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Powdered Red Clover

DEFINITION

Powdered Red Clover is Red Clover reduced to a powder or very fine powder. It contains NLT 0.5% of isoflavones, calculated on the dried basis as the sum of daidzein, genistein, formononetin, and biochanin A.

IDENTIFICATION

• **A.** Powdered Red Clover meets the requirements under *Specific Tests, Botanic Characteristics*.

• **B. THIN-LAYER CHROMATOGRAPHY**

Presence of biochanin A and formononetin

Solvent A: Methanol and water (7:3)

Solvent B: Methanol and water (6:4)

Standard solution A: 0.5 mg/mL of [USP Biochanin A RS](#) in methanol

Standard solution B: 0.5 mg/mL of [USP Formononetin RS](#) in methanol

Standard solution C: 10 mg/mL of [USP Powdered Red Clover Extract RS](#) in *Solvent A*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.

Sample solution: Transfer 0.5 g of Powdered Red Clover to a centrifuge tube, and add 5 mL of *Solvent B*. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant. [NOTE—Reserve a portion of the supernatant for *Identification* test C.]

Chromatographic system

(See [Chromatography \(621\)](#), *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 4 µL each of *Standard solution A*, *Standard solution B*, and *Sample solution*, and 3 µL of *Standard solution C*, as 8-mm bands

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

Developing solvent system: A mixture of 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*, *Standard solution C*, and *Sample solution*

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

System suitability: *Standard solution C* exhibits, at about the middle of the chromatogram, a greenish band corresponding to the band due to biochanin A in the chromatogram of *Standard solution A*, and a bluish band, at about one third of the chromatogram, corresponding to formononetin in the chromatogram of *Standard solution B*. Below the band due to formononetin, the *Standard solution C* chromatogram exhibits a red band in the lower third section of the chromatogram.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following main bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution C*: two greenish bands at about the middle of the chromatogram (distinction from white clover, soy, and alfalfa), one of these greenish bands corresponds to the band due to biochanin A in the chromatogram of *Standard solution A*; and a bluish band at about one third of the chromatogram, corresponding to the band due to formononetin in the chromatogram of *Standard solution B*; below the band due to formononetin, the *Sample solution* chromatogram exhibits a red band in the lower third section of the chromatogram.

• **C. THIN-LAYER CHROMATOGRAPHY**

Presence of flavone glycosides

Solvent A: Methanol and water (7:3)

Solvent B: Methanol and water (6:4)

Standard solution A: 0.1 mg/mL of [USP Hyperoside RS](#) in methanol

Standard solution B: 25 mg/mL of [USP Powdered Red Clover Extract RS](#) in Solvent A. Shake to disperse, heat in a water bath at 60°–80° for 10 min, cool, centrifuge, and use the supernatant.

Sample solution: Use the solution as prepared in *Identification test B*.

Chromatographic system

(See [Chromatography \(621\)](#), *Thin-Layer Chromatography*.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)

Application volume: 4 µL of *Standard solution A*, 8 µL of *Standard solution B*, and 2 µL of *Sample solution*, as 8-mm bands

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

Developing solvent system: A mixture of ethyl acetate, formic acid, glacial acetic acid, and water (100:11:11:27)

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* as bands to a suitable HPTLC plate, and dry in air. Develop the chromatograms in a saturated chamber. Remove the plate from the chamber, heat at 100° for 5 min, derivatize the plate while still warm with *Derivatization reagent*, dry in air, and examine under UV light at 365 nm.

System suitability: *Standard solution B* exhibits, at about the middle of the chromatogram, two yellow bands. The lower band corresponds in color and R_F to the band due to hyperoside in the chromatogram of *Standard solution A* and the upper yellow band is due to isoquercitrin. The solution also exhibits two green bands, or a broad green band, above the yellow bands, and a blue band in the upper third section of the chromatogram. The yellow bands due to hyperoside and isoquercitrin are clearly separated.

Acceptance criteria: The chromatogram of the *Sample solution* exhibits the following bands similar in positions and colors to the corresponding bands in the chromatogram of *Standard solution B*: a yellow band at about the middle of the chromatogram corresponding to the band due to hyperoside in the chromatogram of *Standard solution A*; another yellow band, at an R_F slightly higher than that of hyperoside; two green bands, or a broad green band, above the yellow bands; and a blue band in the upper third section of the chromatogram.

• D. HPLC IDENTIFICATION TEST

Analysis: Proceed as directed for the *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

F = percentage of formononetin

Acceptance criteria: The chromatogram of the *Sample solution* exhibits peaks for daidzein, genistein, formononetin, and biochanin A at retention times that correspond to those in the chromatogram of *Standard solution A*, and the ratio of 5,7-dihydroxyisoflavones to 7-hydroxyisoflavones is between 0.1 and 10.

COMPOSITION

• CONTENT OF ISOFLAVONES

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	0