# Isatidis Folium


**Figure 1** A photograph of Isatidis Folium

## 1. NAMES

Official Name: Isatidis Folium

Chinese Name: 大青葉

Chinese Phonetic Name: Daqingye

## 2. SOURCE

Isatidis Folium is the dried leaf of *Isatis indigotica* Fort. (Brassicaceae). The leaf is collected 2-3 times in summer and autumn, foreign matter removed, then dried under the sun to obtain Isatidis Folium.

## 3. DESCRIPTION

Crumpled and mostly rolled, some are broken, the upper surface dark greyish-green, lower surface with a distinct prominent midvein. When wholly intact, oblong to oblanceolate, 5-20 cm long, 1.5-7 cm wide, base attenuate to the petiole with auricles, margin entire to slightly wavy, apex obtuse; petioles 2-11 cm long, pale brownish-yellow. Texture fragile. Odour slight; taste slightly sour, bitter, and astringent (Fig. 1).

## 4. IDENTIFICATION

### 4.1 Microscopic Identification *(Appendix III)*

**Transverse section**

Upper and lower epidermis consist of 1 layer of rectangular cells. Palisade tissue and spongy tissue are indistinctly differentiated. Vascular bundles collateral, 4-9, with a large one in the middle. Sclerenchyma located immediately outside of the xylem and the phloem. Undefined pigment bodies, bluish-black, scattered in the parenchymatous cells (Fig. 2).

**Powder**

Colour greenish-brown. Bluish-black pigment bodies are scattered in the mesophyll. Crystals with unknown identity, irregular in shape, 30-110 μm in diameter, are also found in the mesophyll. Sclerenchymatous cells occur in bundles. Vessels are in groups, by spiral and reticulate types, 7-55 μm in diameter. Anticlinal walls of lower epidermal cells slightly sinuous and beaded. Stomata anomocytic, with 3-4 subsidiary cells (Fig. 3).

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

**Standard solutions**

*Indigo standard solution*

Weigh 0.1 mg of indigo CRS (Fig. 4) and dissolve in 1 mL of a mixture of methanol and dichloromethane (1:9, v/v).

*Indirubin standard solution*

Weigh 0.1 mg of indirubin CRS (Fig. 4) and dissolve in 1 mL of a mixture of methanol and dichloromethane (1:9, v/v).

**Developing solvent system**

Prepare a mixture of dichloromethane and acetone (97:3, v/v).

**Test solution**

Weigh 2.0 g of the powdered sample and place it in a 50-mL conical flask, then add 20 mL of petroleum ether (60-80°C). Sonicate (240 W) the mixture for 15 min. Filter the mixture. Discard the filtrate. Add 20 mL of dichloromethane to the residue. Sonicate (240 W) the mixture for 15 min. Filter and evaporate the filtrate to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 3 mL of a mixture of methanol and dichloromethane (1:9, v/v).

**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 indigo standard solution (60 µL), indirubin standard solution (10 µL) and the test solution (9 µL) to the plate. 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).

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.

