Rapid Microwave-Assisted Hydrolysis for Determination of Ginkgo Flavonol Glycosides in Extracts of *Ginkgo biloba* Leaves

J. Xiping Chen[1,2] and **Yangde Zhang**[1,*]

[1]National Hepatobiliary & Enteric Surgery Research Center, Ministry of Health, Changsha 410008, China and [2]Hunan Traditional Chinese Medicine College, Changsha 410007, China

Abstract

A rapid microwave-assisted hydrolysis (MAH) method is presented for the sample pretreatment of the determination of ginkgo flavonol glycosides in extracts of *Ginkgo biloba* L. (EGb). By this method, flavonol glycosides can be completely hydrolyzed within 2 min. After investigating the effects of solvents, acidity, microwave power, and microwave radiation time on hydrolysis, the optimal hydrolysis conditions are as follows: 300 W of microwave power, 2 min of hydrolysis time, 5.7% of hydrochloric acid in the hydrolysis solution, and *n*-propanol as the hydrolysis solvent. After MAH of the samples, three flavonol aglycones, such as quercetin, kaempferol, and isorhamnetin are analyzed by high-performance liquid chromatography. Compared with conventional reflux hydrolysis, this method owns offers several advantages: it saves time, costs less, and is environmentally friendly.

Introduction

Modern pharmacological research into the active constituents of ginkgo leaves began in the late 1950s. Today, a standardized, concentrated extract of ginkgo leaves (EGb) is mainly used as a preparation for improving blood circulation. Critical to the extraction process and final product is the standardization of ginkgo flavone glycosides and terpene lactones. These extracts contain approximately 6% terpene lactones and 24% flavone glycosides primarily composed of quercetin, kaempferol, and isorhamnetin (Figure 1). The quercetin, kaempferol, and isorhamnetin in these flavone glycosides consist of a carefully measured balance (1–3). Because the three flavonol aglycones appear in significant concentrations in acid hydrolysis products of EGb, the applied method for the quantitative analysis of flavonol glycosides in Ginkgo leaves and extracts is an acid hydrolysis to result in aglycones, followed by high-performance liquid chromatography (HPLC). Acid hydrolysis of flavonol glycosides to the corresponding aglycones is a well-developed, simple procedure. The three main aglycones are all commercially available and easy to analyze using reversed-phase HPLC (RP-HPLC). A review of the current methodologies for the chemical analysis of *Ginkgo biloba* leaves and extracts was well-presented by van Beek (4).

The conventional reflux acid hydrolysis (CRH) procedure is time-consuming, requiring about 40–60 min. If a new, rapid, acid hydrolysis method is developed, the analysis throughput of ginkgo flavone glycosides would be increased significantly.

Microwave energy holds great potential for rapidly heating materials. In comparison with conventional heat sources, in which energy must first be conducted through the walls of the

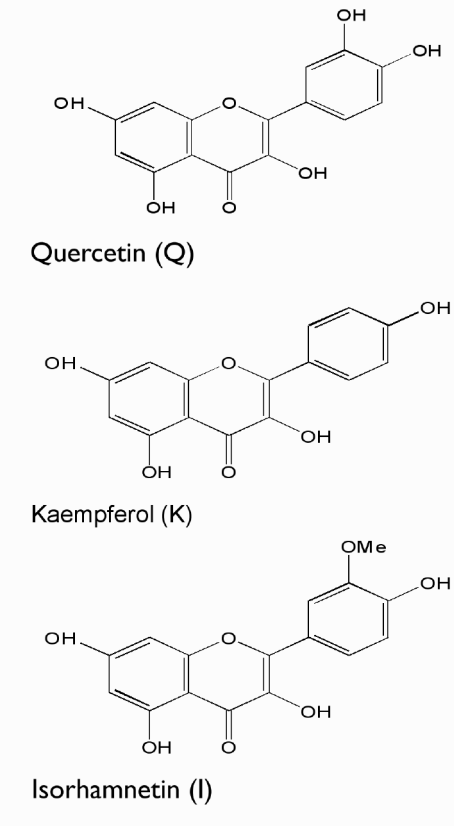

Figure 1. Chemical structures of quercetin, kaempferol, and isorhamnetin.

