# Analysis of Ginkgolides and Bilobalides in *Ginkgo biloba* L. Extract for Its Production Process Control by High-Performance Liquid Chromatography

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## Abstract

**A high-performance liquid chromatographic (HPLC) method is developed for the determination of the pharmacologically active terpenoids Ginkgolide A, B, and C and bilobalide in *Ginkgo biloba* L. leaves extract (GBE). The extracts are dissolved in methanol–water (1:4) and extracted by ethyl acetate after evaporation of the organic solvent, the residue is dissolved in methanol, and the terpenoids in the resultant solution are determined by means of HPLC on a $C_{18}$ column with methanol–water (23:77) as an eluent. The recovery provided by the method is above 96.5%, and the minimum concentration that can be determined is 80 µg of terpenoid per gram of GBE. The method is suitable for GBE production process control.**

## Introduction

The dried ripe seeds and leaves of *Ginkgo biloba* L. are two commonly used traditional Chinese herbal medicines. According to the principles of traditional Chinese medicine, they can be sweet, bitter, or astringent in taste, neutral in nature, and toxic, act on the lung meridian, and have astringing effects on the lung that relieve asthma, stopping leukorrhea and turbid urine, and reducing the frequency of urination (1).

*Ginkgo biloba* L. extracts (GBE) are commonly used in Europe for the treatment of cerebrovascular and peripheral circulatory problems of the elderly (2). These extracts contain flavonol glycosides (2,3), biflavones (4), and terpenes (5,6). The phamacologically active components, the diterpenes Ginkgolide A, B, and C (GA, GB, and GC) and the sesquiterpene bilobalide (BB), are reported to be only present in *Ginkgo biloba* L. (6,2). Thus, the contents of GA, GB, GC, and BB are often used as the quantitative indices of GBE, and the analysis of these constituents is of great importance in production process control (6,2).

High-performance liquid chromatography (HPLC) has been successfully applied to analyze flavonol glycosides (3) and biflavones (7) in *Ginkgo biloba* L. However, the qualitative and quantitative determination of the terpenes (8) is still problematic and limited. In 1991, Van Beek et al. (9) proposed a reversed-phase HPLC method with ultraviolet (UV) detection

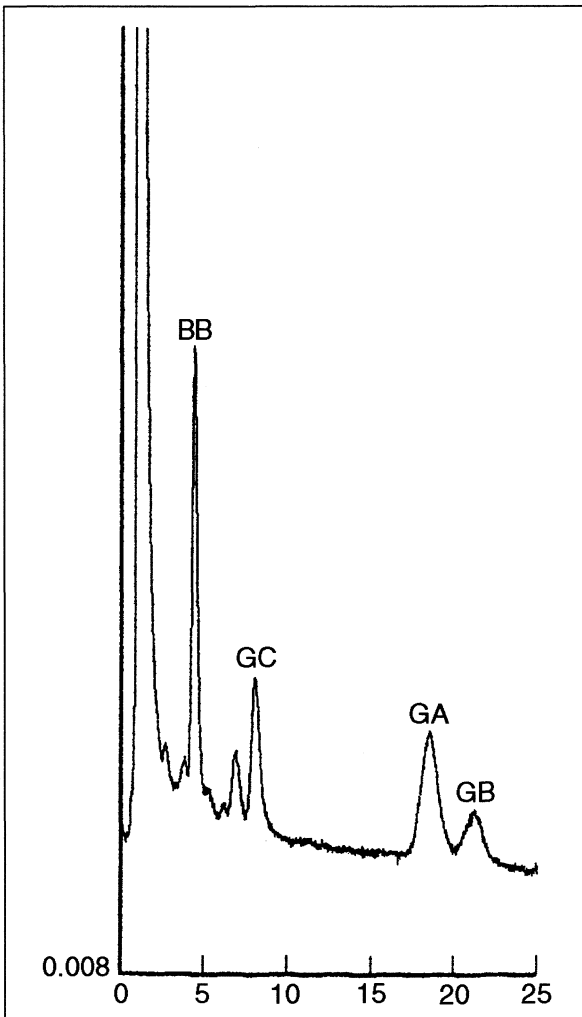


**Figure 1.** Chromatogram of the *Ginkgo biloba* L. extract on a Nova-pak $C_{18}$ column.

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**Table I. Method Reproducibility**

