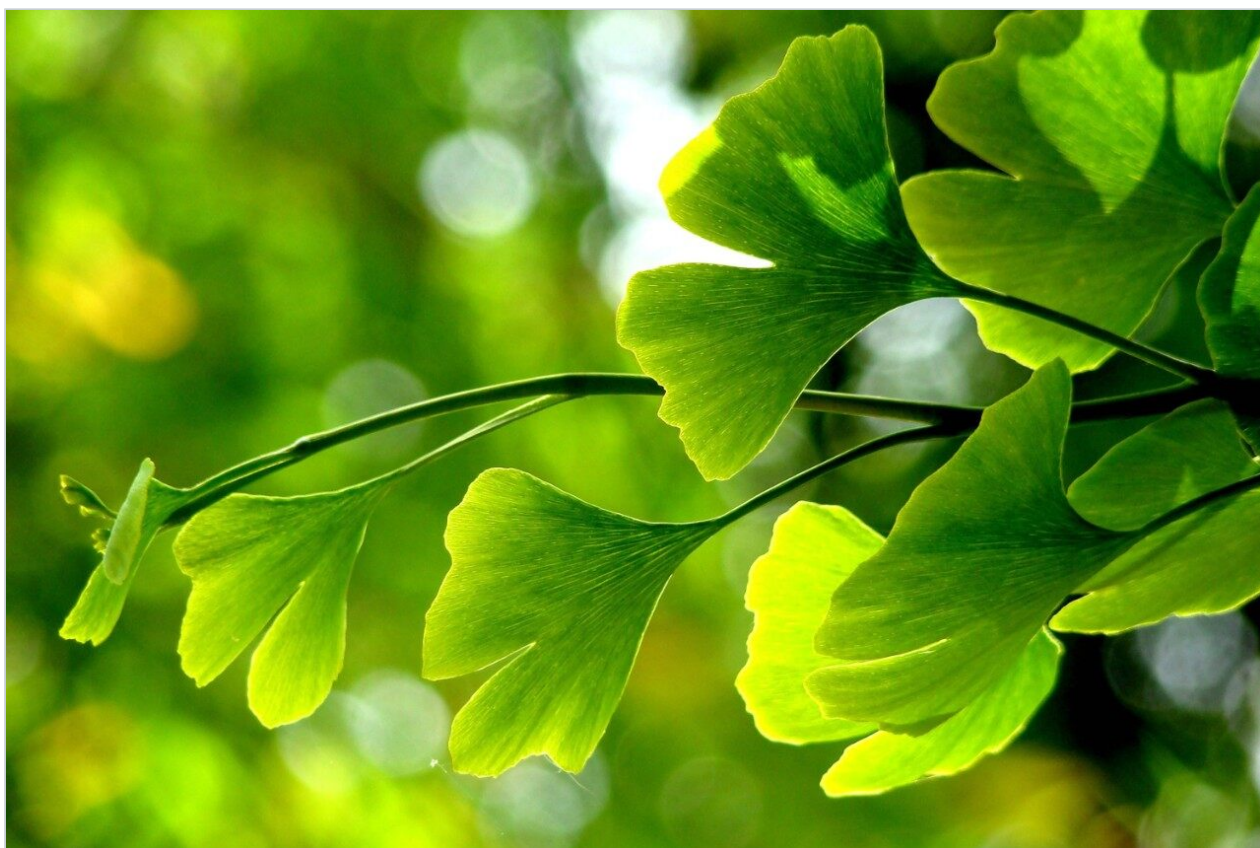


Nota applicativa

Isolation of Flavonoids from Ginkgo Biloba Leaf using the Waters Prep 150 LC System

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Abstract

This application note describes the isolation of the flavonoids from Ginkgo biloba leaf powder using a Waters Prep 150 LC System

Benefits

- A preparative HPLC system that provides high performance with intuitive, easy-to-use software
- Total software control of all system components with ChromScope Software

Introduction

Ginkgo biloba leaf has a long history of medicinal use for the treatment of numerous conditions, going back thousands of years.¹ Extracts of Ginkgo biloba leaves contain a wide variety of active compounds and are a particularly rich source of flavonoids, primarily quercetin, kaempferol, and isorhamnetin (Figure 1).

