Aurantii Fructus Immaturus

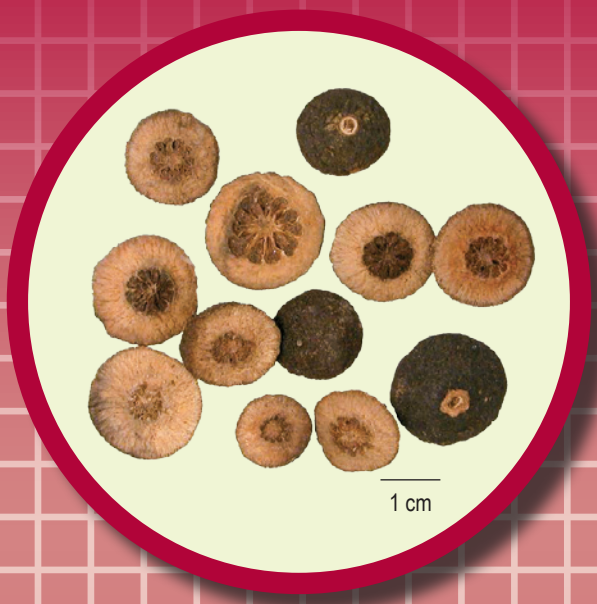


Figure 1 (i) A photograph of dried young fruit of *Citrus aurantium* L.

Figure 1 (ii) A photograph of dried young fruit of *Citrus sinensis* Osbeck

1. NAMES

Official Name: Aurantii Fructus Immaturus

Chinese Name: 枳實

Chinese Phonetic Name: Zhishi

2. SOURCE

Aurantii Fructus Immaturus is the dried young fruit of *Citrus aurantium* L. and its cultivated varieties or *Citrus sinensis* Osbeck (Rutaceae). The fallen young fruit is collected in May and June, removed from foreign matter, whole or cut into two parts or pieces, then dried under the sun or at ambient temperature to obtain Aurantii Fructus Immaturus.

3. DESCRIPTION

***Citrus aurantium* L.:** Semi-spheroidal, 8-35 mm in diameter. Exocarp dark green to dark brownish-green, with granular protuberances and wrinkles; remain of style or fruit stalk scar distinct. Mesocarp in transverse section slightly prominent, 4-8 mm thick, with 1-2 rows of oil cavaties at the outer part of mesocarp. Pulp segments (carpels) 7-13, juice vesicles brown. Centre core 2-5 mm in diameter. Texture hard. Odour aromatic; taste bitter and slightly sour [Fig. 1 (i)].

***Citrus sinensis* Osbeck:** Mostly spheroidal to semi-spheroidal or as a round slice, 5-25 mm in diameter. Exocarp yellowish-brown to dark brownish-green, remains of style or fruit stalk scar distinct. Texture hard. Odour aromatic; taste slightly bitter [Fig. 1 (ii)].

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

***Citrus aurantium* L.:** Exocarp consists of 1 layer of cells covered with thick cuticles and incrusted with stomata. Oil cavities of 1-2 layers are irregularly located in the outer portion of the mesocarp, relatively large in size, the shape subglobular to long-ellipsoid. Mesocarp consists of numerous layers of parenchymatous cells with unevenly thickened cell walls, scattered with prisms of calcium oxalate. Vascular bundles collateral, small, scattered in

the mesocarp. Endocarp consists of 1 layer of parenchymatous cells and shrinking, as is the membrane of segments which is filled with juice vesicles. Juice vesicle consists of parenchymatous cells [Fig. 2 (i)].

***Citrus sinensis* Osbeck:** Ratio of mesocarp in the transverse section is smaller than that in *Citrus aurantium* L. Fan-shaped crystals can be found in the parenchymatous cells of the mesocarp [Fig. 2 (ii)].

Powder

***Citrus aurantium* L.:** Colour yellowish-white. Mesocarp cells subrounded or irregular, most cell walls unevenly thickened and beaded. Epidermal cells of exocarp polygonal, subsquare to rectangular in surface view; stomata actinocytic, 22-32 μm in diameter, subsidiary cells 5-9, in lateral view appearing covered with thick cuticle. Fragments of oil cavity brownish-yellow, often broken, sometimes with droplets of oil. Vessel elements are mostly spiral or reticulate, 4-24 μm in diameter. Prisms of calcium oxalate crystals abundant, rhombic, double-conical or irregularly polygonal, 2-35 μm in diameter, occuring in cells of the mesocarp and inside the juice vesicle; crystals appear intensely white to polychromatic under the polarized microscope [Fig. 3 (i)].

