

PM FLUID EXTRACT METHOD

Draft P. mirifica Fluid Extract Method

Scope

This analytical procedure is intended for the determination of five target analytes in alcohol fluid extracts of P. mirifica roots. The target analytes are: puerarin, daidzin, genistin, daidzein, and genistein. The applicable concentration range is ca. 0.05 to 5 mg/g for each target analyte.

Summary

A 1 gram portion of the fluid extract is dissolved in 100mL ethanol/water (8:2,v:v). An aliquot is taken for HPLC analysis with UV detection at 255nm. The target analytes are quantified by external standardization against reference standards.

Instrumentation

Hewlett Packard Series 1100 HPLC with autosampler and UV/Vis diode array detector.

Equipment

Analytical Balance. Accurate to 0.0001g. Denver Instrument
Autosampler vials. National Scientific Company.
Graduated cylinders. 200mL and 1000mL.
HPLC column: Prodigy 5u ODS(3) 100A, 250 X 4.6mm. Phenomenex #00G-4097-EO.
Pipettes, transfer
Syringe. Gas tight, 1.0mL
Volumetric Flasks. 10.0, 100.0, and 1000mL, class A
Vortex mixer. VWR Scientific, Vortex Genie 2.

Reference Standards

Puerarin.
Daidzin. Chromadex
Daidzein. Chromadex
Genistin. Chromadex
Genistein. Chromadex

Reagents

Acetic Acid
Acetonitrile
Ethanol
Methanol
Water

Reagent Solutions

Dilution Solvent: Ethanol/water (8:2, vol:vol). For 1L of solution, combine 800mL ethanol and 200mL water. Note: methanol can be substituted for ethanol.

Mobile Phase A: 0.1% acetic acid – For 1.0L solution, add 1.0mL glacial acetic acid to a 1.0L volumetric flask containing ca 300mL water. Mix, dilute to volume with water, and mix again.

Mobile Phase B: acetonitrile.

Standard Solutions

Individual Stock Standard Solutions. Prepare separate individual stock standard solutions at ca. 1 mg/mL as follows: For daidzein and genistein, use methanol as the solvent. For the others, use the diluting solvent. Accurately weigh ca 10 mg reference standard and transfer into a 10mL volumetric flask. Dissolve in a small amount (ca. 5-7mL) of solvent (sonication and/or warming of the solution may be required to completely dissolve), dilute to volume, and mix well.

Mixed Stock Standard Solution (ca 500 ug/mL each). Transfer 0.5mL of each stock standard (puerarin, daidzin, daidzein, genistin, and genistein) to a 10.0mL volumetric flask, dilute to volume with dilution solvent and mix well. (Use this level for root powder)

High Level Calibration Standard (ca 50 ug/mL). Transfer 1.0mL of the mixed stock standard to a 10.0mL volumetric flask, dilute to volume with dilution solvent and mix well.

Mid Level Calibration Standard (ca 5.0 ug/mL). Transfer 1.0mL of the high level calibration standard to a 10.0mL volumetric flask, dilute to volume with dilution solvent and mix well.

Low Level Calibration Standard (ca 0.5 ug/mL).). Transfer 1.0mL of the mid level calibration standard to a 10.0mL volumetric flask, dilute to volume with dilution solvent and mix well.

Sample Preparation

1. Accurately weigh 1g fluid extract into a 100mL volumetric flask.
2. Add 70mL of the dilution solvent, swirl flask contents to ensure thorough dissolution of the sample (use vortex mixer if necessary), dilute to volume with dilution solvent and mix well.

3. Transfer an aliquot of the sample solution to an autosampler vial for HPLC analysis.

HPLC Analysis

Column: Prodigy 5u ODS(3) 100A, 250 X 4.6mm. Phenomenex #00G-4097-EO.

Mobile Phase A: 0.1% acetic acid

Mobile Phase B: acetonitrile

Gradient:	Time (min.)	%A	%B
	Initial	90	10
	21.00	48	52
	22.00	0	100
	28.00	0	100
	29.00	90	10
	40.00	90	10

Flow Rate: 1.0 mL/minute

Column Temperature: 30 C

Injection Volume: 5uL

Run Time: 40 minutes

Detector: UV/Vis Diode Array at 255nm, bandwidth = 20nm

Data Analysis (see example chromatogram)

Target analyte identification: Retention time and UV spectral comparison against standards.

	retention time (minutes), 0.2%
puerarin	8.22
daidzin	9.80
genistin	12.13
daidzein	16.52
genistein	20.02

Quantification: The target analytes are quantified against a three point external standard calibration (see Standard Solutions). The calibration data are fit to a linear model, ignoring the origin.

Calculations

Results are expressed as mg/g. To determine the amount of target analyte in the sample, use the following formula:

$$\text{mg/g} = (X \text{ ug/mL})(V \text{ mL}) / (S \text{ g})(1000 \text{ ug/mg})$$

Where: X = target analyte concentration from the instrument print out in ug/mL
V = dilution volume in mL (usually 100)

S = sample weight in g

Method Performance

Six replicates of a partially fortified fluid extract were analyzed according to the method. RSD values and spike recovery were determined. The actual concentrations measured ranged from a low of 0.16 mg/g (genistin) to a high of 0.86 mg/g (puerarin). The fluid extract was fortified as follows:

puerarin	0 ug
daidzin	202ug
daidzein	218ug
genistin	0ug
genistein	418ug

Results:	<u>Analyte</u>	<u>RSD (%)</u>	<u>Recovery (%)</u>
	puerarin	1.05	NA
	daidzin	0.90	93.0
	daidzein	1.95	98.8
	genistin	1.55	NA
	genistein	3.03	92.0

During the course of an actual analysis of a sample group a duplicate and spiked sample were run with the following results: (spike level ca. 0.2 mg each analyte)

<u>Analyte</u>	<u>RSD (%)</u>	<u>Recovery (%)</u>
puerarin	0.03	99.9
daidzin	0.18	100.0
daidzein	0.93	100.0
genistin	0.44	99.6
genistein	0.17	99.8

Current Chromatogram(s)

