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HPLC-UV Analysis of Flavonoids from Ginkgo Biloba Extracts

Method Status: Scientifically Valid per cGMPs for Dietary Supplements

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ChromaDex: Steve Baugh**Introduction**

The identity testing of raw materials for potency and verification of label claim data in nutraceutical formulations are requirements of dietary supplement cGMPs. Manufacturers and contract testing labs alike are looking for accurate and scientifically valid methods that are suitable for use with different formulations. The complex nature of nutraceuticals and botanicals often requires long analysis and difficult sample cleanup steps to resolve matrix interferences.

There were two primary goals of this project: 1) to optimize our pre-existing method to reduce total analysis time using newer high efficiency HPLC technologies and 2) demonstrate the suitability for analysis of two commercially available formulations of Ginkgo Biloba.

Botanical Information

Ginkgo

Botanical Name*Ginkgo biloba***Common Names**

Maidenhair tree

Plant Description

Standing 60-100 feet in average height, ginkgo are unique in their leaf shape and structure. Its insect and disease resistant traits make it a long lived species with some specimens estimated at over 2,500 years old. Native to China, ginkgo is also widely cultivated in Japan, Korea, Europe, and the United States. Ginkgo is a unique species with no other living representatives of its family and is linked to fossils dating back over 200 million years.

Therapeutic Use Overview

Ginkgo has a long history of use in traditional Chinese medicines. It is used for asthma, memory and concentration enhancement, and to protect against oxidative cell damage.

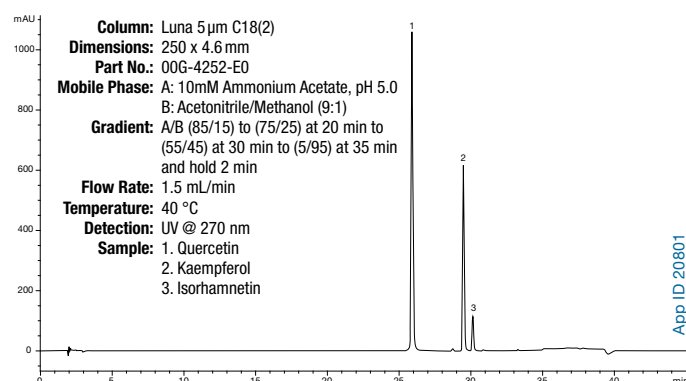
**Experimental**

HPLC analysis was performed using an Agilent® 1100 LC System (Agilent Technologies Inc., Palo Alto, CA, USA). The system was optimized in order to reduce dead volume and improve performance including increasing the UV scan rate, changing the injector needle seat, re-plumbing the system with red PEEKsil™ Tubing (SGE), and using a semi-micro flow cell. The fully porous Luna® 5µm C18(2) 250 x 4.6mm and the Kinetex® Core-Shell Technology 5µm C18 150 x 4.6mm were from Phenomenex, Torrance, CA. All chromatographic conditions are specified on their corresponding chromatograms in **Figures 1** through **5**.

The Ginkgo Biloba leaf extract reference material, Isorhamnetin, Quercetin, Kaempferol, and the analytical method on the Luna C18(2) HPLC column were provided by ChromaDex®. The formulated products were purchased from a local health food store.

Samples were hydrolyzed to remove the glycoside prior to analysis. A 2 g sample was weighed and placed in a 50 mL centrifuge tube and sonicated with 5 mL of methanol for 5 minutes. Samples were then diluted with 10 mL of water and 2.5 mL of HCl and vortexed for 2 minutes. Tubes were then placed in a boiling water bath for 30 minutes and allowed to react. After completion, samples were cooled to room temperature and transferred into a 25 mL volumetric flask. The centrifuge tube was rinsed with methanol and the flask diluted to volume with methanol. An aliquot of the solution was filtered through a Phenex™ 0.45µm PTFE syringe filter into an HPLC vial for analysis.

