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Original Article

Bioactive metabolites in of *Ginkgo biloba* leaves: variations by seasonal, meteorological and soil

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(With 6 figures)

Abstract

Ginkgo biloba is a traditional Chinese herbal medicine containing multiple components that contribute to its notable bioactivities. Variations of seasonal, meteorological and planting soil on the phytochemicals contents in *G. biloba* leaves due to the effects of growth meteorological and soil parameters were investigated in this study. The leaves of *G. biloba* were collected from different months and place in Zhejiang province, the contents of flavones (quercetin, kaempferol and isorhamnetin) and terpene lactones (bilobalide, ginkgolides A, B and C) were quantified by high performance liquid chromatography (HPLC) and the evaporative light scattering detector (ELSD) method. The established methods were validated with good linearity, precision, repeatability, stability, and recovery. Comprehensive analysis suggested the proper harvest time for *G. biloba* was in October of Zhejiang province. The result of correlation analysis with meteorological factors shows that the temperature and precipitation have non-significant effect on the main components of *G. biloba*. In addition, the type and content (Mn and Zn) of the soil showed significantly effect on the content of flavonoids and terpene lactones. This study enriched the knowledge on the development and utilization value of the *G. biloba* leaves and was useful for determining the optimal harvest time and growing condition.

Keywords: Ginkgo biloba leaves, flavonol, terpene lactones, meteorological parameters, soil parameters.

Metabólitos bioativos em folhas de *Ginkgo biloba*: variações sazonais, meteorológicas e do solo

Resumo

Ginkgo biloba é um fitoterápico tradicional da medicina chinesa que contém vários componentes que contribuem para suas notáveis bioatividades. Variações sazonais, meteorológicos e de plantio do solo sobre os teores fitoquímicos em folhas de *G. biloba*, devido aos efeitos do crescimento meteorológico e parâmetros do solo, foram investigadas neste estudo. As folhas de *G. biloba* foram coletadas em diferentes meses e na província de Zhejiang, os teores de flavonas (quercetina, kaempferol e isorhamnetina) e lactonas terpênicas (bilobalida, ginkgolídeos A, B e C) foram quantificados por Cromatografia Líquida de Alta Eficiência (CLAE) e pelo método do detector de dispersão da luz por evaporação. Os métodos estabelecidos foram validados com boa linearidade, precisão, repetibilidade, estabilidade e recuperação. Uma análise abrangente sugeriu que o tempo de colheita adequado para *G. biloba* foi em outubro na província de Zhejiang. O resultado da análise de correlação com fatores meteorológicos mostra que a temperatura e a precipitação não têm efeito significativo sobre os principais componentes de *G. biloba*. Além disso, o tipo e composição (Mn e Zn) do solo apresentaram efeito significativo sobre o teor de flavonoides e lactonas terpênicas. Este estudo enriqueceu o conhecimento sobre o valor de desenvolvimento e utilização das folhas de *G. biloba* e foi útil para determinar o melhor tempo de colheita e condição de crescimento.

Palavras-chave: folhas de *Ginkgo biloba*, flavonol, lactonas terpênicas, parâmetros meteorológicos, parâmetros do solo.

Abbreviations

HPLC, high performance liquid chromatography; ELSD, evaporative light scattering detector; TTL, total terpene lactones; TFG, total flavonol glycosides; GA, ginkgolide A; GB, ginkgolide B; GC, ginkgolide C; BB, bilobalide; RSD, relative standard deviation.

1. Introduction

Ginkgo is one of the oldest documented traditional Chinese medicines, and it is also considered to be a complimentary and alternative medicine nowadays in the United States and Europe (Belwal et al., 2019). *G. biloba* extract has been extensively used therapeutically to increase peripheral and cerebral blood flow as well as for the treatment of dementia all over the world for several decades (Krieglstein et al., 1986; Le Bars et al., 1997). As is well-known, *G. biloba* leaves are rich in flavonol glycosides, terpene lactones, biflavones, and proanthocyanidins, and the former two have been considered to be the main components for their beneficial effects and have gotten by far the most attention (Xie et al., 2014). So the quality control of *Gingko* is usually standardized based on the content of ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), bilobalide (BB), quercetin, kaempferol and isorhamnetin (Ding et al., 2006).

