Determination of Flavonoids in Ginkgo Biloba Using Bond Elut Plexa Solid Phase Extraction Sorbent for Cleanup and HPLC-DAD Analysis

Application Note

Dietary Supplement

Authors

Kediemetse Mothibedi, Janes Mokgadi and Nelson Torto
Department of Chemistry
Rhodes University
P. O. Box 94
Grahamstown 6140
South Africa

Abstract

Sample cleanup of quercetin, kaempferol and isorhamnetin in ginkgo biloba tablets was achieved using Agilent Bond Elut Plexa solid phase extraction sorbent. Analysis was carried out by an Agilent 1200 Series High Performance Liquid Chromatograph coupled to a diode array detector (HPLC-DAD). Good peak separation was achieved using an Agilent ZORBAX Eclipse Plus C18 column (4.6 × 75 mm, 3.5 µm) using a 0.5% phosphoric acid: methanol (40:60) mobile phase. Separation of the compounds was achieved within 4 minutes at a column temperature of 35 °C. The calibration curves exhibited linearity up to 120 µg/mL with correlation coefficients of 0.9989, 0.9992, and 0.9992 for quercetin, kaempferol and isorhamnetin, respectively. The recoveries ranged from 106–107%, 103–109%, and 73–88% for quercetin, kaempferol, and isorhamnetin, respectively, with a relative standard deviation of less than 5% for all analytes.

Agilent Technologies

Introduction

Ginkgo biloba is an ancient Chinese phytomedicine used to treat various ailments such as circulatory and demential disorders [1]. The major active ingredients of ginkgo biloba extracts are flavonoids; in particular aglycone derivatives such as quercetin, kaempferol and isorhamnetin (see structures in Figure 1). Flavonoids are a group of polyphenolic compounds that are present in most plants, exhibiting higher concentrations in seeds, fruit skin or peel, bark, and flowers. They are used mostly for their antioxidant behavior for scavenging free radicals [1] as they are known to exhibit vasodilatory, antithrombotic, antineoplastic, antiviral, antimutagenic, antiallergic, anti-inflammatory, and antibacterial activities [2]. Therefore, flavanoids are very popular as dietary supplements.

For qualitative and quantitative monitoring of the quality of dietary supplements available in the market, it is necessary to employ extraction methods that can cope with the complexity of the sample matrix. It is evident that traditional liquid–liquid or Soxhlet extraction does not fulfill most of the current sample preparation requirements, thus it has been displaced from analytical laboratories by sorbent-based techniques such as solid phase extraction (SPE) [3]. The demand for SPE material with improved analyte recoveries, sorptive capacity, and selectivity is increasing. In addition, the need for simplicity, automation, and increased throughput has stimulated interest towards the development of different SPE formats and configurations [4].

This application note presents the use of an Agilent Bond Elut Plexa solid phase extraction sorbent for the cleanup and preconcentration of flavonoids in ginkgo biloba. It has been designed for simplicity, improved analytical performance and ease-of-use [5].

Experimental

Materials and Chemicals

Quercetin, kaempferol and isorhamnetin reference standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany). Phosphoric acid, hydrochloric acid, and potassium hydroxide pellets were purchased from Merck Chemicals (Johannesburg, South Africa) while ammonia solution (25%) was purchased from Saarchem Analytic (Krugersdorp, South Africa). Solgar ginkgo biloba capsules and ginkgo biloba leaves were purchased from a local herb store (Grahamstown, South Africa). Water was purified using Milli-Q system (Millipore, Bedford, MA, USA). The pH meter used was Jenway 3510 (Bibby Scientific Ltd., Dunmow, Essex, UK). Solid phase extraction cartridges used were Agilent Bond Elut Plexa (1 mL/30 mg, p/n 12109301). Analysis was carried out on an Agilent 1200 Series High Performance LC system with an Agilent 1200 Series Diode Array Detector. The analytical column was an Agilent ZORBAX Eclipse Plus C18 (4.6 × 75 mm, 3.5 µm, p/n 959933-902)

Preparation of Stock and Working Standard Solutions

The stock solution of quercetin, kaempferol, and isorhamnetin, 1,000 µg/mL each, was prepared in methanol and stored at 4 °C. All standard solutions were prepared from the stock solution as required.

Optimization of Parameters

Separation of Peaks

A 5-µL amount of a standard mixture of 50 µg/mL of quercetin and kaempferol and 100 µg/mL of isorhamnetin was injected into the HPLC and the conditions optimized for their separation. The HPLC conditions used are outlined in Table 1 (see Figure 3).

