Polygoni Tinctorii Folium

Figure 1 A photograph of Polygoni Tinctorii Folium

A. Polygoni Tinctorii Folium B. Magnified image of upper surface of intact leaf
C. Magnified image of lower surface of intact leaf

1. NAMES

Official Name: Polygoni Tinctorii Folium

Chinese Name: 蓼大青葉

Chinese Phonetic Name: Liaodaqingye

2. SOURCE

Polygoni Tinctorii Folium is the dried leaf of *Polygonum tinctorium* Ait. (Polygonaceae). The leaf is collected once in summer and once in autumn when foliage branch growing luxuriantly, stem and foreign matter removed, then dried under the sun to obtain Polygoni Tinctorii Folium.

3. DESCRIPTION

Mostly crumpled and broken, when intact flattened out, elliptical, 3-8 cm long, 2-5 cm wide, bluish-green to blackish-blue, apex obtuse, base attenuate, margin entire. Vein pale yellowish-brown, slightly protuberant on the lower surface. Petioles flattened, occasionally with membranaceous ocrea. Texture fragile. Odour slight; taste slightly astringent and bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Upper and lower epidermis consists of 1 layer of rectangular cells. Several layers of collenchymatous cells beneath the inner side of both epidermal layers in midvein region. Vascular bundles collateral, 6-8, arranged in an interrupted ring, the upper one in the middle relative large. Fibre bundles located on the outer side of the phloem, wall thickened and lignified. Palisade tissue consists of 2-3 layers of cells. Mesophyll cells contain clusters of calcium oxalate, polychromatic under the polarized microscope (Fig. 2).

Powder

Colour dark green. Upper epidermal cells subrectangular, anticlinal walls straight, stomata paracytic. Lower epidermal cells polygonal, anticlinal walls straight or slightly sinuous, stomata paracytic, a few anomocytic. Mesophyll tissues contain numerous blue to bluish-black pigment granules. Glandular hairs consist of 4- to 8-celled head and a bicellular or multicellular stalk, 11-45 µm in diameter. Non-glandular hairs multiseriate, with lignified and thickened walls, 112-370 µm long. Clusters of calcium oxalate numerous, 12-80 µm in diameter; polychromatic under the polarized microscope (Fig. 3).