Figure 1.
Analytical Reference Standards Using Luna C18(2) 5 µm Column



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Figure 2.
Analytical Reference Standards using Kinetex® 5 µm Core-Shell Technology Column

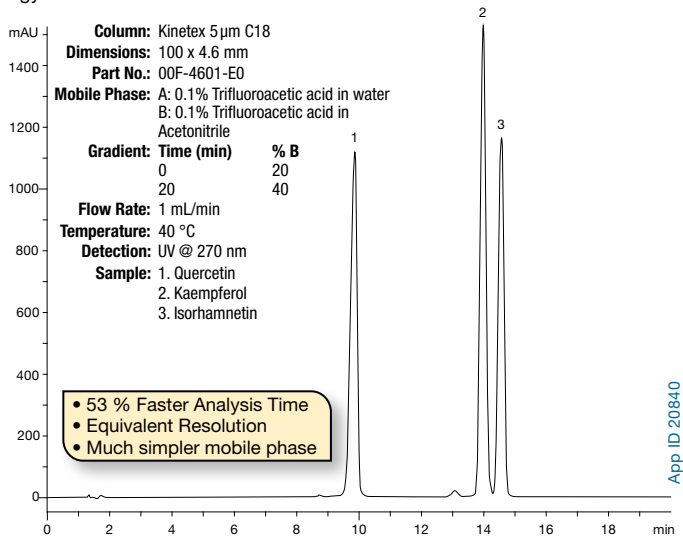


Table 1.
Calibration Curve from Kinetex 5 µm Core-Shell Technology Column Based on ChromaDex® Reference Materials

Compound	R ²	LOQ (µg/mL)	S/N
Quercetin	0.9988	0.4800	S/N = 10.2
Kaempferol	0.9986	0.4800	S/N = 17.05
Isorhamnetin	0.9987	0.4800	S/N = 13.3

Table 2.
Determination of Method Accuracy at 50 µg/mL

	Quercetin		Kaempferol		Isorhamnetin	
	T _R	Area	T _R	Area	T _R	Area
1	9.757	1172.7	13.834	1518.4	14.429	1162.9
2	9.763	1175.1	13.846	1520.2	14.443	1170.8
3	9.753	1173.1	13.838	1516.8	14.44	1167.9
4	9.762	1190.4	13.852	1544.4	14.453	1186.1
5	9.751	1172.5	13.832	1521.6	14.429	1169.1
Total	48.786	5883.8	69.202	7621.4	72.194	5856.8
Mean	9.7572	1176.76	13.8404	1524.28	14.4388	1171.36
STDEV	0.0053	7.6947	0.0084	11.3926	0.0102	8.7497
CV%	0.05	0.65	0.06	0.75	0.07	0.75

Accuracy based on five replicated injections of the 50 µg/mL standard

Figure 3.
Ginkgo Biloba Leaf Sample Using Kinetex 5 µm Core-Shell Technology Column

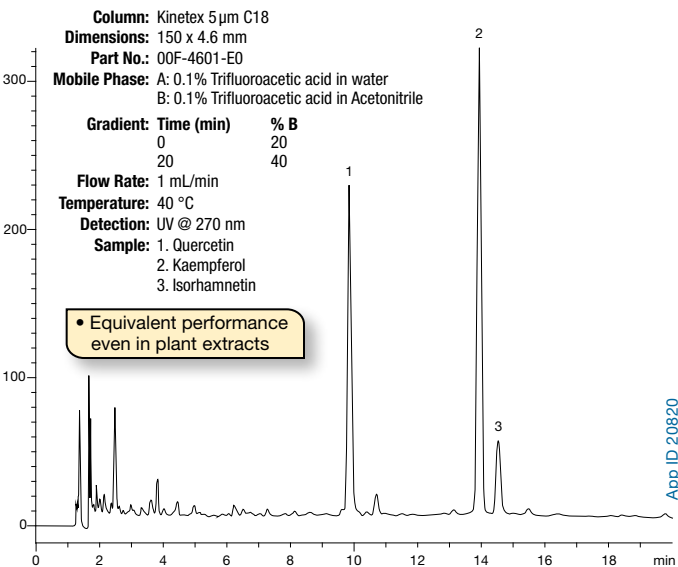


Figure 5.
Ginkgo Biloba Leaf Sample Using Kinetex 5 µm Core-Shell Technology Column

