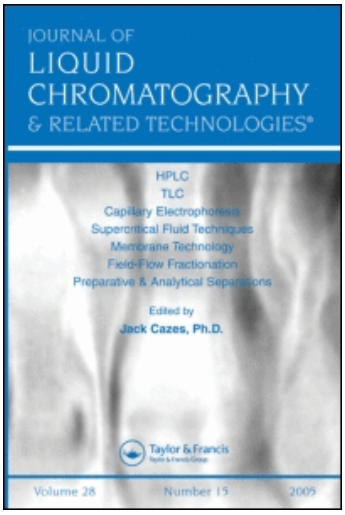

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HPLC DETERMINATION OF FLAVONOIDS AND TERPENE LACTONES IN COMMERCIAL *GINKGO BILOBA* PRODUCTS

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ABSTRACT

Ginkgo biloba products are one of the top ten botanical dietary supplements in the USA. The active constituents include flavonoids and terpene lactones (ginkgolides and bilobalide). Ginkgo flavonoids have been associated with reduced lipid peroxidation in vascular walls and nerve cells. Ginkgolides are well known to be antagonists of platelet-activating factor (PAF). Usually, enriched ginkgo extracts used for the preparation of ginkgo products are standardized to contain 24% flavonoids and 6% terpene lactones. In the present work, we examined nine commercial ginkgo products for the content of total flavonoids and terpene lactones by using high performance liquid chromatography

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(HPLC) with ultraviolet (UV) and evaporative light scattering detection (ELSD), respectively. The methods are reliable and sensitive with detection limits of 2 ng for flavonoids on column with HPLC-UV and 20–35 ng for terpene lactones on column with HPLC-ELSD. The results show that most of the commercial ginkgo products tested contain flavonoids and terpene lactones as claimed on the label.

INTRODUCTION

Currently, there is growing evidence about the benefits of ginkgo (*Ginkgo biloba* L.). Extracts from ginkgo leaves have been widely used for the treatment of cerebrovascular insufficiency, peripheral circulatory problems, and for slowing the progression of cognitive deficits, including Alzheimer's-type and multi-infarct dementia.[1–3] Flavonoids and structurally unique terpene lactones (ginkgolides and bilobalide) are believed to be the pharmacologically active constituents in the extracts.

Ginkgo containing products usually contain enriched ginkgo extracts and accessory agents, and are in the form of capsules, caplets, tablets, or softgels. Under the Dietary Supplement Health and Education Act (DSHEA) of 1994, ginkgo products are marketed as dietary supplements in the USA. Since DSHEA only places the responsibility of safety, but not the burden of proof for the product on the manufacturers, ginkgo products have not been subjected to mandated quality assurance (QA) standards. As a consequence, the product might differ from brand to brand, or even from lot to lot.[4]

Generally, enriched ginkgo extracts for the preparation of ginkgo products are standardized to contain 24% flavonoids (22–27%) and 6% terpene lactones,[5] and, therefore, the content of flavonoids and terpene lactones is one of the important parameters to assess the quality of ginkgo products. For the determination of flavonoids, the method based on the hydrolysis of flavonoid glycosides and their spectrophotometric detection as an aluminium chloride complex is not very specific and permits only an approximate estimation of the total flavonoids in the samples. It cannot give information on individual flavonoid aglycones and is not reproducible due to the high amount of interfering constituents, such as proanthocyanidins.[5] High performance liquid chromatography (HPLC) and subsequent UV or photodiode array detection (PDA) allows selective analysis of the ginkgo flavonoids. Although, there have been more than 30 flavonoids reported from ginkgo leaves,[6–7] most of the genuine flavonoid glycosides can be reduced by hydrolysis to three major flavonoid aglycones, quercetin, kaempferol, and isorhamnetin, which could be easily quantified by using HPLC-UV.[7–8] The total flavonoid content in ginkgo products could be

calculated by converting the content of quercetin, kaempferol, and isorhammetin found in the hydrolyzed sample to that of a flavonol coumaroyl ester glycoside, 3-O-{-2-O-[6-O-(*p*-hydroxy-*trans*-cinnamoyl)-*β*-*D*-glucosyl}-α-*L*-rhamnosyl}-quercetin (MW 756.7), with conversion factors of 2.51, 2.64, and 2.39 for quercetin (MW 302.2), kaempferol (MW 286.24), and isorhamnetin (MW 316.27), respectively.[7] This method has already been applied for the quality evaluation of ginkgo leaves, standardized extracts, and products in Europe[5–7] and China.[9] However, to our knowledge, there has been no data reported for the content of total flavonoids in the commercial ginkgo products sold in the USA.