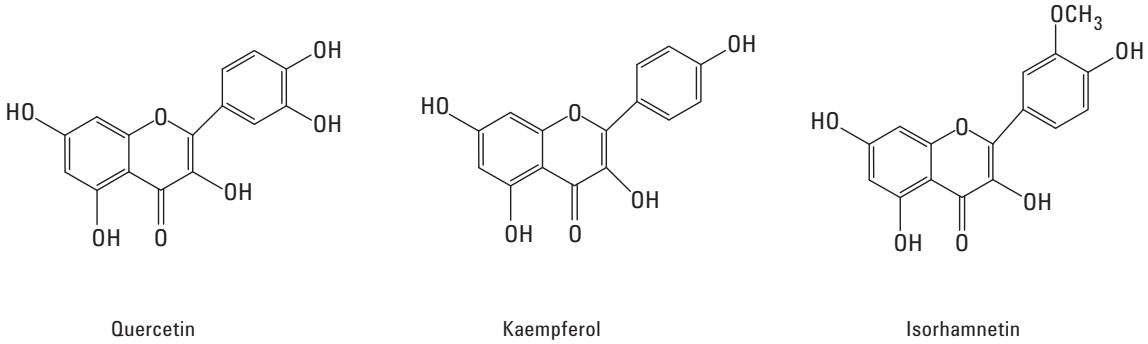

Figure 1. Chemical structures of quercetin, isorhmnetin, and kaempferol.

Table 1. HPLC Conditions

Parameter	Condition
Column	Agilent ZORBAX Eclipse Plus C18 4.6 mm x 75 mm, 3.5 µm (p/n 959933-902)
Flow rate	1 mL/min
Injection volume	5 µL
Column temperature	35 °C
Mobile phase	A: 0.5% phosphoric acid B: methanol
Run time	4 min
Isocratic	40% A:60% B
Detection	UV, 370 nm

Sample Preparation

The contents of ginkgo biloba capsules were first homogenized. The 2 g of the homogenized capsule contents were refluxed with 40 mL of methanol and 40 mL of 5.5% HCl (v/v) while continuously stirring for 1 hour. The mixture was left to cool to room temperature and then filtered using a Whatman filter paper (125 mm diameter). The filtrate was diluted to 1:3 (v/v) with 2% ammonia solution. The pH was adjusted to approximately 7 with 1 M KOH. A 1-mL aliquot was filtered using a 0.45-µm membrane filter and analyzed with HPLC-DAD (see Figure 4). Ginkgo biloba leaves were ground and homogenized using a mortar and a pestle. A 1-g amount was weighed and the same extraction procedure for ginkgo biloba capsules was followed for ginkgo biloba leaves.

Sample Cleanup

SPE Conditions

Conditioning, loading, washing and elution steps of the SPE procedure were studied systematically to obtain optimum SPE conditions as outlined in Figure 2.

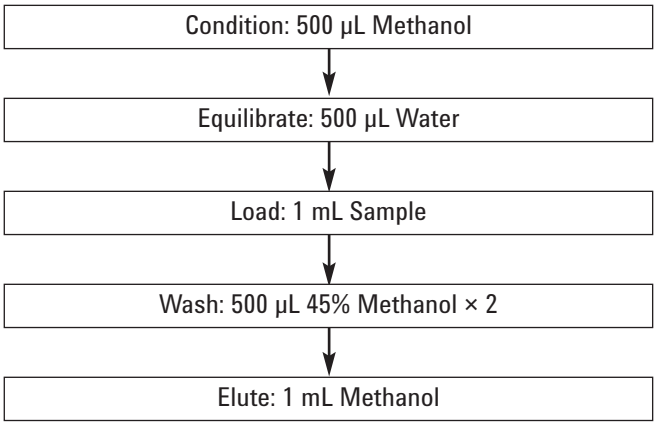

Figure 2. SPE procedure for cleaning flavonoids using Agilent Bond Elut Plexa Sorbent.

Results and Discussion

Separation

Good separation of peaks for a standard mixture of quercetin, kaempferol, and isorhamnetin was achieved using the HPLC conditions outlined in Table 1 (see Figure 3).

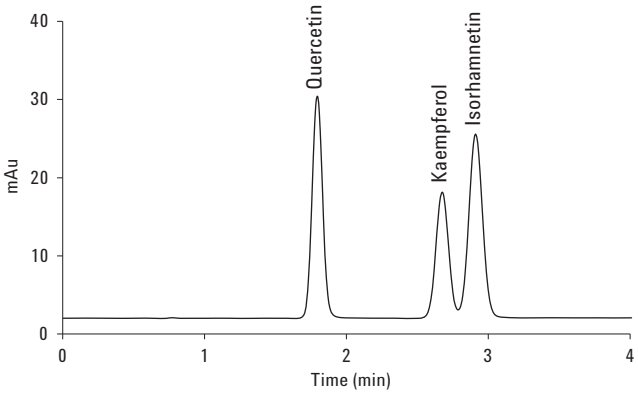

Figure 3. Chromatogram of quercetin, kaempferol, and isohamnetin standard mixture.