It is generally known that external factors (soil, temperature, precipitation and so on) affected some processes associated with the growth and development of the plant, even influencing the ability to synthesize secondary metabolites, resulting in the change of the overall phytochemical profile that plays a strategic role in the production of bioactive substances (Flesch et al., 1992; Wink, 2003). Thus, the production of phytochemical components critically depends on environmental conditions, and the yields in plants gathered from different seasons and areas might be discrepant. The quality and content of bioactive components are influenced significantly by harvest time, cultivation sources and the climate.

In the previous studies, people mainly focused on the analysis of the change of single species composition of *G. biloba by* seasonal (Ding et al., 2007; Lobstein et al., 1991), and few studies involved the external environmental impact (Li and Fitzloff, 2002; Yao et al., 2012; Zhou et al., 2017). Based on our previous research (Lin et al., 2017), the highest TFG in G. biloba leaves harvested in April and Wen'ling. Now, we collected the plant materials consecutive monthly and the data of meteorological and soil parameters in 11 various regions of Zhejiang province, analyzed the content of flavonoids (quercetin, kaempferol and isorhamnetin) and terpene lactones (GA, GB, GC and BB) by using the validated HPLC-ELSD and HPLC-DAD methods, and established the correlation between phytochemical content, meteorological and soil parameters. It could provide a scientific basis for determine the optimal collecting time and growing condition of *Gingko* for medicinal use.

2. Material and Methods

2.1. Plant materials and chemicals

All *Ginkgo* samples were collected consecutive monthly (April-November) in 2013 from 10-20 year old trees located at the Zhejiang Province (China) (Supplementary Material Table S1). Each time, the leaf of different plant was collected at similar site (middle position) for three samples. All samples were dried in the oven at 60 °C to a constant weight, grounded into powder using herbal pulverizer and sieved through 250 μm filter. In addition, quercetin, kaempferol, isorhamnetin, BB, GA, GB and GC (98% purity) were purchased from National Institute for the Control of Pharmaceutical & Biological Products (Beijing, China). Methanol (spectra analyzed grade) came from Merck Chemicals (Darmstadt, Germany). All other chemicals and solvents in analytical grade were purchased from commercial sources.

2.2. Sample determination

All of the samples were prepared and determined thereafter according to the previously reports (Ding et al., 2007; Xie et al., 2014; Zhou et al., 2017). Quantification of each compound was performed on the basis of linear calibration plots of the peak areas versus the corresponding concentration (Detailed information on Supplementary material). The content of TFG was the sum of quercetin, kaempferol and isorhamnetin. The sum of GC, BB, GA and GB defined the TTL.

2.3. Meteorological and soil data

We obtained meteorological data of monthly meteorological variables from the National Meteorological Information Center (2018). The data were collected from a meteorological station in Zhejiang. Two monthly meteorological variables were included in this study: average temperature and average rainfall. The soil data come from two sources: first, the second Zhejiang province soil census, and second, the Second National Soil Census. Based on the two sources, the data was sorted and the relevant attribute data of the soil system was obtained in our study.

2.4. Soil sampling and analysis

Using mixed soil sample collection method, according to the principle of "random" multi-point mixing, randomly take 5 sampling points within 1 m distance of *G. biloba* growth ground, remove surface humus, take 20-40 cm soil layer, and take 5 sampling points. Mix the soil, each weighing about 1kg, air-dried and then heated at 105 °C till they reached constant weight. Phosphorus is extracted with hydrochloric acid and sulfuric acid solution, determined by plasma emission spectrometry; Copper, Zinc, Iron, Magnesium, Calcium, Potassium and Manganese were determined by flame atomic adsorption spectrometer (240AA, Varian Inc., USA), equipped with a GTA 120 graphite tube atomizer and a programmable sample dispenser PSD120. The pH was measured using a FiveEasy Plus pH meter (Mettler-Toledo LLC, Columbus, OH, USA).

2.5. Statistical analysis

All resulting data were presented as the mean standard error of three replications. SPSS version 13.0 (IBM, Armonk, NY, USA) was used for statistical analysis. ANOVA was conducted to compare the compound content every month. A correlation was considered to be significant when $p < 0.01$. The results of ANOVA showed the significant differences at the 0.01 significance level for multiple comparisons among the different months. Correlation between the content of detected compounds and climatic parameters were determined using Pearson's correlation analysis by SPSS.