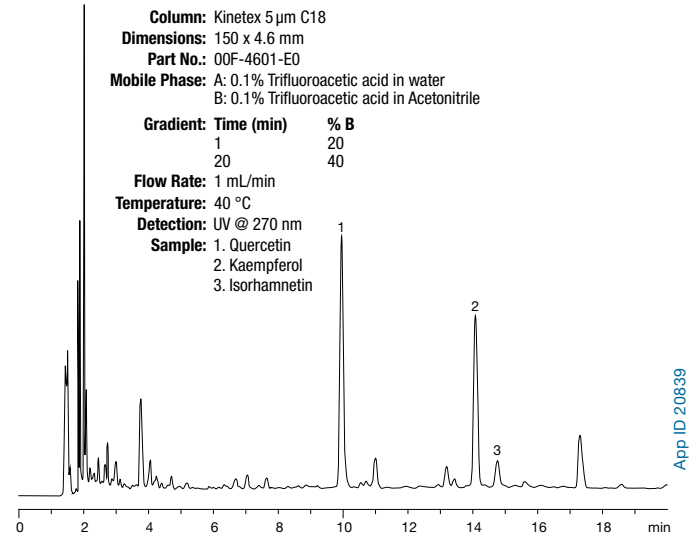
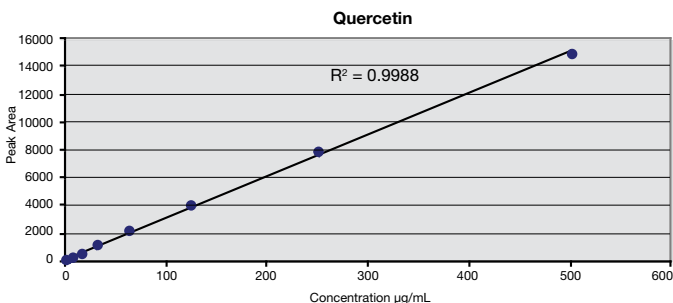


Figure 4.
Representative Calibration Curve for Quercetin from 0.244 to 500 µg/mL



Compound	Concentration (mg)
Quercetin	8.54
Kaempferol	5.36
Isorhamnetin	1.00
Total	14.91
Label Claim	>28.8



Equation 1:
Individual flavonol glycoside (% w/w) = (C)(FV)(D)(F)(100 %) / (W)

Where:

- C = Sample's ginkgoside concentration (mg/mL) from linear regression
- 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 to the glycoside
 - Quercetin: F = 756.7/302.2 = 2.504
 - Kaempferol: F = 740.7/286.2 = 2.588
 - Isorhamnetin: F = 770.6/316.2 = 2.437
- W = The sample weight (mg)

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Results and Discussion

The original method supplied by ChromaDex®, required more than 40 minutes to achieve complete separation for the Kaempferol and Isohamnetic isomers. This separation is based primarily on the efficiency of the column rather than the selectivity of the C18 stationary phase, making it difficult for traditional particle technologies to separate these compounds within a reasonable amount of time.

Kinetex® Core-Shell Technology columns allow scientists to achieve substantially higher chromatographic efficiencies at much lower pressures than the equivalent fully porous material, making it ideal for this separation. The Kinetex Core-Shell Technology enhances the performance of any existing HPLC platform, including UHPLC systems. For those labs that have older HPLC systems with pressure limitations, Kinetex 5 µm core-shell columns allow for a substantial improvement in chromatography.

The increased efficiency of the Kinetex Core-Shell Technology 5µm even in a 150 mm length allowed the method to be shortened by more than 50 %. The mobile phase system was also simplified to a standard water/ACN with 0.1 % TFA mobile phase system. Resolution and sensitivity were similar to the existing method (**Figures 1 and 2**).

When analyzing botanicals and nutraceuticals, the separation of standards can often be misleading since the plant extract can contain many other endogenous components that could lead to poor results. To demonstrate specificity of the new method, Ginkgo Biloba leaf reference materials were run using the new method and no coelutions were observed (**Figure 3**).

Having demonstrated that the new method provided equivalent results, we performed experiments to determine linearity, accuracy, range, and limit of quantitation (LOQ) using the Kinetex Core-Shell Technology column. Methods were shown to be linear over a range of 0.244 to 500 µg/mL (**Figure 4**). The LOQ was determined to be 0.480 µg/mL for all of the compounds (**Table 1**). This methodology produced a Signal to Noise ratio of 10.2 for Quercetin, which was the smallest peak of interest from the isolated standard. Accuracy and precision were determined for each compound at the 50 µg/mL level and found to be less than 1 % CV (**Table 2**).

The final experiment was to analyze a commercially available formulation and determine if the results we obtained were similar to label claims. To ensure that we properly tested our new assay, we attempted to choose difficult formulations such as those with multiple active components and those with gel cap pills.