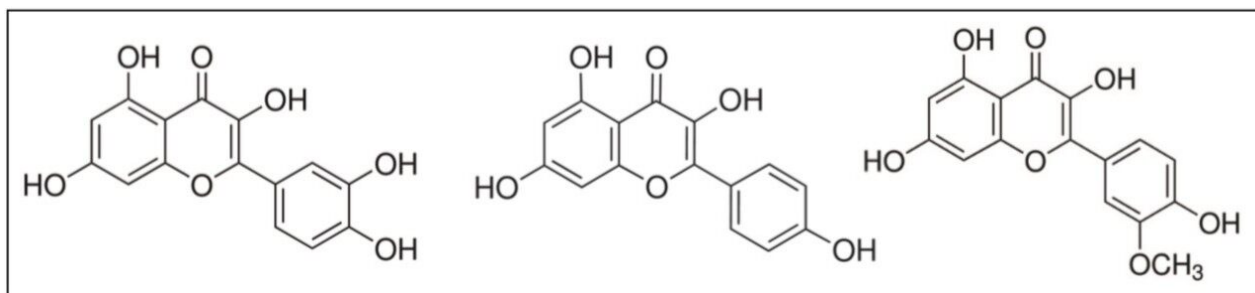


Figure 1. Chemical structures of quercetin, kaempferol, and isorhamnetin.

Often, the goal in natural product purification is to isolate individual component compounds that may have biological activity. Sufficient amounts of these compounds are isolated for a wide variety of purposes, such as preparation of standards, or for use in other studies (e.g. clinical trials, bioassays). Any isolated compound needs to be of as high purity as possible and needs to be obtained quickly and efficiently. Many techniques for extraction, isolation, and purification of natural products have been previously described.² Preparative reversed-phase high performance liquid chromatography (RP-HPLC) is a separation technique that is widely used in this kind of endeavor. It is considered to be a rapid, reliable, and robust technique that has wide applicability over many classes of compounds. This application note describes the isolation of the flavonoids from Ginkgo biloba leaf powder using a Waters Prep 150 LC System (Figure 2).



Figure 2. Waters Prep 150 LC System.

Experimental

Separations

Preparative chromatographic separations were carried out using the Prep 150 LC System (Figure 2) consisting of the following Waters components:

Pump:	2545 Binary Gradient Module
Detector:	2489 UV/Visible Detector with Semi-Prep TaperSlit Flow Cell
Injector:	Preparative Injector configured with a 5 mL loop
Collector:	Fraction Collector III
Software:	ChromScope Version 1.4

Analytical chromatographic separations (for method development and final purity checks) were carried out using an ACQUITY UPLC H-Class System equipped with an ACQUITY PDA Detector controlled with Empower 3 Software. Two initial analytical scale separations were developed (one gradient for purity checks and one isocratic for purification) with the conditions described below.

Analytical gradient conditions (for purity checks)

System:	ACQUITY UPLC H-Class
Column temp.:	50 °C
Flow rate:	0.50 mL/min
Mobile phase A:	0.2% Formic acid in water
Mobile phase B:	Acetonitrile
Gradient:	85:15 to 30:70 over 13 minutes, then to 5:95 13 to 15 minutes
Detection:	UV @ 371 nm
Column:	ACQUITY UPLC BEH C ₁₈ Column, 130Å, 1.7 µm, 2.1 mm x 100 mm

Analytical Isocratic Conditions (for prep scale up)

System:	ACQUITY UPLC H-Class
Column temp.:	Ambient
Flow rate:	1.4 mL/min
Mobile phase A:	0.2% Formic acid in water
Mobile phase B:	Acetonitrile
Composition:	73% A and 27% B
Detection:	UV @ 371 nm
Column:	XBridge BEH C ₁₈ Column, 130Å, 5 µm, 4.6 mm x 100 mm

The preparative separation was geometrically scaled from the analytical method to the 19 x 100 mm XBridge Column and is described below.