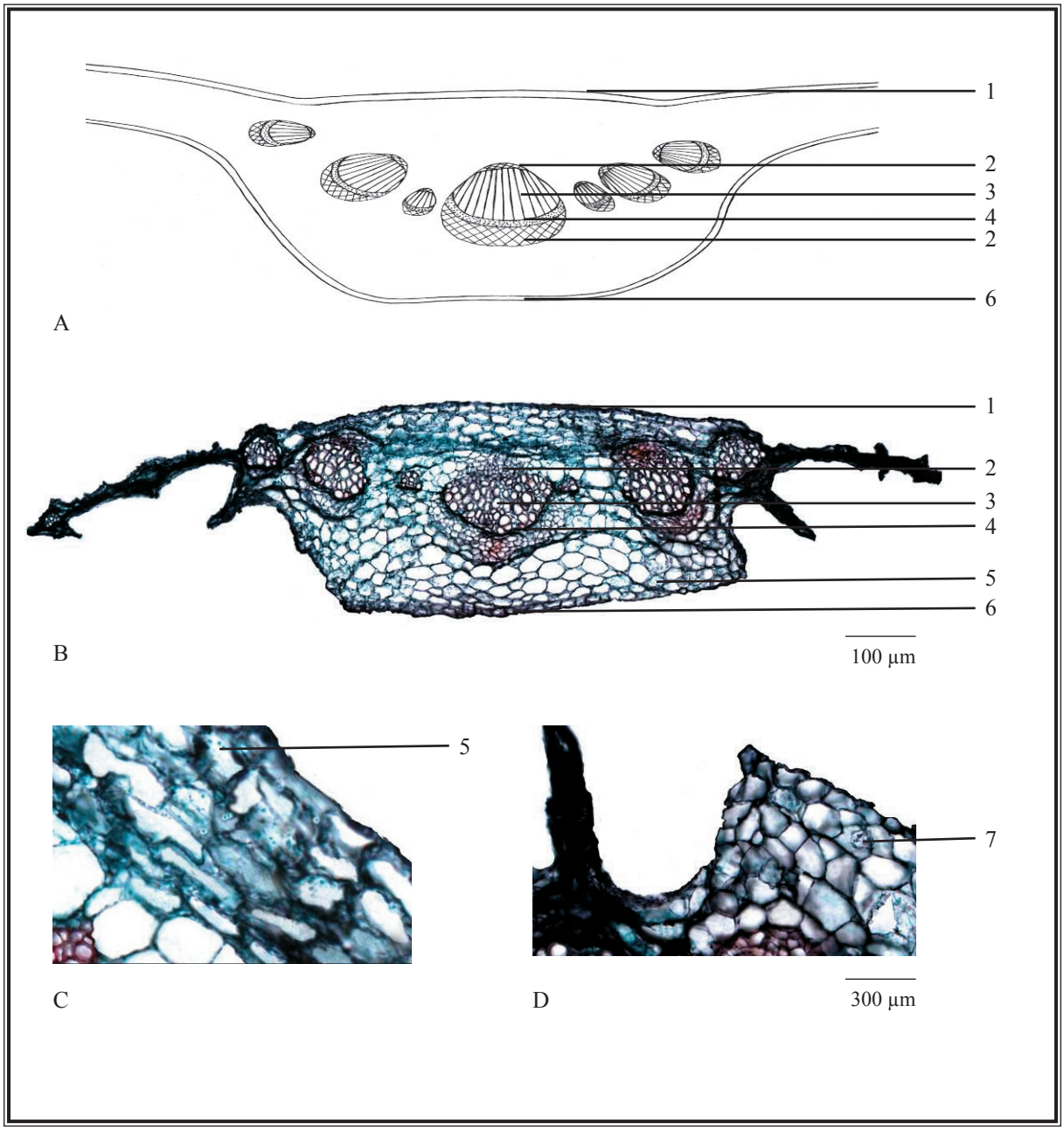



**Figure 2** Microscopic features of transverse section of Isatidis Folium

A. Sketch B. Section illustration C. Bluish-black pigment bodies
D. Crystals with unknown identity

1. Upper epidermis 2. Sclerenchyma 3. Xylem 4. Phloem
5. Bluish-black pigment bodies 6. Lower epidermis 7. Crystal with unknown identity

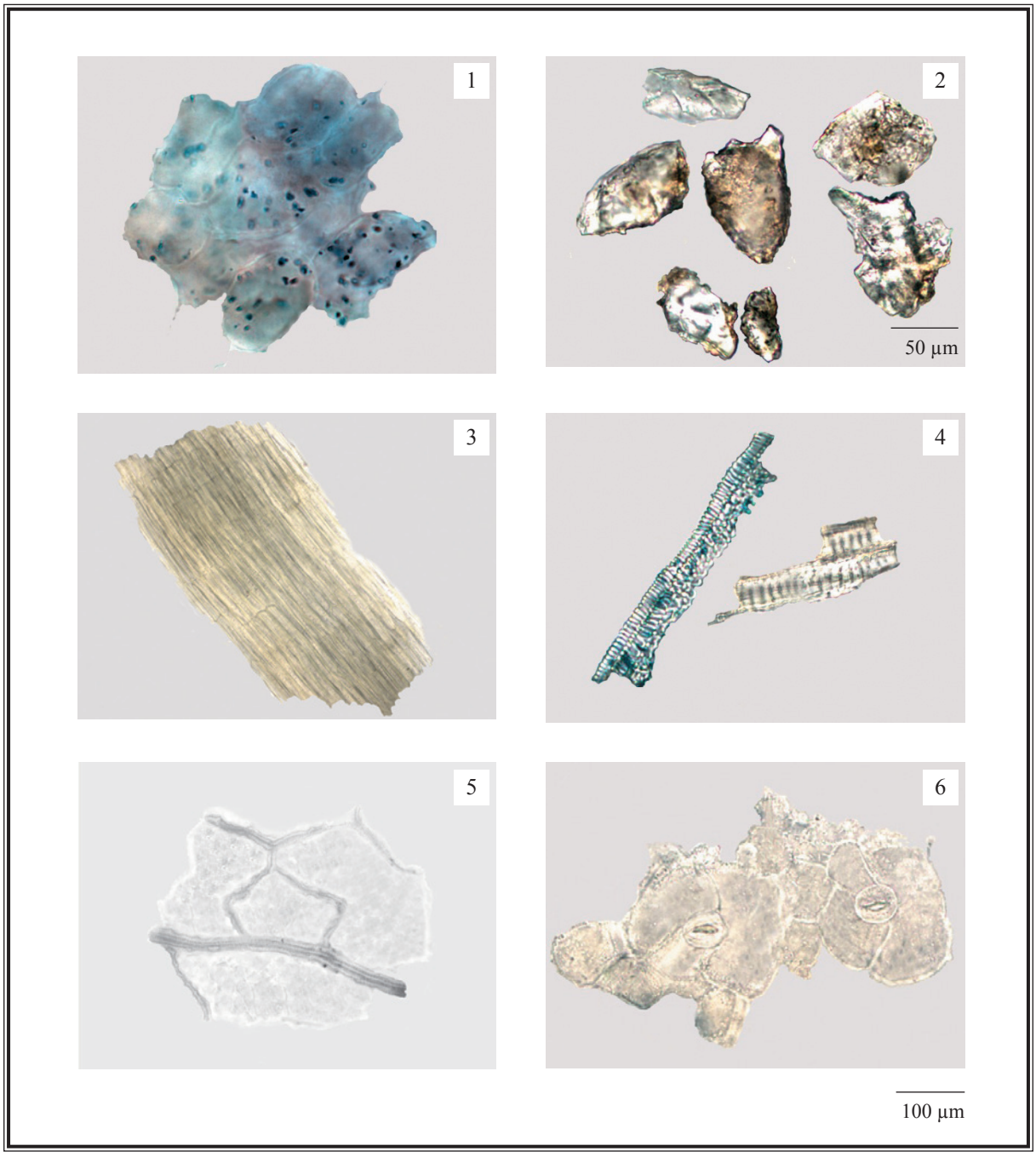
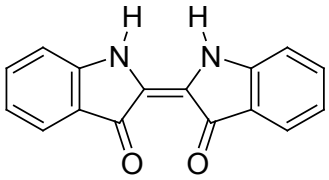