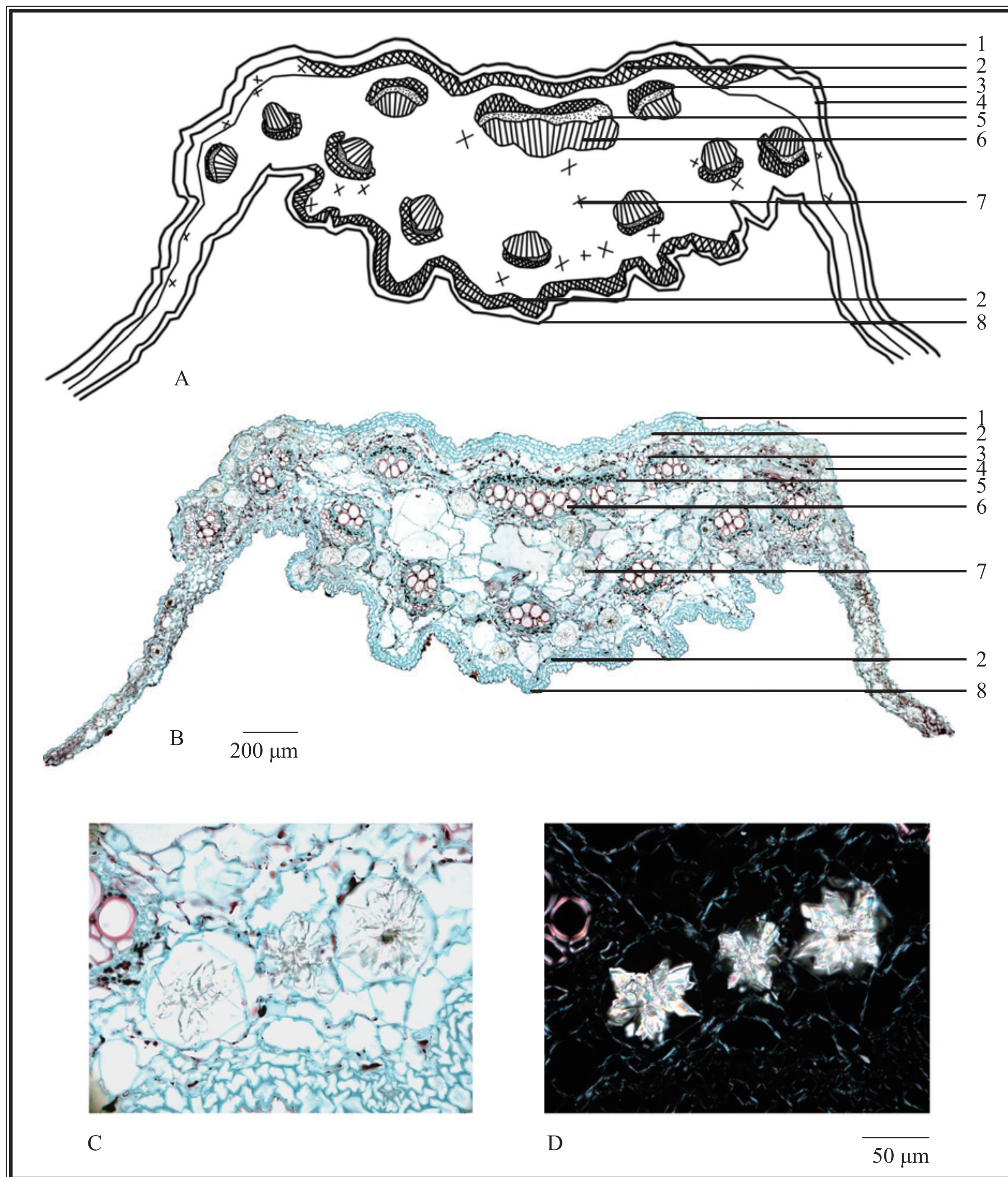

Figure 2 Microscopic features of transverse section of Polygoni Tinctorii Folium

A. Sketch B. Section illustration
C. Clusters of calcium oxalate (under the light microscope)
D. Clusters of calcium oxalate (under the polarized microscope)

1. Upper epidermis 2. Collenchyma 3. Fibre bundles 4. Palisade tissue
5. Phloem 6. Xylem 7. Clusters of calcium oxalate 8. Lower epidermis