***Citrus sinensis* Osbeck:** Colour brownish-yellow. Fan-shaped crystals yellow, sometimes irregular in shape [Fig. 3 (ii)].

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

Standard solution

Synephrine standard solution

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

Developing solvent system

Prepare a mixture of water, n-butanol and acetic acid (5:4:1, v/v) in a separating funnel. Shake well and allow to stand for 30 min. Use the upper layer.

Spray reagent

Weigh 1 g of ninhydrin and dissolve in 100 mL of ethanol.

Test solution

Weigh 0.5 g of the powdered sample and place it in a 50-mL conical flask, then add 20 mL of methanol. Sonicate (240 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 methanol.

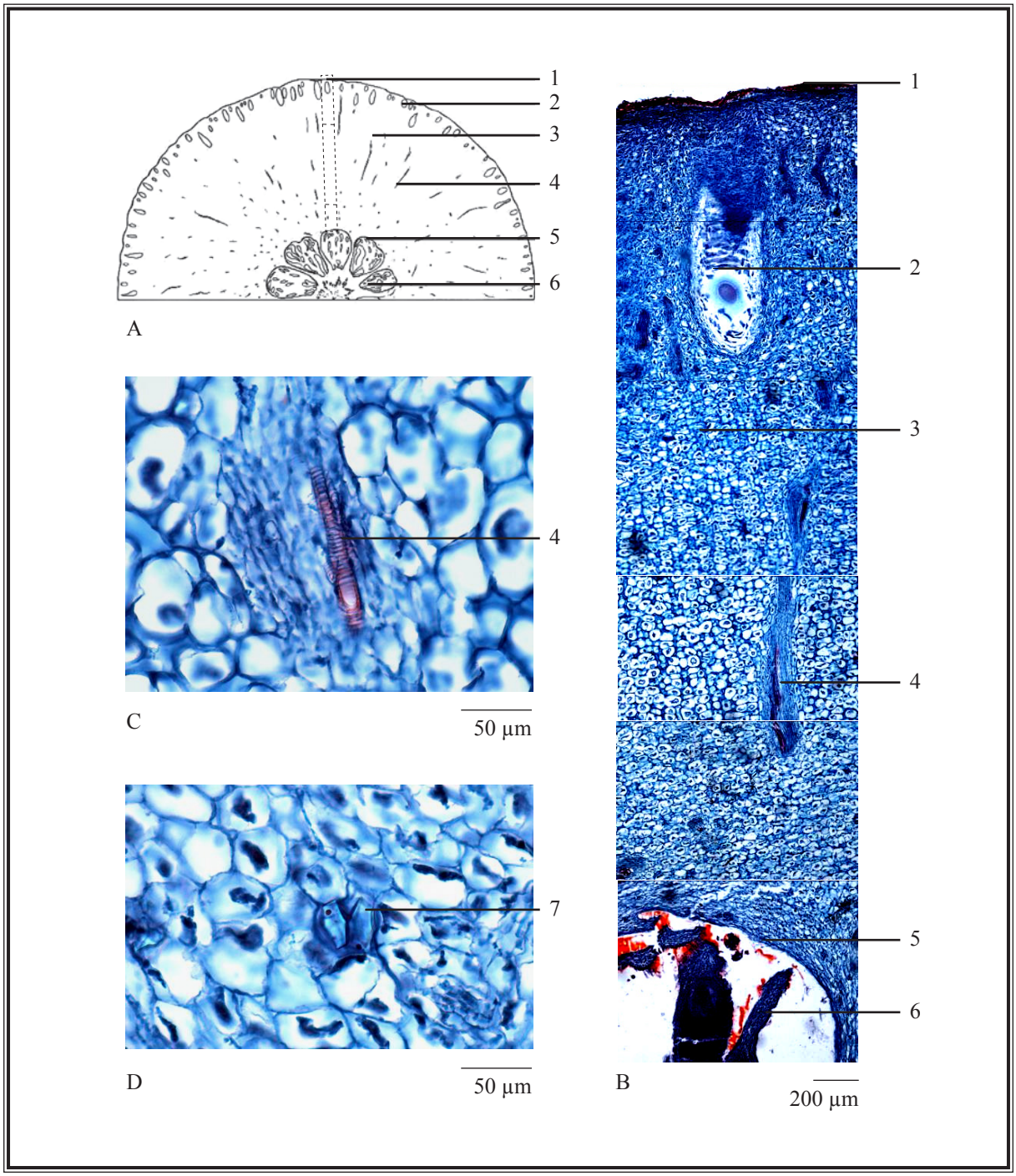


Figure 2 (i) Microscopic features of transverse section of dried young fruit of *Citrus aurantium* L.