With regard to ginkgo terpene lactones, many investigations have been carried out on their separation and determination. However, the terpene lactones are poor chromophores with very weak absorption in the 200–220 nm range. Even trace impurities interfere with the detection of these compounds by HPLC-UV.[10–11] HPLC-IR was proposed and has been used with considerable success in some cases.[12] But the sensitivity and baseline stability of the method remain a problem. GC-FID might be very reliable for the sensitivity and reproducibility, but it requires tedious purification and sample derivatization before analysis. Multi-step manipulations are time consuming and might introduce additional sources of error.[13–16] GC-MS,[13,17] HPLC-MS,[13,18] and NMR[19] methods have one or more advantages in terms of simplicity, sensitivity, and selectivity. However, the instruments are expensive and might not be available in all QA/QC laboratories. The alternative choice is HPLC coupled with the relatively inexpensive evaporative light scattering detection (ELSD). Camponovo et al.[13] first introduced the HPLC-ELSD to the determination of ginkgolides and bilobalide in ginkgo products, with the limits of detection (LOD) of 1100 ng for ginkgolide A, B, C, J, and bilobalide on-column, respectively. Recently, a more sensitive HPLC-ELSD method has been proposed with the LOD of 203 ng for ginkgolides and bilobalide on the column.[20] Unfortunately, the reported sample preparation method only employed a single extraction step with methanol (3×3 mL), which might introduce increased baseline and/or increased signals for the compounds of interest in the HPLC-ELSD chromatogram. It is well known that the ELSD is a mass detection method. Any constituent in the sample co-eluted with the compound of interest will increase the peak area of the compound of interest and result in over-quantification. In the case of ginkgo, besides trace amounts of ginkgolides and bilobalide, there are at least 12 phenolic compounds,[21] more than 30 flavonoids,[7] and variable amounts of tannins, anacardic acids, chlorophyll, and lipids[12] in the crude extract; therefore, a simple refinement step is necessary to remove these non-terpene lactone constituents and matrix as well.

In the present study, HPLC-UV and HPLC-ELSD methods were developed and validated for the determination of total flavonoids and terpene lactones (ginkolide A, B, C, J, bilobalide) in nine commercial ginkgo products. The variability of flavonoids and terpene lactones in these products are also presented.

EXPERIMENTAL

Standards and Samples

Reference standards of ginkgolide A, B, C, J, bilobalide, quercetin, kaempferol, and isorhamnetin (Fig. 1) were from the repository of natural products isolated in our laboratories, and their purity was determined to be more than 99% by employing HPLC-UV and HPLC-ELSD analysis. Ginkgo products, including caplets, capsules, and tablets were purchased at local pharmacies, Chicago, IL, USA.

Reagents

HPLC grade acetonitrile, ethyl acetate, and methanol, and analytical grade trifluoroacetic acid (TFA) and hydrochloric acid (HCl) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized (DI) water was obtained with an in-house Nano-pure® water system (Barnstead, Newton, MA, USA).

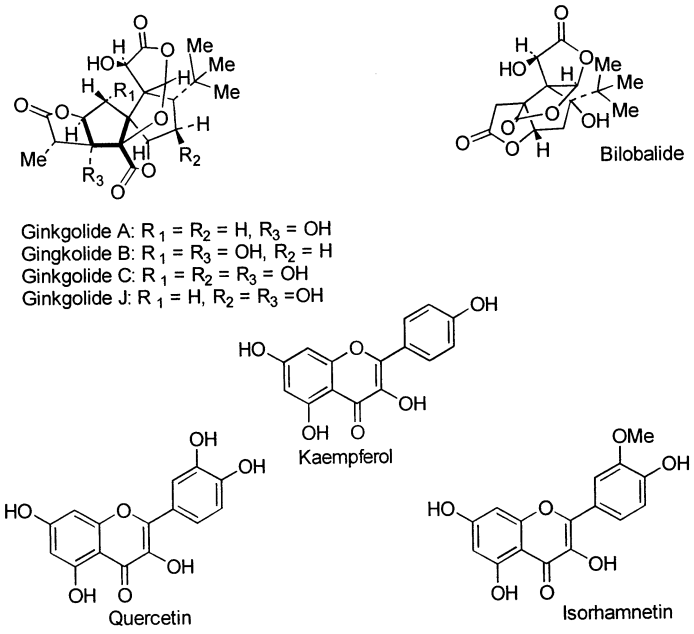

Figure 1. Chemical structures of quercetin, kaempferol, isorhamnetin, ginkgilode A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide.

