1.33	±0.03

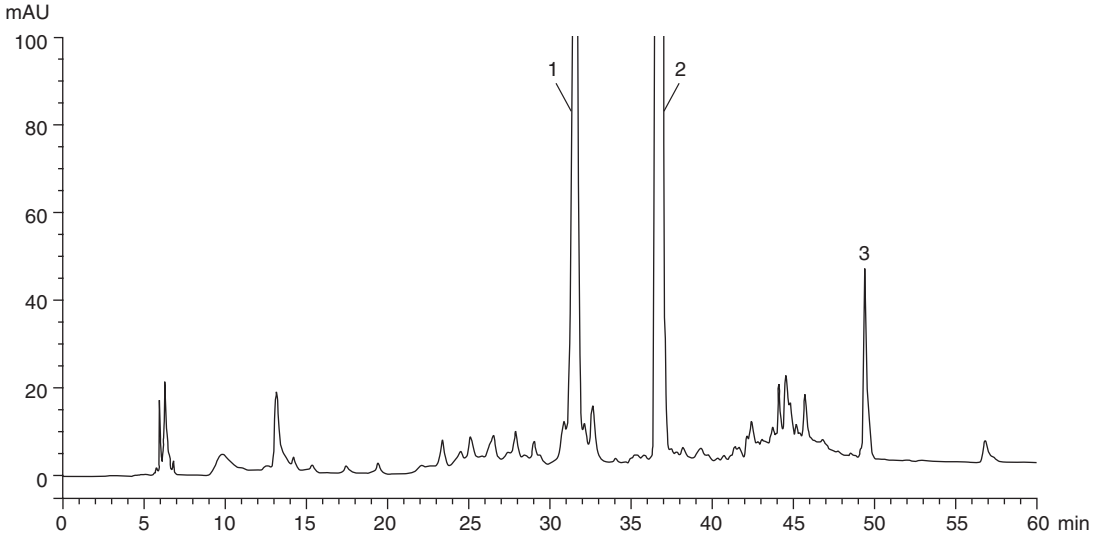


Figure 5 A reference fingerprint chromatogram of Rhizoma Anemarrhenae extract

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. 5).

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 XV)*: meet the requirements.

5.5 Foreign Matter *(Appendix VIII)*: not more than 1.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 5.5%.

Acid-insoluble ash: not more than 1.5%.

5.7 Water Content *(Appendix X)*: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 51.0%.

Ethanol-soluble extractives (hot extraction method): not less than 49.0%.

7. ASSAY

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

Standard solution

Sarsasapogenin standard stock solution, Std-Stock (1000 mg/L)

Weigh accurately 5.0 mg of sarsasapogenin CRS and dissolve in 5 mL of methanol.

Sarsasapogenin standard solution for assay, Std-AS

Measure accurately the volume of the sarsasapogenin Std-Stock, dilute with methanol to produce a series of solutions of 30, 50, 100, 200, 300 mg/L for sarsasapogenin.

Test solution

Weigh accurately 0.25 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 40 mL of ethanol (95%). Shake the mixture for 15 h. Sonicate (220 W) the mixture for 40 min. Centrifuge at about 3000 × *g* for 3 min. Transfer the supernatant to a 250-mL conical flask. Repeat the sonication extraction for two more times each with 40 mL of ethanol (95%). Combine the extracts. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Add 20 mL of water and then 2 mL of hydrochloric acid to the residue. Reflux the mixture for 2 h. Cool to room temperature. Adjust the pH to neutral by adding dropwise of sodium hydroxide solution (10%, w/v) (about 8 mL) with shaking in an ice bath. Transfer the solution to a separating funnel. Extract for three times each with 50 mL of dichloromethane. Combine the extracts. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 25-mL volumetric flask and make up to the mark with methanol. Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with an ELSD [drift tube temperature: 70˚C; nebulizer gas (N_2) flow: 1.8 L/min] and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 0.5 mL/min. The mobile phase is methanol. The elution time is about 30 min.

System suitability requirements

Perform at least five replicate injections, each using 20 μL of sarsasapogenin Std-AS (100 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of sarsasapogenin should not be more than 5.0%; the RSD of the retention time of sarsasapogenin peak should not be more than 2.0%; the column efficiency determined from sarsasapogenin peak should not be less than 20000 theoretical plates.

The *R* value between sarsasapogenin 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 sarsasapogenin Std-AS (20 μL each) into the HPLC system and record the chromatograms. Plot the natural logarithm of peak areas of sarsasapogenin against the natural logarithm of the corresponding concentrations of sarsasapogenin Std-AS. Obtain the slope, y-intercept and the r^2 value from the 5-point calibration curve.

Procedure

Inject 20 μL of the test solution into the HPLC system and record the chromatogram. Identify sarsasapogenin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of sarsasapogenin Std-AS. The retention times of sarsasapogenin peaks from the two chromatograms should not differ by more than 2.0%. Measure the peak area and calculate the concentration (in milligram per litre) of sarsasapogenin in the test solution by using the following equation –

$$\text{Concentration of sarsasapogenin in the test solution} = e^{[\text{Ln (A)}-I]/m}$$

Where A = the peak area of sarsasapogenin in the test solution,

I = the y-intercept of the 5-point calibration curve,

m = the slope of the 5-point calibration curve.

Calculate the percentage content of sarsasapogenin in the sample by using the equations indicated in Appendix IV(B).

Limits

The sample contains not less than 1.3% of sarsasapogenin ($C_{27}H_{44}O_3$), calculated with reference to the dried substance.