JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Efficient ^{1}H Nuclear Magnetic Resonance Method for Improved Quality Control Analyses of Ginkgo Constituents

CHIA-YING LI,† CHUN-HUA LIN,† CHIA-CHE WU,‡ KUO-HSIUNG LEE,§ AND TIAN-SHUNG WU*,†

Department of Chemistry, National Cheng Kung University, Tainan 701, Taiwan, Department of Forestry, National Taiwan University, Taipei 106, Taiwan, and Natural Products Laboratory, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599

We developed an analytical method using ^{1}H nuclear magnetic resonance (NMR) spectrometry to resolve analytical problems with Ginkgo. After a simple hydrolysis step, an NMR analysis of the terpene trilactone H-12 signals and the flavonol aglycone H-2′ (or H-2′/6′ for kaempferol) signals was performed. By comparing the solvent effects on the resolution of these signals, methanol-d_4−benzene-d_6 (65:35) was selected as the optimal ^{1}H NMR solvent. The amounts of terpene lactones and flavonol aglycones in various commercial Ginkgo products and Ginkgo leaves were determined. This newly developed ^{1}H NMR method enables the simultaneous analysis of terpene trilactones and flavonols and allows simple, rapid quantification of these compounds in pharmaceutical Ginkgo preparations.

KEYWORDS: ^{1}H NMR; *Ginkgo biloba*; terpene trilactone; flavonol

INTRODUCTION

Ginkgo biloba, one of the oldest known medicinal plants, has been of great interest to mankind and used as a phytotherapeutic agent since at least 1300 A.D. The use of Ginkgo preparations for the treatment of cough, bronchial asthma, irritable bladder, and alcohol abuse can be traced back to the origins of Chinese herbal medicine (*1*). However, the modern use of Ginkgo phytomedicines is not derived from the traditional medicinal use. In 1965, Schwabe, a German pharmaceutical company, launched the modern medicinal use of Ginkgo leaf extracts for the treatment of circulatory diseases resulting from older age (*2*). Numerous studies have shown that Ginkgo leaves possess many pharmacological properties, including radical scavenging, improvement of blood flow, vasoprotection, cognition enhancing, and anti-PAF (platelet activating factor) activities (*2*−*4*). Among the constituents of *G. biloba*, terpene trilactones and flavonoids have been identified as the active constituents (*4*). However, according to the literature, the quantities of terpene trilactones can vary greatly with small changes in such parameters as collection site, harvest time, and plant growth stage (*5*−*7*). Therefore, the quantity of these active ingredients must be carefully controlled in commercial preparations of Ginkgo products, and reliable chemical analysis is crucial.

Nonetheless, the chemical analysis of terpene trilactones, including bilobalide and ginkgolides A, B, C, and J (**Figure 1**), has long been difficult because of poor UV absorption due to the lack of a good chromophore, relatively low concentrations in leaves, difficult separation of some ginkgolides, and occurrence of many other ultraviolet (UV) active substances in high concentrations in Ginkgo leaves. For the analysis of these bioactive compounds, various quantitative methods based on high-performance liquid chromatography (HPLC) with UV (*8*, *9*), refractive index (RI) (*10*), or ELSD (*11*) detection and gas chromatography (GC)-flame ionization detection (FID) (*12*−*14*) and GC-mass spectrometry (MS) (*15*, *16*) methods after derivatization have been described. In addition, biological standardization using the PAF antagonist properties of ginkgolides has also been developed (*17*). However, disadvantages such as low sensitivity, baseline instability, and the need for extensive