Sample Cleanup

Extracts from ginkgo biloba capsules were filtered using a 25 mm diameter, 0.45 µm porosity PVDF syringe filter and were injected into the HPLC before and after the SPE procedure. An overlay of chromatograms for the two extracts is shown in Figure 4. The chromatogram for the extract without SPE shows some interfering peaks while they were significantly removed after SPE indicating a successful cleanup by the Bond Elut Plexa SPE sorbents.

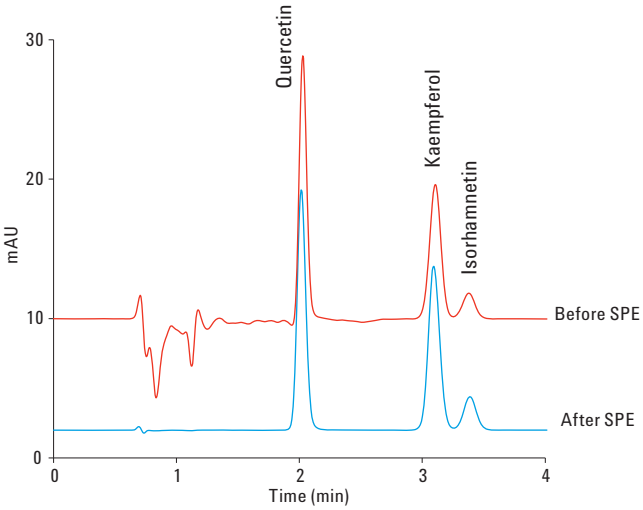

Figure 4. Chromatogram of ginkgo biloba sample before and after SPE.

Recovery and Reproducibility

Six replicates of the extracts from ginkgo biloba capsules were spiked at three different concentration levels for each analyte. The spiked samples were taken through the whole SPE cleanup procedure. The actual concentration of the spiked sample was determined by subtracting the spiked concentration of the sample from the obtained concentration of the spiked sample. The percentage recovery was calculated by comparing the actual concentration with the original spiking concentration. The relative standard deviation (RSD) was calculated for all the spiking levels. The results for recoveries and RSDs are summarized in Table. 2.

Table 2. Recovery and Reproducibility Data of Quercetin, Kaempferol, and Isorhamnetin from Spiked Samples

Analyte	Spiking level (µg/mL) n=6	% Recovery	% RSD
Quercetin	10	107	4.35
	20	106	3.35
Kaempferol	10	109	2.53
	20	103	1.14
	40	108	4.39
Isorhamnetin	10	88	4.11
	20	73	4.50
	40	79	1.73

Calibration Curves

Data for calibration curves were obtained by preparing different concentration levels of a standard mixture containing all the three compounds with concentration ranges up to 120 µg/mL. Each concentration level was run in triplicates and the resultant calibration curves were found to be linear over the concentration range with correlation coefficients of 0.9989, 0.9992, and 0.9992 for quercetin, kaempferol, and isorhamnetin, respectively (see Figure 5).

Linearity of SPE Method

The linearity range of the Bond Elut Plexa SPE sorbent was studied by spiking the ginkgo biloba capsules with increasing concentrations of the standard mixture followed by SPE cleanup. Linearity was no longer observed for quercetin and kaempferol at concentrations above 400 µg/mL because the sorbent was overloaded with analytes. Therefore some of it was lost during the loading and washing steps. However, linearity for isorhamnetin was still observed up to 500 µg/mL (see Figure 6).

