SampliQ OPT Solid Phase Extraction Sorbent in the Cleanup of Flavonoids in Ginkgo Biloba by HPLC-DAD

Application Note

Traditional Chinese Medicine

Authors

Janes Mokgadi, Lesego C. Mmualefe, Nelson Torto
Department of Chemistry
Rhodes University
P. O. Box 94, Grahamstown 6140, South Africa

Abstract

Sample cleanup of flavonoids (quercetin, kaempferol and isorhamnetin) from *Ginkgo biloba* tablets was achieved by solid phase extraction employing Agilent's SampliQ Optimized Polymer Technology (OPT) sorbents. Separation of the flavonoids with a 0.5% phosphoric acid/methanol mobile phase was carried out on an Agilent 1200 Series HPLC coupled with a diode array detector (DAD). The HPLC used an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm) under isocratic conditions and a 9-min total run time. The recoveries for quercetin ranged from 80 to 92% (n=6), kaempferol 83% to 94% (n=6) while those of isorhamnetin ranged from 75% to 102% (n=6), each with %RSD of less than 5. The limits of detection and quantification were 0.57 and 1.91 µg/mL for quercetin, 0.76 and 2.15 µg/mL for kaempferol and 0.88 and 2.55 µg/mL for isorhamnetin, respectively.

Agilent Technologies

Introduction

Ginkgo biloba is a dioecious tree whose seeds have been used historically in Traditional Chinese Medicines (TCMs) against coughs, asthma and skin infections [1]. Recently, leaf extracts have been widely used as a phytomedicine in Europe and China and as a dietary supplement in the United States [2]. Notable examples of commercially available extracts include EGB761 (France), Tebonin (Germany), Tanakan (Japan) and TruNature (USA) [3]. The major active constituents of *G. biloba* extracts are flavonoids, which include three main aglycone derivatives (quercetin, kaempferol and isorhamnetin shown in Figure 1) and six bioflavonones [3]. Flavonoids are a large group of naturally occurring polyphenols with a large range of pharmacological activities [4]. There is evidence that a flavonoid-rich diet inversely correlates with the risk of coronary heart disease and that the antioxidant properties of some flavonoids can protect against certain types of cancer [5].

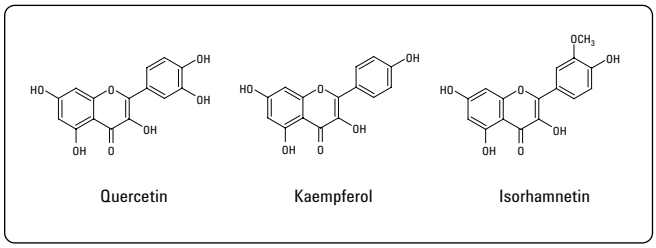

Figure1. Structures of quercetin, kaempferol and isorhamnetin.

Often, the active constituents of a pharmaceutical drug or an extract are present in minute quantities, and separation from the bulk requires HPLC columns which offer high-resolution capabilities. Prior to that, there is a growing need for robust, sensitive analytical methods involving sample handling (sampling, cleanup and preconcentration).

Sample handling is considered to be a fundamental step in the analytical procedure because it helps to achieve the low detection limits set by regulatory authorities [6]. Solid-phase extraction (SPE) is one of the most popular sample cleanup techniques used in sample handling prior to the analysis of environmental, food, pharmaceutical, and biological samples by high performance liquid chromatography (HPLC) or gas chromatography (GC). SPE has many advantages over traditional liquid-liquid extraction, such as the use of minimal amounts of organic solvent, ease of automation, lower cost, and reduced volumes of toxic residues [7]. In recent years, many reports have described the development of new SPE materials, for example mixed-mode sorbents as well as restricted access sorbents [8], immunoaffinity extraction sorbents [9], molecularly imprinted polymers, and conductive polymers [10].