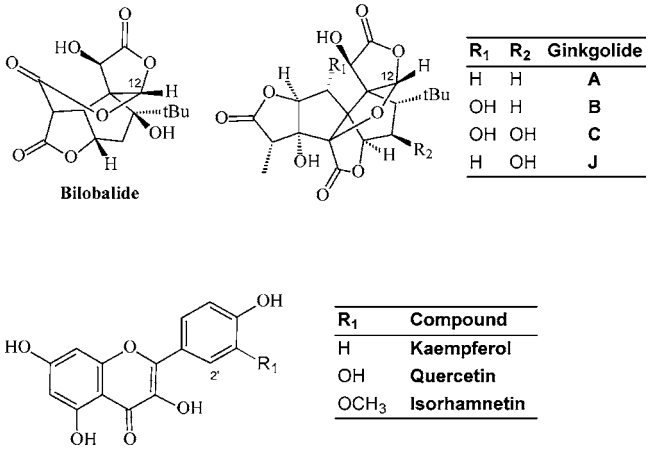

Figure 1. Structures of bilobalide, ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, and the major flavonol aglycones in Ginkgo leaves: kaempferol, quercetin, and isorhamnetin.

* To whom correspondence should be addressed. Tel: +886-6-2757575 ext. 65333. Fax: +886-6-2740552. E-mail: tswu@mail.ncku.edu.tw.
† National Cheng Kung University.
‡ National Taiwan University.
§ University of North Carolina.

10.1021/jf049920h CCC: $27.50
Published on Web 05/06/2004

clean up or derivatization steps still remain problematic in the reported methods. In addition, the above methods require pure reference compounds of terpene trilactones, which are difficult to obtain commercially due to their limited purity and high price. Consequently, Van Beek et al. established a more attractive procedure for the quantitative determination of terpene trilactones using nuclear magnetic resonance (NMR), after a chromatographic preparation step (*18*).

The flavonoids of Ginkgo leaves include great varieties of flavonol glycosides based on kaempferol, quercetin, and isorhamnetin and are found as mono-, di-, and triglycosides as well as their cinnamic acid esters. Thus, the flavonoid profile of Ginkgo is very complex (*19*), and analysis during pharmaceutical quality control is rather tedious. In addition, most of the reference compounds are not commercially available. In the pharmaceutical industry, Ginkgo extracts have long been standardized on flavonol glycosides. A simple HPLC method was established for the analysis of flavonol aglycones after hydrolysis of the glycosides in Ginkgo and sample clean up (*20*).

There are two methods reported in the literature, which can analyze terpene trilactones and flavonoids simultaneously. One is the GC-MS method (*15*), and the other is the HPLC-ELSD method (*11*). The GC-MS method needs numerous preparation and derivatization steps, and the chromatographic running time was more than 40 min. The numerous steps and the poor reproducibility due to the output of the light source and/or detector response stability remain problematic in the HPLC-ELSD method. At present, it appears that there is no suitable method for the simultaneous analysis of both active components of Ginkgo, terpene trilactones, and flavonols. Thus, to resolve the analytical problems of Ginkgo terpene trilactones and simultaneously analyze flavonol glycosides, we investigated a reliable analytical method using ^{1}H NMR spectrometry after a simple hydrolysis step as an alternative to the conventional analyses. The method was applied to the quantitative analysis of 10 commercial Ginkgo products containing terpene trilactones and flavonols and three samples of Ginkgo leaves collected from different regions.

MATERIALS AND METHODS

Chemicals and Instrument. HPLC grade methanol, cyclohexane, and methylethyl ketone were purchased from E. Merck. Methanol-d_4 (99.8%), dimethylsufoxide-d_6 (99.9%), pyridine-d_5 (99.5%), acetone-d_6 (99.9%), benzene-d_6 (99.6%), and toluene-d_8 (99.5%) were obtained from Aldrich. 1,3,5-Trimethoxybenzene was prepared by methylation of phloroglucinol (Sigma) (*21*). The reference compounds (bilobalide; ginkgolides A, B, C, and J; kaempferol; quercetin; and isorhamnetin) were isolated from *G. biloba* leaves in a prior study (*22*). The purity of all of these reference compounds was established by NMR and HPLC. ^{1}H NMR spectra were recorded in various solvent systems using a Varian UNITY plus 400 MHz spectrometer. For each sample, 100 scans were recorded with the following parameters: 0.187 Hz/point; spectra width, 3600 Hz; pulse width, 4.0 μs; relaxation delay, 1 s; and acquiring time, 2.67 μs. For quantitative analysis, the peak area was used and the start and end points of the integration of each peak were selected manually.