| Sample | | Recovered amounts (%) | | | | | Mean (%) | CV (%) |
|---|---|---|---|---|---|---|---|---|
| 1 | BB | 0.77 | 0.79 | 0.74 | 0.77 | 0.76 | 0.77 | 2.3 |
| | GC | 1.14 | 1.17 | 1.13 | 1.11 | 1.15 | 1.14 | 1.9 |
| | GA | 3.26 | 3.31 | 3.21 | 3.32 | 3.20 | 3.26 | 1.7 |
| | GB | 1.43 | 1.43 | 1.39 | 1.40 | 1.47 | 1.42 | 2.2 |
| 2 | BB | 0.93 | 0.95 | 0.93 | 0.92 | 0.97 | 0.94 | 2.1 |
| | GC | 1.00 | 1.05 | 1.05 | 1.04 | 1.00 | 1.03 | 2.5 |
| | GA | 2.17 | 2.23 | 2.18 | 2.24 | 2.25 | 2.21 | 1.6 |
| | GB | 1.10 | 1.15 | 1.13 | 1.08 | 1.09 | 1.11 | 2.6 |
| 3 | BB | 0.53 | 0.53 | 0.50 | 0.54 | 0.51 | 0.52 | 3.2 |
| | GC | 1.10 | 1.13 | 1.08 | 1.08 | 1.14 | 1.11 | 2.5 |
| | GA | 2.97 | 2.91 | 3.02 | 2.95 | 2.95 | 2.96 | 1.4 |
| | GB | 1.92 | 1.97 | 1.98 | 1.94 | 1.90 | 1.94 | 1.7 |
| 4 | BB | 1.27 | 1.20 | 1.29 | 1.24 | 1.31 | 1.26 | 3.4 |
| | GC | 0.73 | 0.78 | 0.77 | 0.71 | 0.73 | 0.74 | 4.1 |
| | GA | 2.76 | 2.69 | 2.79 | 2.68 | 2.73 | 2.73 | 1.7 |
| | GB | 2.12 | 2.05 | 2.13 | 2.07 | 2.07 | 2.09 | 1.7 |
| 5 | BB | 0.62 | 0.67 | 0.69 | 0.69 | 0.63 | 0.66 | 5.0 |
| | GC | 1.37 | 1.28 | 1.35 | 1.31 | 1.28 | 1.32 | 3.1 |
| | GA | 1.93 | 1.91 | 2.02 | 1.98 | 1.97 | 1.96 | 2.2 |
| | GB | 1.51 | 1.47 | 1.51 | 1.56 | 1.58 | 1.53 | 2.9 |

