Citri Exocarpium Rubrum

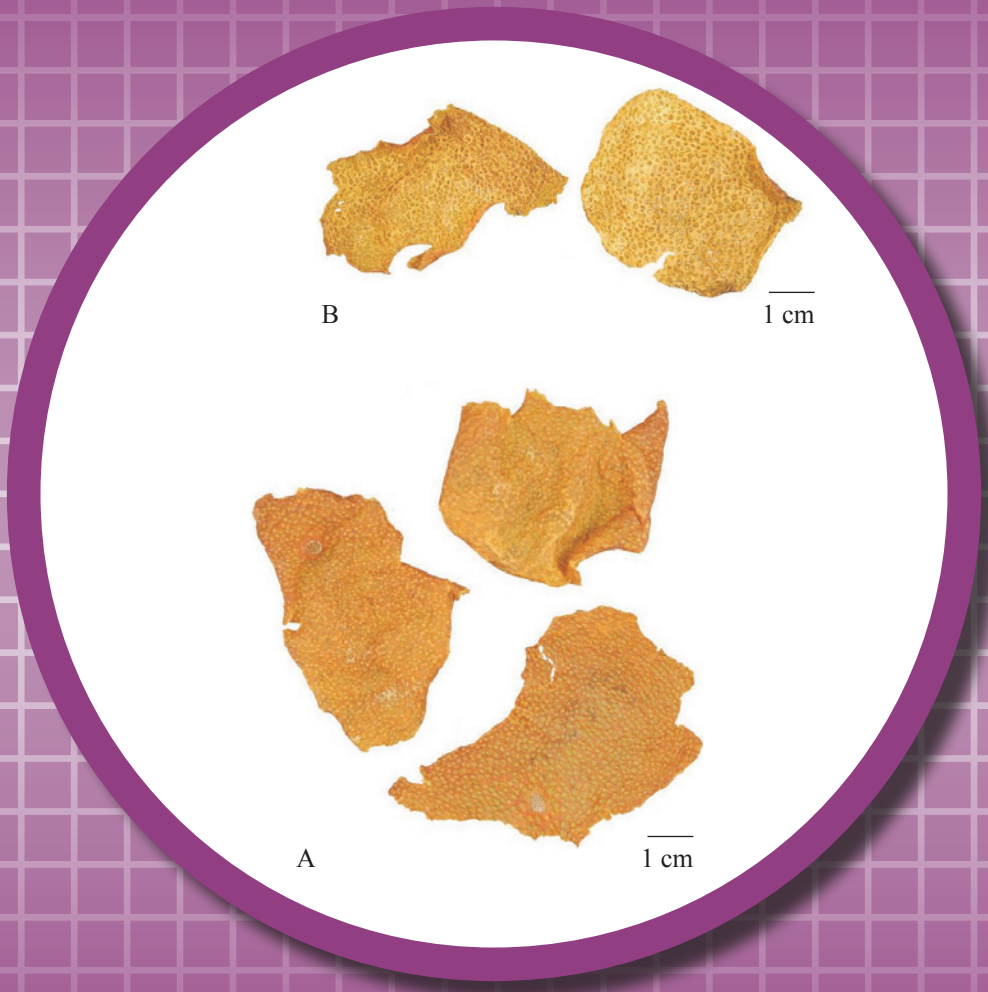

Figure 1 A photograph of Citri Exocarpium Rubrum

A. Outer surface of exocarps B. Inner surface of exocarps

1. NAMES

Official Name: Citri Exocarpium Rubrum

Chinese Name: 橘紅

Chinese Phonetic Name: Juhong

2. SOURCE

Citri Exocarpium Rubrum is the dried exocarp of *Citrus reticulata* Blanco (Rutaceae) and its cultivated varieties. The ripe fruit is collected in late autumn and early winter, the pericarp is split, exocarp collected after endocarp scratched off, then dried under the sun or in a shaded area to obtain Citri Exocarpium Rubrum.