* Author to whom correspondence should be addressed: email zhangyangde123@126.com.

vessel containing the reactants, microwaves heat the contents directly, allowing the temperature to rise much faster, boosting reaction rates (5). Using microwave heating, a series of methods and techniques have been developed for sample preparation in environmental organic analysis, such as: microwave-assisted extraction, focused microwave-assisted Soxhlet extraction, microwave-assisted headspace analysis, microwave-assisted derivation, microwave-assisted saponification, and microwave-assisted decomposition (6). These novel techniques possess many advantages over traditional methods, including increased efficiency for sample preparation, saving of energy and time, and environmental friendliness. Microwave hydrolysis has already been successfully used in food sample analysis, such as determination of amino acids, lysine, furosine, choline, and vitamin B12, etc. (7–10).

In this paper, a rapid microwave-assisted hydrolysis method for ginkgo flavonol glycosides was developed and found to be advantageous.

Table I. Effect of Different Solvents on Hydrolysis Yields of Flavonol Aglycones*

Solvent	Aglycones contents (%)			Recovery of flavonol glycosides (%)
	Quercetin	Kaempferol	Isorhamnetin	
Methanol	4.14	1.56	0.47	59.6
n-Propanol	6.89	2.40	1.19	101.1
Acetone	4.44	1.72	0.58	65.1
n-Butanol	3.23	2.32	1.12	64.4

* Content of flavonol glycosides was calculated according to: sum of three flavonol aglycines × 2.51 (13).

Table II. Effect of Concentration of Hydrochloric Acid Aqueous Solution on Hydrolysis Efficiency*

Hydrochloric acid concentration (%)	Aglycones contents (%)			Recovery of flavonol glycosides (%)
	Quercetin	Kaempferol	Isorhamnetin	
36	6.81	2.41	1.16	100.2
20	5.81	1.97	0.88	83.6
10	5.29	1.59	0.65	72.7
5	3.37	0.97	0.33	45.1

* Volume of hydrochloric acid is 7.5 mL.

Table III. Effect of Hydrochloric Acid Volume on the Yield of Flavonol Aglycones*

Volume of hydrochloric acid	Aglycones contents (%)			Recovery of flavonol glycosides (%)
	Quercetin	Kaempferol	Isorhamnetin	
20	6.76	2.38	1.20	99.8
15	6.77	2.39	1.15	99.5
10	6.81	2.38	1.17	100.0
5	6.79	2.40	1.14	99.7

* Concentration of hydrochloric acid is 36%.

Table IV. Results of Sample Analysis and Method Comparison

Sample	MAH		CRH	
	Flavonol glycosides (%) (n = 5)	RSD (%)	Flavonol glycosides (%) (n = 5)	RSD (%)
EGb 1	21.6 ± 0.20	0.91	21.4 ± 0.24	1.12
EGb 2	17.9 ± 0.20	1.13	18.1 ± 0.24	1.35
EGb 3	24.8 ± 0.55	2.22	24.5 ± 0.25	1.04
Gb leaves 1	2.12 ± 0.023	1.10	2.10 ± 0.046	2.17
Gb leaves 2	1.24 ± 0.026	2.13	1.23 ± 0.022	1.78

Experimental

Materials

EGb was purchased from Hunan Phytoway Pharmaceutical Co., (Changsha, China). Reference samples of EGb (26% of flavonol glycosides), quercetin, kaempferol, and isorhamnetin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). HPLC-grade methanol (Tedia Company Inc. OH) and ultrapure water prepared by a Milli-Q purification system (Millipore Corp. Bedford, MA) were used as the mobile phases of HPLC. Other reagents were analytical-grade.