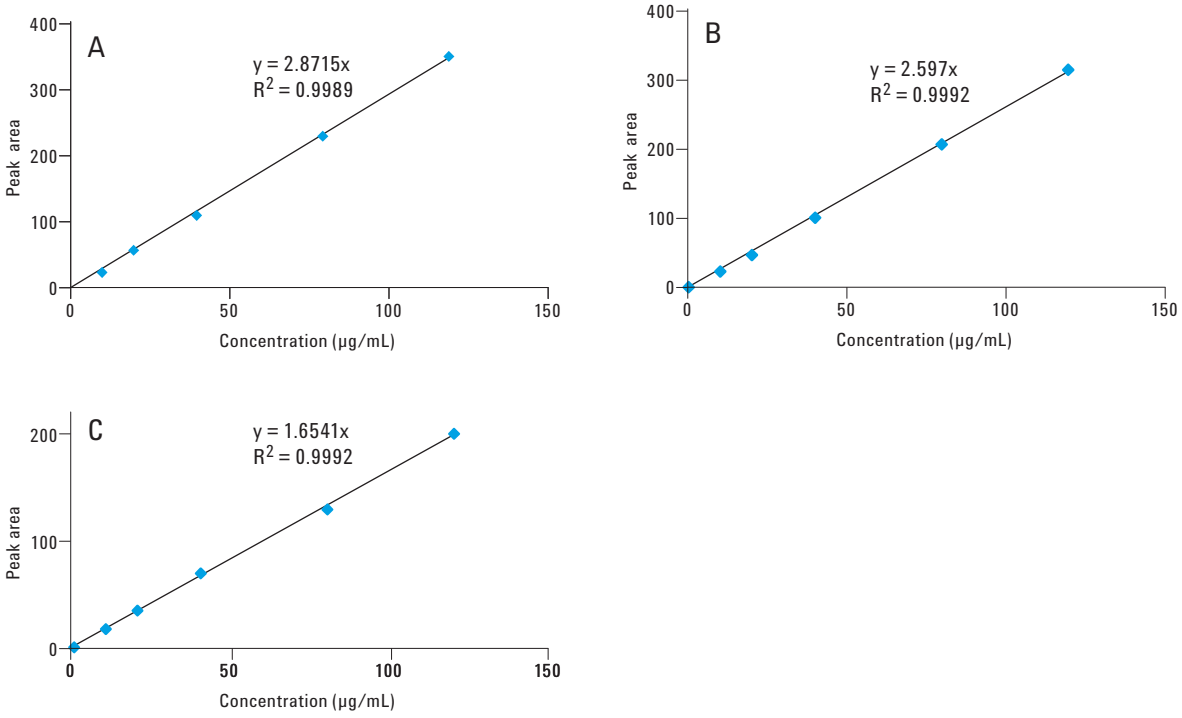

Figure 5. Calibration curves of [A] quercetin, [B] kaempferol, and [C] isorhamnetin.