This application note presents a method that has been optimized for SPE of quercetin, kaempferol and isorhamnetin in *Ginkgo biloba* employing Agilent SampliQ Optimized Polymer Technology (OPT) cartridges. The method uses polymeric sorbents, and results in a significant reduction of matrix interferences, and improved analysis.

Experimental

Materials and Chemicals

Quercetin, kaempferol and isorhamnetin standards were purchased from Sigma-Aldrich (Saint Louis MO, USA). Hydrochloric acid (HCl), phosphoric acid (H_3PO_4), ammonium acetate (NH_4COOCH_3) and potassium hydroxide (KOH) were purchased from Merck Chemicals (Gauteng, South Africa) while HPLC grade methanol (CH_3OH) was purchased from Merck KGaA (Darmstadt, Germany). *Ginkgo biloba* tablets manufactured by A. Vogel (Irvine, N. Ayrshire, Scotland, UK), were purchased from a local herbal store (Grahamstown, South Africa). SPE cartridges were Agilent SampliQ OPT, 30-mg tubes, p/n 5982-3013, obtained from Agilent Technologies (Santa Clara, CA, USA). Analysis was performed on an Agilent 1200 Series HPLC coupled with a diode array detector (DAD). The analytical column was an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm × 75 mm, 3.5 µm, p/n 959933-902). Water was purified using the Milli-Q system (Millipore, Billerica, MA, USA) and a Jenway 3510 pH meter manufactured by Bibby Scientific Ltd (Dunmow, Essex, UK) was used to adjust the pH values.

Preparation of stock and working solutions

The stock solutions of quercetin, kaempferol and isorhamnetin (1000 µg/mL each), were freshly prepared in methanol and stored at 4 °C when not used. All other standard solutions were prepared from the stock solutions as required.

Separation

A 5-uL aliquot of quercetin, kaempferol and isorhamnetin mixed standard solution (40 µg/mL each) was injected into the HPLC column to optimize their separation. The HPLC conditions are as outlined in Table 1.

Table 1. HPLC Conditions

Column	Agilent ZORBAX Eclipse Plus C18, column, 4.6 mm × 75 mm, 3.5 µm
Flow rate	1 mL/min
Injection volume	3 µL
Column temperature	35 °C
Mobile Phase	A: 0.5% Phosphoric acid, pH 2.7 B: Methanol
Run time	6 min
Post time	3 min
Isocratic	40% A 60% B
Detection	370 nm

Sample Preparation

The tablets were ground and homogenized using a mortar and a pestle. A 0.5-g amount of ground tablets was weighed into a boiling flask and then 40 mL of methanol was added. A 40-mL amount of 1.5 M HCl solution was added to the mixture, refluxed for 2 h and then left to cool at room temperature. The solution was transferred into a 100-mL volumetric flask and filled to the mark with methanol. The extracts were then filtered using a hydrophobic PVDF 0.45-µm Millipore Millex - HV membrane filter (Billerica, MA, USA). The methanolic extracts were diluted 1:9 with water and the pH adjusted to approximately 7 with 1 M potassium hydroxide solution.

SPE Procedure

A systematic study of a series of conditioning, loading, washing and elution solvents was performed. The SPE procedure was optimized by evaluating the isolation of quercetin, kaempferol and isorhamnetin from a mixed standard solution. Figure 2 shows a schematic outline of the optimized SPE procedure.