3. DESCRIPTION

Thin slices of exocarp, long stripes, elliptic to ovate or irregular in shape, the margin shrunken and curved inward. The outer surface yellowish-brown to orange-red, becoming dark brown on storage, with numerous yellowish-white protuberant or sunken oil cavities. The inner surface yellowish-white, with numerous sunken and transparent small spots. Texture fragile, easily broken and some slightly pliable. Odour fragrant; taste slightly bitter and pungent (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

The outermost layer of exocarp consists of 1 layer of small subsquare epidermal cells, covered with cuticle. Parenchymatous cells below the epidermis tangentially elongated, rectangular or irregular in shape. Fragments of oil cavities numerous, scattered in the exocarp. Prisms of calcium oxalate located inside the parenchymatous cells, polygonal or rhombic in shape. Fan-shaped cluster crystals inside parenchymatous cells sometimes visible, usually aggregated into spheroid or amorphous masses (Fig. 2).

Powder

Colour pale yellowish-brown. Epidermal cells of exocarp polygonal, subsquare or rectangular in surface view, anticlinal walls thickened, stomata subrounded, subsidiary cells indistinct. The walls of parenchymatous cells of fragments of oil cavities slightly thickened. Prisms of calcium oxalate abundant, scattered in parenchymatous cells, rhombic to double-conical to irregularly polygonal, 4-31 μm in diameter; polychromatic under the polarized microscope. Fan-shaped cluster crystals yellow, sometimes visible, usually aggregated into spheroid or amorphous masses; polychromatic under the polarized microscope (Fig. 3).

