Aurantii Fructus

Figure 1 A photograph of Aurantii Fructus

1. NAMES

Official Name: Aurantii Fructus

Chinese Name: 枳殼

Chinese Phonetic Name: Zhiqiao

2. SOURCE

Aurantii Fructus is the dried immature fruit of *Citrus aurantium* L. (Rutaceae) and its cultivated varieties. The immature fruit is collected in July while the fruit is green, cut into two parts, then dried under the sun or at ambient temperature to obtain Aurantii Fructus.

3. DESCRIPTION

Semi-spheroidal, 30-60 mm in diameter. Exocarp brown to dark brown, with rigidly precise convexes and sunken oil cavity spots on the surface; remains of style at the apex and fruit stalk scar at base distinct. Mesocarp in transverse section yellowish-white, smooth and slightly prominent, 3-10 mm thick, with 1-2 rows of oil cavities on the outer part of pericarp. Pulp segments (carpels) 7-14, juice vesicles dried and shrunken, brown to dark brown, containing seeds. Centre core 5-9 mm in diameter. Texture hard, uneasily broken. Odour aromatic; taste bitter and slightly sour (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

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, subrounded to long-elliptic. 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).

Powder

Colour yellowish-white. Mesocarp fragments abundant, cells subrounded or of irregular shapes, mostly with uneven thickened walls. Epidermal cells of exocarp subpolygonal to subsquare on surface view; stomata actinocytic, 22-32 µm in diameter, subsidiary cells 5-9. Fragments of oil cavity brownish-yellow, often broken, sometimes with droplets of oil. Fragments of juice vesicles pale brownish-yellow, the cells long and shrinking as lines, scattered with smaller prisms of calcium oxalate. Vessel elements mostly spiral, 4-24 µm in diameter. Prisms of calcium oxalate abundant, dispersed singly in the parenchymatous cells, rhombic to double-conical to irregularly polygonal, 2-35 µm across; intensely white to polychromatic under the polarized microscope (Fig. 3).

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

Standard solutions

Naringin standard solution

Weigh 1.0 mg of naringin CRS (Fig. 4) and dissolve in 50 mL of methanol.

Neohesperidin standard solution

Weigh 1.0 mg of neohesperidin CRS (Fig. 4) and dissolve in 50 mL of methanol.

Developing solvent system

Prepare a mixture of water and acetone (3:2, v/v).

Spray reagent

Weigh 2 g of aluminium trichloride and dissolve in 100 mL of ethanol.

Test solution

Weigh 0.1 g of the powdered sample and place it in a 250-mL conical flask, then add 100 mL of methanol. Sonicate (240 W) the mixture for 30 min. Filter the mixture.

Procedure

Carry out the method by using a TLC polyamide plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately naringin standard solution, neohesperidin standard solution and the test solution (2 µL each) 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 15 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 7.5 cm. After the development, remove the plate from the chamber, mark the solvent front and