Preparative Isocratic Conditions

System:	Prep 150 LC
Column temp.:	Ambient
Flow rate:	24.8 mL/min
Mobile phase A:	0.2% Formic acid in water
Mobile phase B:	Acetonitrile
Composition:	73% A and 27% B

Injection vol.:	0.25 mL
Detection:	UV @ 371 nm
Column:	XBridge BEH C ₁₈ OBD Prep Column, 130Å, 5 µm, 19 mm x 100 mm

Extraction

Ginkgo biloba leaf powder (20 g) was added to 100 mL of methanol and sonicated for 60 minutes. Following the addition of 40 mL of 3N hydrochloric acid, the mixture was brought to a boil and allowed to reflux for 90 minutes. Using this reflux technique provided hydrolysis of the flavone glycosides to the respective flavonoids. After cooling to room temperature, this extract passed through a Whatman #1 filter paper and was used without further treatment.

Results and Discussion

Gradient UPLC analysis of the prepared extract indicated quercetin and kaempferol concentrations of 0.088 and 0.104 mg/mL, respectively (a quantitative standard of isorhamnetin was not available), and an approximate overall purity of 11%, 14%, and 5% for the 3 compounds by UV (371 nm) area percent (Figure 3). Using a previously developed analytical isocratic HPLC method (Figure 4), the flow rate for the preparative separation was scaled to a 19 mm I.D. column using the Analytical to Prep Gradient Calculator built into the ChromScope Software (Figure 5). The Analytical to Prep Gradient Calculator is an easy-to-use tool that aids in analytical-to-preparative scaling calculations providing both flow rate and gradient time calculation information. Proper scaling of analytical methods to prep allows for method development at an analytical scale, preserving sample and lowering solvent usage during method development. Scaled preparative chromatography will be very similar to the analytical chromatography, increasing confidence in the ability of the preparative method to collect peaks of interest. A previously published application note³ describes in greater detail these scaling calculations.

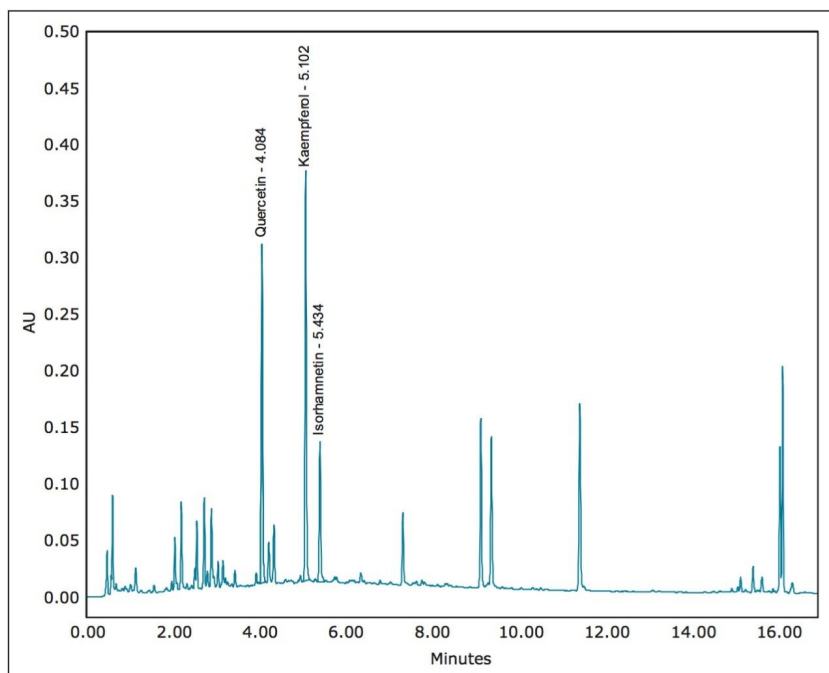


Figure 3. UPLC chromatogram of *Ginkgo biloba* leaf powder extract.