3. Results

3.1. Dynamic accumulation of chemical components in leafs of G. biloba

As we all know, terpene lactones and flavonoids were considered as the most effective constituents in *G. biloba*, which have been the focus of academic research (Li et al., 2009). The 7 investigated constituents were well separated under the HPLC conditions and identified based on comparing with standards. In our research, the contents of flavones (quercetin, kaempferol and isorhamnetin) (Figure 1) and terpene lactones (GA, GB, GC and BB) (Figure 1) were found in all the 264 samples collected from 11 main production areas of Zhejiang in this study. Each sample was analyzed of these chemical compounds, and the results were shown in Figure 2. It was easy to find that the flavones glycosides and terpene lactones being more abundant in all 264 samples with the total contents varied from 3.44 to 18.72 mg/g and 2.08 to 7.15 mg/g. The contents of these detected constituents were in the order of GC > kaempferol glycosides > quercetin glycosides > GA > isorhamnetin > GB > BB. Among the major flavones glycosides and terpene lactones, kaempferol glycosides (0.763-3.23 mg/g) and GC (1.04-3.67 mg/g) were the most abundant in all the samples.

The main flavones glycosides and terpene lactones increased from the early season and reached to a peak high in October, then declined markedly towards the end of the season (Figure 2). For an individual sample, in general, the lowest amount of flavones glycosides and terpene lactones in each place appeared in November and the highest amount in April. The concentration of GC, quercetin, kaempferol and isorhamnetin fluctuated most by seasons, and BB,GB were relatively constant over the whole vegetative year in *G. biloba* leaves. Consequently, the optimum harvest time of leaf from *G. biloba* should be the before turning yellow stage in October. This is consistent with the traditional harvesting time and previously reports (Shi et al., 2012; Zhou et al., 2017).

3.2. Climate fluctuation of the chemical content

There is a certain growth correlation between cells, tissues, and organs in the plants: they are independent of each other, meanwhile, closely related. In addition, the environmental factors, such as moisture, temperature, and light, show significant influence during plant growth and component accumulation (Akula and Ravishankar, 2011; Gairola et al., 2010).

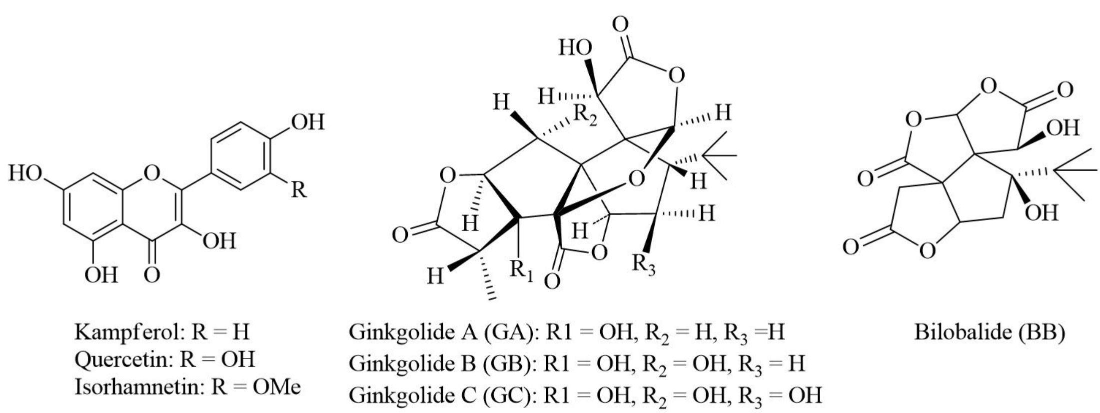

Figure 1. The structures of Kaempferol, Quercetin, Isorhamnetin, GA, GB, GC and BB.

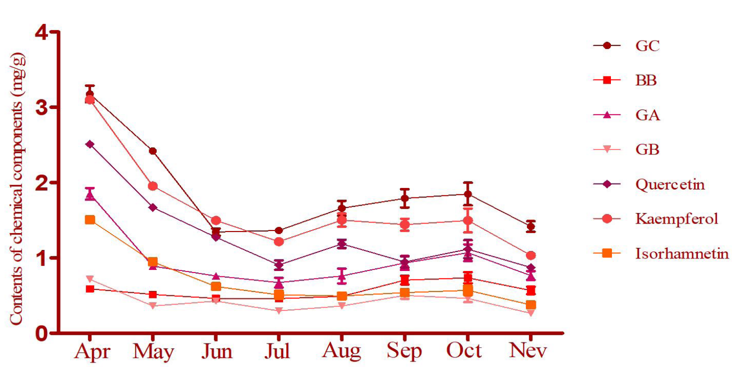

Figure 2. Main components in *G. biloba* leaves during the whole vegetative year.