**Figure 3** Microscopic features of powder of Isatidis Folium (under the light microscope)

1. Bluish-black pigment bodies 2. Crystals with unknown identity 3. Sclerenchymatous cells
4. Spiral vessels 5. Lower epidermal cells 6. Anomocytic stomata

(i)



(ii)

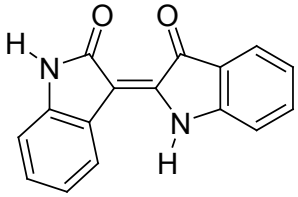


**Figure 4** Chemical structures of (i) indigo and (ii) indirubin

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

### Standard solutions

*Indigo standard solution for fingerprinting, Std-FP (2 mg/L)*

Weigh 0.2 mg of indigo CRS and place it in a 50-mL volumetric flask, then dissolve in 5 mL of a mixture of methanol and dichloromethane (1:9, v/v). Make up to the mark with methanol. Pipette 1 mL of the solution to a 2-mL volumetric flask and make up to the mark with methanol.

*Indirubin standard solution for fingerprinting, Std-FP (6 mg/L)*

Weigh 0.3 mg of indirubin CRS and dissolve in 50 mL of methanol.

### Test solution

Weigh 0.6 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 40 mL of ethyl acetate. Sonicate (240 W) the mixture for 1 h. Centrifuge at about 3000 × *g* for 10 min. Transfer the supernatant to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 25 mL of methanol. Filter through a 0.45-µm PTFE filter.

### Chromatographic system

The liquid chromatograph is equipped with a DAD (250 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) | Water (%, v/v) | Acetonitrile (%, v/v) | Elution |
|---|---|---|---|
| 0 – 10 | 80 → 78 | 20 → 22 | linear gradient |
| 10 – 20 | 78 → 45 | 22 → 55 | linear gradient |
| 20 – 45 | 45 → 43 | 55 → 57 | linear gradient |
| 45 – 60 | 43 → 40 | 57 → 60 | linear gradient |

**System suitability requirements**

Perform at least five replicate injections, each using 20 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 60000 and 40000 theoretical plates respectively.

The *R* value between peak 2 and the closest peak; and the *R* value between peak 3 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 5).

**Procedure**

Separately inject indigo Std-FP, indirubin Std-FP and the test solution (20 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 three characteristic peaks (Fig. 5) 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 three characteristic peaks of Isatidis Folium extract are listed in Table 2.

