Bull. Environ. Contam. Toxicol. (2003) 71:662–667

DOI: 10.1007/s00128-003-0184-0

Environmental Contamination and Toxicology

Novel Atoxic Method of Flavonoid Extraction from *Ginkgo biloba* Leaves

C. X. Zhang,[1] X. L. Hu[2]

[1] National Laboratory for Molecular and Biomolecular Electronics, Chien-Shiung Wu Laboratory, Southeast University, Nanjing, 210096, People's Republic of China
[2] Department of Chemical Engineering, Northwestern Polytechnical University, Xi'an, 710072, People's Republic of China

Received: 31 August 2002/Accepted: 16 June 2003

Ginkgo biloba leaves have been one of the most popular traditional Chinese medicines for several thousand years (Kleijen and Kripschild 1992; Jacobs and Browner 2000). The indications for *Ginkgo biloba* extracts (GBE) are primarily related to diseases appearing with advanced age (Logani et al. 2000; Winther et al. 1998; Simonetti et al. 2000). As the most active ingredients in GBE, the flavonoids are only a little in *Ginkgo biloba* leaves. To obtain flavonoids from *Ginkgo* leaves, a relatively tedious work and purification procedure comprising many steps like liquid/liquid extraction, precipitation, and concentration (DeFeudis 1991) were used.

Organic solvents used as leaching liquors to extract flavonoids have volatility and flammability, which makes the processing more complicated and costly. Since *Ginkgo* flavonoids are water-soluble, it is possible to use water as a leaching liquor (Liu and Chen 1996; Zhang et al. 1999). Water is safe and inexpensive for use, no contaminative to the environment.

Aqueous two-phase systems were reported firstly in the literature as early as 1896 (Diamond and Hsu 1990). Albertsson first applied aqueous two-phase systems to preparative biomolecular purification (Albertsson 1986). Aqueous two-phase systems consist of polyethylene glycol (PEG), potassium phosphate, and water, and the system are suitable especially for purification of biologically active materials, because each phase contains 70% to 90% water, and commonly used polymers PEG is atoxic, nonimmunogenic, and nondenaturing (Mahadevan and Hall 1990). The systems have been demonstrated to provide a protective environment for biological activity of biomolecules, and offer different physical and chemical environments that allow for the selective partitioning of solutes. Several experimental and theoretical studies have described phase separation of protein aqueous two-phase systems (Vlachy et al. 1993). These studies are motivated by an increasing demand for pure proteins in pharmaceutical and related industries. Thus, aqueous two-phase extraction has the potential of being used for the isolation and concentration of biomaterials (Kenkare and Hall 1996). The systems have been demonstrated to provide a protective environment for biological activity of biomolecules, and a safe condition for production and use.

In this paper, a novel method in which water substituted for organic solvents as leaching liquor was studied to reduce contamination and cost of production. Aqueous two-phase systems were also investigated to purify flavonoids in the leachate.

Correspondence to: C. X. Zhang

MATERIALS AND METHODS

A Model 721 spectrophotometer (Shanghai, China) was used for quantification. A PHS-3C precise pH meter was used for pH measurement of the solution. A GSP-84-02 magnetic stirrer with constant temperature was used for liquid-liquid extraction separation. A vacuum filter was used for removing undissolved impurities. The water used was double distilled water, chemicals including ethylbutyl ketone, sodium nitrite, aluminum nitrate, PEG, and potassium phosphate were analytical grade. Rutin was used as a standard in the study. Flavonoids can react with aluminum nitrate in solution to produce a coloured liquid, so that we can use a spectrophotometer to measure the absorption at 510 nm to calculate the concentration by comparison with rutin as a standard (Wu et al. 1995; Sha 1995).

An atoxic method to extract flavonoids from *Ginkgo* leaves was developed. There are two main steps to extract flavonoids from *Ginkgo* leaves: leaching and purification.

