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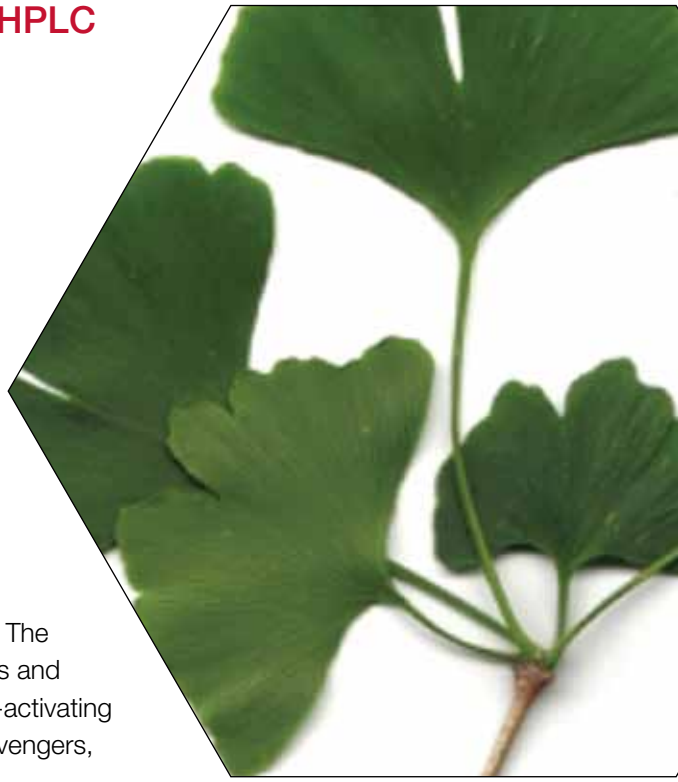
Application Note

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

0047 - Ginkgo for Flavonoids and Terpenes by HPLC



Botanical Name: *Ginkgo biloba L.*

Common Names: Maidenhair tree, Ying-Xing-Ye

Parts of Plant Used: Leaves

Uses: Treatment of poor memory, memory loss, Alzheimer's disease, peripheral vascular disease.

Modes of Action:

More than 100 clinical trials have investigated the bioactivities of standardized ginkgo leaf extracts. Most of them showed effectiveness for memory loss, Alzheimer's disease, and peripheral vascular disease. The compounds responsible for the activities of ginkgo are terpene lactones and flavonol glycosides. The terpene lactones were found to inhibit platelet-activating factors and the ginkgo flavonoids were found to act as free radical scavengers, inhibiting nitric oxide formation.

Chemical Markers:

Various compounds have been identified from ginkgo leaves including terpenes, flavonoids, long-chain hydrocarbon derivatives (long-chain hydrocarbons, alcohols, aldehydes, ketones, acids), alicyclic acids and cyclic compounds (shikimic acid, quinic acid, ascorbic acid, ginkgolic acids), carbohydrates, sterols, and carotenoids. Terpenes lactones and flavonoids are the major compounds in ginkgo. The terpene lactones from ginkgo have one of two characteristic skeletons: they have a diterpene structure as in ginkgolide A, B, C, J, and M, or they have a sesquiterpene structure as in bilobalide.

Ginkgo flavonoids are represented by flavonol glycosides and biflavonoids. Ginkgo leaves contain about 20 flavonol glycosides, mainly mono-, di-, and triglycosides, and p-coumarin esters of glycosides of kaempferol, quercetin, and isorhamnetin. The biflavonoids in ginkgo are all 3',8"- biflavones (bilobetin, ginkgetin, isoginkgetin, amentoflavone, sciadopitysin, 5'-methoxybilobetin).

Ginkgo also contains other types of flavonoids including glycosides of myricetin, 3'-methyl myricetin, apigenin, luteolin, and catechins.[1,2] Currently, the content of terpene lactones (ginkgolides, bilobalide) and flavonol glycosides (glycosides of quercetin, kaempferol, and isorhamnetin) are used as the marker compounds for quality control of ginkgo leaf extract. The ginkgolic acids also are analyzed and generally the amount of ginkgolic acids is required to be lower than 5 ppm because they are possible toxic compounds.