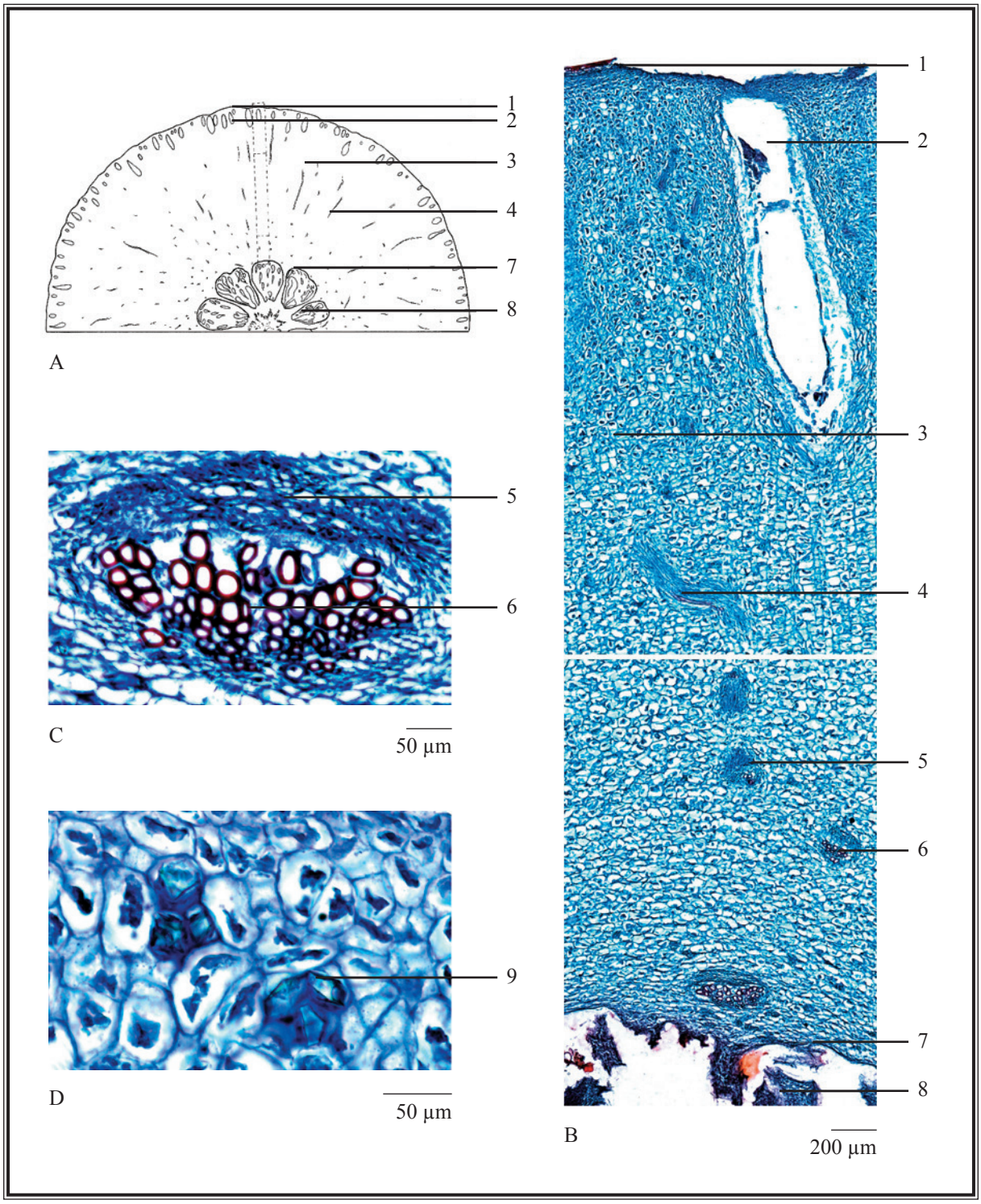


Figure 2 Microscopic features of transverse section of Aurantii Fructus

A. Sketch of transverse section B. Transverse section C. Vascular bundle D. Prisms of calcium oxalate

1. Exocarp 2. Oil cavity 3. Mesocarp 4. Vascular bundle 5. Phloem 6. Xylem 7. Endocarp 8. Juice vesicle 9. Prisms of calcium oxalate

