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Application Note

As published in "The Handbook of Analytical Methods for Dietary Supplements"

0060 - Red Clover for Isoflavones by HPLC

Botanical Name: *Trifolium pratense L.*

Common Names: Cow clover, meadow clover, purple clover

Parts of Plant Used: Flowers, leaves, and stems

Uses: Treatment of age-related and hormone-dependent diseases including menopausal symptoms and osteoporosis.

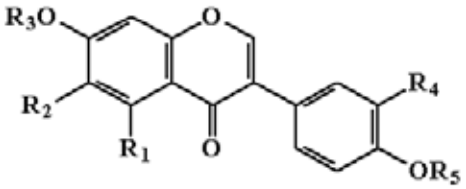
Modes of Action:

The isoflavone content in red clover was found to have estrogenic properties that may play an important role in moderation of menopausal symptoms and prevention of cancers as well as other health benefits.[1]

Chemical Markers:

Various flavonoids and isoflavonoids as well as glycoside and glycoside malonate derivatives of flavonoids and isoflavonoids have been identified in red clover and related species. The major isoflavonoids include biochanin A, formononetin, sissotrin (biochanin A-7-O-glycoside), ononin (formononetin-7-O-glycoside), biochanin A-7-O-glycoside-6"-O-malonate, formononetin-7-O-glycoside-6"-malonate, and the minor isoflavonoids are daidzein, glycitein, calycosin, genistein, pratensein, pseudobaptigenin, irilone, prunetin, and their glycosides and glycoside malonates.

The identified flavonoids include quercetin, isoquercetin, hyperoside, apigenin, luteolin, apigenin-7-glycoside, and luteolin-7-glycoside.[1–4] Cyanogenic glycosides, caffeoyl derivatives, saponins, and sterols also were found in red clover.[5–7] The essential oils from flowers of red clover were studied by GC–MS and the main components were identified as maltol, linalool, 1-phenylethyl alcohol, phenol, phenylethyl acetate, acetophenone, and (Z)-3-hexenyl acetate.[8] As the isoflavonoids are believed to be the active components in red clover, they are used as marker compounds for quality control of red clover extracts.



Name	R_1	R_2	R_3	R_4	R_5
Daidzein	H	H	H	H	H
Formononetin	H	H	H	H	Me
Genistein	OH	H	H	H	H
Pseudobaptigenin	H	H	H		OCH_{2-}
Biochanin A	OH	H	H	H	Me
Prunetin	OH	H	Me	H	H
Glycitein	H	OMe	H	H	H
Calycosin	H	H	H	OH	Me
Irilone	H	OMe	H	H	H
Glycitein	OH	H	H	OH	Me

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Methods of Analysis

Several HPLC and LC–MS methods have been published to identify and analyze the isoflavones in red clover and red clover extracts. Due to the instabilities of isoflavone glycoside malonates, for quality control purposes, they should be converted to glycosides or converted to aglycones before analysis.

Method 1:

The method of Lin et al.[4] was used. This method separates and identifies more than 30 compounds in red clover.

Sample Preparation:

For plant materials, extract about 0.5 g of flowers or 0.25 g of leaves with 10 mL of methanol–water (9:1) by sonication at room temperature for 60 minutes.

To get malonate-free samples, filter the resulting solution and heat in a sealed vial at 80°C to 85°C for 16 hours.

To get a hydrolyzed sample, filter, then mix 2 mL of the resulting solution with concentrated hydrochloric acid (37%, 0.4 mL), and heat in a sealed vial at 80°C to 85°C for 1.5 hours.

Chromatography:

Column: Waters SymmetryShield, 5 µm, 150 x 39 mm with a guard column, (SymmetryShield, 5 µm, 3.9 × 20 mm).
Mobile phase: Solvent A = water containing 0.25% acetic acid, solvent B = acetonitrile containing 0.25% acetic acid.
Gradient: 14%B to 22%B in 36 minutes, then 22%B to 52%B in 64 minutes.
Flow rate: 0.2 mL/minute
Injection volume: 10 µL
Detection wavelength: 255 nm
Column temperature: 45°C

Validation Data:

Not available

Method 2:

The method of Klejdus et al.[1] separates and identifies about 50 compounds in red clover.

Sample Preparation:

Extract about 0.5 g of material with methanol–water (7:3) using a magnetic stirrer and a “fex IKA Werke 50” extractor. Then clean-up the sample using solid-phase extraction.

Chromatography:

Column: Metachem Polaris C18A, 3 µm, 150 × 2.0 mm.
Mobile phase: Solvent A = water containing 0.2% acetic acid, solvent B = acetonitrile.
Gradient: 15%B to 25%B in 36 minutes, then 25%B to 55%B in 54 minutes, and finally 55%B to 15%B in 10 minutes.
Flow rate: 0.3 mL/minute
Injection volume: 10 µL
Detection wavelength: 255 nm

Column temperature: 40°C

Validation Data:
Not specified

Method 3:
The method of Krenn et al.[3] can be used to analyze the hydrolyzed aglycone daidzein, genistein, formononetin, and biochanin A in red clover.

Sample Preparation:
Extract 200 mg of pulverized drug with 30 mL of 80% methanol (acidified to pH 3 with trifluoroacetic acid) for 15 minutes under reflux at 85°C. Filter, wash the residues with 20 mL of 80% methanol. Evaporate the extraction solution to dryness under reduced pressure and dissolve the residue in 2 mL of dimethyl sulfoxide. To 500 µL of the dimethyl sulfoxide solution, add 40 µL of standard solution (15 mg of 6-methoxyflavanone/mL).