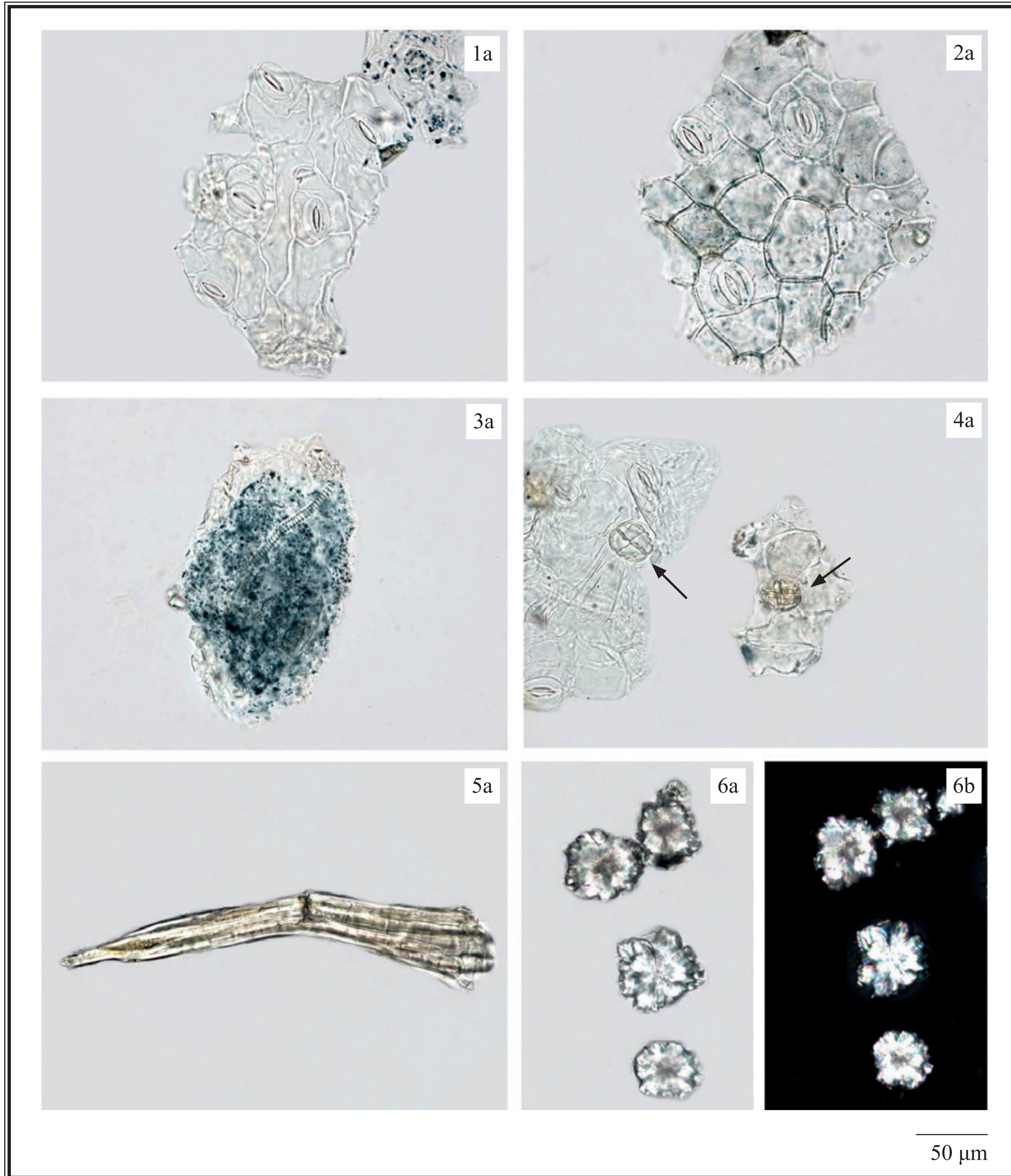

Figure 3 Microscopic features of powder of Polygoni Tinctorii Folium

1. Upper epidermal cells with stomata 2. Lower epidermal cells with stomata
3. Mesophyll tissue contains pigment granules 4. Glandular hairs
5. Non-glandular hair 6. Clusters of calcium oxalate

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

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

Standard solutions

Indigo standard solution

Weigh 2.0 mg of indigo CRS (Fig. 4) and dissolve in 1 mL of ethanol.

Indirubin standard solution

Weigh 0.2 mg of indirubin CRS (Fig. 4) and dissolve in 1 mL of ethanol.

Developing solvent system

Prepare a mixture of dichloromethane and acetone (40:1, v/v).

Test solution

Weigh 1.0 g of the powdered sample (through a No. 5 sieve) and place it in a 50-mL conical flask, then add 20 mL of ethanol. Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 50-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 1 mL of ethanol.

Procedure

Carry out the method by using a TLC silica gel F_{254} plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately indigo standard solution (2 μL), indirubin standard solution (2 μL) and the test solution (8 μL) to the plate. Before the development, add the developing solvent to one of the troughs of the chamber and place the TLC plate in the other trough. Cover the chamber with a lid and let equilibrate for about 5 min. Carefully tilt the chamber to allow sufficient solvent to pass from the trough containing the solvent to the other containing the TLC plate for development. Develop over a path of about 6 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Examine the plate under visible light. Calculate the R_f values by using the equation as indicated in Appendix IV (A).