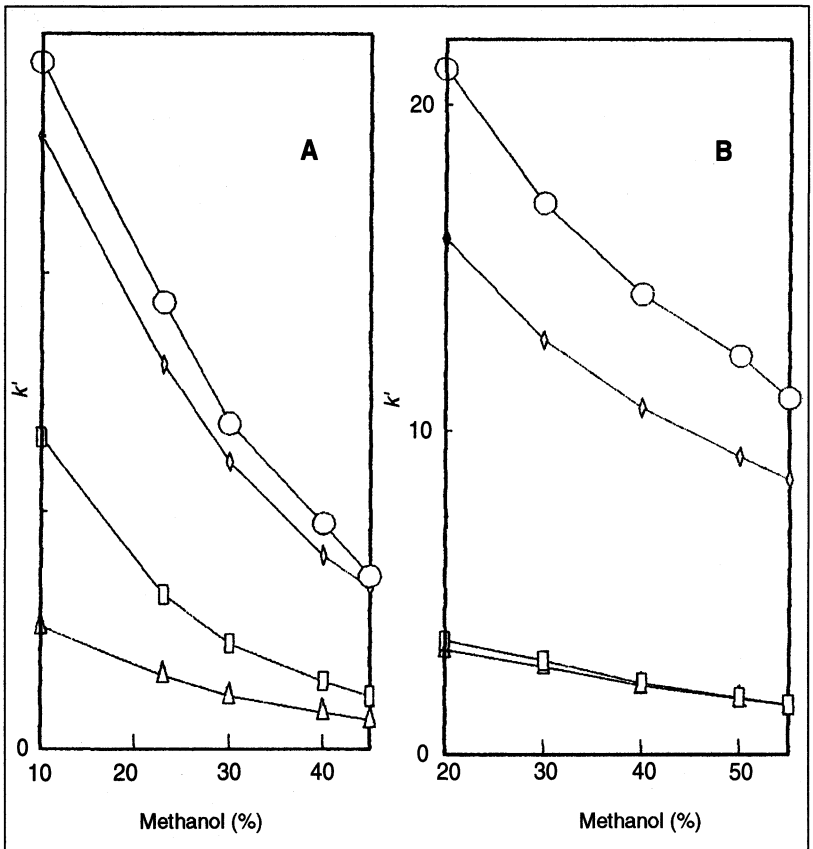


**Figure 2.** Retention characteristics of the terpenoids on Nova-pak $C_{18}$ (A) and YWG $C_{18}$ (B) columns. BB (triangle), GC (rectangle), GA (diamond), and GB (circle).

and refractive index detection for the analysis of these compounds. Unfortunately, their purification procedure was very complex and difficult to reproduce. To our knowledge, capillary electrophoresis (CE), which has been considered as an alternative to the HPLC method (9), has not been applied to analyze real samples yet. This paper describes an efficient and simple method to analyze GA, GB, GC, and BB in GBE for control of the manufacturing process.

## Experimental

### Materials

Standards of GA, GB, GC, and BB were obtained from the Kunmin Plant Research Institute of the Chinese Academy of Science (Kunmin, China). The purity was checked by reversed-phase HPLC, UV spectroscopy, and 200-MHz proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy. The methanol and ethyl acetate were of analytical reagent grade, and the water was purified using a Milli-Q purifier (Millipore, Milford, MA).

### Chromatographic conditions

Chromatographic runs were performed on a Nova-pak $C_{18}$ column (150 × 3.5 mm) from Waters Associates (Milford, MA) with a model 510 pump, a model U6K universal injector, and a model R401 refractive index (RI) detector (Waters) connected to a Baseline 810 chromatographic workstation (Waters). To preserve the life of the analytical column, a Pre-pak $C_{18}$ column (Waters) was used. The eluent was methanol–water (23:77) at a flow rate of 1.5 mL/min. The peaks were recorded and integrated by a Baseline 810 chromatographic workstation.

### Sample preparation

GBE (300 mg) was dissolved in methanol (3 mL) in an ultrasonic bath. Water (12 mL) was then added into the sample solution and mixed well. Ethyl acetate (20 mL) was added to the resultant mixture, and the mixture was shaken on a vortex mixer for 40 min and then centrifuged at 3000 rpm; the ethyl acetate phase was transferred to a glass tube. The extraction procedure was repeated five times. Upon extraction, the combined ethyl acetate layer was evaporated by nitrogen at 50°C. The residue was dissolved in 2 mL of methanol, and 20 µL of the sample solution was injected into the HPLC system for analysis.

## Results and Discussion

### Optimization of the chromatographic system

To analyze the terpenes in GBE, we used a Nova-pak $C_{18}$ column (Waters) and a YWG $C_{18}$ column (Chromatography Research Center of China, Dalian, China), the packings of which were 4-µm octadecylsilyl-bonded amorphous silica and 10-µm octadecylsilyl-bonded amorphous microparticular, respectively. A typical chromatogram of GBE on the Nova-pak $C_{18}$ column is shown in Figure 1, in which a successful separation of BB, GC, GA, and GB was obtained with retention times of 4.4, 8.1, 18.5, and 21.3 min, respectively. The result was similar to that obtained with the RP-$C_{18}$ column by Van Beek et al. (9). There was also an unknown independent peak at 6.8 min that corresponded to the peak of Ginkgolide J (GJ) in Beek's chromatogram. Without a GJ standard, the peak could not be identified. Figure 2 shows the retention characteristics of the terpenes on different columns. In contrast to the results obtained on the Nova-pak $C_{18}$ column, BB, GC, and the peak at 6.8 min could not be separated completely on the YWG $C_{18}$ column, which may be ascribed to the differences in stationary phase coverage and retention volumes between these two packings. Additionally, the ratio of the eluent components had to be adjusted according to the column used.