In current work, the total terpene lactones (TTL) and total flavonol glycosides (TFG) were analyzed for any correlation to temperature and rainfall (Figure 3). The investigation found that there was not any significant correlation between these compound levels and meteorological parameters. Under natural conditions, the natural factors affected are more affected, and the experimental design conditions will be different. The different results had showed in previous report, the correlation between flavonoid compounds levels and climate parameters has been reported that dry climate resulted in an increase in flavonoids content (Zhu et al., 2010). As seen in the present study, the content of flavonoids was slightly elevated in August, but TTL does not have this phenomenon, which deserves our further study.

3.3. Variation of the chemical content with the effect of soil

In consideration of soil as the only variable, we selected samples from the harvesting period to evaluate the impact of the soil parameters (Figure 4, Table 1 and Supplementary Material Figure S3). Three major flavones were quantified, kaemferol being the more abundant in all 11 samples (Figure 5). The levels of kaempferol ranged from 1.04 to 2.71 mg/g, quercetin and isorhamnetin

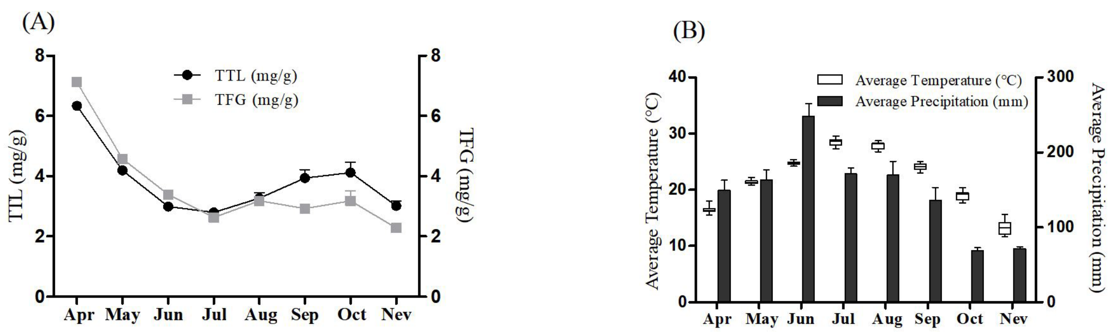

Figure 3. Effect of climate parameters on the mean contents of TFG and TTL in leaves of *G. biloba*. (A) The contents of TTL and TFG; (B) The average monthly temperature and precipitation.

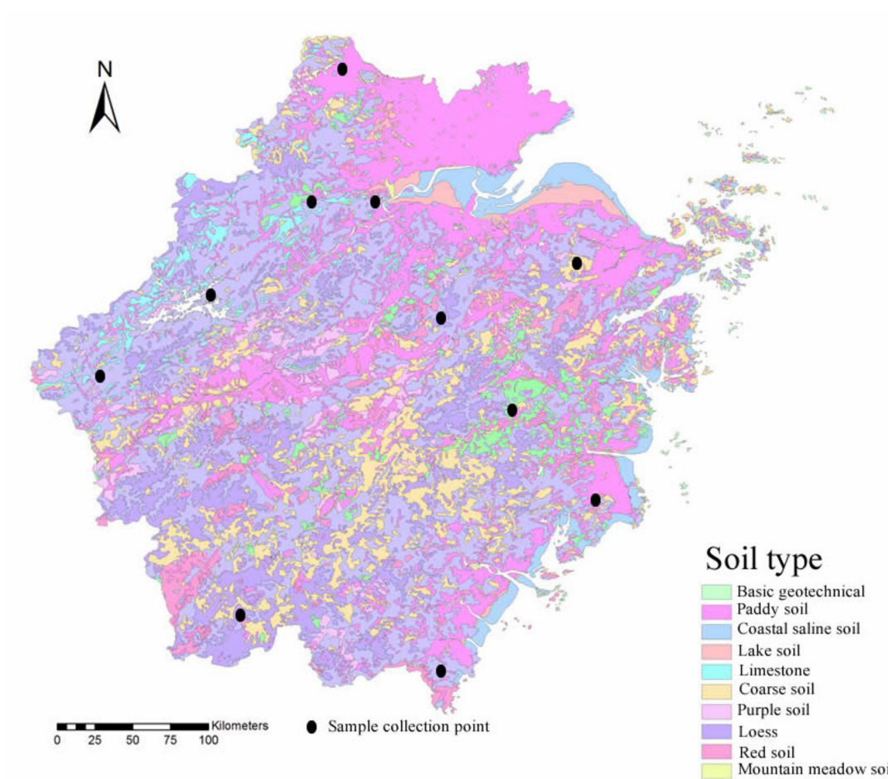

Figure 4. Sample collection information and soil map of Zhejiang province.