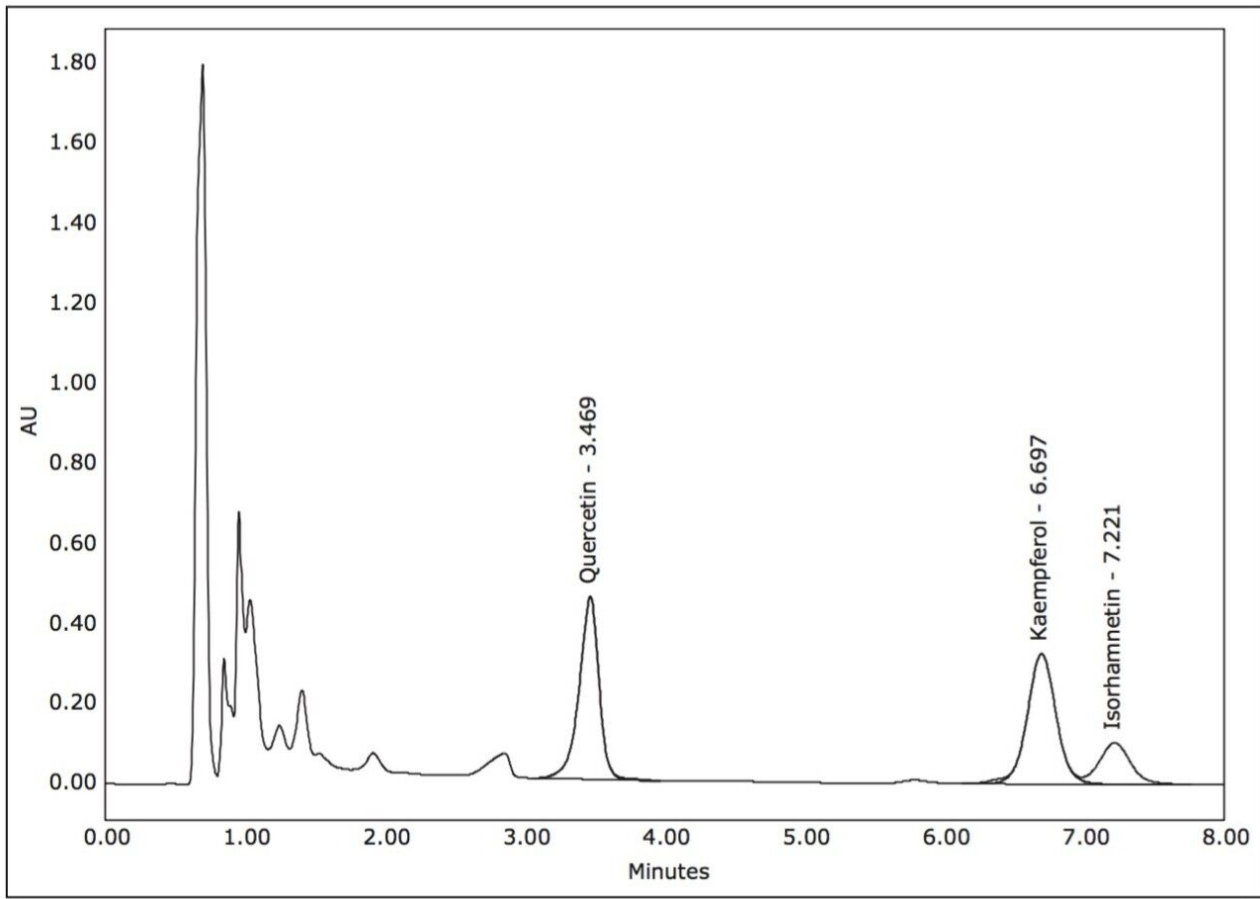


Figure 4. Analytical scale isocratic HPLC chromatogram of *Ginkgo biloba* leaf powder extract.