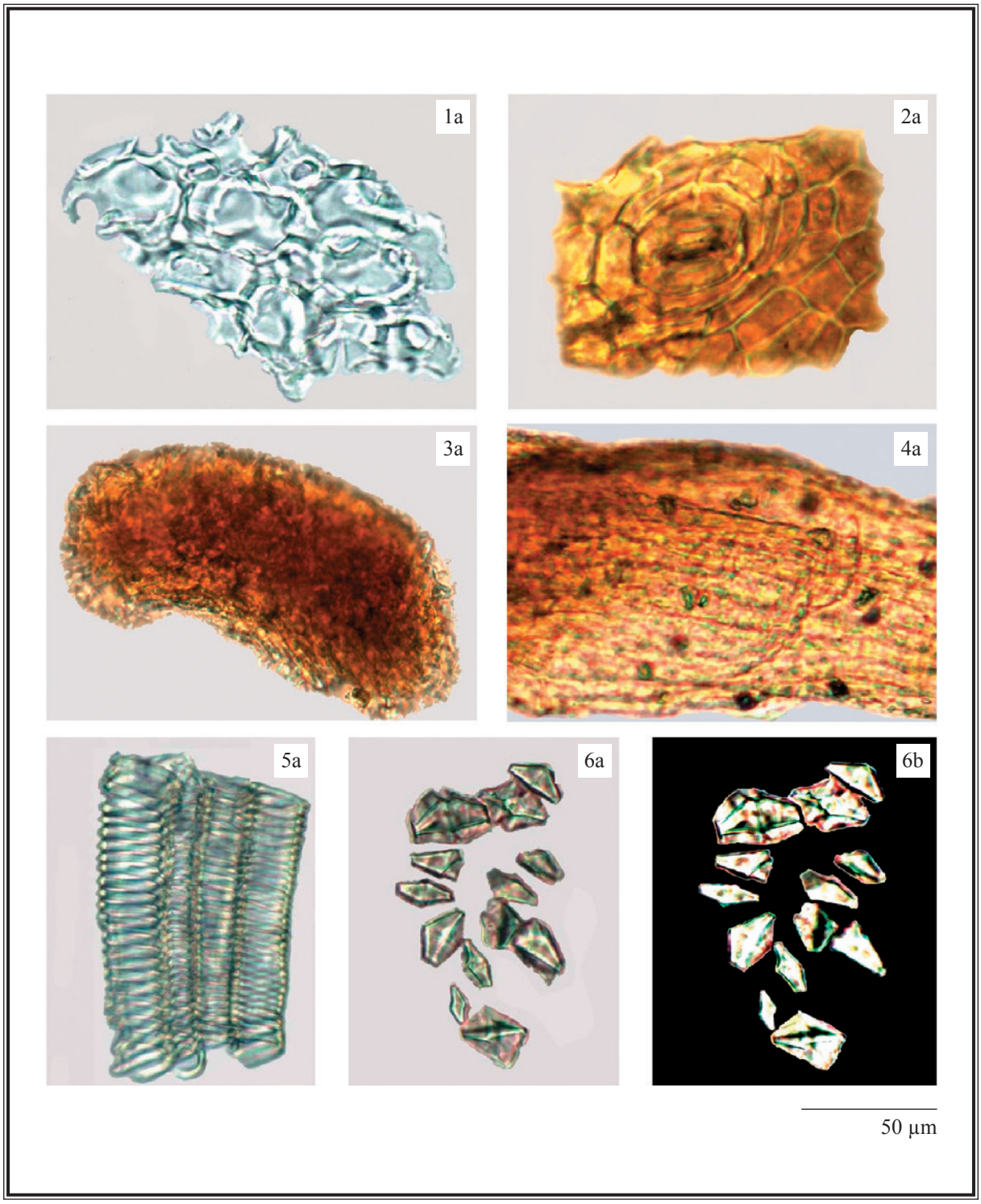


Figure 3 Microscopic features of powder of Aurantii Fructus

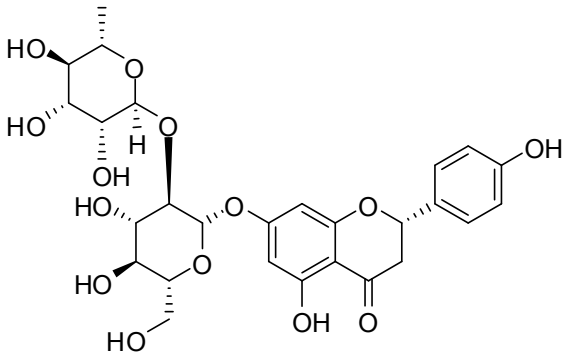
1. Mesocarp cells 2. Exocarp epidermal cells 3. Fragments of oil cavity 4. Fragments of juice vesicles
5. Spiral vessels 6. Prisms of calcium oxalate

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

dry in air. Spray the plate evenly with the spray reagent and dry in air. Examine the plate under UV light (366 nm). 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 naringin and neohesperidin.