### Linear range, detection limits, and precision

To verify the linearity of the proposed method, peak response (area) was assessed by serial dilutions of 1% stock solutions of BB, GA, GB, and GC in methanol. Peak response was found to be linear relative to the concentrations of BB between 5 and 50 µg (four replicates, correlation coefficient [$r$] = 0.999) and to GA, GB, and GC between 4 and 60 µg (four replicates, $r$ = 0.999); the curves passed through the origin, and the detection limits for BB, GA, GB, and GC were 0.5, 0.4, 0.4, and 0.2 µg, respectively.

Reproducibility was determined when the method was applied to extracts with high ginkgolide contents. For this purpose, five 300-mg samples of GBE were analyzed by the proposed procedure (five replicates). The results are given in Table I.

### Sample preparation

In Van Beek's work, a $C_{18}$ column and a polyamide solid-phase extraction column were employed to purify the sample solution. After purification, the sample solution could be analyzed successfully by HPLC–UV. Because of this cleanup procedure, the operation became complex, and the recovery depended on the content of the terpenoids in the sample.

In the proposed HPLC–RI method, ethyl acetate was used to extract the terpenoids from the sample solution, and no peak interference was found in the determination of terpenoids in real samples. In order to determine the recovery and reproducibility of the extraction procedure, the standard solutions of BB, CA, GB, and GC in five different respective concentrations were analyzed by the above procedure (five replicates), and the results are shown in Table II. The recovery was above 96.5%, and the maximum coefficient of variation (CV) was 2.3%. Therefore this simple extraction procedure can provide high recovery and accuracy. Additionally, as described below, this method can also be used to monitor the manufacturing process effectively (see Table IV).

### Analytical applications and production process control

The method described above was applied to determine ginkgolides and bilobalide in GBE, the production of which involved Schwabe patented technology and resin technology. The results are shown in Table III. Additionally, for manufacturing process control, the analyses were completed on the different manufacturing steps, and the results are shown in Table IV. From these two tables, it is shown that the quality of GBE as well as the efficiency of extract production in different steps can be monitored effectively by the proposed method.

A growing application area for supercritical fluid extraction (SFE) is the extraction of natural products. SFE technology has many advantages such as high extraction efficiency and a lack of solvent residue. We used the proposed HPLC–RI method to evaluate SFE extraction of *Ginkgo biloba* L. The results shown in Table V demonstrate that the extraction efficiency of terpenoids using SFE without modifier is low and slightly influ-

**Table II. Recovery and the Reproducibility of the Sample Extraction**

| Sample | Amount of standard added (%) | Recovered amounts (%) | | | | | Mean recovery (%) | CV (%) |
|---|---|---|---|---|---|---|---|---|
| 1 BB | 0.40 | 0.38 | 0.38 | 0.40 | 0.39 | 0.41 | 98.0 | 3.3 |
| GC | 0.40 | 0.40 | 0.40 | 0.41 | 0.38 | 0.39 | 99.0 | 2.9 |
| GA | 0.40 | 0.37 | 0.41 | 0.39 | 0.39 | 0.41 | 98.5 | 4.2 |
| GB | 0.40 | 0.40 | 0.41 | 0.41 | 0.39 | 0.39 | 100 | 2.5 |
| 2 BB | 0.80 | 0.79 | 0.79 | 0.80 | 0.82 | 0.79 | 99.8 | 1.6 |
| GC | 0.80 | 0.78 | 0.77 | 0.80 | 0.78 | 0.80 | 98.3 | 1.7 |
| GA | 0.80 | 0.76 | 0.76 | 0.79 | 0.77 | 0.80 | 97.0 | 2.3 |
| GB | 0.80 | 0.77 | 0.78 | 0.79 | 0.75 | 0.76 | 96.5 | 1.9 |
| 3 BB | 1.20 | 1.17 | 1.20 | 1.18 | 1.22 | 1.19 | 99.3 | 1.6 |
| GC | 1.20 | 1.20 | 1.21 | 1.21 | 1.17 | 1.18 | 99.5 | 1.5 |
| GA | 1.20 | 1.17 | 1.22 | 1.21 | 1.19 | 1.18 | 99.5 | 1.7 |
| GB | 1.20 | 1.21 | 1.19 | 1.19 | 1.19 | 1.18 | 99.3 | 0.9 |
| 4 BB | 1.60 | 1.54 | 1.57 | 1.62 | 1.59 | 1.61 | 99.1 | 2.0 |
| GC | 1.60 | 1.57 | 1.57 | 1.62 | 1.58 | 1.61 | 99.4 | 1.5 |
| GA | 1.60 | 1.55 | 1.55 | 1.59 | 1.61 | 1.59 | 98.6 | 1.7 |
| GB | 1.60 | 1.54 | 1.55 | 1.60 | 1.57 | 1.59 | 98.1 | 1.6 |
| 5 BB | 2.00 | 2.05 | 2.02 | 1.96 | 1.99 | 1.97 | 99.9 | 3.7 |
| GC | 2.00 | 2.00 | 2.00 | 2.06 | 2.01 | 1.95 | 100.2 | 2.0 |
| GA | 2.00 | 1.95 | 1.98 | 1.99 | 2.00 | 1.95 | 98.7 | 1.2 |
| GB | 2.00 | 2.01 | 2.01 | 2.00 | 1.96 | 1.99 | 99.7 | 1.0 |