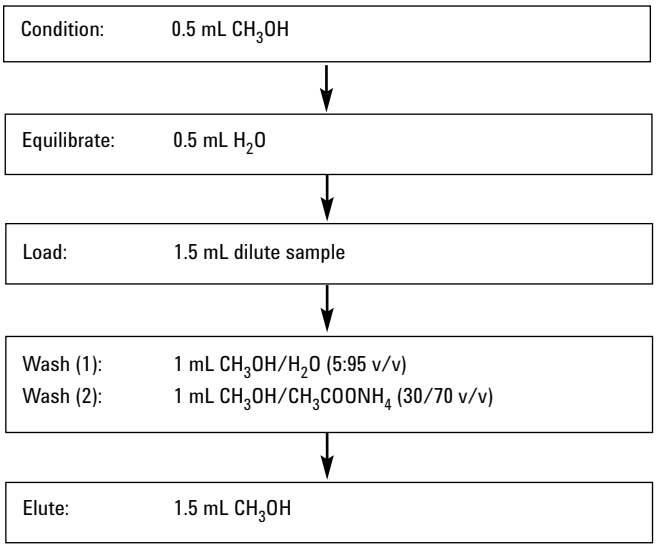

Figure 2. SPE procedure for the cleanup of flavonoids.

Results and Discussion

Separation and Cleanup

The chromatogram of a mixture of quercetin, kaempferol and isorhamnetin (Figure 3), shows a good separation for the compounds, using the HPLC conditions outlined in Table 1. The peaks are all symmetrical and the separation was obtained in less than 4 min. No interfering peaks appeared at the peak positions of quercetin, kaempferol and isorhamnetin in the standard sample.

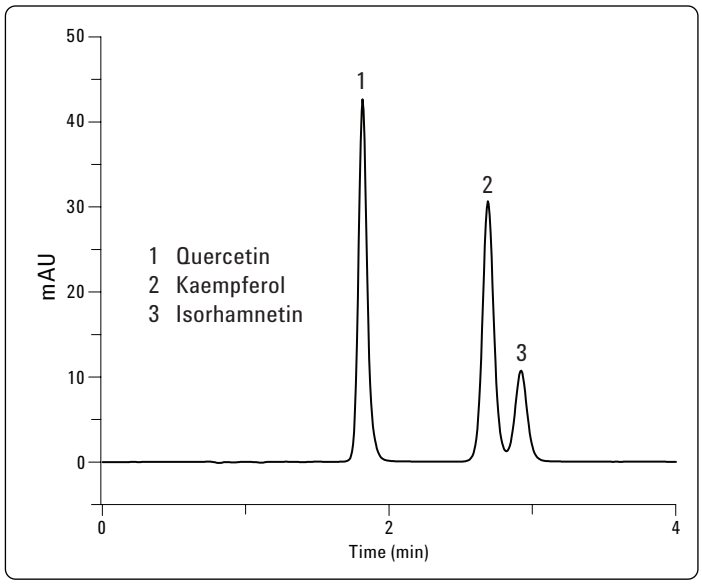

Figure 3. Chromatogram of a standard mixture containing quercetin, kaempferol and isorhamnetin (40 µg/mL each).

A filtered extract from A. Vogel *Ginkgo biloba* tablets was injected into the HPLC before (Figure 4a) and after (Figure 4b) sample cleanup using the SPE procedure outlined in Figure 2. There was a significant decrease in the number of small peaks in Figure 4b, indicating that the SPE cleanup removed potentially interfering peaks.

A commercial *Ginkgo biloba* sample was also spiked with a mixed standard of quercetin, kaempferol and isorhamnetin and injected into the column before (Figure 5a) and after (Figure 5b) sample cleanup. Again, interfering peaks were removed by the cleanup and the peak intensities of the flavonoids were also increased.