The leaves were washed after impurities were removed from them, dried in an oven, and crushed. Take a certain amount of crushed leaves, put them in a container, add a certain volume of solvent, adjust the pH of the solution, and keep the container in a constant temperature for a given time. After that, let the solution through a filter to remove undissolved impurities to get a clean leachate. Leaching efficiencies can be calculated as following:

$$\text{Leaching efficiency} = (C_2 / C_1) \times 100\% \qquad (1)$$

Where C_1 is the total content of the flavonoids in the leaves before extraction and C_2 is the content leached from the leaves.

For extraction of flavonoids with aqueous two-phase system, add the leachate into an appropriate system first, and shake the mixture for several minutes, then place the mixture for 15-20 minutes at a constant temperature. After that, the flavonoids entered into the upper phase and the impurities into the lower phase. The extraction efficiency is calculated as following, where C_3–C_4 is the concentration of flavonoids in the upper phase while C_4 is that in the lower.

$$\text{Extraction efficiency} = [(C_3 - C_4) / C_3] \times 100\% \qquad (2)$$

RESULTS AND DISCUSSION

Ethanol has been used for many years as a leaching liquor to extract flavonoids although it has some disadvantages, such as volatility, flammability, which make the processing more complicated, high costly. *Ginkgo* flavonoids are water- soluble, it is possible to use water as a leaching liquor. Water is safe and inexpensive for use, no contamination to the environment. We have studied the leaching efficiencies by comparing water with ethanol as leaching liquors (Table 1).

Table 1 indicates that temperature is the main factor which effects the leaching efficiency. To get the highest efficiency, the efficiencies at different temperatures must be determined. Figure 1 shows that higher temperature gives the higher leaching efficiency. However, if the temperature is too high, the structures of the active

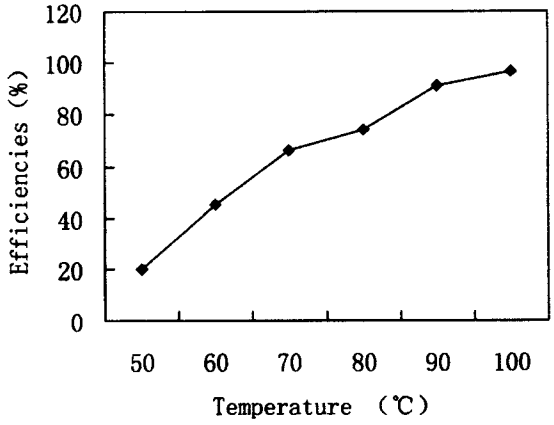

Figure 1. Effect of temperatures on leaching efficiencies
Experimental conditions: leaves:water = 1:40 (in weight), duration 12 hours, pH 6.0

Table 1. Leaching efficiencies of water and ethanol*.

Temperatures	Leaching liquors	Leaching efficiencies (%)
50℃	Ethanol	65.4
	Water	21.5
	Ethanol:water(1:1)	35.4
70℃	Ethanol	92.3
	Water	65.8
	Ethanol:water(1:1)	73.1
90℃	Ethanol	----**
	Water	89.6
	Ethanol:water(1:1)	78.1

* Ethanol and water were used as leaching liquors at different temperatures (from 50℃ to 90℃).
**At 90℃, there is no data because ethanol is volatilized at 90℃.

flavonoids will be destroyed, thus the activity will not be remained. Accordingly, 90℃ was chosen as the optimal temperature in the experimental study. At 90℃, lots of impurities such as proteins and saccharides were leached together with flavonoids, at the same time, the flavonoids were wrapped by the impurities so that they were protected to avoid to be hydrolyzed (Liu and Chen 1996).

The hydroxyl and carboxyl groups in flavonoids that makes them weakly acidic. Thus alkaline water will improve the leaching efficiencies. We used the waters with pH of 4.0, 6.0, and 8.0, respectively, to leach flavonoids from *Ginkgo* leaves and found that