Analytical to Prep Gradient Calculator

Analytical or Scouting Column: 4.6 mm Diameter, 50 cm Length, 5 µm Particle Size, L/dp ratio 10

Preparative Column: 19 mm Diameter, 100 cm Length, 5 µm Particle Size, L/dp ratio 20

System Dwell volume (mL)

Analytical: 0.88 mL

Prep: 1 mL

[How to calculate Dwell volume](#)

Enter Analytical Gradient Conditions

	Time	Flow	%A	%B
	0.00	1.40	98	2
Hold step	0	1.40	98	2
	10.00	1.40	50	50
	11.00	1.40	98	2

Solvent used per gradient (mL): 12.0

Total solvent per gradient (mL): 17 mL

A: 12.0 mL

B: 5 mL

Constant Linear Velocity Gradients

Scaled Preparative Gradient (without dwell volumes)

	Time	Flow	%A	%B
	0.00	23.88	98	2
Hold step	0.00	23.88	98	2
	20.00	23.88	50	50
	22.00	23.88	98	2

Solvent used per gradient: 389 mL

Total solvent per gradient: 526 mL

A: 389 mL

B: 137 mL

Scaled Preparative Gradient (with Dwell volumes)

	Time	Flow	%A	%B
	0.00	23.88	98	2
Hold step	1.22	23.88	98	2
	21.22	23.88	50	50
	23.22	23.88	98	2

Solvent used per gradient: 418 mL

Total solvent per gradient: 556 mL

A: 418 mL

B: 138 mL

Tips:

- 1) If you input the dwell volumes of your systems the second table will become active and show an adjusted Gradient table that will more accurately align the retention times between analytical and Prep
- 2) To keep the same resolution between Analytical and Prep choose columns with the same L/dp ratio
- 3) To isolate selected peaks or to calculate a shallow focus gradient for a key gradient area use the separate UPLC to Prep Focus Gradient Calculator

Prep Flow Rate Override

If the scaled Prep flow rate is higher than you would like, enter the flow rate you would prefer and it will calculate a new gradient table

Figure 5. Waters ChromScope Analytical to Prep Gradient Calculator.

Using a 0.25 mL injection, acceptable preparative separations were achieved (Figure 6) with the isocratic purification separation method and threshold based fraction collection method. Fraction collection was easily set up through the Chromscope Software for threshold collection within windows (Figure 7). In this example, a pair of collection windows is defined (3.0 to 4.0 and 6.0 to 7.5 minutes), and any peaks that eluted outside of that window are not collected. To be collected, peaks eluting within the window must meet peak threshold criteria (in this case, 25 mAU for the first window, 15 mAU for the second window). Purity analysis of the collected fractions showed UV area% purity of 87, 96, and 85% for the three compounds, respectively, based on UPLC analysis (data not shown).