Sample Preparation

Five units (capsules, tablets, or caplets) of ginkgo product were exactly weighed into a 50 mL tared flask and extracted with 80% methanol (v/v) (25 mL) by sonication at room temperature for 60 min. After cooling, the mixture was filtered through a filter paper (Whatman # 1) into a 250 mL round-bottom flask, and the residue was returned to the flask. Another 25 mL of 80% methanol (v/v) was added and the mixture was sonicated at room temperature for 30 min. The extract was filtered through a filter paper (Whatman # 1) into the same round-bottom flask. The above procedure was repeated one more time. The combined methanolic extracts were evaporated under reduced pressure at 45°C. The resulting residue was dissolved in 80% methanol (v/v), and transferred into a 50 mL volumetric flask and made up to the volume with 80% methanol (v/v). Triplicate sample solutions were prepared for each product.

For the analysis of total flavonoids, 5 mL of the sample solution was transferred into a 250 mL round-bottom flask and refluxed with 25 mL of HCl (4N) : MeOH (1 : 4) (v/v) for 30 min. After cooling, the mixture was transferred into a 50 mL volumetric flask and made up to the volume with methanol. After centrifugation, 10 μL of the supernatant was subjected to HPLC–UV analysis.

For the analysis of terpene lactones, 10 mL of the sample solution was evaporated under reduced pressure at 45°C, and the resulting residue was suspended in 20 mL of hot water and extracted with acetyl acetate (3 × 20 mL) by liquid–liquid extraction. The combined acetyl acetate extracts were evaporated under reduced pressure at 45°C and the resulting residue was re-dissolved and transferred with methanol to a 10 mL volumetric flask and made up to the volume with methanol. The sample solution was centrifuged before 10 μL of the supernatant was subjected to HPLC–ELSD analysis.

HPLC-UV and HPLC-ELSD Analysis

A Waters 2690 Alliance HPLC system (Milford, MA, USA), equipped with an on-line degasser, an autosampler, and a 996 photodiode array detector, was used for solvent delivery and detection. The separations were carried out on a Supelco Discovery RP-18 column (250 × 4.6 mm, 5 μm particle size, col # 24855-08, bonded phase lot # 3651, silica lot # PS 183) (Supelco, Bellefonte, PA, USA) protected by a Waters Delta-Pak RP-18 guard column (Waters Technology Ireland, Ltd, Wexford, Ireland) and set at 20°C. The solvents used for chromatography were water (containing 5% methanol and 0.05% TFA), solvent A, and methanol (containing 0.05% TFA), solvent B. For the analysis of flavonoids, the mobile phase was run isocratically with solvent B held at 50% over 20 min.

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Table 1. Linearity Data of Reference Standards

Compound	Method	Slope (a)	Intercept (b)	Correlation Coefficient (r^2)	Concentration Range (ng)
Quercetin	HPLC–UV	3,513.3	8,827.7	0.9994	4–2,000
Kaempferol	HPLC–UV	3,845.1	16,640	0.9992	4–2,000
Isorhamnetin	HPLC–UV	3,935.8	51,409	0.9953	4–2,000
Ginkgolide A	HPLC–ELSD	1.587	1.603	0.9982	40–2,000
Ginkgolide B	HPLC–ELSD	1.5444	1.7144	0.9978	40–2,000
Ginkgolide C	HPLC–ELSD	1.6113	1.5219	0.9975	40–2,000
Ginkgolide J	HPLC–ELSD	1.6678	1.2802	0.9992	70–3,500
Bilobalide	HPLC–ELSD	1.6063	1.5519	0.9976	40–2,000

Concentrations (X) expressed in ng for flavonoids and terpene lactones, the calibration curves for terpene lactones were obtained as Log (concentration, ng) vs. Log (peak area).

For the analysis of terpene lactones, the mobile phase was run as a gradient with solvent B from 25% to 50% over 20 min. After the PDA, the column effluent was directed to a Sedex 75 evaporative light scattering detector (ELSD) (Cedex 94141, Alfortville, France). Nebulization of the eluent in the ELSD was provided by a stream of pressured air at 2.9 bar. The nebulization was performed at room temperature, and the nebulized effluents were evaporated at 61°C. The detector output was interfaced, using a SATIN box, to the Waters Millennium 2000® chromatographic manager system (Waters, Milford, MA, USA) loaded on a Compaq 6400X/10,000/CDS computer (Houston, TX, USA) for data handling and chromatogram generation.