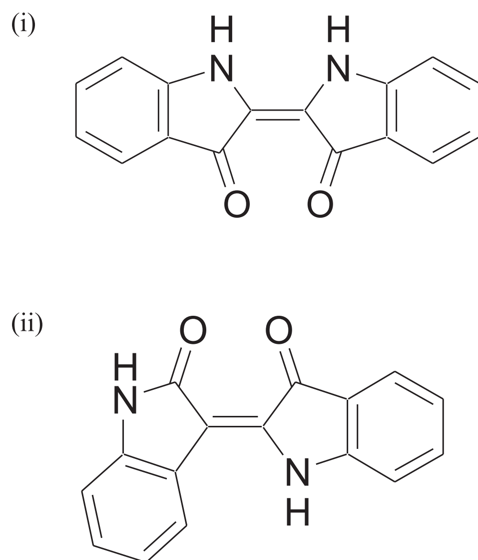

Figure 4 Chemical structures of (i) indigo and (ii) indirubin

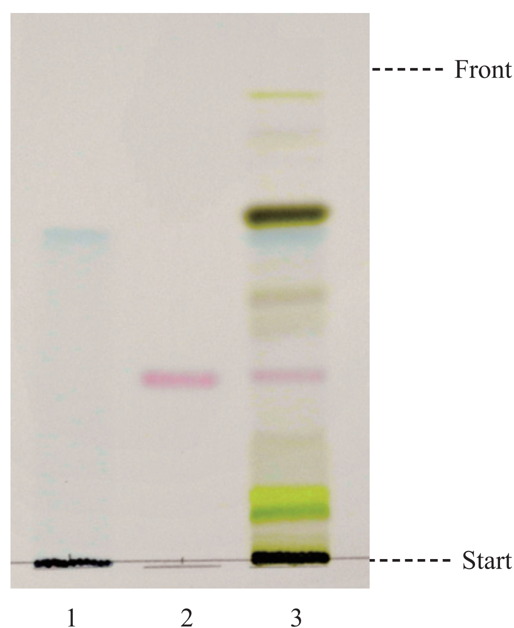

Figure 5 A reference TLC chromatogram of Polygoni Tinctorii Folium extract observed under visible light

1. Indigo standard solution 2. Indirubin standard solution 3. Test solution

For positive identification, the sample must give spots or bands with chromatographic characteristics, including the colour and the R_f values, corresponding to those of indigo and indirubin (Fig. 5).

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

Standard solutions

Indigo standard solution for fingerprinting, Std-FP (5 mg/L)

Weigh 2.5 mg of indigo CRS and dissolve in 250 mL of chloral hydrate-dichloromethane (2%, w/v). Pipette 5 mL of the solution to a 10-mL volumetric flask and make up to the mark with methanol.

Indirubin standard solution for fingerprinting, Std-FP (0.4 mg/L)

Weigh 0.1 mg of indirubin CRS and dissolve in 250 mL of methanol.

Standard solution

Weigh 0.25 g of the powdered sample (through a No. 5 sieve) and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (100 W) the mixture for 1 h. Centrifuge at about 3000 x *g* for 10 min. Filter through a 0.45-µm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (290 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (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)	Acetonitrile (%, v/v)	Water (%, v/v)	Elution
0 – 15	28	72	isocratic
15 – 30	28 → 50	72 → 50	linear gradient
30 – 40	50 → 60	50 → 40	linear gradient
40 – 60	60	40	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of indigo Std-FP and indirubin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of indigo and indirubin should not be more than 5.0%; the RSD of the retention times of indigo and indirubin peaks should not be more than 2.0%; the column efficiencies determined from indigo and indirubin peaks should not be less than 80000 theoretical plates.

The *R* value between peak 3 and the closest peak; and the *R* value between peak 4 and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 6).

Procedure

Separately inject indigo Std-FP, indirubin Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention times of indigo and indirubin peaks in the chromatograms of indigo Std-FP, indirubin Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify indigo and indirubin peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of indigo Std-FP and indirubin Std-FP. The retention times of indigo and indirubin peaks in the chromatograms of the test solution and the corresponding Std-FP 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 four characteristic peaks of Polygoni Tinctorii Folium extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of Polygoni Tinctorii Folium extract

Peak No.	RRT	Acceptable Range
1	0.71	± 0.03
2	0.91	± 0.03
3 (marker, indigo)	1.00	-
4 (indirubin)	1.07	± 0.03