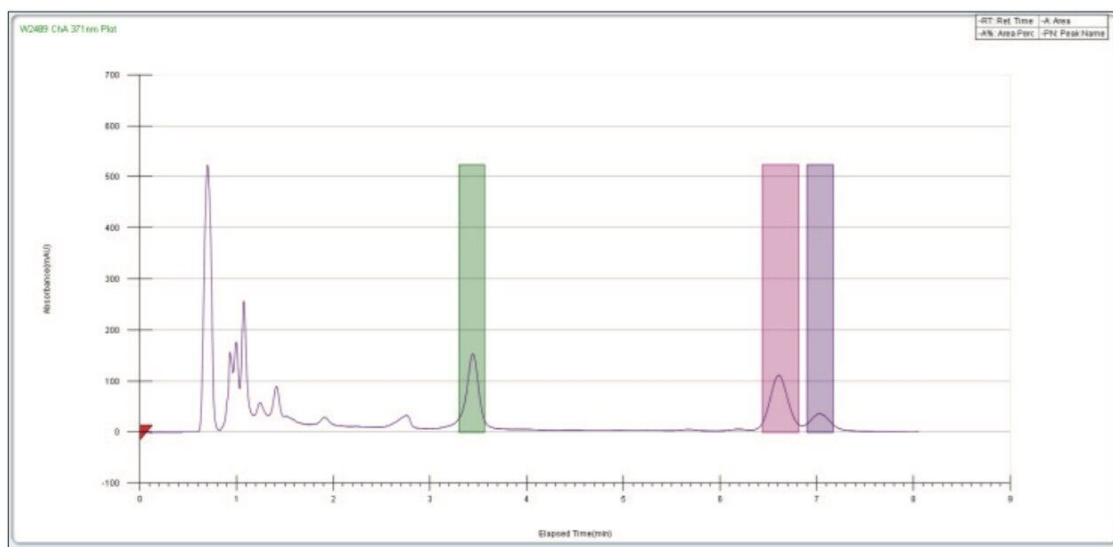


Figure 6. ChromScope Preparative HPLC chromatogram showing three collected peaks.

Fraction Collection Parameters										
Current selected window: 2										
	Window #	Single Fraction	Collection Type	Select Detector Signal	Threshold Start (mAU)	Threshold Stop (mAU)	Time Window Start (min.)	Time Window Stop (min.)	Collect waste within time window	Fraction is Inverted
	1	<input type="checkbox"/>	Signal Threshold	(w/2489) 2DChannel_1	25.0000	25.0000	3.000	4.000	<input type="checkbox"/>	<input type="checkbox"/>
▶	2	<input type="checkbox"/>	Signal Threshold	(w/2489) 2DChannel_1	15.0000	15.0000	6.000	7.500	<input type="checkbox"/>	<input type="checkbox"/>
*		<input type="checkbox"/>							<input type="checkbox"/>	<input type="checkbox"/>

Figure 7. ChromScope Fraction Collection Method.

Conclusion

- Using a Waters Prep 150 LC preparative HPLC system, material from an extract of Ginkgo biloba leaf powder was isolated and purified.
- Total system software control through Waters ChromScope software (Version 1.4) provided system flexibility by allowing easy modification of all method parameters.
- Separations were developed analytically and then geometrically scaled to a 19 x 100 mm preparative column on the Prep 150 LC System using the Analytical to Prep Gradient Calculator.

- Easy-to-set up fraction collection allowed for precise collection of target compounds.
- The initial extract contained 15% quercetin and 18% kaempferol. Each component was purified to greater than 87% on this easy-to-use system.

References

1. *PDR for Herbal Medicines*, 4th Ed., 2007, Thompson Healthcare Inc, Montvale NJ, U.S.A.
2. *Natural Products Isolation* 2nd edition, edited by S.D. Sarker, Z. Latif, A.I., 2006 Humana Press Inc, Totowa, NJ, U.S.A.
3. "Analytical HPLC to Preparative HPLC: Scale Up Techniques using a Natural Product Extract." Andrew Aubin and Ronan Cleary. Waters Application Note 720003120en, 2009

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Prep 150 LC System <<https://www.waters.com/134727002>>

ACQUITY UPLC H-Class PLUS System <<https://www.waters.com/10138533>>

2489 UV/Visible (UV/Vis) Detector <<https://www.waters.com/515198>>

ACQUITY UPLC PDA Detector <<https://www.waters.com/514225>>

ChromScope Software <<https://www.waters.com/134647658>>

Empower 3 Chromatography Data Software <<https://www.waters.com/10190669>>

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