**Table III. GBE Sample Analysis Results**

| Sample | BB (%) | GC (%) | GA (%) | GB (%) | Total (%) |
|---|---|---|---|---|---|
| 1 | 1.14 | 0.72 | 3.27 | 0.93 | 6.06 |
| 2 | 1.10 | 0.81 | 3.12 | 0.99 | 6.02 |
| 3 | 0.72 | 0.73 | 2.41 | 0.32 | 4.18 |
| 4 | 0.81 | 0.52 | 2.04 | 0.65 | 4.02 |
| 5 | 0.51 | 1.31 | 2.87 | 1.37 | 6.06 |
| 6 | 0.52 | 1.29 | 3.10 | 1.13 | 6.04 |

**Table IV. Sample Analysis Results from Different Manufacturing Steps***

| Step | BB (%) | GC (%) | GA (%) | GB (%) | Total (%) |
|---|---|---|---|---|---|
| 1 | 0.51 | 0.23 | 0.72 | 0.51 | 1.97 |
| 2 | 0.72 | 0.27 | 0.87 | 0.56 | 2.42 |
| 3 | 0.87 | 0.54 | 0.98 | 0.84 | 3.23 |
| 4 | 1.07 | 0.72 | 2.37 | 1.01 | 5.17 |
| 5 | 1.12 | 0.83 | 2.67 | 1.43 | 6.05 |

* Resin technology.

**Table V. SFE Extract Analysis Results**

| | BB (%) | GC (%) | GA (%) | OB (%) | Total (%) |
|---|---|---|---|---|---|
| Pure $CO_2$ (#1) | < 0.10 | 0.11 | 0.21 | 0.14 | < 0.56 |
| Pure $CO_2$ (#2) | < 0.10 | 0.11 | 0.24 | 0.14 | < 0.59 |
| Pure $CO_2$ (#3) | 0.11 | 0.13 | 0.25 | 0.17 | 0.66 |
| Modifier A | 0.23 | 0.20 | 0.51 | 0.31 | 1.25 |
| Modifier B | 0.31 | 0.21 | 0.63 | 0.29 | 1.44 |
| Modifier C | 0.17 | 0.25 | 0.58 | 0.34 | 1.34 |

enced by the temperature and pressure of extraction. This can be explained by the fact that the polarity of the extracts obtained by SFE without any modifier is situated between *n*-hexane and chloroform, and the terpenoids are polar compounds; therefore, it is impossible to extract the terpenoids with pure $CO_2$. However, adding some solvent modifiers could raise the efficiency of extraction, and different modifiers could bring about different extraction recoveries. In addition, when using the proposed method to analyze the samples prepared with SFE technology, fat-soluble compounds in the samples should be removed with *n*-hexane. Otherwise, the sample would not dissolve in a solvent mixture of methanol and water, and recoveries would be low.

## Conclusion

This method is simple, sensitive, and reproducible for the determination of terpenes in GBE. The sample preparation offered in the proposed method is very simple because only the extraction procedure, instead of the conventional cleanup steps, is required. Good precision and accuracy were achieved with no need for any internal standard. The assay is suitable for the production process control of GBE.

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