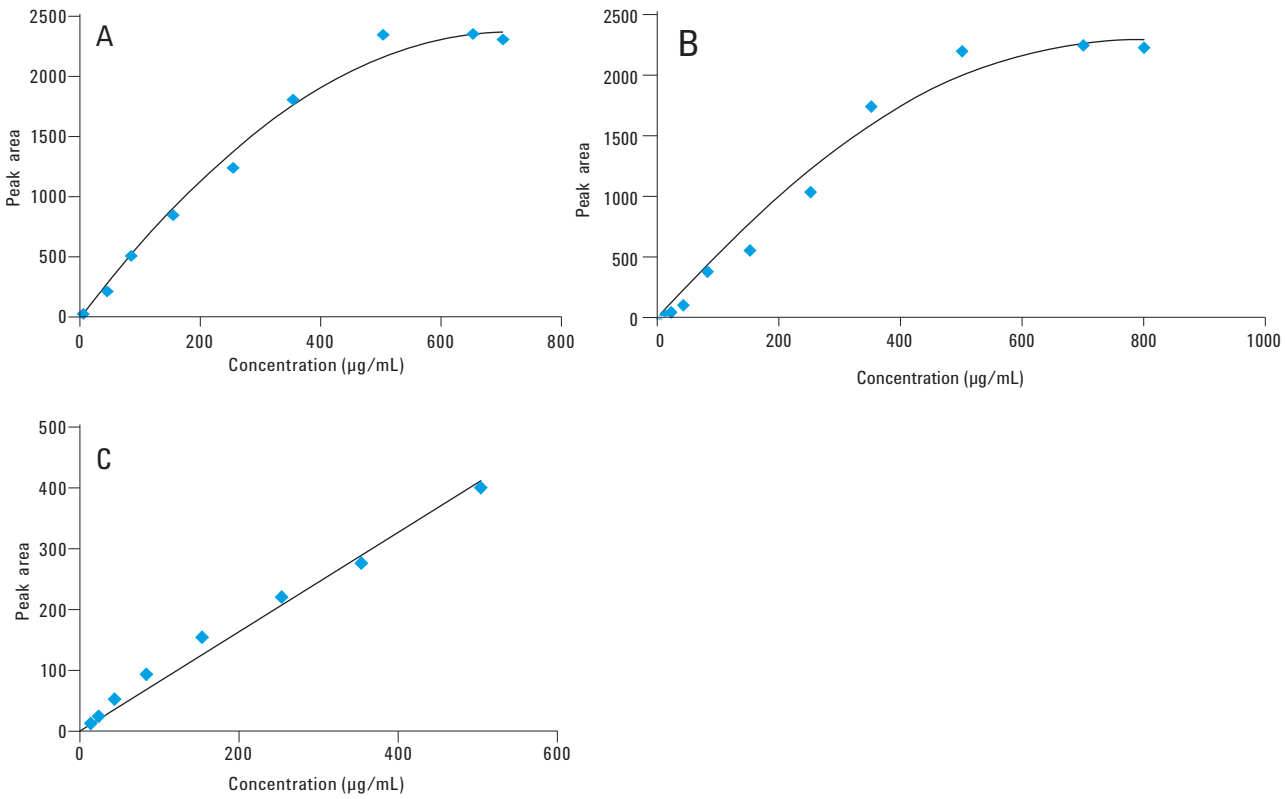

Figure 6. Linearity range of Bond Elut Plexa SPE for (A) quercetin, (B) kaempferol and (C) isorhamnetin.

Limit of Detection and Limit of Quantification

The limits of detection (LOD) were calculated with the intercept, y_B, and the standard error of the regression line, S_B at 3 times standard error using Equations 1 and 2.

$$y_{LOD} = y_B + 3S_B \quad \text{Equation 1}$$

$$LOD = (y_{LOD} - y_B)/m \text{ where m = gradient slope} \quad \text{Equation 2}$$

Limit of quantification (LOQ) values were calculated using the same method as in Equations 1 and 2, but using 10 times the standard error of regression line, (Equations 3 and 4).

$$y_{LOQ} = y_B + 10S_B \quad \text{Equation 3}$$

$$LOQ = (y_{LOQ} - y_B)/m \quad \text{Equation 4}$$

The LOD and LOQ for quercetin, kaempferol, and isorhamnetin are summarized in Table 3.

Table 3. LOD and LOQ for Quercetin, Kaempferol, and Isorhamnetin

	LOD (µg/mL)	LOQ (µg/mL)
Quercetin	1.47	4.67
Kaempferol	0.80	2.65
Isorhamnetin	3.25	10.8

Application to Real Samples

Ginkgo biloba tablets were analyzed using the developed method and the concentrations were found to be 14.54 µg/mL, 20.20 µg/mL and 3.09 µg/mL for quercetin, kaempferol, and isorhamnetin, respectively. The concentrations for flavonoids ginkgo biloba leaves were below the LOQ, therefore could not be measured.

Conclusion

Agilent Bond Elut Plexa solid phase extraction sorbent was found to be effective in cleanup of flavonoids in ginkgo biloba. A fast, accurate and reproducible method was developed employing the sorbent. Good recoveries were obtained ranging from 106–107%, 103–09%, and 73–88% for quercetin, kaempferol and isorhamnetin, respectively, and a relative standard deviation of less than 5% was achieved. The LOD for quercetin, kaempferol, and isorhamnetin were found to be 1.47 µg/mL, 0.80 µg/mL, and 3.25 µg/mL respectively while the LOQ were found to be 4.67 µg/mL, 2.65 µg/mL, and 10.8 µg/mL, respectively. Ginkgo biloba tablets contained concentrations of 14.54 µg/mL, 20.20 µg/mL, and 3.09 µg/mL for quercetin, kaempferol, and isorhamnetin, respectively. The flavanoid levels in whole ginkgo biloba were below the LOQ and, therefore, couldn't be measured.

References

1. M.-J. Dubber, V. Sewram, N. Mshicileli, G. S. Shephard, and I. Kanfer, *J of Pharm and Biomed Ana*. 37 (2005) 723-731.
2. P. Chan, Q. Xia and P. P. Fu, *J of Environmental Science and Health Part C*, 25 (2007) 211-244.
3. J. Mokgadi, L. C. Mmualefe and N. Torto, "Agilent SampliQ OPT solid phase extraction sorbent in the cleanup of Flavonoids in ginkgo biloba by HPLC-DAD," Agilent Technologies. Santa Clara, CA, United States of America, Publication No. 5990-4799EN, October 21, 2009.
4. http://www.chem.agilent.com/Library/brochures/5989-9334EN.pdf
5. http://www.chem.agilent.com/enUS/Products/columns-supplies/samplepreparation/spe/bondelutplexa/pages/default.aspx

For More Information

These data represent typical results. For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
December 16, 2011
5990-9547EN

Agilent Technologies