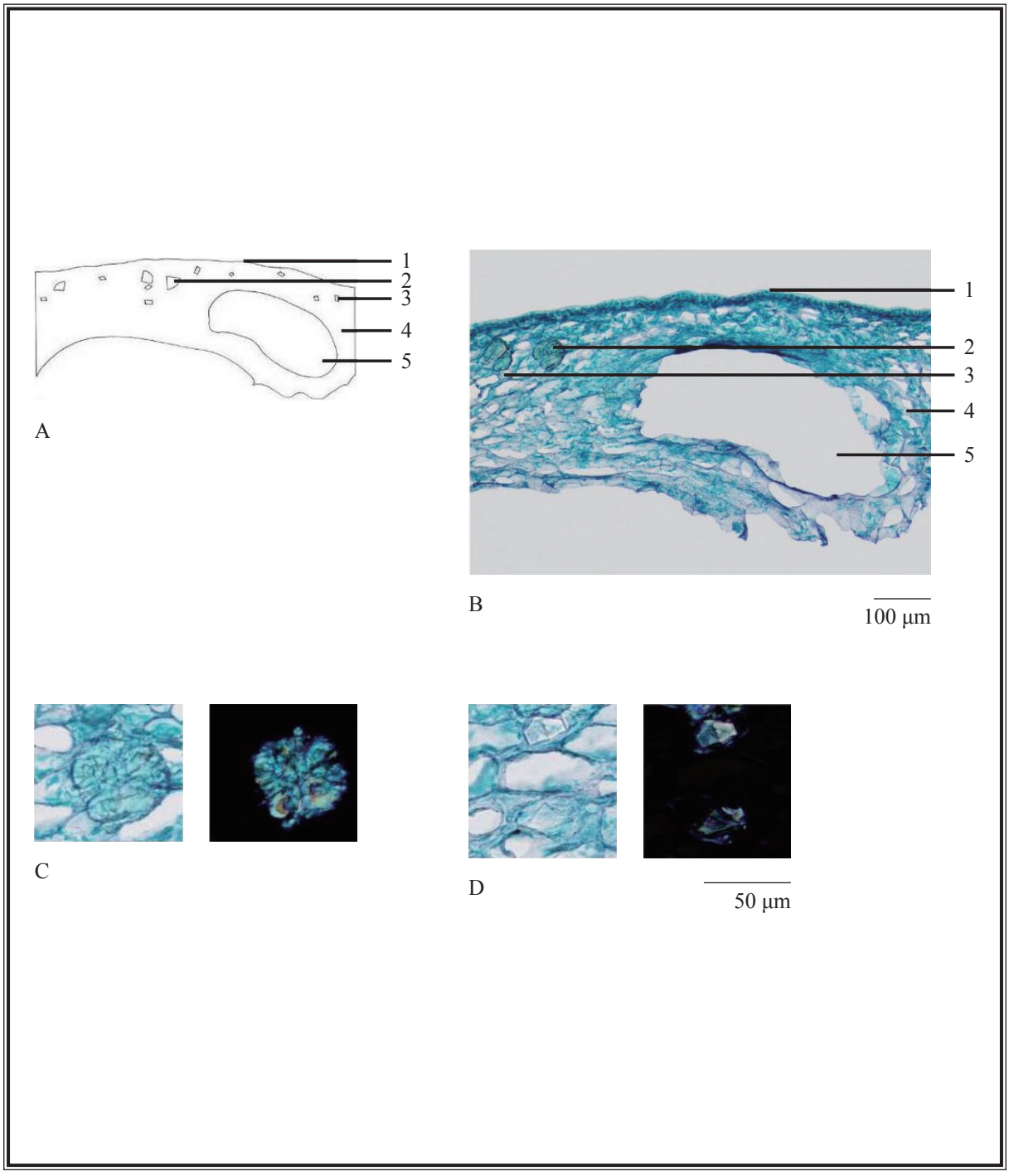

Figure 2 Microscopic features of transverse section of Citri Exocarpium Rubrum

A. Sketch B. Section illustration C. Fan-shaped cluster crystals D. Prisms of calcium oxalate

1. Epidermis 2. Fan-shaped cluster crystals 3. Prisms of calcium oxalate 4. Parenchymatous cells 5. Fragments of oil cavity