Prior to each run, the HPLC-UV-ELSD system was allowed to warm up for 20–30 min and the pumps were primed using the protocol suggested by the manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

Identification and Quantification

The chromatograms for flavonoids and terpene lactones were monitored with PDA in the UV range of 200–400 nm and ELSD with gain of 11, respectively. Their identification in the samples was carried out by comparing the retention times of reference standards with those obtained in the sample chromatograms. The concentrations of flavonoid aglycones and terpene lactones identified were measured using the external standard method by calibration curves (peak area at 365 nm vs. concentration for flavonoids in UV, log-transformed peak area vs. log-transformed concentration for terpene lactones in ELSD) obtained for these compounds over the range of concentrations observed (Table 1). The concentration of total flavonoids in ginkgo product, expressed as 3-O-{-2-O-[6-O-(*p*-hydroxy-*trans*-cinnamoyl)-*β*-*D*-glucosyl}-α-*L*-rhamnosyl}-quercetin, was calculated by converting the content of total flavonoid aglycones (quercetin, kaempferol, and isorhamnetin) with a conversion factor of 2.51.[7]

RESULTS AND DISCUSSION

HPLC Identification

Figure 2 shows a typical HPLC-UV chromatogram of the HCl-hydrolyzed methanolic extract of a ginkgo product at 365 nm. Three major flavonoid aglycones, quercetin, kaempferol, and isorhamnetin were identified by their PDA (200–400 nm) spectra and by their retention times in comparison with those of reference standards, and also by the method of standard addition to the samples.

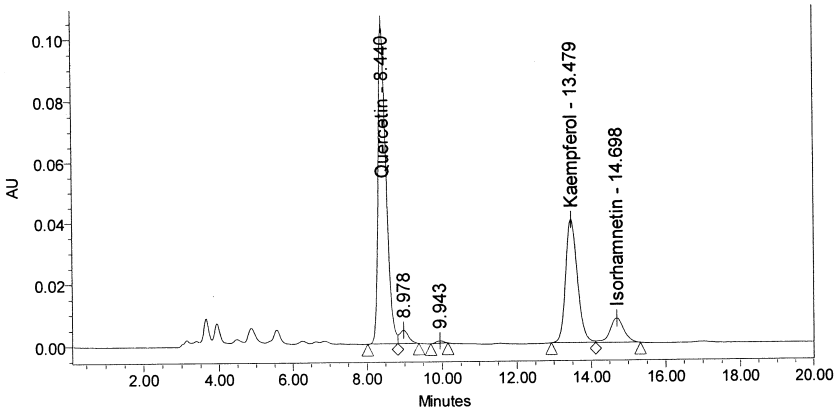

Figure 2. Typical HPLC-UV chromatogram of the HCl-hydrolyzed methanolic extract of a ginkgo product with the retention times of quercetin, kaempferol, and isorhamnetin of 8.44, 13.47, and 14.70 min, respectively.

In the same way, the terpene lactones in ethyl acetate extract were observed in HPLC-ELSD chromatograms (Fig. 3) by their retention times, in comparison with that of reference standards, and by the method of standard addition to the samples.

Method Validation

The current methods were validated for precision, linearity, sensitivity (limit of detection and limit of quantification), and recovery.

Precision

Six aliquots of the same sample were analyzed at a time for flavonoids by using HPLC-UV and terpene lactones by using HPLC-ELSD, respectively. The precision value was expressed as coefficient of variation (CV %), which was 3.18, 2.08, 0.18, 1.63, 2.53, 1.60, 3.77, and 3.37 for quercetin, kaempferol, isorhamnetin, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide, respectively.