(i)



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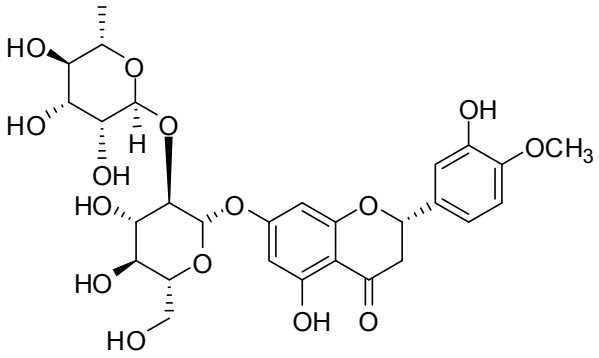

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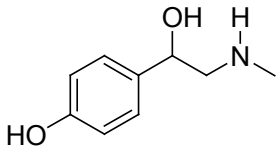


Figure 4 Chemical structures of (i) naringin (ii) neohesperidin and (iii) synephrine

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

Standard solutions

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

Weigh 2.5 mg of naringin CRS and dissolve in 25 mL of methanol.

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

Weigh 2.5 mg of neohesperidin CRS and dissolve in 25 mL of methanol.

Synephrine standard solution for fingerprinting, Std-FP (50 mg/L)

Weigh 2.5 mg of synephrine CRS (Fig. 4) and dissolve in 50 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, neohesperidin Std-FP and synephrine Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of naringin, neohesperidin and synephrine should not be more than 5.0%; the RSD of the retention times of naringin, neohesperidin and synephrine peaks should not be more than 2.0%; the column efficiencies determined from naringin, neohesperidin and synephrine peaks should not be less than 8000, 8000 and 70000 theoretical plates respectively.

The *R* value between peak 2 and the closest peak; 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 1.0 (Fig. 5).

Procedure

Separately inject naringin Std-FP, neohesperidin 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, neohesperidin and synephrine peaks in the chromatograms of naringin Std-FP, neohesperidin Std-FP, synephrine Std-FP and the retention times of the four characteristic peaks (Fig. 5) in the chromatogram of the test solution. Identify naringin, neohesperidin and synephrine peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of naringin Std-FP, neohesperidin Std-FP and synephrine Std-FP. The retention times of naringin, neohesperidin 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 extract are listed in Table 2.

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

Peak No.	RRT	Acceptable Range
1	0.84	± 0.03
2 (marker, naringin)	1.00	-
3 (neohesperidin)	1.29	± 0.03
4 (synephrine)	2.46	± 0.05

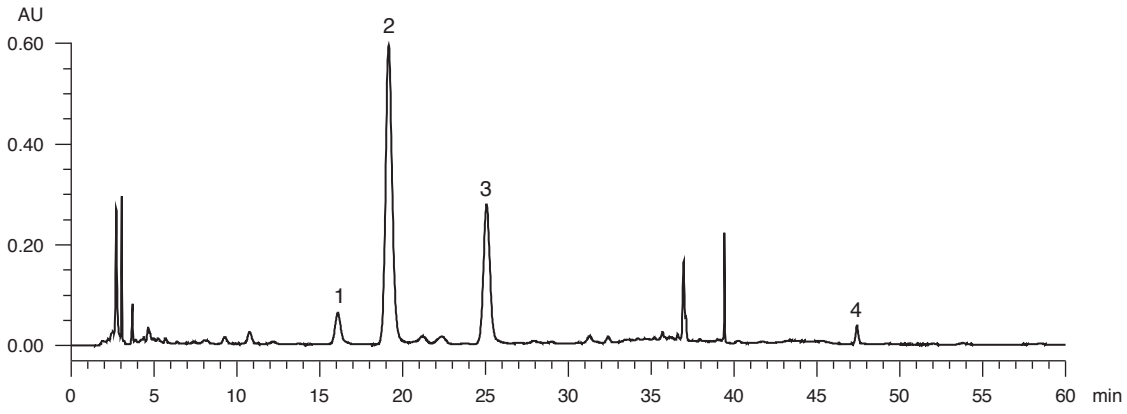


Figure 5 A reference fingerprint chromatogram of Aurantii Fructus extract

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. 5).

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 5.5%.
Acid-insoluble ash: not more than 1.0%.

5.7 Water Content *(Appendix X)*

Toluene distillation method: not more than 9.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 32.0%.
Ethanol-soluble extractives (hot extraction method): not less than 25.0%.