Commercial Ginkgo Extracts, Ginkgo Products, and Ginkgo Leaves. Ginkgo extracts were purchased from Chemax (Ellis Bridge, Ahmedabed, India), Chart (Paterson, NJ), Tokiwa PhytoChemical (Sakura-shi, Chiba, Japan), YBS (Tokyo, Japan), CONBA (Lanxi, Zhejiang, China), Indena (Milan, Italy), J C Bright M. (Vancouver, BC, Canada), Ningbo (Ningbo, Zhejiang, China), and USA NutraSource (Eugene, OR). The Ginkgo product (Cebonin) was sold by Nang Kuang Pharmaceutical Company (Tainan, Taiwan). Two samples of Ginkgo leaves were purchased from a Taiwan market, and one sample was collected at Chi-Tou, Taiwan.

Sample Preparation. For extraction, 1 g of dry Ginkgo leaves was extracted three times with 30 mL of 70% MeOH by sonication for 30 min. The three extracts were combined and evaporated to dryness using a rotary evaporator. The crude extract was dissolved in 30 mL of 5% HCl, heated to reflux for 30 min (*23*), and then partitioned three times with cyclohexane (30 mL). The remaining water layer was partitioned by methylethyl ketone (30 mL $\times$ 3 times). The methylethyl ketone fractions were evaporated and dissolved in 0.6 mL of a mixture of methanol-d_4 and benzene-d_6 (65:35). All experiments were performed in triplicate. The commercial Ginkgo extracts (50 mg) and Ginkgo product (1 tablet, about 265 mg) were dissolved in 30 mL of 5% HCl for hydrolysis and processed as described above.

Recovery Test. The recovery test of reference compounds was according to Choi's method (*24*). One gram of filter paper disks (5892 white ribbon ashless, Schleicher & Schuell, GmbH, Cassel, Germnay) was cut into a 1 cm diameter and placed in the extraction vessel. Each standard of bilobalide; ginkgolides A, B, C, and J; kaempferol; quercetin; and rutin (3.0 mg) was spiked into the filter paper disks. Then, the spiked samples were dried in a vacuum oven at 40 °C for 24 h. These cellulose papers were extracted, hydrolyzed, processed as described above, and quantified by the ^{1}H NMR method.

RESULTS AND DISCUSSION

In the ^{1}H NMR quantification of terpene trilactones and flavonol glycosides in Ginkgo leaves and commercial preparations, it would be desirable to quantify each individual terpene trilactone and flavonol by means of the integral of a specific proton signal. However, the ^{1}H NMR peaks of other Ginkgo constituents may interfere with the target signals of the active compounds. In previous studies, tedious cleaning or preparation steps, changing NMR solvents, controlling the pH value, or the addition of a shift reagent were evaluated to solve this problem. Van Beek et al (*18*). reported a one column clean up method, and Choi et al (*24*). changed the NMR solvents to determine ginkgolide A, B, and C and bilobalide contents. Choi's method resolved the problems of low recoveries and time-consuming column preparation steps. However, poor solubility in the solvent system (acetone-d_6−benzene-d_6 50:50) and degradation of the internal standard (phloroglucinol) remained problematic. To analyze terpene trilactones and flavonol glycosides efficiently and simultaneously, we selected a suitable solvent system with a high solubility and a more stable internal standard.

In previous reports (*18*, *24*), the H-12 protons of bilobalide and ginkgolides appeared as well-separated singlets with little interference. Because of these special characteristics, the H-12 signal was selected as the target signal for determining terpene trilactones in the present study. In the previous report, it was found that these terpene trilactones were extraordinarily stable even in boiling HNO_3 (*25*). Utilizing this unique stability, we can hydrolyze the Ginkgo extracts to simultaneously analyze the flavonol aglycones. The many varieties of flavonol glycosides in Ginkgo can be reduced in number by hydrolysis to the corresponding aglycones (**Figure 1**). Each aglycone has its own characteristic H-2′ signal (or H-2′/6′ for kaempferol) in the ^{1}H NMR spectrum due to the different substitution patterns. In addition, this aglycone signal appears between 7.8 and 8.8 ppm, which generally is a noncrowded spectroscopic region and thus shows relatively little interference with other compounds. Therefore, the H-2′ signal (or H-2′/6′ for kaempferol) was selected to be the target signal to quantify the main aglycones: kaempferol, quercetin, and isorhamnetin.