The standard convention for Ginkgo flavonoid is to report them as glycosides. In this method, we hydrolyze the samples using acid to convert all compounds back to the parent form before analysis. This process improves retention of the three main components and simplifies the number of components that must be analyzed since each flavonoid has many possible glycosides species. To correct our values to match standard conventions, we used the equation and correction factors included in INA Method 102.000 (**Figure 5**).

Analysis of a formulated product obtained results that were slightly lower than label claim. The lower results could be explained in part due to the fact that our standard for Quercetin was corrected for water. Most standards for Quercetin are provided as a dehydrate. Since Quercetin is the most abundant flavonoid, using uncorrected standards can lead to high results that are biased (**Figure 5**).

Conclusion

Analysis time was reduced by over 50 % by adapting the current method to a Kinetex Core-Shell Technology column. We performed experiments to determine that the methodology was suitable for nutraceutical formulations. Final results indicate that this method is robust and ready for routine sample analysis.

Analysis of Ginkgo Biloba leaf reference materials provided results that were consistent with the supplied certificate of analysis. Analysis of formulated products resulted in values that were slightly lower than label claims. This might be explained in part due to the differences in the reference standards that were used. However, the label claims were quite hard to interpret, making it very difficult for a consumer to understand the value of the botanical they are taking. Providing standardized reference methods for analysis is the first step to ensuring quality in nutraceutical products.



Section 21CFR111.320 of cGMPs for Dietary Supplements requires you to "identify and use an appropriate scientifically valid method for each established specification for which testing or examination is required to determine whether the specification is met". The FDA does not elaborate on what is considered a scientifically valid method in the cGMPs. ChromaDex has defined scientifically valid as a method that meets minimum linearity, precision, sensitivity and range requirements. These requirements are outlined in an FDA laboratory document, ORA LABORATORY PROCEDURE Food and Drug Administration, ORA-LAB.5.4.5. This laboratory guidance document defines minimal performance attributes for selected methods of analysis and has been applied by ChromaDex to the selection of methods that are fit for purpose in the dietary supplements industry. According to the above definition, the method detailed in this document is considered scientifically valid as application to the cGMP requirements. Product specific, full method validations according to AOAC guidelines can be applied to customer samples upon request, to further document method performance in specific samples and matrices.

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ChromaDex Ordering Information
Phytochemical Reference Standards

Description	Quantity	Part No.
Isorhamnetin (P)	5 mg	ASB-00009520-005
Isorhamnetin (P)	10 mg	ASB-00009520-010
Isorhamnetin (P)	25 mg	ASB-00009520-025
Isorhamnetin (P)	100 mg	ASB-00009520-100
Kaempferol (AHP)	5 mg	ASB-00011020-005
Kaempferol (AHP)	10 mg	ASB-00011020-010
Kaempferol (AHP)	25 mg	ASB-00011020-025
Quercetin (P)	5 mg	ASB-00017030-005
Quercetin (P)	10 mg	ASB-00017030-010
Quercetin (P)	25 mg	ASB-00017030-025
Quercetin (P)	100 mg	ASB-00017030-100
Ginkgo Flavonoids Standards Kit	3x 10 mg	KIT-00007184-010
Ginkgo Flavonoids Standards Kit	3x 25 mg	KIT-00007184-025

Botanical Reference Materials

Description	Quantity	Part No.
Ginkgo (Ginkgo biloba) Leaf AHP-BRM	5 g	ASB-00030298-005
Ginkgo (Ginkgo biloba) Leaf XRM	5 g	ASB-00030299-005
Ginkgo (Ginkgo biloba) Leaf BRM	5 g	ASB-00030831-005
Ginkgo (Ginkgo biloba) Leaf RGBRM	5 g	ASB-00030996-005

Phenomenex Ordering Information
Kinetex® Core-Shell HPLC Columns

5 µm Columns (mm)	SecurityGuard™ ULTRA Cartridges*				SecurityGuard™ ULTRA Cartridges*		
	50 x 2.1	3/pk	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	3/pk
XB-C18	00B-4605-AN	AJO-8782	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJO-8768
C18	00B-4601-AN	AJO-8782	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJO-8768
PFP	00B-4602-AN	AJO-8787	00B-4602-E0	00D-4602-E0	00F-4602-E0	00G-4602-E0	AJO-8773
Phenyl-Hexyl	00B-4603-AN	AJO-8788	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJO-8774

* SecurityGuard ULTRA cartridges require holder, Part No. AJO-9000.
for 2.1 mm ID for 4.6 mm ID



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