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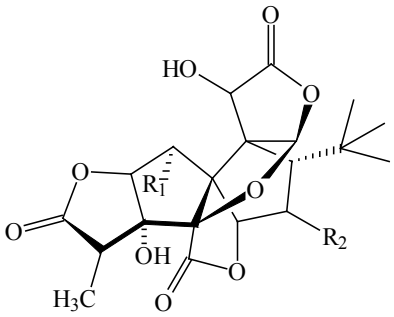
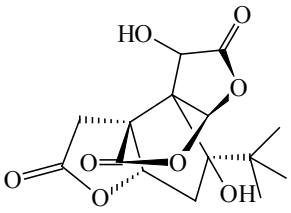
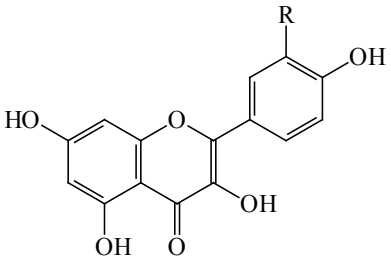


Ginkgolide A: R_1 = H, R_2 = H
Ginkgolide B: R_1 = OH, R_2 = H
Ginkgolide C: R_1 = OH, R_2 = OH
Ginkgolide J: R_1 = H, R_2 = OH



Bilobalide



Kaempferol: R = H
Quercetin: R = OH
Isorhamnetin: R = OCH_3

Methods of Analysis

As one of the best-selling herbal medicines in the world, ginkgo has been studied extensively and reviewed by van Beek.[3] Extraction is key for accurate analysis of ginkgo compounds. Various extraction solvents have been compared for the analysis of terpene lactones aqueous methanol, ethanol, and acetone were all found to be suitable extraction solvents. The flavonoid glycosides in ginkgo also can be extracted with aqueous alcohol.

Method 1:

The method found at www.nsfina.org can be used to determine the flavonoid glycosides content in ginkgo. The glycosides are hydrolyzed with acid to produce the aglycones, kaempferol, quercetin, and isorhamnetin. The aglycones are quantified externally against pure reference standards and then converted back to glycosides using a cofactor.

Sample Preparation:

For plant material, accurately weigh approximately 1.0 g of ground ginkgo leaves into a 250-mL flask; add 50 mL of ethanol, 20 mL of water, and 8 mL of concentrated hydrochloric acid; and reflux at moderate heat for 2.25 hours. Decant the solution into a 100-mL volumetric flask. Add 20 mL of methanol to the hydrolysis flask, sonicate for 30 minutes at 30 to 40°C, then transfer the solution with filtering to the 100-mL volumetric flask, and carefully wash the solids to final volume.

For extracts, weigh about 300 mg into a 250-mL flask; add 50 mL of ethanol, 20 mL of water (deionized distilled), and 8 mL of concentrated hydrochloric acid; sonicate for 5 minutes; and reflux at moderate heat for 2.25 hours. Dilute the extraction solution to 100 mL.

Chromatography:

Column: Phenomenex Prodigy ODS(3), 5 µm, 100Å, 4.6 × 250 mm.
Mobile phase: Methanol–0.5% phosphoric acid (50:50), isocratic.
Flow rate: 1.2 to 1.5 mL/minute
Injection volume: 10 µL
Column temperature: 35°C
Detection wavelength: 270 nm

Correction factors for conversion of the aglycone to the glycoside:
For quercetin, F = 756.7/302.2 = 2.504.
For kaempferol, F = 740.7/286.2 = 2.588.
For isorhamnetin, F = 770.6/316.2 = 2.437.

Method 2:

The method at www.nsfina.org also can be used to analyze the terpene lactones in ginkgo.

Sample Preparation:

For plant materials, accurately weigh 600 to 800 mg into a 50-mL round-bottom flask fitted with a reflux condenser. Reflux with 5 mL of methanol–water (90:10) for 15 minutes. Filter the sample through a Büchner funnel and extract the leaf material with another 5 mL of methanol–water (90:10). Filter and combine the collected filtrates and washes, then dry under a stream of nitrogen. Finally dissolve the residues in 1 mL of methanol for HPLC analysis.

For the extract, accurately weigh approximately 400 mg into a 50-mL volumetric flask along with 40 mL of methanol–water (90:10). Sonicate the sample at 60°C for 20 minutes or until completely dissolved. Filter the solution before HPLC analysis.

Chromatography:

Column: Phenomenex Prodigy ODS(3), 5 µm, 100Å, 4.6 × 250 mm.
Mobile phase: Solvent A = water, solvent B = methanol.
Gradient:

Time (minutes)	%A	%B
0	75	25
23	52	48
25	25	75
30	25	75
35	10	90
40	75	25
50	75	25

Column temperature: 25°C
Flow rate: 1.0 mL/minute
Injection volume: 8 µL
Detector: ELSD

Method 3:

The HPLC method of Ganzera et al.[4] was used for the analysis of terpene lactones (ginkgolides A, B, C, and J and bilobalide) in ginkgo.

Sample Preparation:

Extract about 500 mg of sample three times with 3 mL of methanol by sonication for 10 minutes. Centrifuge after each extraction and combine the supernates. Fill to 10 mL with methanol.