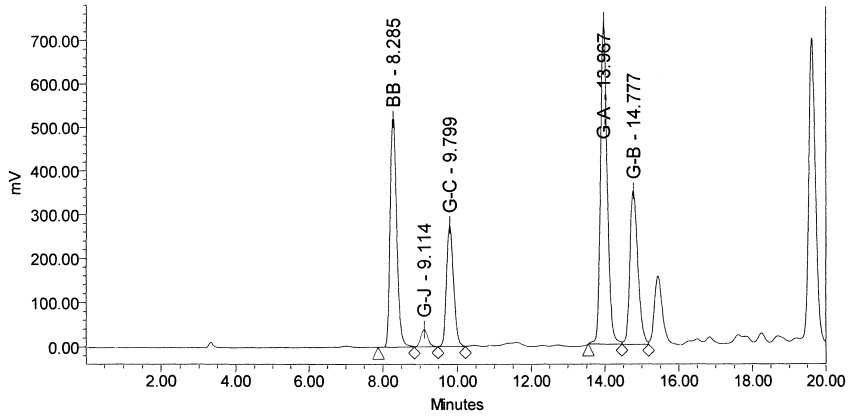

Figure 3. Typical HPLC-ELSD chromatogram of the refined methanolic extract of a ginkgo product with the retention times of bilobalide, ginkgolide J, C, A, and B of 8.29, 9.11, 9.80, 13.97, and 14.78 min, respectively.

Linearity

Seven to nine non-zero point linearity of the standard curves was studied for all reference standards tested. As shown in Table 1, the correlation coefficients (r^2) were better than 0.998.

Sensitivity

The limits of detection (LOD, $S/N > 3$) for quercetin, kaempferol, isorhamnetin, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and bilobalide were found to be 2, 2, 2, 20, 20, 20, 35, and 20 ng on the column, respectively. The limits of quantification (LOQ, $S/N > 10$) were determined to be as follows: quercetin (4 ng), kaempferol (4 ng), isorhamnetin (4 ng), ginkgolide A (40 ng), ginkolide B (40 ng), ginkgolide C (40 ng), ginkgolide J (70 ng), and bilobalide (40 ng).

Recovery

Accuracy of the methods was calculated as the percentage of analytes recovered by the assay. Ginkgo product powder (3.0 g) was extracted in a 50 mL flask with 30 mL of 80% methanol (v/v) by means of sonication for 60 min. After

filtration, the residue was returned to the same flask and extracted with 30 mL of fresh 80% methanol (v/v) with a sonicator for 60 min. After filtration, the above extraction procedure was repeated until no peaks were detected in the filtrate by HPLC-PDA and HPLC-ELSD as described above. The residue was dried before use. A portion of the dried residue powder (0.5 g) was accurately weighed into a 20 mL PTFE capped sample vial. To the vial, 1 mL of standard recovery working solution (containing quercetin, kaempferol, and isorhamnetin at concentrations of 1.375, 1.95, and 1.75 mg/mL, respectively, and ginkgolide A, B, C, J, and bilobalide at a concentration of 0.5 mg/mL, respectively) was added. The mixed sample was extracted with 15 mL of 80% methanol (3×15 mL) as described. The combined extracts were evaporated under reduced pressure and the residue was dissolved in methanol into a 10 mL volumetric flask and made up to the volume with methanol. For the recovery of flavonoids, 5 mL of it was subjected to hydrolysis, as described above, to prepare the recovery sample solution. Triplicate recovery samples were prepared and analyzed as described. The percentage recoveries were 98.48 ± 1.55, 97.55 ± 2.95, and $97.77 \pm 1.62\%$, for quercetin, kaempferol, and isorhamnetin, respectively. For the recovery of terpene lactones, 4 mL of the above solution was evaporated under vacuum, and the resulting residue was subjected to liquid–liquid extraction. Triplicate recovery samples were prepared and analyzed as described above. The recovery was observed to be 93.24 ± 0.52, 94.84 ± 2.59, 97.91 ± 1.48, 94.27 ± 6.25, and $95.08 \pm 4.59\%$ for bilobalide, ginkgolide J, C, A, and B, respectively.

Application

The current methods were applied to analyze nine commercial ginkgo products in the form of caplets, tablets, and capsules. Tables 2 and 3 show the content of flavonoids and terpene lactones, respectively.

As shown in Table 2 and Fig. 2, generally, quercetin and kaempferol are the main peaks and the concentration of isorhamnetin is approximately five times lower. There are some very small minor peaks that represent further possible aglycones, such as apigenin, luteolin, etc.[7] Our investigation has shown that the commercial ginkgo products contain total flavonoid aglycone content of 2.93–6.17 mg/unit, corresponding to "ginkgo flavonoid" content of 7.35–15.48 mg/unit, which matched most of the manufacturers' claims. The ratio of quercetin/kaempferol and quercetin/isorhamnetin were observed in the range of 1.4–2.3 and 6.7–30, respectively, indicating the variability of flavonoid content in ginkgo products.

