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Red Clover Aerial Parts Isoflavone Aglycones Dry Extract

DEFINITION

Red Clover Aerial Parts Isoflavone Aglycones Dry Extract is prepared from dried aerial parts of *Trifolium pratense* L. (Fam. Fabaceae) by extraction with hydroalcoholic mixtures or other suitable solvents. It contains NLT 36.0% and NMT 44.0% of isoflavones, predominantly as aglycones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A. It contains NMT 1.0% of daidzein and NMT 1.0% of genistein, both on the dried basis. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.9 and 1.7. It may contain suitable excipients.

IDENTIFICATION

• **A. HPTLC for Articles of Botanical Origin ⟨203⟩**

Presence of biochanin A and formononetin

Solvent: Methanol and water (7:3)

Standard solution A: 1 mg/mL each of USP Biochanin A RS and USP Formononetin RS in methanol

Standard solution B: 10 mg/mL of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS in *Solvent*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, centrifuge, and use the supernatant.

Sample solution: 10 mg/mL of Red Clover Aerial Parts Isoflavone Aglycones Dry Extract in *Solvent*. Shake to disperse, heat on a water bath at 60°–80° for 10 min, centrifuge, and use the supernatant.

Chromatographic system

Adsorbent: Chromatographic silica gel with an average particle size of 5 µm (HPTLC plate)[1]

Application volume: 2 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, as 8-mm bands

Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Temperature: Ambient, not to exceed 30°

Developing solvent system: Ethyl acetate, toluene, and formic acid (30:70:1)

Developing distance: 6 cm

Derivatization reagent: 5 mg/mL of 2-aminoethyl diphenylborinate in ethyl acetate

Analysis

Samples: *Standard solution A*, *Standard solution B*, and *Sample solution*

Apply the *Samples* and dry in air. Develop in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize while warm with the *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution A* exhibits a bluish band at about one-third of the chromatogram due to formononetin, and a greenish band due to biochanin A at about the middle of the chromatogram. *Standard solution B* exhibits prominent bands of similar color and position to those of formononetin and biochanin A in *Standard solution A*. A red band appears below the formononetin band in *Standard solution B*.

Acceptance criteria: The *Sample solution* exhibits the following prominent bands similar in position and color to those in *Standard solution B*: two greenish bands at about the middle of the chromatogram (a distinction from white clover, soy, and alfalfa), one of them corresponding to biochanin A in *Standard solution A*; a bluish band at about one-third of the chromatogram, corresponding to the formononetin band in *Standard solution B*; and a red band below that due to formononetin.

• **B. HPLC**

Analysis: Proceed as directed in the test for *Content of Isoflavones*.

Using the values obtained in the test for *Content of Isoflavones*, calculate the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones:

$$\text{Result} = (B + G)/(D + F)$$

B = percentage of biochanin A

G = percentage of genistein

D = percentage of daidzein

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F = percentage of formononetin

Acceptance criteria: The *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times corresponding to those in *Standard solution A* and *Standard solution B*. The ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.9 and 1.7.

COMPOSITION

• **Content of Isoflavones**[2]

Solution A: Acetonitrile and water (1:3) containing 0.05% trifluoroacetic acid

Solution B: Acetonitrile containing 0.05% trifluoroacetic acid

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
2	100	0
2.5	87	13
7.5	80	20
7.8	73	27
8.0	55	45
11.0	50	50
13.0	40	60
15.0	26	74
16.0	0	100
18.1	100	0
23.0	100	0

Diluent: Alcohol and water (1:1)

Standard solution A: Prepare a 0.10–0.15-mg/mL solution of USP Red Clover Aerial Parts Isoflavone Aglycones Dry Extract RS in *Diluent*, sonicate briefly, and filter through a membrane of 0.45-µm or finer pore size.

Standard solution B: 0.1 mg/mL of USP Formononetin RS, 0.02 mg/mL of USP Genistein RS, 0.02 mg/mL of USP Daidzein RS, and 0.1 mg/mL of USP Biochanin A RS in a mixture of *n*-propanol and water (1:1)

Sample solution: Accurately weigh a quantity of Red Clover Aerial Parts Isoflavone Aglycones Dry Extract, equivalent to 12 mg of the labeled content of isoflavones, and transfer to a 100-mL volumetric flask. Add 50 mL of *Diluent*, sonicate to disperse, and dilute with *Diluent* to volume. Before injection, pass through a filter of 0.45-µm or finer pore size.

Chromatographic system

(See *Chromatography ⟨621⟩, System Suitability*.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 25-cm; end-capped 5-µm packing L1

Column temperature: 45°

Flow rate: 1.0 mL/min

Injection volume: 10 µL

System suitability

Samples: *Standard solution A* and *Standard solution B*

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