A. Sketch B. Section illustration C. Vascular bundle D. Crystals of calcium oxalate

1. Exocarp 2. Oil cavity 3. Mesocarp 4. Vascular bundle 5. Endocarp 6. Juice vesicles
7. Prisms of calcium oxalate

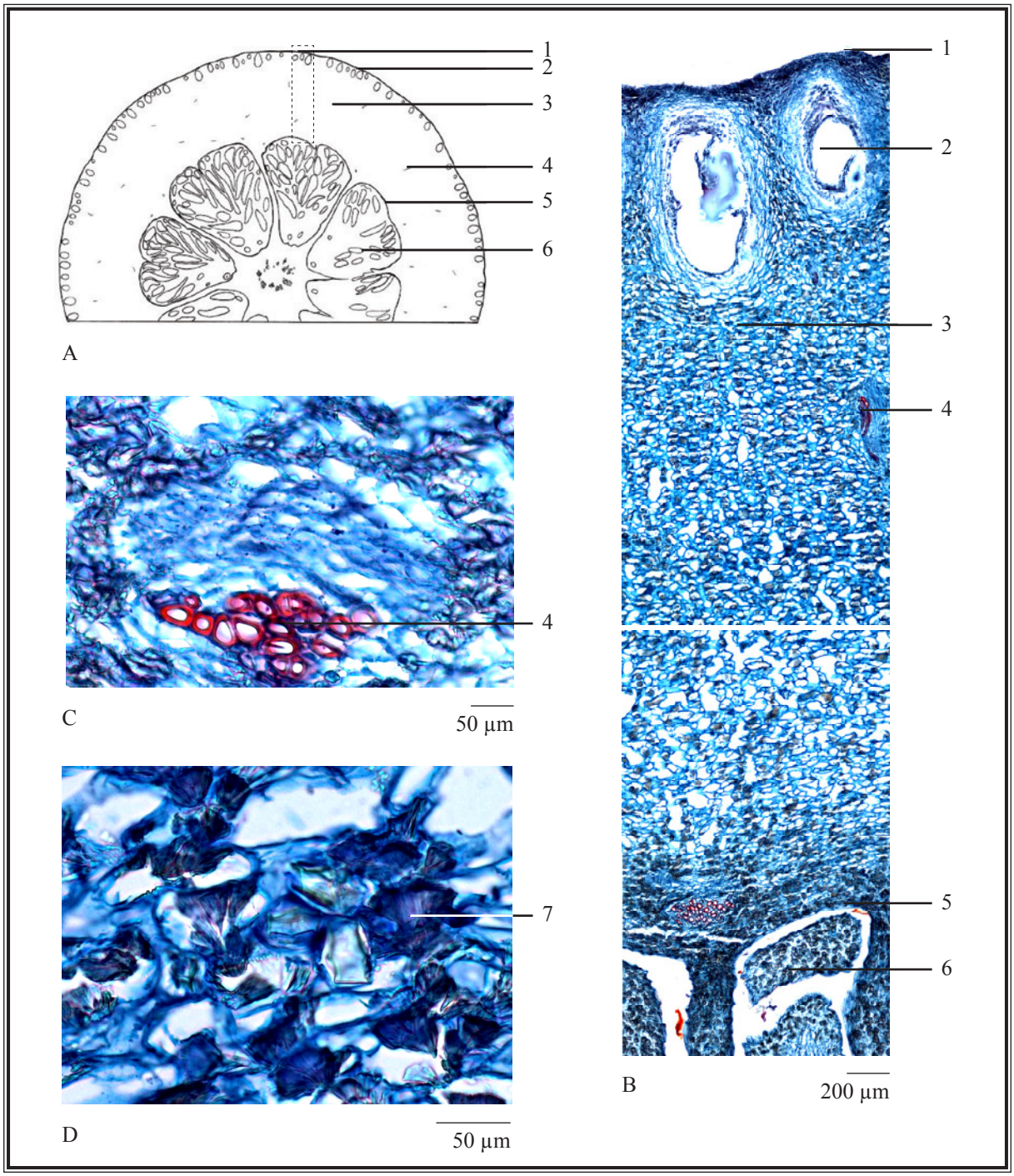


Figure 2 (ii) Microscopic features of transverse section of dried young fruit of *Citrus sinensis* Osbeck