Table 1. Chemical properties of the selected soil in 11 producing area.

Producing area	pH	Fe	Mg	Ca	K	P	Mn[a,b]	Cu	Zn[a,b]
Kai'hua	5.6	133.4	60.9	1180.4	130.5	357.1	19.0	3.4	1.5
Chang'xin	5.2	82.8	60.3	8985.3	221.3	277.9	28.8	1.6	1.2
Qin'yuan	5.4	119.6	41.3	2882.2	184.3	289.8	15.6	0.9	1.5
Chun'an	5.8	241.5	72.6	8466.6	83.1	417.8	28.4	4.8	1.8
Zhu'ji	5.8	71.3	60.1	1073.5	324.5	360.5	77.5	1.4	3.5
Lin'an	5.5	108.1	62.1	6893.0	472.3	165.0	15.2	1.6	2.1
Hang'zhou	5.7	151.8	72.2	5453.6	141.3	517.0	33.5	2.5	1.2
Cang'nan	5.1	119.6	122.1	8513.2	241.5	720.3	21.8	4.9	2.3
Yu'yao	5.5	144.9	202.3	8135.8	290.5	623.1	31.7	4.9	2.1
Pan'an	5.8	75.9	41.0	2105.3	102.0	532.9	6.1	2.0	0.9
Wen'ling	6.0	232.3	91.2	2653.3	134.8	555.1	92.1	5.7	2.7

[a]Significantly correlated with TTL ($p < 0.01$); [b]Significantly correlated with TFG ($p < 0.01$). Data are mean values of three replications (mg/kg).

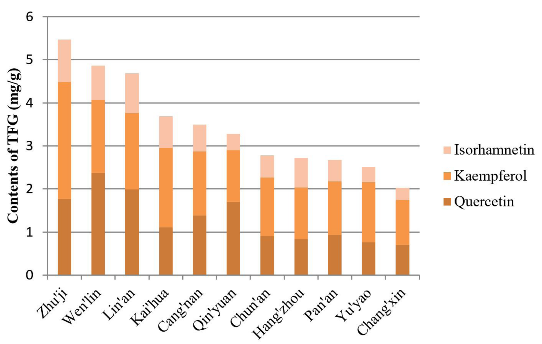

Figure 5. Effects of different cultivation sources on TFG of *G. biloba.*

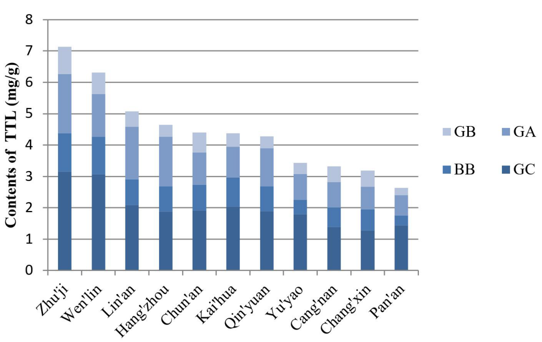

Figure 6. Effects of different cultivation sources on TTL of *G. biloba.*

ranged from 0.70 to 2.38 mg/g and 0.35 to 0.99 mg/g. As the results (Figure 5 and 6), samples from Zhu'ji major contained the highest level of terpene lactones and flavones (7.13 and 13.72 mg/g). Samples contained the lowest terpene lactones (2.64 mg/g) and flavones (5.08 mg/g) from Pan'an and Chang'xin, respectively.

Meanwhile, the variance in contents of terpene lactones from 11 producing areas were showed in Figure 6. It can be seen that the TTL in *G. biloba* analyzed varied from 2.64 to 7.13 mg/g. Most samples contained terpene lactones between 3.2 and 4.6 mg/g, but several samples had extremely high levels of the compound, e.g. samples collected from Zhu'ji (7.13 mg/g). However, BB was hardly detected in samples from Pan'an (0.32 mg/g), and also existed in trace amounts in several samples from other producing areas, e.g. in Qin'yuan (0.47 mg/g).