**Table 2** The RRTs and acceptable ranges of the three characteristic peaks of Isatidis Folium extract

| Peak No. | RRT | Acceptable Range |
|---|---|---|
| 1 | 0.71 | ± 0.03 |
| 2 (indigo) | 0.87 | ± 0.03 |
| 3 (marker, indirubin) | 1.00 | - |

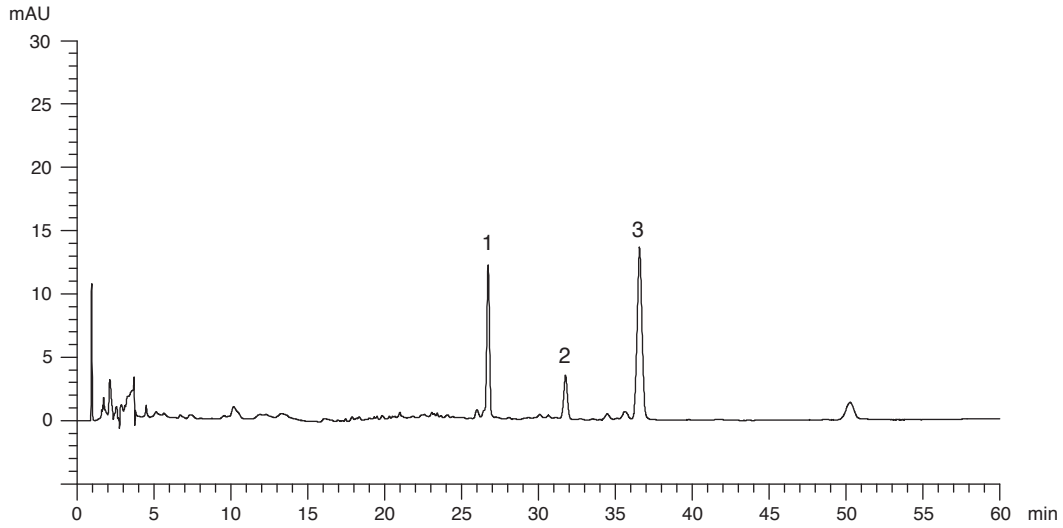



**Figure 5** A reference fingerprint chromatogram of Isatidis Folium extract

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 XVIII)*: meet the requirements.

**5.5 Foreign Matter** *(Appendix VIII)*: not more than 2.0%.

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

Total ash: not more than 21.0%.
Acid-insoluble ash: not more than 7.5%.

**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 3.0%.
Ethanol-soluble extractives (cold extraction method): not less than 22.0%.

## 7. ASSAY

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

### 7.1 Assay of indirubin

**Standard solution**

*Indirubin standard stock solution, Std-Stock (30 mg/L)*

Weigh accurately 1.5 mg of indirubin CRS and dissolve in 50 mL of methanol.

*Indirubin standard solution for assay, Std-AS*

Measure accurately the volume of the indirubin Std-Stock, dilute with methanol to produce a series of solutions of 0.1, 1, 1.5, 3, 4 mg/L for indirubin.

**Test solution**

Weigh accurately 0.25 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (240 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 10 min. Transfer the supernatant to a 50-mL volumetric flask. Repeat the extraction for one more time. Combine the supernatants and make up to the mark with methanol. Filter through a 0.45-μm PTFE filter.

**Chromatographic system**

The liquid chromatograph is equipped with a DAD (291 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 acetonitrile and water (60:40, v/v). The elution time is about 30 min.

**System suitability requirements**

Perform at least five replicate injections, each using 20 μL of indirubin Std-AS (1.5 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of indirubin should not be more than 5.0%; the RSD of the retention time of indirubin peak should not be more than 2.0%; the column efficiency determined from indirubin peak should not be less than 12000 theoretical plates.

The *R* value between indirubin 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 indirubin Std-AS (20 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of indirubin against the corresponding concentrations of indirubin 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 indirubin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of indirubin Std-AS. The retention times of indirubin 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 indirubin in the test solution, and calculate the percentage content of indirubin in the sample by using the equations indicated in Appendix IV(B).

**Limits**

The sample contains not less than 0.020% of indirubin ($C_{16}H_{10}N_2O_2$), calculated with reference to the dried substance.

## 7.2 Assay of indigo

**Standard solution**

*Indigo standard stock solution, Std-Stock (27 mg/L)*

Weigh accurately 2.7 mg of indigo CRS and dissolve in 100 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 0.44, 1.76, 4.40, 6.60, 8.80 mg/L for indigo.

**Test solution**

Weigh accurately 0.1 g of the powdered sample and place it in a 50-mL conical flask, then add 25 mL of chloral hydrate-dichloromethane (2%, w/v). Sonicate (240 W) the mixture for 90 min. Filter and transfer the filtrate to a 25-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 (604 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 (62:38, v/v). The elution time is about 20 min.

**System suitability requirements**

Perform at least five replicate injections, each using 10 µL of indigo Std-AS (4.40 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 6000 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 2.0%. 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 indicated in Appendix IV(B).

**Limits**

The sample contains not less than 0.030% of indigo ($C_{16}H_{10}N_2O_2$), calculated with reference to the dried substance.