Instrumentation

Rapid microwave-assisted hydrolysis experiments were performed with a MARS 5 experimental microwave system (1200 W, CEM, USA). It has a built-in computer with a ramp-to-temperature/pressure option and programmable methods. Pressure vessels at 1500 psi were used for the MARS in this experiment.

Analyses of samples were performed on a Waters (Milford, MA) Alliance 2695 liquid chromatographic system interfaced to a Waters 2487 dual absorbance detector equipped with a Johnson (Dalian, P. R. China) Spherigel analytical column (4.6 × 250 mm) packed with 5 µm C_{18} sillica. The data obtained was recorded and treated with Millennium^{32} chromatographic software (Waters). The mobile phase of elution was of methanol + 0.4% phosphoric acid aqueous solution (50 + 50, v + v). The flow-rate was 1 mL/min. The column temperature was kept constant at 30°C. The detection wavelength was set at 360 nm. The injection volume was 5 µL.

Microwave-assisted hydrolysis

Fifty milligrams of EGb, 40 mL of *n*-propanol, and 7.5 mL of concentrated hydrochloric acid (36%) aqueous solutions

were transferred in a microwave vessel. After mixing the solution well, the vessel was airproofed with its special cap and placed on the turntable of the microwave oven. Hydrolysis progressed for 2 min using 300 W of microwave radiation power. After completing hydrolysis, the volume of the sample solution was raised to 50 mL with *n*-propanol. Five microliters of the sample solution was injected into the HPLC system.

Results and Discussion

Effect of solvents on microwave-assisted hydrolysis

The principle of heating using microwave energy is based on the direct effect of microwaves on molecules by ionic conduction and dipole rotation. At 2450 MHz, which is frequently used in commercial systems, the dipoles align and randomize 4.9 × 109 times per second. This forces molecular movement, resulting in heat. The ability of a solvent to absorb microwave energy and pass it on in the form of heat to other molecules partly depends on the dissipation factor of the molecules (6,11,12). Therefore, the selection of solvent for hydrolysis is very important.

We investigated the effects of different solvents on hydrolysis of ginkgo flavonol glycosides in a standard reference sample of EGb. Methanol, acetone, *n*-propanol, and *n*-butanol were tested using the hydrolysis conditions previously described in the microwave-assisted hydrolysis section. The results are shown in Figure 2 and Table I. *n*-Propanol resulted in optimal hydrolysis efficiency, and methanol, *n*-butanol, and acetone resulted in low recoveries of hydrolysis. In accordance with the results, n-propanol was chosen as a hydrolysis solvent to optimize other hydrolysis conditions.

Effect of acidity on microwave-assisted hydrolysis

Based on the hydrolysis conditions described in the microwave-assisted hydrolysis section, different concentrations and volumes of hydrochloric acid aqueous solutions were investigated to evaluate the effect of acidity on the hydrolysis efficiency. The results are listed in Tables II and III, and present that the concentration of hydrochloric acid aqueous solutions can affect the hydrolysis efficiency dramatically. Thirty-six percent of concentration resulted in optimal hydrolysis recovery. The volume from 5–20 mL of the acid does not have an obvious effect on hydrolysis efficiency. Considering a higher content flavonol glycosides sample, 7.5 mL of concentrated hydrochloric acid was finally used to hydrolyze. The concentration of hydrochloric acid in whole hydrolysis solution is approximately 5.7%.

Effects of power and time of microwave radiation on microwave-assisted hydrolysis

Optimization of power and time on microwave radiation for hydrolysis can decrease reactive time, saving energy. The results are shown in Figure 3. The conditions were same as those previously described in the microwave-assisted hydrolysis section except power and time.

The results show that hydrolysis reaction occurs more rapidly during a 2-min time period at a higher energy level of microwave radiation.

