1. Purpose:

To determine flavonol glycosides of quercetin, kaempferol, and isorhamnetin in plant materials and dried extracts of *Ginkgo biloba*.

2. Principle:

Plant material and extract are extracted with ethanol/water. The glycosides are hydrolyzed with acid to produce the aglycones quercetin, kaempferol, and isorhamnetin. The aglycones are quantified externally against quercetin as pure reference standard.

3. Materials:

*The following materials are needed to carry out the analysis.*

3.1 Standards:

- Quercetin, Sigma Chemical Co.
- Kaempferol, Sigma Chemical Co.
- Isohamnetin, Sigma Chemical Co.

3.2 Apparatus:

- HPLC instrument: Hewlett-Packard HP 1100 Series HPLC equipped with autosampler, DAD detector, and HP ChemStation Software or equivalent.
• HPLC column: Phenomenex® Prodigy ODS (3), 5 \( \mu \), 150 x 3.2 mm.
• Calibrated analytical balance accurate to \( \pm 0.01 \) mg
• Sonicator, with temperature control
• Volumetric flasks, appropriate sizes
• Syringes, 3-cc disposable with Luer-lok tip
• Filters, 0.45-\( \mu \)-m, glass PVDF or Cellulose Acetate
• Pipettes, class A, assorted sizes
• Refluxing apparatus, 250 mL

3.3 Reagents:

- Water, HPLC grade
- Phosphoric acid, 85%, reagent grade
- Methanol, HPLC grade
- Ethanol, 95%, food grade
- Hydrochloric acid, reagent grade, VWR # VWR 3110-3 or equivalent

4. HPLC conditions:

The following HPLC condition should be used when carrying out this analysis:

4.1 Project and location: Ginkgo HPLC program (Ginkgo.m) for separating and checking and quantifying isoflavones is in the C:\HPCHEM\1\Methods\ directory of HP ChemStation software system. Sequence: Ginkgo.s.
4.2 HPLC column: Phenomenex® Prodigy ODS (3), 5 \( \mu \), 150 x 3.2 mm.
4.3 Column temperature: 35 °C.
4.4 Mobile phase: The mobile phase was consisted of solvent A (0.1% phosphoric acid solution) and solvent B (methanol) 50:50.
4.5 Flow rate: 1.2 mL/min.
4.6 Injection volume: 10 \( \mu \)L.
4.7 Detection wavelength: 270 nm.
4.8 Running time: 40 minutes.
3.9 DAD detector option: Diode-array Detector (DAD) data were acquired on a mode 1100 series DAD detector over the wavelength range 200-400 nm at a rate of 1 spectrum/second and 1.2 nm resolution.

5. Preparation of Standard
Prepare a stock standard in methanol at a final concentration of approximately 0.600 mg/mL quercetin.

**NOTICE:** Store the standard solutions in the refrigerator when not in use. Upon storage, solid may precipitate out. To dissolve the solids, warm the standard preparation under hot tap water for several minutes before use. The standard solutions must not be cloudy or hazy when used.

Dilute the stock standard to create a minimum of a three-point standard curve response that includes the stock standard. Suggested standard dilutions of the stock standard are 1:5 and 1:10 using methanol. These standard solutions are stable for at least 2 weeks at refrigerated temperatures. If required, a low concentration standard solution may be created by diluting the stock 1:100 using methanol. *However, this standard solution will degrade within 48 hours.*

**6. Preparation of Samples**

**6.1 Plant Material:**

Accurately weigh out approximately 1.0 g of ground ginkgo leaves. Place the leaves into a 250-mL flask along with 50 mL of ethanol, 20 mL of DI water, and 8 mL concentrated hydrochloric acid using a fume hood. Reflux at moderate heat for 2.25 hours protected by N₂. Cool to room temperature.

**NOTE:** The solution will turn burgundy color during the reflux. The color of the solution is not a definitive indication of reaction completeness.

Separate the extracted leaves from the solvent by decanting the solution into a 100-mL volumetric flask. Add 20 mL methanol to the hydrolysis flask and sonicate for 30 minutes at 30 - 40°C. Transfer the solution with filtering to the 100-mL volumetric flask and carefully wash the solids to final volume. Filter an aliquot through 0.45µm PVDF into a HPLC vial and cap.

**6.2 Extract:**

Accurately weigh 300 mg of dry extract (equivalent to 72 mg total ginkgo flavonol glycosides). Place the dry extract into a 250-mL flask along with 50 mL of ethanol
and 20 mL of DI water. Sonicate for 5 minutes. Add 8 mL of concentrated hydrochloric acid using a fume hood. Add boiling chips. Reflux at moderate temperature for 2.25 hours protected by N\textsubscript{2}. Cool to room temperature.

*NOTE:* The solution will turn burgundy color during the reflux. The color of the solution is not a definitive indication of reaction completeness.

Quantitatively transfer the solution from the flask into a 100-mL volumetric flask. Dilute the solution to volume using DI water and mix. Filter an aliquot through 0.45µm PVDF into an HPLC vial and cap.

7. Procedure

- Prepare reference standard solutions and sample preparations as directed.
- Make a single injection of an extraction solution blank.
- Make single injection of the standard preparations.
- Prepare a linearity curve for the standard, quercetin with the origin ignored, using peak area vs. concentration (mg/mL). Perform linear regression analysis on the data. The $r^2$ must be $\geq 0.999$ for each analyte's calibration curve.
- Make single injections of sample preparations.

*NOTICE:* If there is not baseline separation of all peaks in the standard preparation's chromatogram, adjust the mobile phase by increasing its polarity to optimize the separation.

- Calculate the percent flavonol glycosides in the sample(s).

8. Quality Assurance

- A duplicate sample preparation and standard preparation should be analyzed with each set of 20 or less samples.
- To monitor method variance, a ginkgo control sample of known composition should be assayed with each batch and the assay results control charted.

9. Calculations:

Express all the ginkgo flavonolglycosides present as coumaroyl derivative of the aglycone, quercetin.
Individual flavonolglycoside (%w/w) = \frac{(C)(FV)(D)(F)(100\%)}{(W)}

Where:

C = Sample's glycoside concentration (mg/mL) from linear regression analysis.

FV = The final volume of the sample preparation (mL).

D = The dilution factor of the sample preparation (if needed).

F = The correction factor for conversion of the aglycone, quercetin to the glycosides.

Quercetin glycoside: F = 2.504
Kaempferol glycoside: F = 2.940
Isorhamnetin glycoside: F = 2.372

W = The sample weight (mg).

Total Flavonol Glycosides (% w/w) in Sample Extract = QG + KG + IG

Where:

QG = % Quercetin glycosides
KG = % Kaempferol glycosides
IG = % Isorhamnetin glycosides

References:

Stricher, O., Quality of Ginkgo Preparations *Planta Med.* 1993, 59, 1-11
**APPENDIX I:**

*Structures of the Standard Compounds:*

![Quercetin (C_{15}H_{10}O_{7}, MW: 302)]

![Kaempferol (C_{15}H_{10}O_{6}, MW: 286)]

![Isohamnetin (C_{16}H_{12}O_{7}, MW: 316)]