Chromatography:
Column: Hypersil BDS C18, 5 µm, 250 × 4.0 mm.
Mobile phase: Solvent A = water (adjusted to pH 2.7 with sulfuric acid), solvent B = acetonitrile.
Gradient: 20%B to 37%B in 35 minutes, then 37%B to 100%B in 10 minutes, keep at 100%B for 10 minutes, then from 100%B to 20%B in 10 minutes, and keep at 20%B for 10 minutes.
Flow rate: 1 mL/minute
Injection volume: 10 µL
Detection wavelength: 254 nm

Validation Data:
Linearity: 1.0 to 260, 1.5 to 260, 0.5 to 60, and 2.0 to 300 mg/L for daidzein, genistein, biochanin A, and formononetin, respectively, with correlation coefficients over 0.9988.
Accuracy: The percent recoveries were 99.2 for biochanin A and 98.2 for formononetin.
Precision: The RSD was less than 5.1% for all four compounds.
Selectivity: Peak identification was determined against standards.
Ruggedness: Not specified
Robustness: Not specified
LOD/LOQ: LOD = 1.6, 3.1, 0.6, and 3.7 ng for daidzein, genistein, biochanin A, and formonometin, respectively. LOQ = 5.0, 8.5, 2.0, and 10.0 for daidzein, genistein, biochanin A, and formonometin, respectively.

Method 4:
The method of Wu et al.[2] identifies 31 isoflavones in red clover and also analyzes the hydrolyzed aglycone daidzein, genistein, formononetin, biochanin A, pseudobaptigenin, glycitein, calycosin, prunetin, irilone, and pratensein.

Sample Preparation:
For qualitative studies, extract about 100 mg of powder with 10 mL of 80% methanol using sonication for 1 hour.

For quantitative analysis of total aglycones, hydrolyze about 500 mg of herb powder with 50 mL of ethanol, 20 mL of water, and 8 mL of concentrated hydrochloric acid for 2 hours under nitrogen protection.

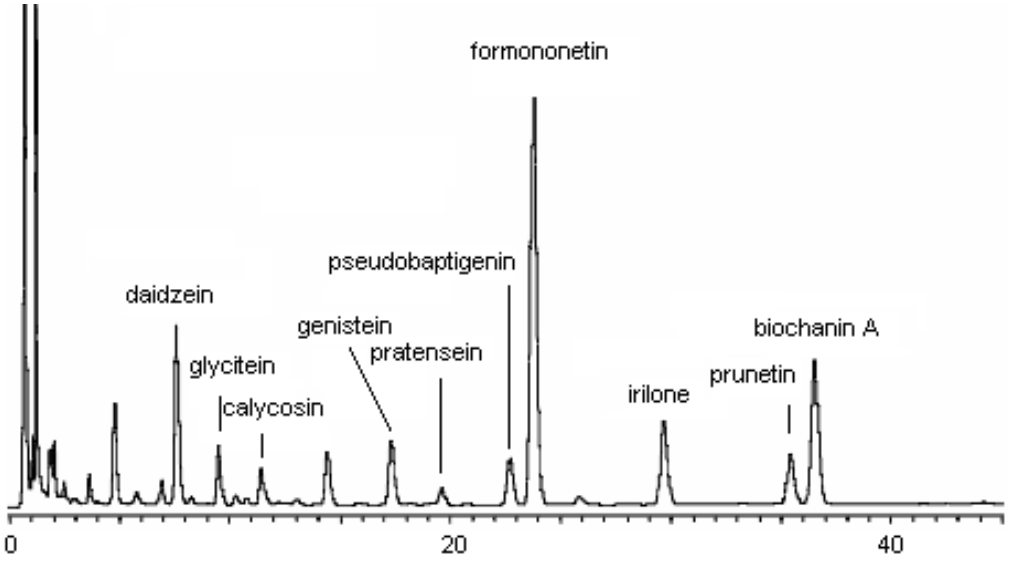
Chromatography:

Column: Phenomenex Prodigy ODS3, 5 µm, 150 × 3.2 mm.
Mobile phase: Solvent A = water with 0.1% formic acid (vol/vol), solvent B = acetonitrile with 0.1% formic acid (vol/vol).
Gradient: 20%B to 40%B in 40 minutes.
Flow rate: 1 mL/minute
Injection volume: 5 µL
Detection wavelength: 254 nm

Validation Data:

Linearity: 28.32 to 14,500, 32.23 to 16,500, 30.76 to 15,750, 21.97 to 11,250, 23.44 to 12,000, 25.39 to 1300, 25.39 to 1300, 25.39 to 1300, 23.44 to 12,000, and 23.44 to 12,000 ng/mL for daidzein, formononetin, genistein, pseudobaptigenin, glycitein, calycosin, prunetin, biochanin A, irilone, and pratensein, respectively, with correlation coefficients over 0.9995.
Accuracy: Not available
Precision: The RSD was less than 8% for all 10 aglycones at various concentrations.
Selectivity: Peak identification was determined against standards.
Ruggedness: Not specified
Robustness: Not specified
LOD/LOQ: LOQ = About 24 ng/mL for all 10 compounds

Representative HPLC Chromatogram of Red Clover Run by Method 4



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