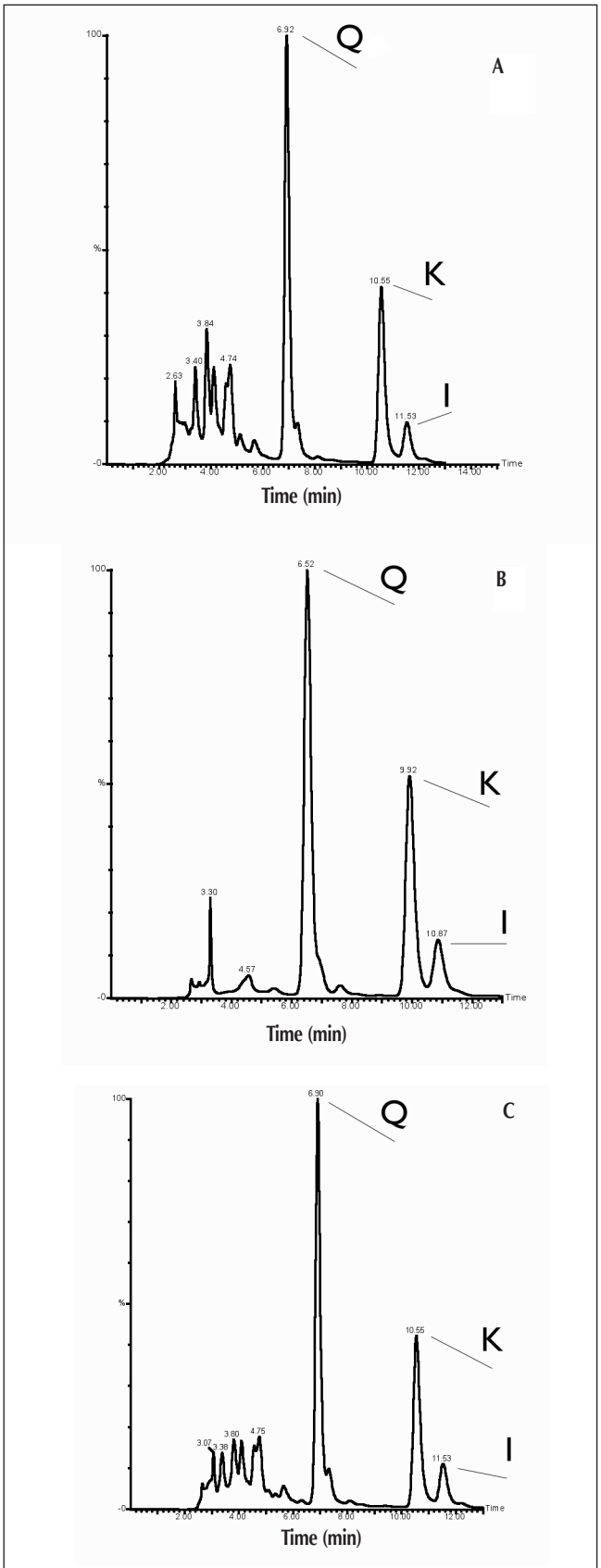

Figure 2. Chromatograms in different hydrolysis solvents: methanol (A), *n*-propanol (B), acetone (C). Note: mobile phase was of methanol + 0.4% phosphoric acid aqueous solution (65:35, v/v). Quercetin (Q), kaempferol (K), and isorhamnetin (I).