To improve solubility, polar deuterated solvents such as methanol-d_4, dimethyl sulfoxide-d_6, and pyridine-d_5 were used. To more readily resolve the target signals and reduce interference from other Ginkgo constituents, the addition of benzene-d_6 or toluene-d_8 to the selected high polar solvent was evaluated.

Table 1. Contents of Bilobalide (BB), Ginkgolide A (gA), Ginkgolide B (gB), ginkgolide C (gC), Ginkgolide J (gJ), Kaempferol (K), Quercetin (Q), and Isorhamnetin (I) Determined by an ^{1}H NMR Spectrum in Commercial Ginkgo Products and Ginkgo Leaf Extracts and the Recoveries of BB, GA, GB, GC, GJ, K, Q, and I after Hydrolysis and Partitioning with Cyclohexane and Methylethyl Ketone Followed by the ^{1}H NMR Method[a]

sample	BB	gA	gB	gC	gJ	K	Q	I
sample 1[b]	2.84 (0.9)[e]	5.70 (0.7)	2.22 (1.7)	1.14 (0.9)	0.56 (1.8)	5.88 (0.9)	6.40 (2.1)	1.44 (2.4)
sample 2[b]	5.02 (1.3)	3.60 (1.4)	1.34 (1.5)	1.32 (1.3)	0.84 (1.6)	6.10 (0.6)	5.88 (1.9)	1.52 (1.3)
sample 3[b]	3.06 (1.1)	1.78 (1.5)	0.80 (1.1)	1.02 (1.6)	0.68 (1.4)	3.40 (1.1)	3.34 (2.4)	0.96 (1.6)
sample 4[b]	3.10 (1.5)	1.34 (1.1)	0.42 (1.9)	1.22 (0.8)	0.60 (1.3)	3.78 (1.3)	4.76 (2.0)	1.04 (2.7)
sample 5[b]	3.32 (0.8)	2.70 (1.8)	0.94 (1.6)	1.42 (1.4)	0.88 (1.6)	3.42 (1.1)	3.78 (1.5)	0.96 (1.6)
sample 6[b]	2.08 (1.9)	2.52 (1.6)	1.16 (1.2)	1.44 (1.4)	0.68 (0.9)	3.12 (1.6)	5.36 (1.1)	1.16 (2.1)
sample 7[b]	2.62 (1.5)	6.24 (1.7)	2.66 (1.2)	2.18 (1.9)	1.16 (1.5)	4.38 (1.4)	3.80 (1.7)	0.90 (2.7)
sample 8[b]	2.16 (2.0)	6.02 (1.0)	2.56 (1.5)	2.94 (1.1)	1.30 (1.1)	6.38 (1.3)	4.36 (1.7)	1.16 (2.8)
sample 9[b]	3.36 (1.7)	2.88 (1.2)	0.76 (2.1)	1.46 (1.3)	0.76 (1.7)	3.68 (1.9)	4.18 (1.6)	0.94 (1.8)
product[c]	1.76 (0.9)	0.82 (1.1)	0.40 (1.3)	0.77 (2.2)	0.58 (2.6)	2.48 (1.8)	2.10 (1.4)	0.55 (2.8)
leaves 1[d]	0.84 (1.6)	0.67 (1.7)	0.34 (1.4)	0.39 (1.7)	0.31 (1.7)	0.58 (1.9)	0.79 (2.2)	0.22 (2.7)
leaves 2[d]	0.48 (2.2)	0.29 (2.8)	0.27 (2.2)	0.34 (2.7)	0.07 (2.6)	0.22 (2.1)	0.31 (3.0)	0.14 (2.9)
leaves 3[d]	0.07 (2.1)	0.02 (2.9)	0.10 (3.2)	0.05 (2.8)	Trace	0.36 (2.4)	0.90 (3.1)	0.13 (1.6)
recovery	103.0 (2.9)	98.2 (2.2)	102.3 (1.5)	97.2 (2.2)	98.1 (1.1)	97.3 (2.2)	98.6 (2.9)	96.8 (2.1)

[a] All experiments were based on triplicate measurements. [b] In % of Ginkgo extracts. [c] In mg/1 tablet of Ginkgo product. [d] In mg/1 g of Ginkgo leaves. [e] % RSD.