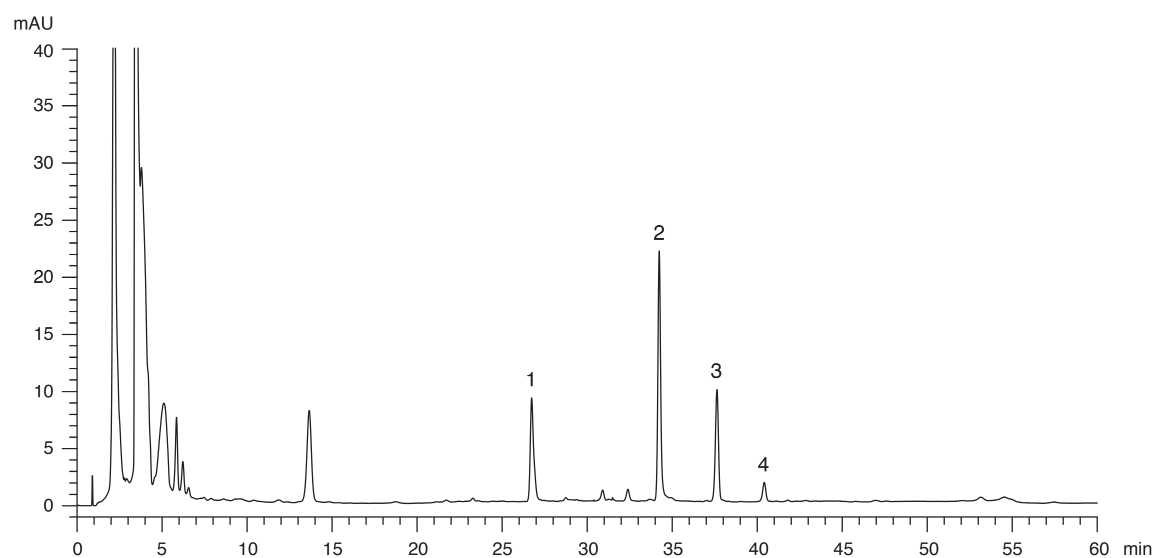

Figure 6 A reference fingerprint chromatogram of Polygoni Tinctorii Folium extract

For positive identification, the sample must give the above four 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 4.0%.

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

Total ash: not more than 23.5%.
Acid-insoluble ash: not more than 12.0%.

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

Oven dried method: not more than 8.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 17.0%.
Ethanol-soluble extractives (hot extraction method): not less than 11.0%.

7. ASSAY

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

Standard solution
Indigo standard stock solution, Std-Stock (10 mg/L)
Weigh accurately 2.5 mg of indigo CRS and dissolve in 250 mL of chloral hydrate-dichloromethane (2%, w/v).
Indigo standard solution for assay, Std-AS
Measure accurately the volume of the indigo Std-Stock, dilute with chloral hydrate-dichloromethane (2%, w/v) to produce a series of solutions of 0.5, 1.5, 3, 5, 7 mg/L for indigo.

Test solution

Weigh accurately 20 mg of the powdered sample (through a No. 5 sieve) and place it in a 50-mL centrifuge tube, then add 50 mL of chloral hydrate-dichloromethane (2%, w/v). Sonicate (100 W) the mixture for 1.5 h. Centrifuge at about 3000 x *g* for 5 min. Transfer the supernatant to a 50-mL volumetric flask. Make up to the mark with chloral hydrate-dichloromethane (2%, w/v). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (290 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 1.0 mL/min. The mobile phase is a mixture of methanol and water (65:35, v/v). The elution time is about 30 min.

System suitability requirements

Perform at least five replicate injections, each using 10 μL of indigo Std-AS (3 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of indigo should not be more than 5.0%; the RSD of the retention time of indigo peak should not be more than 2.0%; the column efficiency determined from indigo peak should not be less than 5000 theoretical plates.

The *R* value between indigo 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 indigo Std-AS (10 μL each) into the HPLC system and record the chromatograms. Plot the peak areas of indigo against the corresponding concentrations of indigo 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 indigo peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of indigo Std-AS. The retention times of indigo 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 indigo in the test solution, and calculate the percentage content of indigo in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 0.55% of indigo ($C_{16}H_{10}N_2O_2$), calculated with reference to the dried substance.