A. Sketch B. Section illustration C. Vascular bundle D. Fan-shaped crystals

1. Exocarp 2. Oil cavity 3. Mesocarp 4. Vascular bundle 5. Endocarp 6. Juice vesicles
7. Fan-shaped crystals

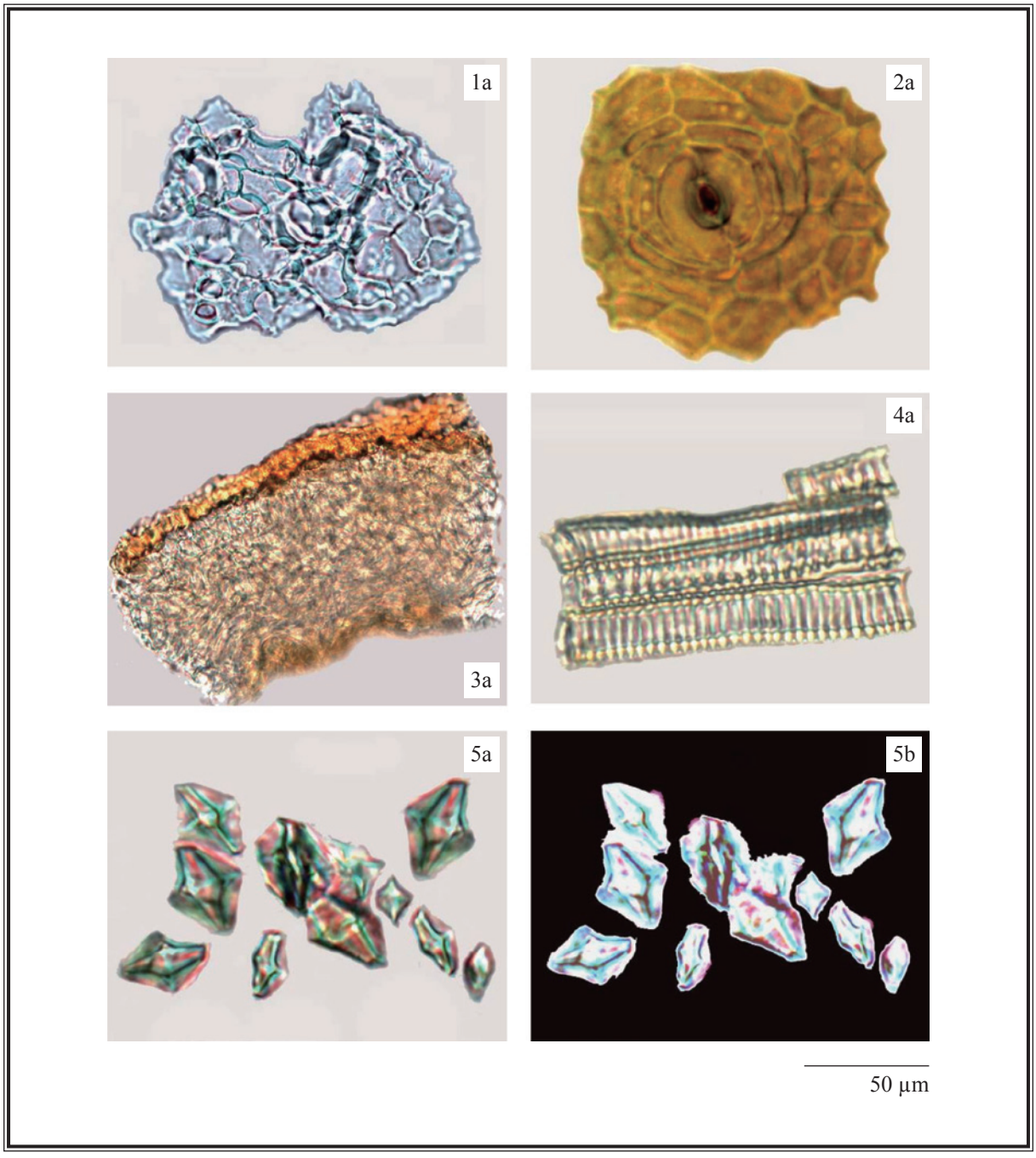


Figure 3 (i) Microscopic features of powder of dried young fruit of *Citrus aurantium* L.