Figure 3 shows a typical HPLC separation of ginkgolides and bilobalide in a ginkgo product methanolic extract after refinement with three-steps of liquid–liquid extraction using ethyl acetate. The quantitative results of the study are

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Table 2. Flavonoids Content in Commercial Ginkgo Products ($n = 3$)

	Flavonoid Aglycones Found				
#	Quercetin (mg/unit)	Kaempferol (mg/unit)	Isorhamnetin (mg/unit)	Total Flavonoid Aglycones (mg/unit)	Total Flavonoids (mg/unit)
1	2.60 ± 0.032	1.806 ± 0.016	0.311 ± 0.005	4.718 ± 0.053	11.841 ± 0.133
2	1.857 ± 0.121	1.259 ± 0.086	0.183 ± 0.031	3.299 ± 0.238	8.280 ± 0.596
3	3.766 ± 0.027	1.617 ± 0.004	0.292 ± 0.002	5.675 ± 0.033	14.245 ± 0.083
4	3.131 ± 0.417	1.906 ± 0.273	0.337 ± 0.089	5.372 ± 0.778	13.485 ± 1.954
5	3.823 ± 0.087	1.968 ± 0.041	0.377 ± 0.07	6.167 ± 0.134	15.480 ± 0.337
6	1.330 ± 0.031	0.682 ± 0.015	0.044 ± 0.004	2.057 ± 0.050	5.162 ± 0.124
7	3.293 ± 0.187	1.712 ± 0.111	0.257 ± 0.037	5.261 ± 0.335	13.207 ± 0.841
8	4.201 ± 0.192	2.161 ± 0.065	0.358 ± 0.033	6.720 ± 0.289	16.867 ± 0.726
9	1.637 ± 0.105	1.049 ± 0.069	0.242 ± 0.032	2.928 ± 0.205	7.349 ± 0.515

Table 3. Content of Terpene Lactones in Commercial Ginkgo Products ($n = 3$)

#	G-A (mg/unit)	G-B (mg/unit)	G-C (mg/unit)	G-J (mg/unit)	BB (mg/unit)	Total (Found) (mg/unit)	Total (Claimed) (mg/unit)
1	1.322 ± 0.025	0.700 ± 0.018	0.630 ± 0.026	0.239 ± 0.002	1.056 ± 0.022	3.946 ± 0.089	N/A
2	0.589 ± 0.021	0.268 ± 0.012	0.467 ± 0.021	0.198 ± 0.005	1.178 ± 0.021	2.700 ± 0.080	N/A
3	2.492 ± 0.002	1.761 ± 0.005	1.324 ± 0.025	0.460 ± 0.003	1.893 ± 0.005	7.930 ± 0.038	3.6
4	2.201 ± 0.053	1.283 ± 0.011	1.326 ± 0.021	0.516 ± 0.007	0.671 ± 0.058	5.996 ± 0.095	4.2
5	2.347 ± 0.185	1.638 ± 0.057	0.901 ± 0.013	0.294 ± 0.023	0.523 ± 0.0006	5.704 ± 0.278	3.6
6	0.787 ± 0.015	0.507 ± 0.009	0.268 ± 0.001	0.093 ± 0.006	0.196 ± 0.124	1.851 ± 0.042	1.2
7	2.500 ± 0.069	1.763 ± 0.051	0.910 ± 0.005	0.306 ± 0.0004	0.497 ± 0.013	5.975 ± 0.135	3.6
8	2.655 ± 0.216	1.847 ± 0.127	0.985 ± 0.058	0.351 ± 0.037	0.592 ± 0.024	6.431 ± 0.264	3.6
9	1.19 ± 0.008	0.598 ± 0.002	0.539 ± 0.003	0.194 ± 0.001	0.909 ± 0.010	3.360 ± 0.009	1.8

[a]G-A: ginkgolide A; G-B: ginkgolide B; G-C: ginkgolide C; G-J: ginkgolide J; BB: bilobalide.

combined in Table 3. The data includes the content (mg) of each individual terpene lactone in a unit of ginkgo product, as well as the total terpene lactones per dosage unit (caplet, capsule, or tablet). Compared with the content claimed by manufacturers, the terpene lactones found were generally higher. Also, by analyzing the ratios of ginkgolide A, B, C, J to bilobalide B, it was found that the ginkgo products were rather heterogeneous. As shown in Table 3, although ginkgolide J is always the minor constituent, the content order for ginkgolide A, B, C, and bilobalide were observed to be variable. In some products, bilobalide and ginkgolide A are the most dominant compounds, but the most dominant terpene lactones overall are ginkgolide A and ginkgolide B.

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