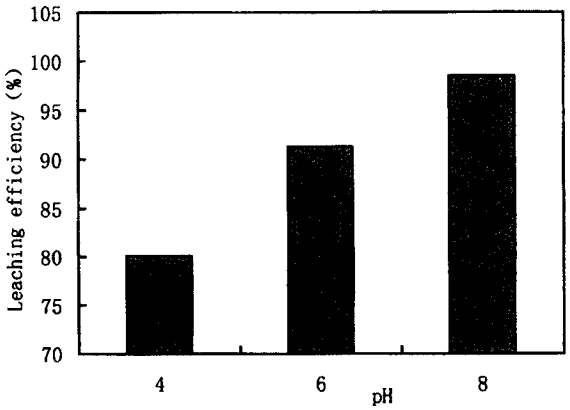

Figure 2. Effect of pHs on leaching efficiencies
Experimental conditions: leaves:water = 1:40 (in weight), duration 12 hours, temperature 90℃

the water with pH 8.0 could give the highest efficiency (Figure 2). At 90℃, with water of pH 8.0 as a leaching liquor, the leaching efficiency can be reached 98.5%, which is higher than that of ethanol at 70℃.

Aqueous two-phase systems consist of polyethylene glycol (PEG), potassium phosphate, and water. The systems have been demonstrated to provide a protective environment for biological activity of biomolecules, and to offer different physical and chemical environments that allow for the selective partitioning of solutes. The partition coefficient depends on many variables including molecular size, electrochemistry, molecular conformation, as well as environmental conditions such as pH, buffer concentrations and temperature. We have done some work to elucidate the effects of molecular weight and concentrations of PEG, salts, and temperatures on flavonoids partitioning.

The molecular weight of PEG influences the solvability in water. Higher molecular weight has a lower solvability and is more difficult to form aqueous two-phase system. Accordingly, PEG with average molecular weight of 1500 was chosen in this study.

As a two-phase system, there exist critical concentrations of PEG and salt used in the system (Figure 3). When the concentrations of PEG and salt are higher than the critical, aqueous two-phase system was formed, otherwise, there is no aqueous two-phase system formed. With the increasing amount of PEG used, the used amount of phosphate is decreased. Simultaneously, the results not only showed that the phase ratios decreased with the increasing concentration of phosphate when PEG is at a fixed concentration, but also the ratios increased with the increasing concentrations of PEG under a fixed concentration of the phosphate. Therefore, to get a higher separating efficiency, the contribution ratio and phase ratio must be considered during aqueous two-phase partitioning.

The aqueous two-phase system varied with temperature because the water-solvability of polymer was influenced significantly by the temperature. Higher temperature is

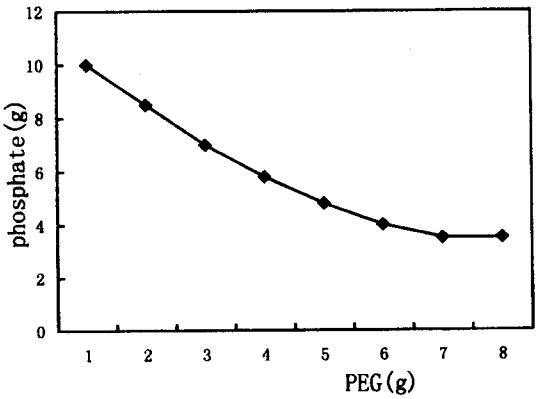

Figure 3. Phase diagram of PEG/phosphate system

Table 2. Extraction efficiencies at different phase ratios.

Phase Ratios (upper/lower)	0.56	0.51	0.40
Extract efficiencies (%)	98.2	97.3	91.8

benefit for the forming of two phases. However, when temperature is too high, such as over 80℃, PEG will be crystallized and precipitated (the data were not shown in this paper). Thus the aqueous two-phase system deformed. Only when the temperature is between 15-70℃, did the aqueous two-phase system form. The temperature range meets well with the requirement of bioactive compounds.

According to the studies above, PEG with average molecular weight of 1500 was chosen as the polymer to get an aqueous two-phase system. As lower temperature is good for bioactive material, 25℃ was chosen as the separating temperature. Table 2 showed us the effect of phase-ratios on separation efficiencies under the above conditions. From the results we know that higher efficiency can be reached at higher phase-ratio. However, a too high phase ratio will cause a larger volume of upper phase, in this case, the flavonoids in the leachate were not concentrated. Meanwhile, the amount o f PEG must be added to get higher phase-ratio, this will raise the production cost.