1. Mesocarp cells 2. Epidermal cells of exocarp 3. Fragments of oil cavity 4. Spiral vessels
5. Prisms of Calcium oxalate

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

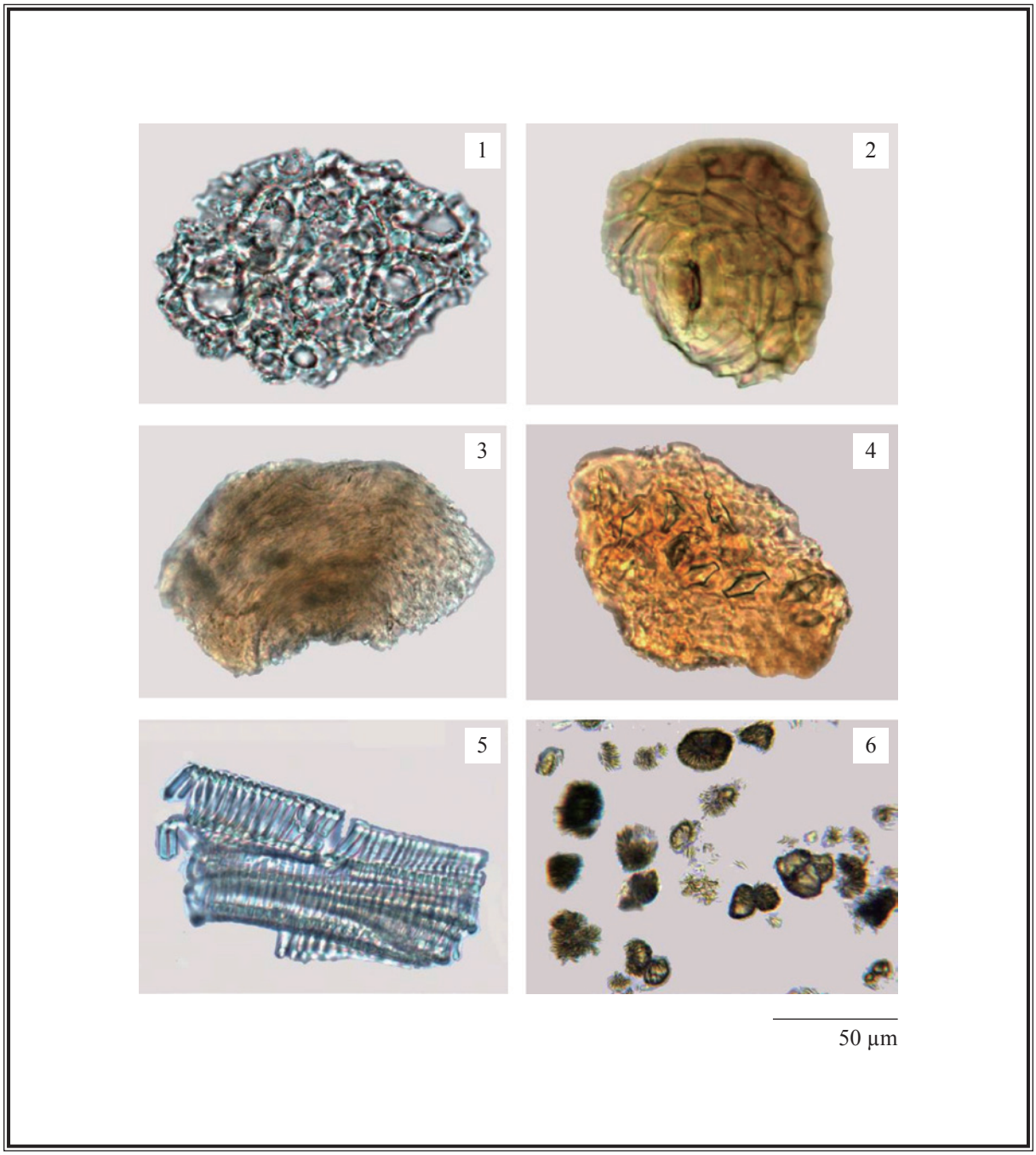


Figure 3 (ii) Microscopic features of powder of dried young fruit of *Citrus sinensis* Osbeck (under the light microscope)

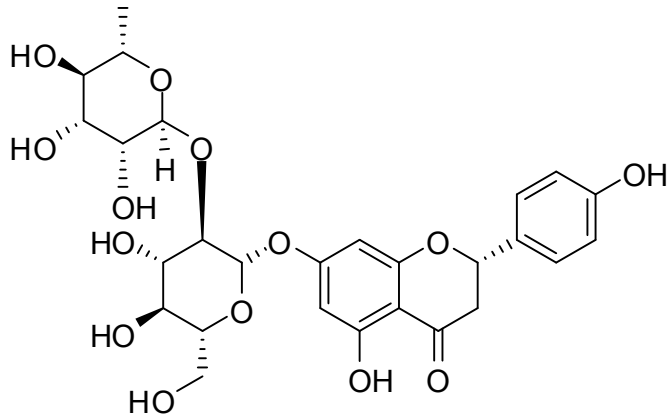
1. Mesocarp cells 2. Epidermal cells of exocarp 3. Fragments of oil cavity 4. Prisms of calcium oxalate
5. Spiral vessels 6. Fan-shaped crystals

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate [immerse in sodium hydroxide solution (1%, w/v) for 20s. Dry the plate at 105°C for 1 h. Cool in a desiccator.], a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately synephrine standard solution and the test solution (3 µL each) 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 7 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Spray the plate evenly with spray reagent and heat at about 105°C 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).