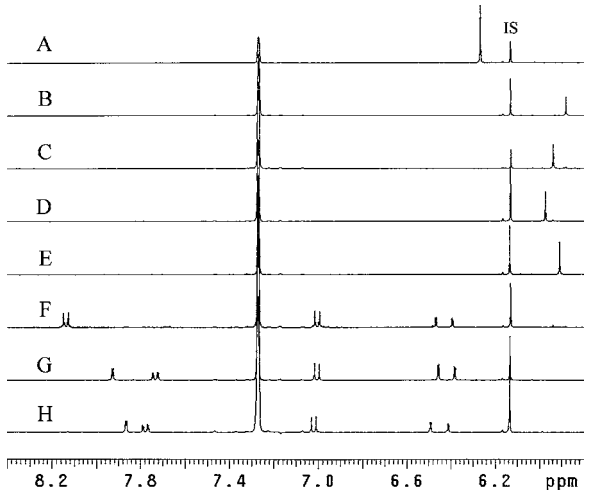

Figure 2. ^{1}H NMR spectra of (**A**) bilobalide, (**B**) ginkgolide A, (**C**) ginkgolide B, (**D**) ginkgolide C, (**E**) ginkgolide J, (**F**) kaempferol, (**G**) quercetin, and (**H**) isorhamnetin in methanol-d_4–benzene-d_6 (65:35) in the range of δ 8.4–5.8. IS: internal standard (1,3,5-trimethoxybenzene).

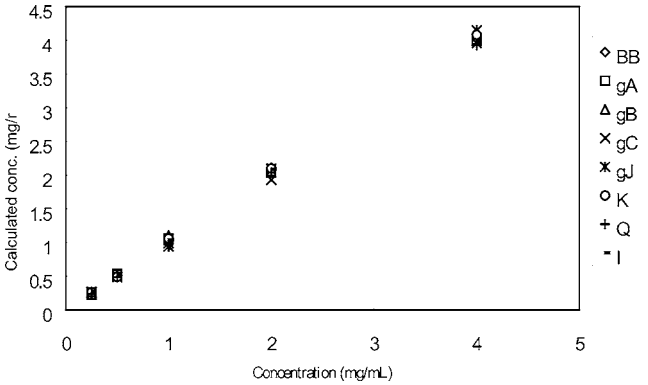

Figure 3. Calibrations for terpene trilactones and flavonoids calculated from the integral of the target signal as compared to the internal standard (1,3,5-trimethoxybenzene). BB, bilobalide; gA, ginkgolide A; gB, ginkgolide B; gC, ginkgolide C; gJ, ginkgolide J; K, kaempferol; Q, quercetin; and I, isorhamnetin.

Among the various combinations (methanol-d_4, methanol-d_4−benzene-d_6, methanol-d_4−toluene-d_8, dimethyl sulfoxide-d_6−benzene-d_6, dimethyl sulfoxide-d_6−toluene-d_8, and pyridine-d_5), methanol-d_4−benzene-d_6 was found to be the optimal NMR solvent system, as it showed a good separation of the H-12 peaks of biolbalide; ginkgolides A, B, C, and J; and H-2′ peaks of kaempferol, quercetin, and isorhamnetin. Accordingly, the effect of the percentage of benzene-d_6 on the separation of these signals was studied. A 65:35 mixture of methanol-d_4 and benzene-d_6 was found to give optimal separation of target peaks originating from flavonols and terpenes (**Figure 2**).