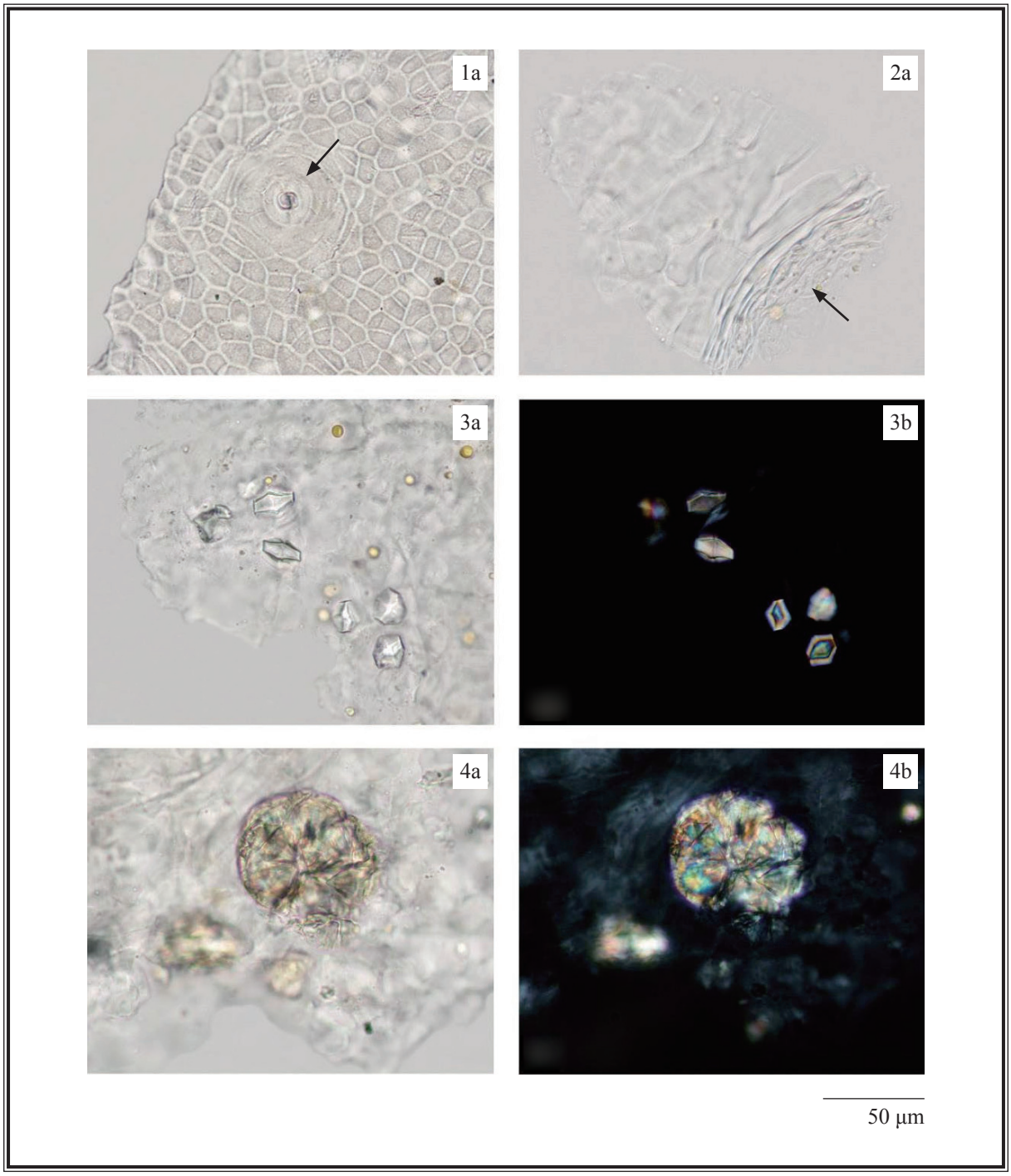

Figure 3 Microscopic features of powder of Citri Exocarpium Rubrum

1. Epidermal cells of exocarp (stoma ⟶) 2. Fragment of oil cavity (⟶)
3. Prisms of calcium oxalate 4. Fan-shaped cluster crystals

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

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

Standard solutions

Hesperidin standard solution

Weigh 1.0 mg of hesperidin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Nobiletin standard solution

Weigh 1.0 mg of nobiletin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of dichloromethane, methanol and water (13:7:2, v/v). Use the lower layer.

Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL conical flask, then add 10 mL of methanol. Sonicate (400 W) the mixture for 30 min. Filter and transfer the filtrate to a 100-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 1 mL of methanol. Filter through a 0.45-μm nylon filter.

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 hesperidin standard solution (10 μL), nobiletin standard solution (2 μL) and the test solution (1 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 8 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Examine the plate under UV light (254 nm). Calculate the R_f values by using the equation as indicated in Appendix IV (A).

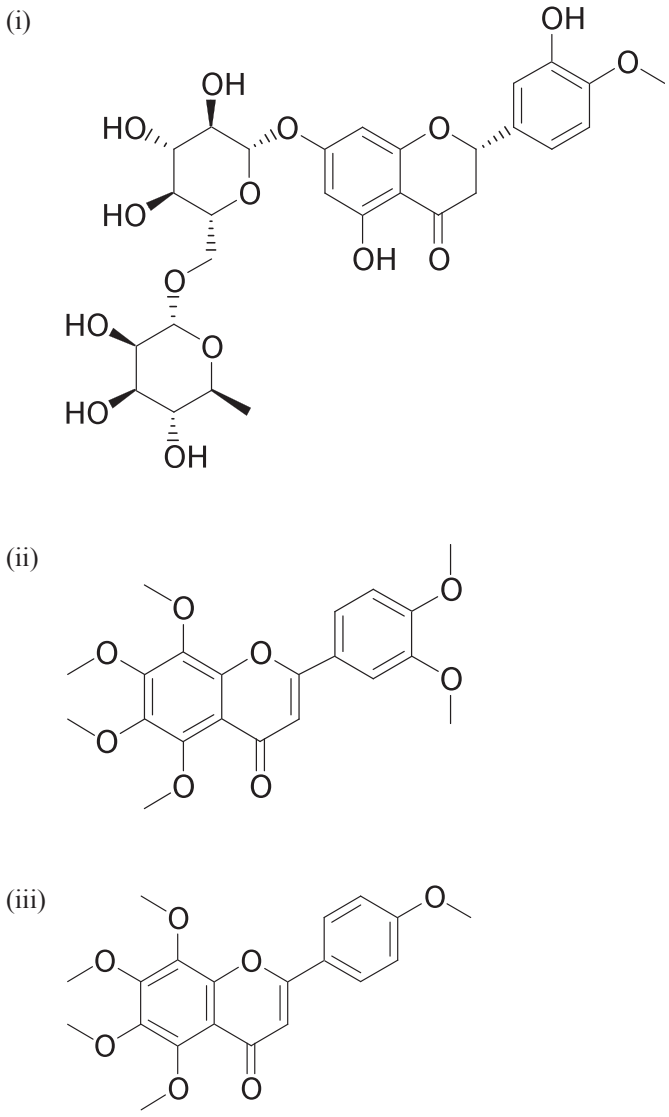

Figure 4 Chemical structures of (i) hesperidin (ii) nobiletin and (iii) tangeretin