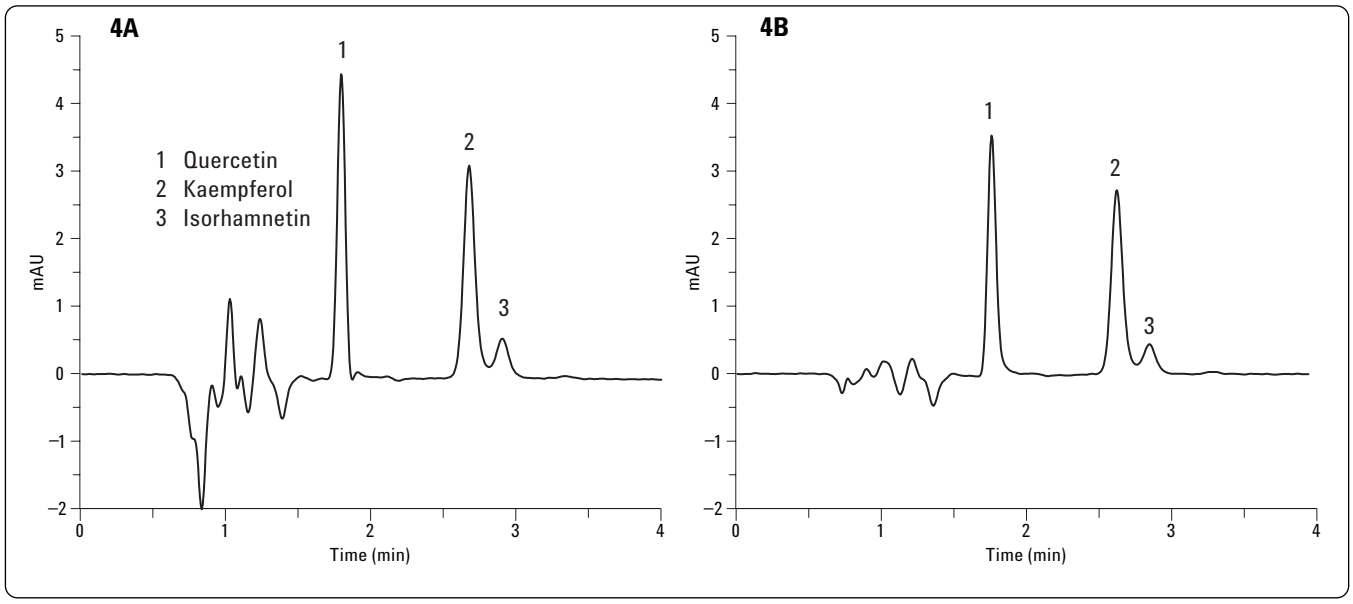

Figure 4. Chromatograms of Ginkgo biloba samples before and after cleanup with SampliQ OPT sorbent.