In the previous study (*24*), phloroglucinol (1,3,5-trihydroxybenzene) was used as an internal standard. Because phloroglucinol was degraded in our experiment, 1,3,5-trimethoxybenzene was used as a more appropriate internal standard. It was stable and gave a sharp singlet in the same area as target signals but remained well-separated (**Figure 2**).

Using the ratio of the integrals of the compound and the internal standard, calibration curves for each compound were determined over a large concentration range in order to evaluate the accuracy of this method depending on the different concentrations. The linearity of all curves was found to be greater than 0.999 (**Figure 3**). Actually, these calibration curves are not really necessary because the area of each signal is proportional to the molar concentration of the corresponding compound in ^{1}H NMR.

Finally, nine commercial Ginkgo preparations, one Ginkgo product, and three samples of Ginkgo leaves were analyzed for biolbalide; ginkgolides A, B, C, and J; kaempferol; quercetin; and isorhamnetin using the newly developed ^{1}H NMR method. For the samples evaluated in this study, the H-12 peak of each Ginkgo terpene trilactone and the H-2′ peak of each flavonol aglycone were well-separated from the remaining peaks, and few interferences were observed in the δ 5.8−8.4 ppm range in the ^{1}H NMR spectrum (**Figure 4**). The repeatability of the ^{1}H NMR method was found to be high with a maximum standard deviation of 3.2% (**Table 1**). In the commercial Ginkgo extracts (samples 1−9), the terpene trilactone content varied greatly between samples. However, the total amounts exceeded the required, standard quantity for commercial Ginkgo extracts (5.4−6.6%). Except for sample 3, the quantities of flavonol aglycones in commercial Ginkgo extracts agreed well with the standard quantity of flavonol aglycones [8.6−10.5%, equivalent to 21.6−26.4% of flavonol glycosides (*20*)] for commercial Ginkgo extracts. The flavonol aglycone content in sample 3 was somewhat less than in the other Ginkgo commercial extracts. In the Ginkgo leaf samples, the terpene trilactone content showed much variation, in particular, the Ginkgo leaves collected at Chi-Tou, Taiwan (leaves 3), contained only traces

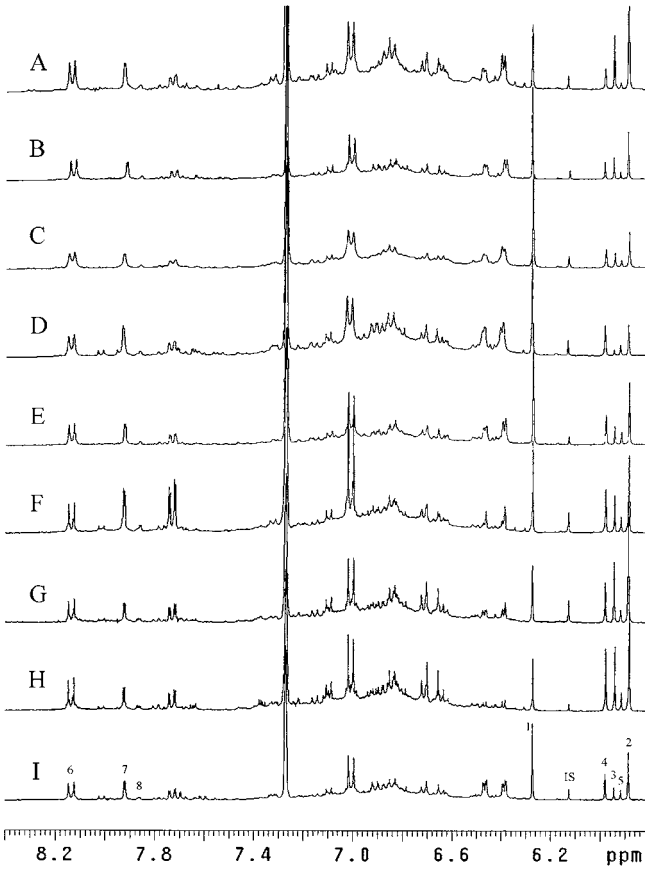

Figure 4. ^{1}H NMR spectra of commercial Ginkgo extracts in the range of δ 8.4–5.8. Sample numbers: (**A**) 1, (**B**) 2, (**C**) 3, (**D**) 4, (**E**) 5, (**F**) 6, (**G**) 7, (**H**) 8, and (**I**) 9. Peak numbers: 1, bilobalide; 2, ginkgolide A; 3, ginkgolide B; 4, ginkgolide C; 5, ginkgolide J; 6, kaempferol; 7, quercetin; 8, isorhamnetin; and IS, internal standard (1,3,5-trimethoxybenzene).