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 synephrine.

(i)



(ii)

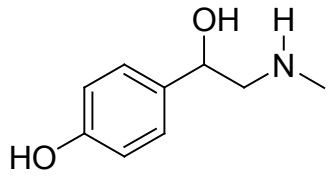


Figure 4 Chemical structures of (i) naringin and (ii) synephrine

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

Standard solutions

Naringin standard solution for fingerprinting, Std-FP (100 mg/L)

Weigh 1.0 mg of naringin CRS (Fig. 4) and dissolve in 10 mL of methanol.

Synephrine standard solution for fingerprinting, Std-FP (100 mg/L)

Weigh 1.0 mg of synephrine 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 20 mL of ethanol (50%). Sonicate (240 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 5 min. Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (224 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)	0.1% Phosphoric acid and 0.1% Sodium dodecyl sulphate (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 15	80	20	isocratic
15 – 25	80 → 75	20 → 25	linear gradient
25 – 30	75 → 67	25 → 33	linear gradient
30 – 60	67 → 65	33 → 35	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 μL of naringin Std-FP and synephrine Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of naringin and synephrine should not be more than 5.0%; the RSD of the retention times of naringin and synephrine peaks should not be more than 2.0%; the column efficiencies determined from naringin and synephrine peaks should not be less than 8000 and 150000 theoretical plates respectively.

The *R* value between peak 2 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 1.0 [Fig. 5 (i) or (ii)].

Procedure

Separately inject naringin Std-FP, synephrine Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of naringin and synephrine peaks in the chromatograms of naringin Std-FP, synephrine Std-FP and the retention times of the four characteristic peaks [Fig. 5 (i) or (ii)] in the chromatogram of the test solution. Identify naringin and synephrine peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of naringin Std-FP and synephrine Std-FP. The retention times of naringin and synephrine 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 Aurantii Fructus Immaturus extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of Aurantii Fructus Immaturus extract

Peak No.	RRT	Acceptable Range
1	0.34	± 0.03
2 (naringin)	0.40	± 0.03
3 (hesperidin)	0.44	± 0.03
4 (marker, synephrine)	1.00	-

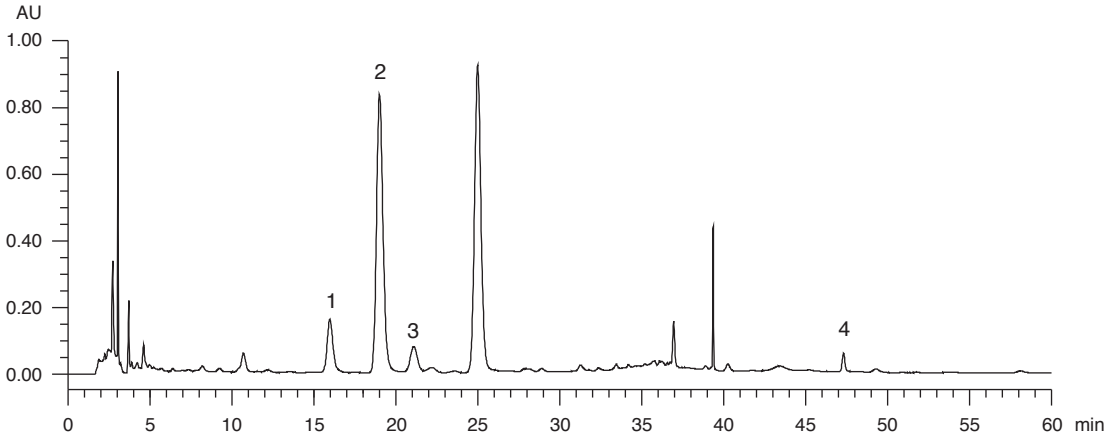


Figure 5 (i) A reference fingerprint chromatogram of dried young fruit of *Citrus aurantium* L. extract