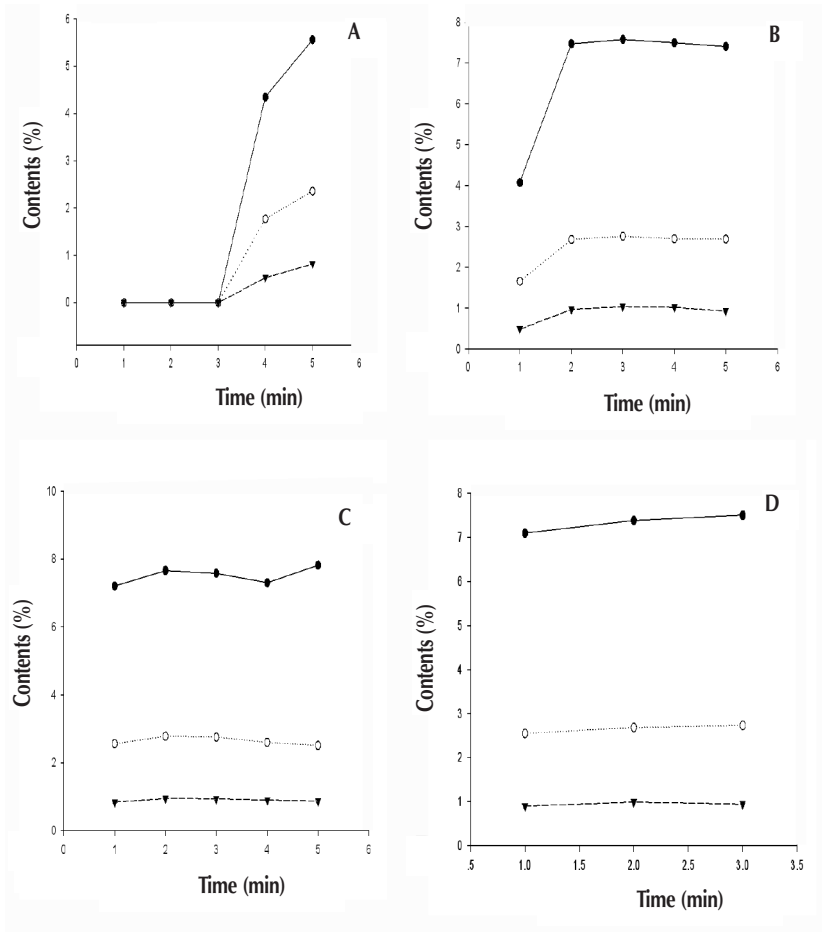

Figure 3. Effects of power and time of microwave radiation on hydrolysis efficiency: 150 W (A), 300 W (B), 450 W (C), 600 W (D). Line label: –•–, quercetin; –o–, kaempferol; –▲–, isorhamnetin.

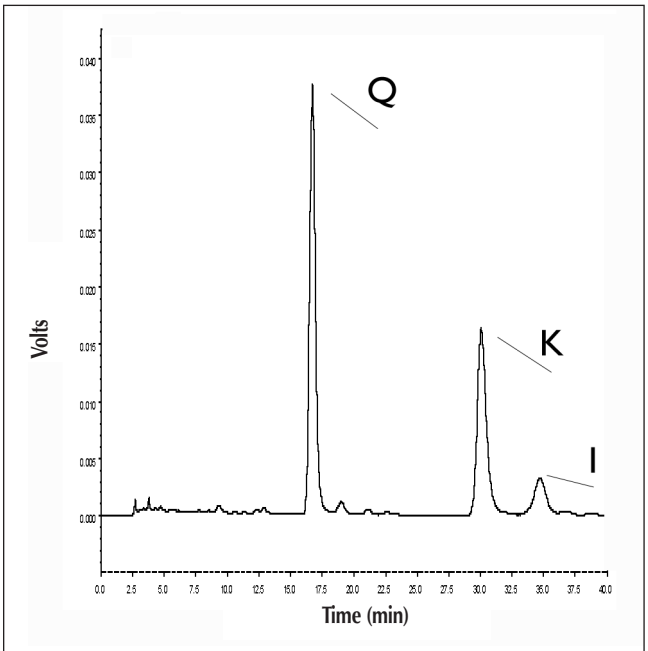

Figure 4. Typical chromatogram of MAH EGb. Note: chromatographic conditions were the same as those described in the Instrumentation section.

The HPLC chromatograms showed that when power was 150 W, there were many obvious peaks except for three flavonol aglycones. This suggested that some flavonol glycosides were not completely hydrolyzed. When power was higher than 150 W, these flavonol glycosides peaks disappeared, and contents of three flavonol aglycones increased to invariable levels. From the radiation time, 2 min is enough when the power is higher than 300 W.