of ginkgolides. The flavonol aglycone content in different Ginkgo leaves also varied significantly, especially in leaf sample 2.

The results of recovery tests (**Table 1**) showed that hydrolysis did not affect the quantity of flavonol aglycones or Ginkgo terpene trilactones. This ^{1}H NMR method allowed rapid, simple, and selective quantification of underivatized terpene trilactones and flavonol aglycones simultaneously, after a straightforward hydrolysis process. Overall profiles of several commercial preparations were successfully obtained using this method. Therefore, the ^{1}H NMR method developed satisfies the requirements for analysis of commercial Ginkgo extract products and is reproducible for Ginkgo pharmaceutical preparations.

ACKNOWLEDGMENT

We thank Professor S. T. Chang (Department of Forestry, National Taiwan University, Taipei, Taiwan) for the collection of fresh Ginkgo leaves.

LITERATURE CITED

(1) State Administration of Traditional Chinese Medicine. *Zhonghua Bencao*; Shanghai Science and Technology Press: Shanghai, 1996; Vol. 1, p 236.

(2) DeFeudis, F. V. *Ginkgo biloba Extract (EGb 761): From Chemistry to Clinic*; Ullstein Medical: Wiesbaden, 1998.

(3) Joyeux, M.; Lobstein, A.; Anton, R.; Mortier, F. Comparative antilipoperoxidant, antinecrotic and scavenging properties of terpenes and biflavones from ginkgo and some flavonoids. *Planta Med.* **1995**, *61*, 126−129.

(4) Sticher, O. Quality of Ginkgo preparations. *Planta Med.* **1993**, *59*, 2−11.

(5) Van Beek, T. A.; Lelyveld, G. P. Concentration of ginkgolides and bilobalide in *Ginkgo biloba* leaves in relation to the time of year. *Planta Med.* **1992**, *58*, 413−416.

(6) Flesch, V.; Jacques, M.; Cosson, L.; Tang, B.; Petiard, V.; Balz, J. Relative importance of growth and light level on terpene content of *Ginkgo biloba*. *Phytochemistry* **1992**, *31*, 1941−1945.

(7) Hasler, A.; Meier, B. *Ginkgo biloba*—Content of flavonoids and terpenes from leaves during the harvest time and from full extracts determined by chromatographic and biologic methods. *Planta Med.* **1993**, *59*, 632.

(8) Xing, S. Y.; Wu, D. J.; Xing, L. F.; You, X. L.; Zhang, Y. P.; Sun, X.; Liu, Y. Q. Quantitative genetic analysis and multiple trait selection of pharmaceutical composition on *Ginkgo biloba* L. leaves. *Yichuan Xuebao* **2002**, *29*, 928−935.

(9) Pietta, P. G.; Mauri, P. L.; Rava, A. Analysis of terpenes from *Ginkgo biloba* L. extracts by reversed phase high-performance liquid chromatography. *Chromatographia* **1990**, *29*, 251−253.

(10) Chen, P.; Su, N. L.; Nie, L. H.; Yao, S. Z.; Zeng, J. G. Analysis of ginkgolides and bilobalides in *Ginkgo biloba* L. extract for its production process control by high performance liquid chromatography. *J. Chromatogr. Sci.* **1998**, *36*, 197−200.

(11) Li, W.; Fitzloff, J. F. Simultaneous determination of terpene lactones and flavonoid aglycones in *Ginkgo biloba* by high-performance liquid chromatography with evaporative light scattering detection. *J. Pharm. Biomed. Anal.* **2002**, *30*, 67−75.