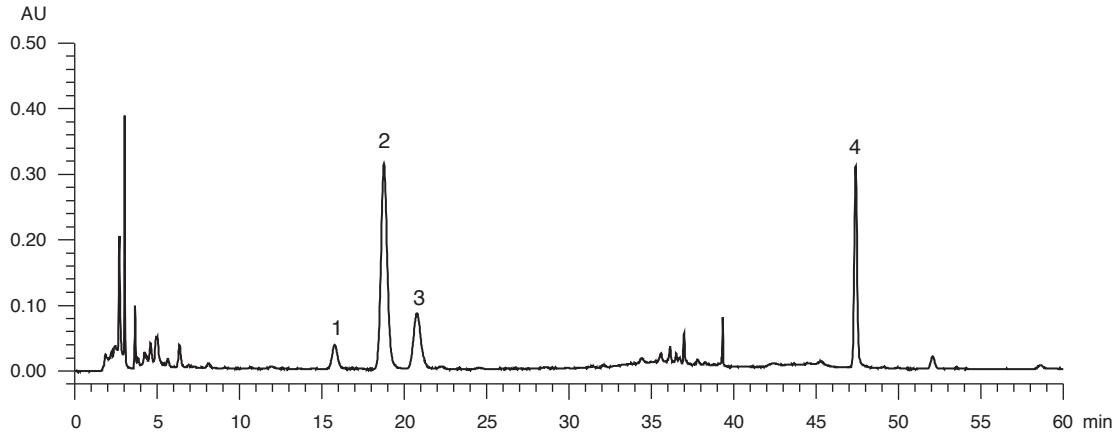


Figure 5 (ii) A reference fingerprint chromatogram of dried young fruit of *Citrus sinensis* Osbeck extract

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 respective reference fingerprint chromatograms [Fig. 5 (i) or (ii)].

5. TESTS

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

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

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

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

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

5.6 Ash *(Appendix IX)*

Total ash: not more than 6.0%.
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 22.0%.
Ethanol-soluble extractives (hot extraction method): not less than 23.0%.

7. ASSAY

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

7.1 Assay of synephrine

Standard solution

Synephrine standard stock solution, Std-Stock (500 mg/L)

Weigh accurately 2.5 mg of synephrine CRS and dissolve in 5 mL of methanol.

Synephrine standard solution for assay, Std-AS

Measure accurately the volume of the synephrine Std-Stock, dilute with methanol to produce a series of solutions of 25, 50, 75, 100, 150 mg/L for synephrine.

Test solution

Weigh accurately 0.5 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 25 mL of methanol (50%). Sonicate (240 W) the mixture for 30 min. Centrifuge at about 2000 × *g* for 10 min. Transfer the supernatant to a 250-mL round-bottomed flask. Repeat the extraction for one more time. Combine the supernatants. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol (50%). Transfer the solution to a 25-mL volumetric flask and make up to the mark with methanol (50%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (224 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 0.075% phosphoric acid with 0.1% sodium dodecyl sulphate and acetonitrile (68:32, v/v). The elution time is about 30 min.

System suitability requirements

Perform at least five replicate injections, each using 10 μL of synephrine Std-AS (50 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of synephrine should not be more than 5.0%; the RSD of the retention time of synephrine peak should not be more than 2.0%; the column efficiency determined from synephrine peak should not be less than 8000 theoretical plates.

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

Limits

The sample contains not less than 0.30% of synephrine ($C_9H_{13}NO_2$), calculated with reference to the dried substance.

7.2 Assay of naringin

Standard solution

Naringin standard stock solution, Std-Stock (500 mg/L)

Weigh accurately 2.5 mg of naringin CRS and dissolve in 5 mL of methanol.

Naringin standard solution for assay, Std-AS

Measure accurately the volume of the naringin Std-Stock, dilute with methanol to produce a series of solutions of 0.2, 6.25, 12.5, 50, 100 mg/L for naringin.

Test solution

Weigh accurately 0.1 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 15 mL of ethanol (50%). Sonicate (240 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 5 min. Transfer the supernatant to a 100-mL volumetric flask. Repeat the extraction for two more times. Combine the supernatants and make up to the mark with ethanol (50%). Filter through a 0.45-µm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (283 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 0.2% phosphoric acid and acetonitrile (79:21, v/v). The elution time is about 30 min.

System suitability requirements

Perform at least five replicate injections, each using 10 µL of naringin Std-AS (12.5 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of naringin should not be more than 5.0%; the RSD of the retention time of naringin peak should not be more than 2.0%; the column efficiency determined from naringin peak should not be less than 8000 theoretical plates.

The *R* value between naringin 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 naringin Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of naringin against the corresponding concentrations of naringin Std-AS. Obtain the slope, y-intercept and the r^2 value from the 5-point calibration curve.