Chromatography:

Column: Phenomenex Synergi Max RP, 80Å, 4 µm, 150 × 4.6 mm.
Mobile phase: Solvent A = 10 mM ammonium acetate buffer adjusted to pH 5 with glacial acetic acid, solvent B = methanol–isobutanol (9:1).
Gradient: in 10 minutes from 10%B to 20%B, then to 25%B in 15 minutes.
Flow rate: 1.0 mL/minute
Injection volume: 20 µL
Column temperature: 25°C
Detection wavelength: 205 nm and ELSD detector

Validation Data:

Linearity: 31.2 to 500 mcg/mL with a correlation coefficient of 0.9988 for all five compounds.
Accuracy: 98.26% and 100.10% recovery for bilobalide and ginkgolide J, respectively.
Precision: 3.07% RSD (three determinations)
Selectivity: Peak identification was determined against standards.
Ruggedness: Not specified
Robustness: Not specified
LOD/LOQ: Between 10 and 20.3 mcg/mL for five standards.

Method 4:

Another analytical method is that of Li and Fitzloff.[5]

Sample Preparation:

Extract five units (capsules, tablets, or caplets) with 25 mL of 80% methanol through sonication for 60 minutes, then extract two more times with 25 mL of 80% methanol and sonicate 30 minutes each time. Combine the extraction solutions and evaporate under reduced pressure. Dissolve the residue in 50 mL of 80% methanol.

For total flavonoid analysis, reflux 5 mL of the sample preparation solution with 25 mL of hydrochloric acid (4 N)–methanol (1:4 vol/vol) for 30 minutes. Cool, then make up to 50 mL with methanol.

For terpene lactone analysis, evaporate 10 mL of the sample preparation solution to dryness, suspend the residue in 20 mL of hot water, and extract with acetyl acetate (3 × 20 mL). Evaporate the acetyl acetate to dryness and dissolve the residue in 10 mL of methanol.

Chromatography:

Column: Supelco Discovery RP-18, 5 µm, 250 × 4.6 mm, with a Waters Delta-Pak RP-18 guard column.
Mobile phase: Solvent A = water (5% methanol and 0.05% trifluoroacetic acid), solvent B = methanol (0.05% trifluoroacetic acid). For flavonoid analysis, mobile phase is 50%B isocratic for 20 minutes.
Column temperature: 20°C
Detection wavelength: 365 nm
Injection volume: 10 µL
Flow rate: Not specified
For terpene lactone analysis, mobile phase is 25%B to 50%B in 20 minutes.
Injection volume: 10 µL
Flow rate: Not specified
Detector: ELSD

Validation Data:

Linearity: For quercetin, kaempferol, and isorhamnetin, 4 to 2000 mcg/mL with correlation coefficient over 0.9953; for ginkgolide A, B, and C and bilobalide, 40 to 2000 mcg/mL with a correlation coefficient over 0.9975; and for ginkgolide J, 70 to 3500 mcg/mL with a correlation coefficient over 0.9992.
Accuracy: For quercetin, kaempferol, and isorhamnetin, over 97.55% recovery; and for bilobalide, ginkgolide A, B, C, and J, between 93.24% and 97.91%.
Precision: Less than 3.77% for all eight standards.
Selectivity: Peak identification was determined against standards.
Ruggedness: Not specified
Robustness: Not specified
LOD/LOQ: LOD for quercetin, kaempferol, isorhamnetin, ginkgolide A, B, C, and J, and bilobalide = 2, 2, 2, 20, 20, 20, 35, and 20 ng, respectively. LOQ for quercetin, kaempferol, isorhamnetin, ginkgolide A, B, C, J, and bilobalide = 4, 4, 4, 40, 40, 40, 70, and 40 ng, respectively.

Method 5:

The method of Fuzzati et al.[6] can be used to analyze ginkgolic acids in ginkgo extract.

Sample Preparation:

Dissolve 500 mg of ginkgo solution in 10 mL of methanol.

Chromatography:

Column: Agilent Zorbax Eclipse XDB-C8, 5 µm, 250 × 4.6 mm.
Mobile phase: Solvent A = water (0.01% trifluoroacetic acid), solvent B = acetonitrile.
Gradient:

Time (minutes)	%A	%B
0	25	75
30	10	90
35	10	90

Flow rate: 1 mL/minute
Detection wavelength: 210 nm
Column temperature: 35°C
Injection volume: 50 µL

Validation Data:

Linearity: For ginkgolic acid C13, 0.05 to 2 mcg/mL; for ginkgolic acid C15, 0.15 to 8 mcg/mL; and for ginkgolic acid C17, 0.06 to 3 mcg/mL. The correlation coefficient was over 0.999 for all.
Accuracy: All recovery values were between 82.5% and 121.2%.
Precision: RSDs were between 0.1% and 9.0%.
Selectivity: Peak identification was determined against standards.
Ruggedness: Not specified
Robustness: Not specified
LOD/LOQ, LOD = About 0.025 mcg/mL for all three compounds. LOQ = about 0.083 mcg/mL for all three compounds.

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