All the results indicated that the contents of the major bioactive constituents in *G. biloba* leaves varied greatly from different producing areas. The contents of these detected constituents were in the order of Loess > Paddy soil > Mountain meadow soil > Red soil > Coastal saline soil. This discrepancy might be accounted by the different soil type. Correlation analysis between soil organic matter and active constituents of *G. biloba* leaves showed that there was no necessary connection. Meanwhile, we analyzed the main element contents of soils in 11 producing areas (Table 1). It was found that in the areas with high active ingredient content of *G. biloba*, the Mn and Zn elements in the soil were also higher, showed a significant positive correlation.

At present, TTL and TFG were chosen as the quality control of *G. biloba* in Chinese Pharmacopoeia (National Pharmacopoeia Committee, 2015). From our experiment results, according to rule of Chinese Pharmacopoeia (the contents of TTL and TFG should not be less than 0.25% and 0.40%), the 264 batches samples analyzed were all met the Pharmacopoeia standard. Moreover, the significant differences in the contents of GC and quercetin were found in *Ginkgo* among different producing areas, suggesting that the current assessment and quality control standard were insufficient to distinguish the quality of *G. biloba*.

4. Discussion

The flavonoid glycosides and terpene lactones in *G. biloba* leaves are secondary metabolites of *G. biloba*. They are not only restricted by secondary metabolic processes, but also closely related to primary metabolism

(Cheng et al., 2009). The influencing factors are many and the process is complicated. Soil properties and climatic factors are important factors affecting the cultivation conditions of *G. biloba*. Firstly, it affects the primary metabolism of plants, directly affects the growth and development of plants, and also has a greater impact on secondary metabolic processes. The impact of accumulation of flavonoids and terpene lactones from *Ginkgo* has been discussed in the past, but the results vary widely (Leng et al., 2001; Zhu et al., 2010). The complexity of the relationship between the metabolism of active ingredients in *G. biloba* leaves and environmental factors was further confirmed. According to the dynamic changes of flavonols and terpene lactones content in the whole growing season of *G. biloba* leaves, the content of active ingredients appeared the first peak in April and the second peak appeared in October. Although the content of the components in *G. biloba* leaves was high in April, it was obviously not feasible to pick leaves at the young leaf stage, and the comprehensive medicinal quality and yield indexes of the leaves did not meet the production requirements. In October, the leaves of *G. biloba* began to decline, and the content of active ingredients was relatively high. Therefore, it is considered that the best time for picking leaves is in October. The results indicates that there is a correlation between the important secondary metabolites and the growth of *G. biloba*, and the secondary metabolism and primary growth are coordinated with each other, showing a certain seasonal variation.

The accumulation of dynamic components in *G. biloba* leaves showed a process of decreasing first, then increasing and then decreasing in different producing areas. This process has nothing to do with the production environment. Therefore, according to the characteristics of dynamic accumulation, timely harvesting can not only ensure the quality of *G. biloba* leaves and the inherent medicinal quality. In addition, the sample selection range of this study is relatively small, and the meteorological information generated is basically the same, so the meteorological factors do not have a significant impact in this study. In the follow-up study, the sampling range can be further expanded to study the relationship between secondary metabolites of *G. biloba* leaves and climatic factors in different provinces.

Nutrients often control the metabolism of plants as a whole and also affect secondary metabolites (Chishaki and Horiguchi, 1997; Forde and Lorenzo, 2001). In this study, the nature and level of inorganic element content distribution were analyzed. The contents of nine inorganic elements such as Fe, Mg, P, K, Ca, Mn, Fe, Zn and Cu in soils from 11 producing areas were determined, and the data were analyzed in combination with the properties of soils to clarify the soils of different producing areas. It was found that *G. biloba* leaves grown in loess and paddy soil had higher content of TFG and TTL. And the main ingredients of *G. biloba* have a significant positive correlation with the content of Mn and Zn in the soil. It may contribute to Mn is a structural component of chloroplasts, which can affect the energy metabolism of cells and the metabolism of carbohydrates (Papadakis et al., 2007). Zn is a component or activator of some enzymes, participates in the hydration of CO_2 in photosynthesis (Ohki, 1976), promotes protein metabolism and production of secondary metabolites of *G. biloba*. The specific impact mechanism of the above findings remains to be further studied.

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Supplementary Material

Supplementary material accompanies this paper.
S1. Preparation of standard solutions.
S2. Preparation of samples solutions
S3. Validation of the methods
S5. Chromatographic conditions and instrumentation
This material is available as part of the online article from http://www.scielo.br/bjb