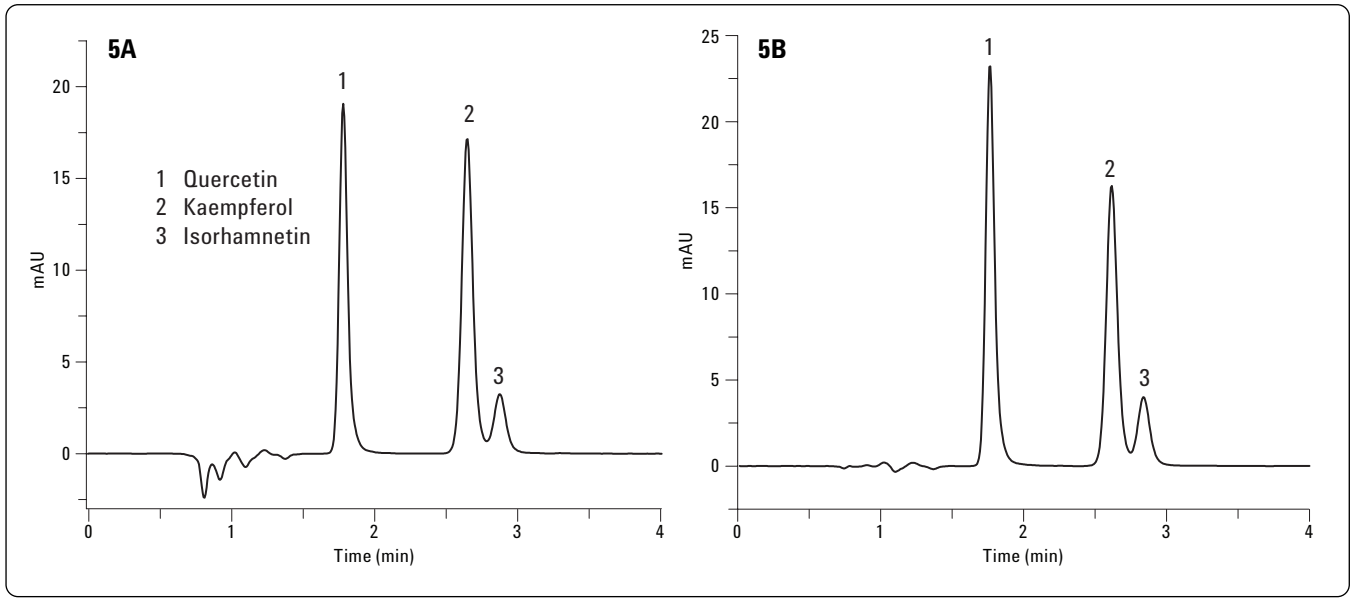

Figure 5. Chromatograms of Ginkgo biloba sample spiked with quercetin, kaempferol and isorhamnetin (40 µg/mL each) before and after cleanup with SampliQ OPT sorbent.

Recovery and Reproducibility

The reproducibility and recovery of the method was evaluated by analyzing six replicates of the commercial product (*Ginkgo biloba* tablets) that were spiked at three concentration levels in a day. Prior to spiking, the background levels of the flavonoids in the tablet were determined so as to calculate the actual recoveries. The interday percentages (%RSD) were all less than 5%. Isorhamnetin showed the highest %RSD, which could be attributed to previously documented lower polarity and solubility [11]. The recovery and reproducibility values for quercetin, kaempferol and isorhamnetin are as outlined in Table 2.

Table 2. Recovery and Reproducibility Data for Quercetin, Kaempferol and Isorhamnetin

Compound	Spiking level (µg/mL) n=6	% Recovery	% RSD
Quercetin	5	92	0.53
	40	90	0.21
	100	80	0.52
Kaempferol	5	94	0.76
	40	90	0.39
	100	83	0.48
Isorhamnetin	10	102	3.64
	60	75	1.18
	100	84	0.80

Calibration Curves

The calibration curves were evaluated by preparing appropriate concentrations in methanol from quercetin, kaempferol and isorhamnetin stock solutions and injecting directly into the HPLC column without SPE procedure. The method was found to be linear in the concentration ranges of 0–120 µg/mL for quercetin, 0–120 µg/mL for kaempferol and 0–150 µg/mL for isorhamnetin with r^2 values of 0.9998, 0.9991 and 0.9995 respectively (Figure 6).

Linearity of the SPE Method

Linearity was studied on the SampliQ OPT sorbent by spiking sample extracts with increasing concentrations of the flavonoids followed by SPE cleanup. At concentrations higher than 400 µg/mL linearity was no longer observed for quercetin and kaempferol (Figure 7). This is because the SPE sorbent could no longer retain the flavonoids due to overload. The analytes were lost during the loading and the washing steps of the SPE procedure. For isorhamnetin, linearity was observed at higher concentrations up to 500 µg/mL.