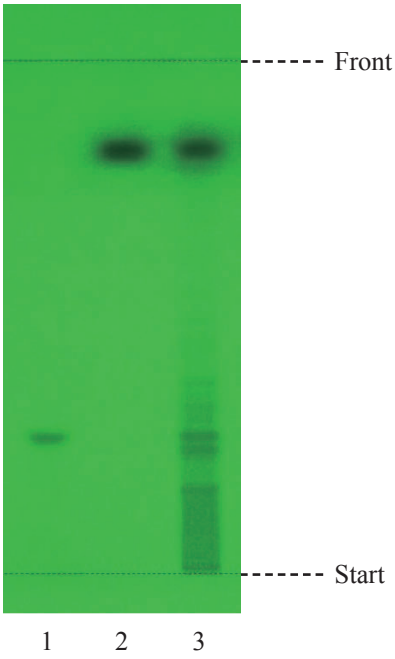

Figure 5 A reference HPTLC chromatogram of Citri Exocarpium Rubrum extract observed under UV light (254 nm)

1. Hesperidin standard solution 2. Nobiletin 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 hesperidin and nobiletin (Fig. 5).

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

Standard solutions

Hesperidin standard solution for fingerprinting, Std-FP (125 mg/L)

Weigh 1.25 mg of hesperidin CRS and dissolve in 10 mL of methanol.

Nobiletin standard solution for fingerprinting, Std-FP (3 mg/L)

Weigh 0.15 mg of nobiletin CRS and dissolve in 50 mL of methanol.

Tangeretin standard solution for fingerprinting, Std-FP (1 mg/L)

Weigh 0.1 mg of tangeretin CRS (Fig. 4) and dissolve in 100 mL of methanol.

Test solution

Weigh 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (400 W) the mixture for 30 min. Centrifuge at about 4000 × *g* for 10 min. Filter and transfer the filtrate to a 50-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 PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (330 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	85 → 80	15 → 20	linear gradient
10 – 25	80	20	isocratic
25 – 35	80 → 60	20 → 40	linear gradient
35 – 55	60 → 45	40 → 55	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of hesperidin Std-FP, nobiletin Std-FP and tangeretin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hesperidin, nobiletin and tangeretin should not be more than 5.0%; the RSD of the retention times of hesperidin, nobiletin and tangeretin peaks should not be more than 2.0%; the column efficiencies determined from hesperidin, nobiletin and tangeretin peaks should not be less than 10000, 100000 and 100000 theoretical plates respectively.

The *R* value between peak 1 and the closest peak; 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 1.5 (Fig. 6).

Procedure

Separately inject hesperidin Std-FP, nobiletin Std-FP, tangeretin Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of hesperidin, nobiletin and tangeretin peaks in the chromatograms of hesperidin Std-FP, nobiletin Std-FP, tangeretin Std-FP and the retention times of the three characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify hesperidin, nobiletin and tangeretin peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of hesperidin Std-FP, nobiletin Std-FP and tangeretin Std-FP. The retention times of hesperidin, nobiletin and tangeretin 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 Citri Exocarpium Rubrum extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the three characteristic peaks of Citri Exocarpium Rubrum extract

Peak No.	RRT	Acceptable Range
1 (hesperidin)	0.44	± 0.03
2 (marker, nobiletin)	1.00	-
3 (tangeretin)	1.10	± 0.03