(12) Van Beek, T. A. Chemical analysis of *Ginkgo biloba* leaves and extracts. *J. Chromatogr. A* **2002**, *967*, 21−55.

(13) Huh, H.; Staba, E. J. Ontogenic aspects of ginkgolide production in *Ginkgo biloba*. *Planta Med.* **1993**, *59*, 232−239.

(14) Yang, C.; Xu, Y. R.; Yao, W. X. Extraction of pharmaceutical components from *Ginkgo biloba* leaves using supercritical carbon dioxide. *J. Agric. Food Chem.* **2002**, *50*, 846−849.

(15) Deng, F.; Zito, S. W. Development and validation of a gas chromatographic-mass spectrometric method for simultaneous identification and quantification of marker compounds including bilobalide, ginkgolides and flavonoids in *Ginkgo biloba* L. extract and pharmaceutical preparations. *J. Chromatogr. A* **2003**, *986*, 121−127.

(16) Chauret, N.; Carrier, J.; Mancini, M.; Neufeld, R.; Weber, M.; Archambault, J. Gas chromatographic-mass spectrometric analysis of ginkgolides produced by *Ginkgo biloba* cell culture. *J. Chromatogr.* **1991**, *588*, 281−287.

(17) Li, C. L.; Wong, Y. Y. The bioavailability of ginkgolides in *Ginkgo biloba* extracts. *Planta Med.* **1997**, *63*, 563−565.

(18) Van Beek, T. A.; Van Veldhuizen, A.; Lelyveld, G. P.; Piron, I.; Lankhorst, P. P. Quantitation of bilobalide and ginkgolides A, B, C and J by means of nuclear magnetic resonance spectroscopy. *Phytochem. Anal.* **1993**, *4*, 261−268.

(19) Hasler, A.; Gross, G. A.; Meier, B.; Sticher, O. Complex flavonol glycosides from the leaves of *Ginkgo biloba*. *Phytochemistry* **1992**, *31*, 1391−1394.

(20) Hasler, A.; Sticher, O.; Meier, B. Identification and determination of the flavonoids from *Ginkgo biloba* by high-performance liquid chromatography. *J. Chromatogr.* **1992**, *605*, 41−48.

(21) Five grams of phloroglucinol was added to the mixture of 4 equiv of Me_2SO_4 and 4 equiv of K_2CO_3 in 20 mL of acetone. The reaction mixture was refluxed under 60 °C for 8 h. After the solvent was removed under reduced pressure, the resulting residue was purified by column chromatography over silica gel (hexane−acetone 19:1) to afford 1,3,5-trimethoxybenzene (5.3 g, 80%) as a crystalline solid. The solid was recrystallized using hexane−acetone (1:1) to obtain a white powder. The purity was verified by HPLC (more than 99.9% total area). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.09 (3H, s), 3.77 (9H, s).

(22) Wu, T. S. Unpublished results.

(23) The condition of hydrolysis of flavonols was evaluated by the ^{1}H NMR spectrum. The different hydrolysis temperatures (60, 80, 100, and 120 °C, extra temperature) and hydrolysis times (15, 30, 45, and 60 min) were used. The reflux (extra temperature, 120 °C) for 30 min can hydrolyze completely.

(24) Choi, Y. H.; Choi, H. K.; Hazekamp, A.; Bermejo, P.; Schilder, Y.; Erkelens, C.; Verpoorte, R. Quantitative analysis of bilobalide and ginkgolides from *Ginkgo biloba* leaves and Ginkgo products using ^{1}H NMR. *Chem. Pharm. Bull*. **2003**, *51*, 158−161.

(25) Nakanishi, K. The ginkgolides. *Pure Appl. Chem.* **1967**, *14*, 89−113.

Received for review January 15, 2004. Revised manuscript received April 6, 2004. Accepted April 8, 2004. The financial support for this research (NSC-92-2323-B-006-002) from the National Science Council, Republic of China, is gratefully acknowledged.

JF049920H