Method comparison and application

Real samples of EGb were analyzed using MAH method. The samples were also analyzed using conventional reflux hydrolysis following HPLC. The results of content and comparison are listed in Table IV. The results show no significant difference between MAH with CRH. However, the hydrolysis time was only 2 min by MAH. Furthermore, every analysis was repeated 5 times, and the relative standard deviation was smaller than 2.22%. A typical chromatogram of MAH EGb is shown in Figure IV.

Conclusions

Compared to the conventional reflux acid hydrolysis method, the MAH proposed in this paper has certain advantages such as saved time and lowered cost. In addition, a laboratorial microwave oven can process up to 14 vessels per run, thus significantly increasing the throughput of hydrolysis of EGb. Furthermore, the hydrolysis is completed in a hermetical environment. The method is also environmentally friendly, and the amount of solvent consumed is smaller than that consumed in the CRH method. These advantages have obvious significance for the rapid analysis of ginkgo flavone glycosides in EGb.

Acknowledgments

This work was supported by the Natural Science Foundation of China (90606012) and the Natural Science Foundation of Hunan Province (06JJ4039).

References

1. A. Hasler and O. Sticher. Identification and determination of the flavonoids from Ginkgo biloba by high-performance liquid chromatography. *J. Chromatogr. A* **605:** 41–48 (1992).
2. S. Jaracz, S. Malik and K. Nakanishi. Isolation of ginkgolides A, B, C, J and bilobalide from *G. biloba* extracts. *Phytochemistry* **65:** 2897–2902 (2004).

3. H. Oberpichler, T. Beck, M.M. Abdel-Rahman, G.W. Bielenberg and J. Krieglstein. Effects of ginkgo biloba constituents related to protection against brain damage caused by hypoxia. *Pharmacol. Res. Comm.* **20:** 349–368 (1988).
4. Teris A. van Beek. Chemical analysis of Ginkgo biloba leaves and extracts. *J. Chromatogr. A* **967:** 21–55 (2002).
5. D. Adam. Out of the kitchen. *Nature* **421:** 571–572 (2003).
6. C. S. Eskilsson and E. Björklund. Analytical-scale microwave-assisted extraction. *J Chromatogr. A* **902:** 227–250 (2000).
7. H. Zhong, S. L. Marcus and L. Li. Microwave-assisted acid hydrolysis of proteins combined with liquid chromatography MALDI MS/MS for protein identification. *J. Am. Soc. Mass Spectrom.* **16:** 471–481 (2005)
8. C. Xu and B. Li. Spectrophotometric determination of paracetamol with microwave assisted alkaline hydrolysis. *Spectrochim. Acta Part A* **60:** 1861–1864 (2004)
9. R. Acquistucci, G. Panfili, and E. Marconi. Application of the microwave hydrolysis to furosine determination in cereal and dairy foods. *J. Agric. Food Chem.* **44:** 3855–3857 (1996).
10. G. Panfili, P. Manzi and D. Compagnone et al. Rapid assay of choline in foods using microwave hydrolysis and a choline biosensor. *J. Agric. Food Chem.* **48:** 3403–3407 (2000).
11. H. M. Kingston and L. B. Jassie. Introduction to microwave sample preparation, 1st ed. H. M. Kingston and L. B. Jassie, Eds. American Chemical Society, Washington, DC, 1988, pp. 3–27.
12. H. M. Skip Kingston and S. J. Haswell. Microwave-enhanced chemistry, 1st ed. H. M. Skip Kingston and S. J. Haswell, Eds. American Chemistry Society, Washington, DC, 1997, pp. 569–612.
13. National pharmacopoeia committee of China. Chinese pharmacopoeia, Book, 1st ed. National pharmacopoeia committee of China, Eds. Chemical industry press, Beijing, 2005, pp. 282.

Manuscript received September 13, 2006;
revision received January 6, 2007.