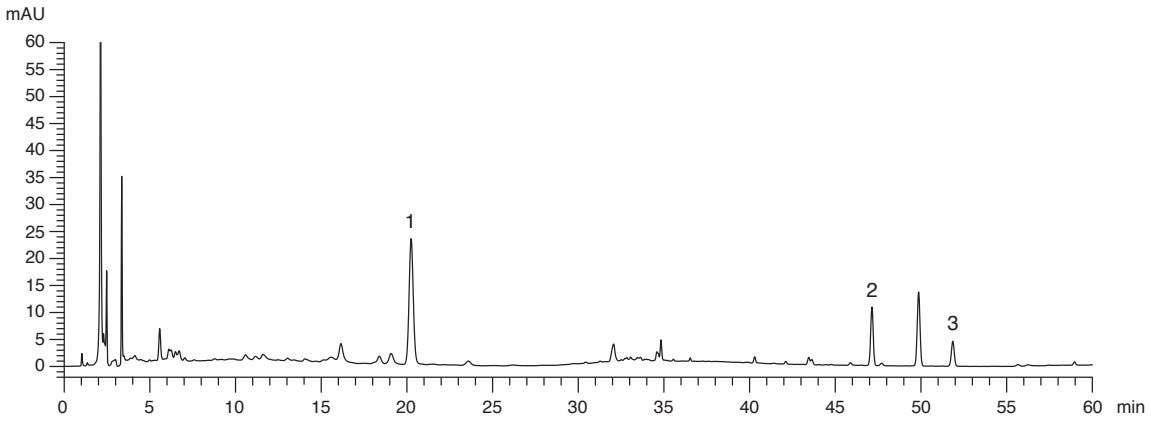

Figure 6 A reference fingerprint chromatogram of Citri Exocarpium Rubrum extract

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 1.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 5.0%.
Acid-insoluble ash: not more than 1.0%.

5.7 Water Content *(Appendix X)*

Toluene distillation method: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 33.0%.
Ethanol-soluble extractives (hot extraction method): not less than 31.0%.

7. ASSAY

7.1 Assay of Hesperidin, Nobiletin and Tangeretin

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

Standard solution

Mixed hesperidin, nobiletin and tangeretin standard stock solution, Std-Stock (500 mg/L for hesperidin, 12 mg/L for nobiletin and 5 mg/L for tangeretin)

Weigh accurately 25.0 mg of hesperidin CRS, 0.6 mg of nobiletin CRS and 0.25 mg of tangeretin CRS, and dissolve in 50 mL of methanol.

Mixed hesperidin, nobiletin and tangeretin standard solution for assay, Std-AS

Measure accurately the volume of the mixed hesperidin, nobiletin and tangeretin Std-Stock, dilute with methanol to produce a series of solutions of 31, 62, 125, 250, 500 mg/L for hesperidin, 0.4, 0.7, 1.5, 3, 6 mg/L for nobiletin and 0.3, 0.6, 1.3, 2.5, 5 mg/L for tangeretin.

Test solution

Weigh accurately 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (400 W) the mixture for 30 min. Centrifuge at about 4000 × *g* for 10 min. Filter and transfer the filtrate to a 50-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 PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (330 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 3) –

Table 3 Chromatographic system conditions

Time (min)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 10	85 → 80	15 → 20	linear gradient
10 – 25	80	20	isocratic
25 – 35	80 → 60	20 → 40	linear gradient
35 – 55	60 → 45	40 → 55	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of the mixed hesperidin, nobiletin and tangeretin Std-AS (125 mg/L for hesperidin, 1.5 mg/L for nobiletin and 1.3 mg/L for tangeretin). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hesperidin, nobiletin and tangeretin should not be more than 5.0%; the RSD of the retention times of hesperidin, nobiletin and tangeretin peaks should not be more than 2.0%; the column efficiencies determined from hesperidin, nobiletin and tangeretin peaks should not be less than 10000, 100000 and 100000 theoretical plates respectively.

The *R* value between hesperidin peak and the closest peak; the *R* value between nobiletin peak and the closest peak; and the *R* value between tangeretin peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curves

Inject a series of the mixed hesperidin, nobiletin and tangeretin Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of hesperidin, nobiletin and tangeretin against the corresponding concentrations of the mixed hesperidin, nobiletin and tangeretin Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify hesperidin, nobiletin and tangeretin peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed hesperidin, nobiletin and tangeretin Std-AS. The retention times of hesperidin, nobiletin and tangeretin peaks in the

chromatograms of the test solution and the Std-AS should not differ by more than 5.0%. Measure the peak areas and calculate the concentrations (in milligram per litre) of hesperidin, nobiletin and tangeretin in the test solution, and calculate the percentage contents of hesperidin, nobiletin and tangeretin in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 3.0% of hesperidin ($C_{28}H_{34}O_{15}$) and not less than 0.060% of the total content of nobiletin ($C_{21}H_{22}O_8$) and tangeretin ($C_{20}H_{20}O_7$), calculated with reference to the dried substance.

7.2 Assay of Volatile Oil

Weigh accurately 80 g of the powdered sample and place it in a 1000-mL round-bottomed flask. Add 500 mL of water and a few glass beads, shake and mix well. Carry out the method as directed in Appendix XIII (Method A).

Limits

The sample contains not less than 0.27% (v/w) of volatile oil.