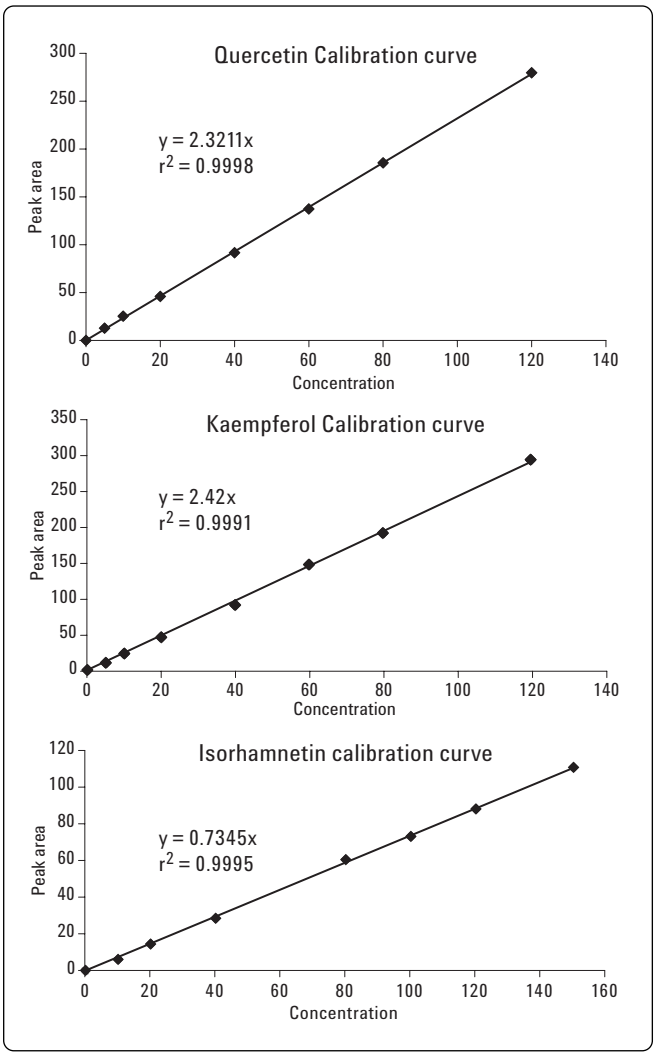

Figure 6. Calibration curves of quercetin, kaempferol and isorhamnetin.

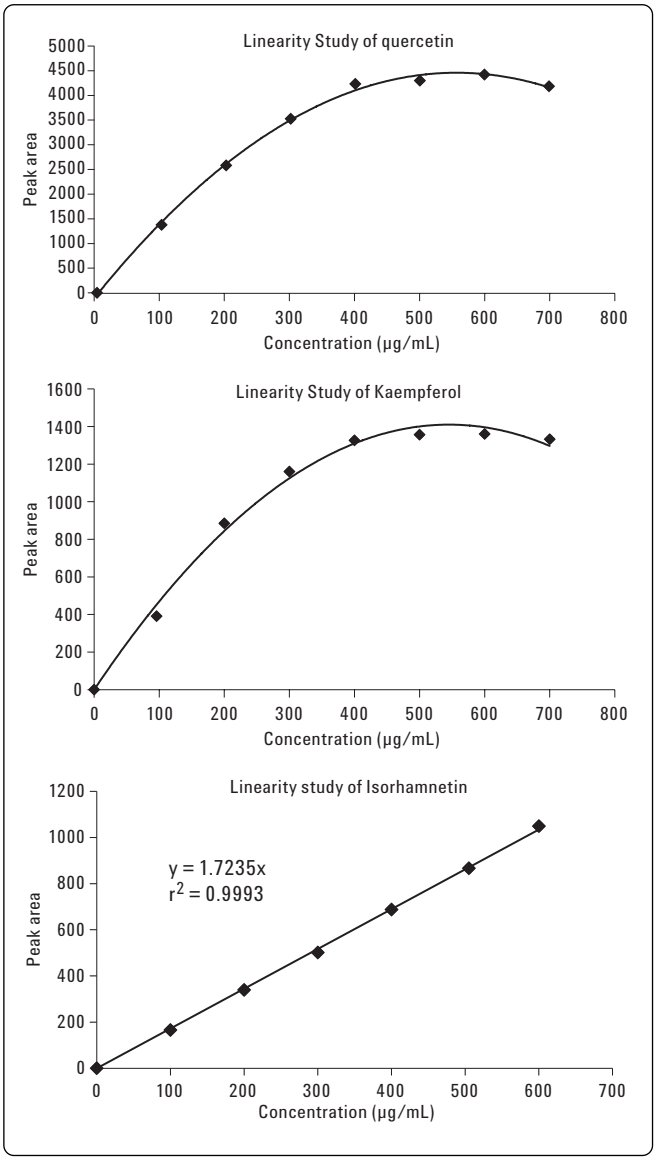

Figure 7. Studies of the linearity of SampliQ OPT 3 mg sorbent to high concentrations of quercetin, kaempferol and isorhamnetin.