The experimental results showed that using aqueous two-phase system to separate *Ginkgo* flavonoids from the leachate of *Ginkgo* leave is superior to other separation methods in some aspects, for instance, lower temperature, shorter separating time, and higher efficiency. In our work, the partitioning efficiency is 98.2%, much higher than that of organic solvent extraction which is 65.8% at 70℃ (Zhang et al. 2001). It supposed to be a new method to extract *Ginkgo* flavonoids.

Compared with organic solvent extraction, aqueous two-phase partition has some advantages, such as simpler partition, shorter separating time, lower operation temperature. More important is that the component used in the systems, including PEG and phosphate, are not volatile, atoxic to the biomaterial, the people, and the

environment.

Acknowledgments We thank Miss Jin Hua Lu for doing some experimental work, and Professors Zu Hong Lu and Nong Yue He for helpful discussion.

REFERENCES

Albertsson, P-A. (1986) Partition of cell particles and macromolecules, 3rd ed., Wiley-Interscience, New York

DeFeudis, F. V (1991) *Ginkgo biloba* extract (EGb 761): pharmacological activities and clinical applications, Editions Scientifiqus Elsevier, Paris: pp.1-187

Diamond AD, Hsu JT (1990) Protein partitioning in PEG/Dextran aqueous two-phase systems, American Institute of Chemical Engineers J 36: 1017-1024

Liu Z, Chen YY (1996) Study on water leaching method of total flavone glycosides in *Ginkgo* leaves, Huaxue Shijie 7: 355-358

Jacobs BP, Browner WC (2000) *Ginkgo Biloba*: a living fossil. American J Med 108: 341–342.

Kenkare PU, Hall KC (1996) Modeling of phase separation in PEG-salt aqueous two-phase systems. American Institute of Chemical Engineers J 42: 3508-3522

Kleijen J, Kripschild P (1992) *Ginkgo biloba.* Lancet 340: 1136-1139

Liang H, Xu B, Pan WM (2000) Analysis of proteins in seeds and leaves of *Ginkgo Biloba* leaves. J Plant Res Environ 9: 5-8

Logani S, Meng CC, Tuyet T, Tam L, Robert BR (2000) Actions of *Ginkgo Biloba* related to potential utility for the treatment of conditions involving cerebral hypoxia. Life Sci 67: 1389-139

Mahadevan H, Hall CK (1990) Statistical-Mechanical model of protein precipitation by nonionic polymer. American Institute of Chemical Engineers J 36: 1517-1528

Sha H, Chen GX, Ma L (1995) Extraction and assay of total flavonoids in Ginkgo leaves. Zhong Guo Yao Fang 6: 13-14

Simonetti P, Gardana C, Mauri P (2000) Trolox equivalent antioxidant capacity (TEAC) of *Ginkgo biloba* flavonol and Camellia sinensis catechin metabolites. J Pharmacol Biomed Anal 23: 223–226

Vlachy V, Blanch HW, Prausnitz JM (1993) Liquid-liquid phase separations in aqueous solutions of globula proteins. American Institute of Chemical Engineers J 39: 215-223

Winther K, Randlov C, Rein E, Mehlsen J (1998) Effects of *Ginkgo Biloba* extract on cognitive function and blood pressure in elderly subjects. Therap Res 59: 881-888

Wu HL, Liu XL, Gong J, Qu YN (1995) Determining the total flavone glycosides of *Ginkgo* leaves in different seasons. Zhong Cao Yao 26: 445

Xin WJ, Wei TT, Chen C, Ni YC, Zhao BL, Hou JW (2000) Mechanisms of apoptosis in rat cerebellar granule cells induced by hydroxyl radicals and the effects of EGb761 and its constituents. Toxicology 148: 103–110

Zhang CX, Hu XL, Yue H (1999) Progress of extraction and analytical methods of flavones in Ginkgo Biloba leaves. Huagong Jinzhan 6: 27

Zhang CX, Hu XL, YUE H, Lü L, Lu JH (2001) Extraction of flavonoes from Ginkgo leaves. Chinese J Appl Chem 13: 454-456