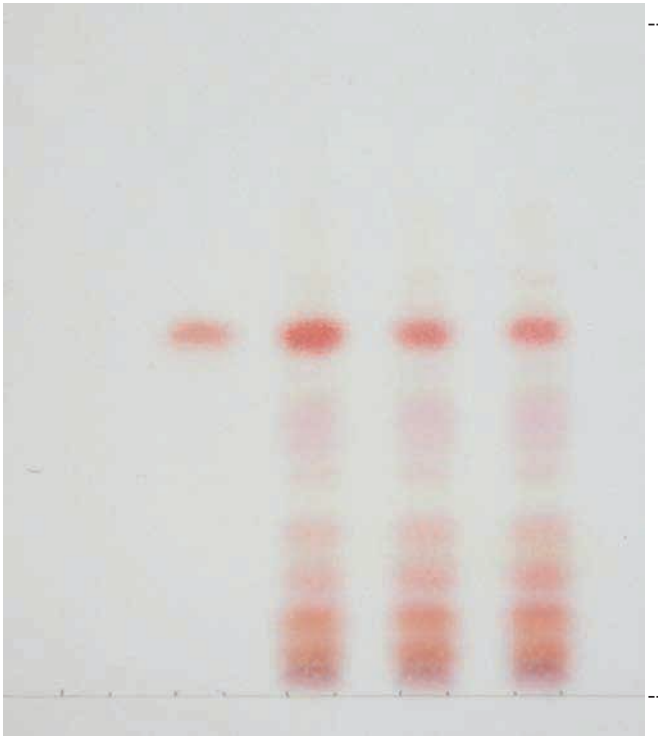
Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify naringin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of naringin Std-AS. The retention times of naringin 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 naringin in the test solution, and calculate the percentage content of naringin in the sample by using the equations indicated in Appendix IV(B).

Limits

The sample contains not less than 0.66% of naringin ($C_{27}H_{32}O_{14}$), calculated with reference to the dried substance.

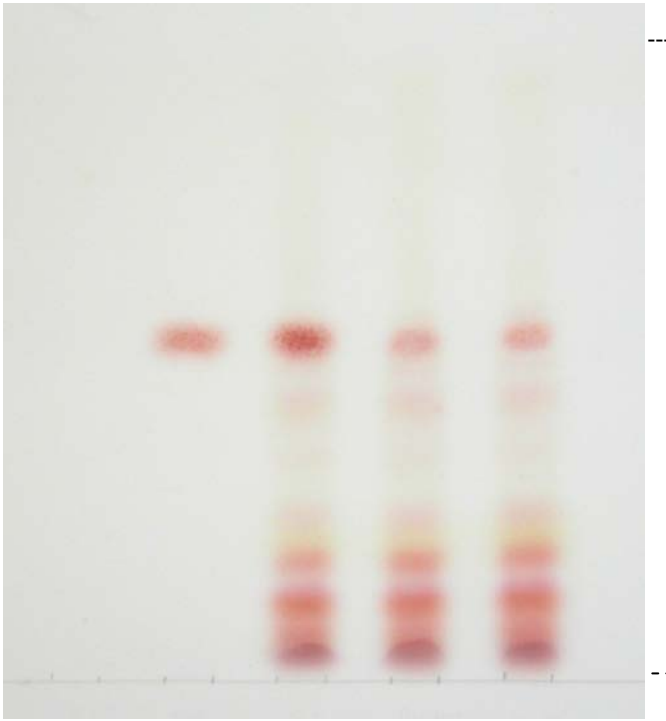
Aurantii Fructus Immaturus　(枳實)



Lane	Sample	Results
1	Blank (Methanol)	Negative
2	Standard (Synephrine)	Synephrine positive
3	Spiked sample (Sample plus synephrine)	Synephrine positive
4	Sample (*Citrus aurantium* L.)	Synephrine positive
5	Sample duplicate (*Citrus aurantium* L.)	Synephrine positive

Figure 1　TLC results of the dried young fruit of *Citrus aurantium* L. extract observed under visible light after staining

Aurantii Fructus Immaturus　(枳實)



Lane	Sample	Results
1	Blank (Methanol)	Negative
2	Standard (Synephrine)	Synephrine positive
3	Spiked sample (Sample plus synephrine)	Synephrine positive
4	Sample (*Citrus sinensis* Osbeck)	Synephrine positive
5	Sample duplicate (*Citrus sinensis* Osbeck)	Synephrine positive

Figure 2　TLC results of the dried young fruit of *Citrus sinensis* Osbeck extract observed under visible light after staining