Limits of detection (LOD) and limit of quantification (LOQ)

The limits of detection were calculated using the intercept, y_B, and the standard error of the regression line, s_B at three times standard error and LOD values were calculated using equations 1 and 2 [11, 12].

$$y(LOD) = y_B + 3s_B \qquad \text{Equation 1}$$

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

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

$$yLOQ = y_B + 10s_B \qquad \text{Equation 3}$$

$$LOQ = (yLOQ - y_B)/m \qquad \text{Equation 4}$$

The limits of detection and quantification for quercetin, kaempferol and isorhamnetin are as summarized in Table 3.

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

	LOD (µg/mL)	LOQ (µg/mL)
Quercetin	0.57	1.91
Kaempferol	0.76	2.15
Isorhamnetin	0.88	2.55

The method was applied to the analysis of the commercial samples of *Ginkgo biloba* tablets and leaves from A. Vogel. It was observed that the product contained quantifiable amounts of the flavonoids as indicated in Table 4. However, the isorhamnetin concentration was too low to be detected in the sample extract from the leaves.

Table 4. Concentrations of Quercetin, Kaempferol and Isorhamnetin in Commercial Samples

	Concentration (µg/mL)	
	Tablet extract	Leaf extract
Quercetin	26.42	12.35
Kaempferol	19.08	10.75
Isorhamnetin	8.99	–

Conclusions

Agilent SampliQ OPT cartridges achieved effective sample cleanup. The results demonstrated that the method was reproducible with good recoveries (80% to 92% for quercetin, 83% to 94% for kaempferol and 75% to 102% for isorhamnetin at n=6 and RSDs less than 5%). The limits of detection and quantification for quercetin were 0.57 and 1.91, and 0.76 and 2.15 µg/mL respectively for kaempferol while those of isorhamnetin were 0.88 and 2.55 µg/mL respectively. The tablet extract and the leaf extract contained measurable but different concentrations of the flavonoids.

References

1. T. A. Van Beek and P. Montoro, *J of Chrom A*. 1216 (2009) 2002-2032
2. Y. Wang, J. Cao, J. H. Weng, S. Zeng, *J of Pharm Biomed Ana.* 39 (2005) 328-333
3. K. L, Chiu, Y. C. Cheng, J.H. Chen, C.J. Chang, P. W. Yang, *J. of Supercritical Fluids*, 24 (2002) 77-87
4. M. J. Dubber, I. Kanfer, *J Pharm Pharmaceut Sci*, 7 (3), (2004) 303-309.
5. D. G. Watson, E. J. Oliveira, *J. of Chrom B*, 723 (1999) 203-210
6. L. Mmualefe, N. Torto, P. Huntsman-Mapila, B. Mbongwe, *Microchemical Journal* 91 (2009), 239-244.
7. L. F. C. Melo, C.H. Collins, I. C. S. F. Jardin, *Journal of Chromatography A*, 1032 (2004), 51-58
8. M. S. Landis, *J. of Pharm. Biomed. Anal*. 44 (2007), 1029-1039
9. K. Bielicka-Daszkiewicz, A. Voelkel, M. Szejner and J. Osypiul, Chemosphere, 62 (2006) 890-898.
10. M. C. Hennion, *J. of Chromatography A*, 856 (1999), 3-54
11. F. Deng and S. W. Zito, *J of Chhrom A*, 986 (2003) 121-127
12. M. A. Alabdalla, *Journal of Clinical Forensic Medicine*, 12 (2005) 310-315

For More Information

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
October 21, 2009
5990-4799EN

Agilent Technologies