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 6.25, 12.5, 25, 37.5, 50 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 (25 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 0.083% of synephrine ($C_9H_{13}NO_2$), calculated with reference to the dried substance.

7.2 Assay of naringin and neohesperidin

Standard solution

Mixed naringin and neohesperidin standard stock solution, Std-Stock (200 mg/L each)

Weigh accurately 2.0 mg of naringin CRS and 2.0 mg of neohesperidin CRS, and dissolve in 10 mL of methanol.

Mixed naringin and neohesperidin standard solution for assay, Std-AS

Measure accurately the volume of the mixed naringin and neohesperidin Std-Stock, dilute with methanol to produce a series of solutions of 12.5, 50, 75, 100, 150 mg/L for both naringin and neohesperidin.

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 the mixed naringin and neohesperidin Std-AS (50 mg/L each). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of naringin and neohesperidin should not be more than 5.0%; the RSD of the retention times of naringin and neohesperidin peaks should not be more than 2.0%; the column efficiencies determined from naringin and neohesperidin peaks should not be less than 8000 theoretical plates.

The *R* value between naringin peak and the closest peak; and the *R* value between neohesperidin 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 naringin and neohesperidin Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of naringin and neohesperidin against the corresponding concentrations of the mixed naringin and neohesperidin Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

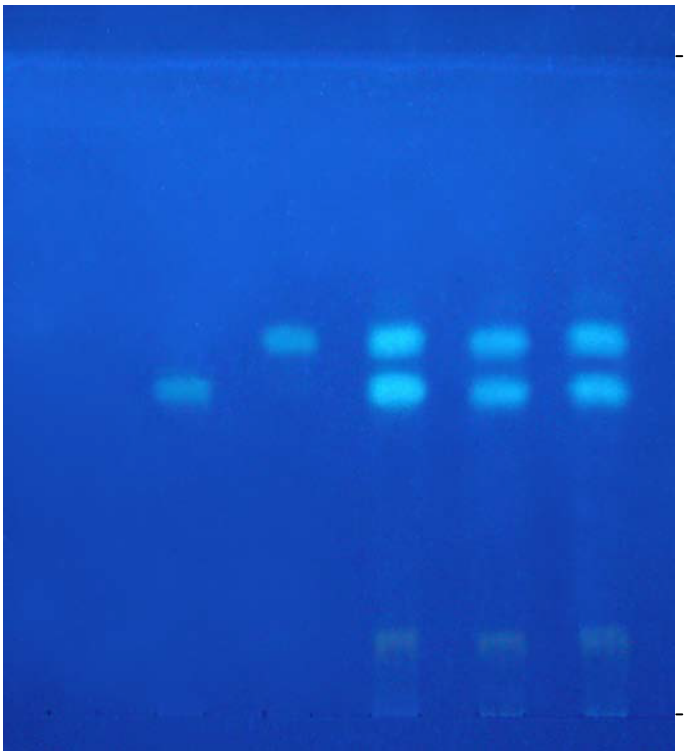
Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify naringin and neohesperidin peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed naringin and neohesperidin Std-AS. The retention times of naringin and neohesperidin peaks in the chromatograms of the test solution and the Std-AS should not differ by more than 2.0%. Measure the peak areas and calculate the concentrations (in milligram per litre) of naringin and neohesperidin in the test solution, and calculate the percentage contents of naringin and neohesperidin in the sample by using the equations indicated in Appendix IV(B).

Limits

The sample contains not less than 4.0% of naringin ($C_{27}H_{32}O_{14}$) and not less than 3.0% of neohesperidin ($C_{28}H_{34}O_{15}$), calculated with reference to the dried substance.

Aurantii Fructus　(枳殼)



Lane	Sample	Results
1	Blank (Methanol)	Negative
2	Standard (Naringin)	Naringin positive
3	Standard (Neohesperidin)	Neohesperidin positive
4	Spiked sample (Sample plus naringin and neohesperidin)	Naringin and neohesperidin positive
5	Sample (Aurantii Fructus)	Naringin and neohesperidin positive
6	Sample duplicate (Aurantii Fructus)	Naringin and neohesperidin positive

Figure 1　TLC results of Aurantii Fructus extract observed under UV light (366 nm) after staining