Efficient Extraction of Ginkgolides and Bilobalide from *Ginkgo biloba* Leaves

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An efficient and rapid protocol has been developed for extracting ginkgolides and bilobalide (terpene trilactones) from *Ginkgo biloba* leaves. The procedure takes advantage of the extraordinary stability of the terpene trilactone structure to a variety of chemical treatments, especially oxidation, despite the presence of multiple oxygen functions. The protocol involves boiling the aqueous extract of leaves with dilute hydrogen peroxide, extraction with ethyl acetate, washing with basic solutions, and charcoal filtration to yield an off-white powder, terpene trilactone content 60−70%. It is likely that the hydrogen peroxide treatment degrades the undesired leaf constituents that lead to intense emulsification during extractions. Further reversed-phase chromatography of the extracts with polymeric resins removes the undesirable ginkgolic acids to amounts less than 10 ppm. The extracts are suited for pure terpene trilactone preparation, enrichment of terpene trilactone content in nutraceuticals, and preparations of low-flavonoid/high-terpene trilactone controls in medicinal studies. The four ginkgolides (ginkgolides A, B, C, J) and bilobalide isolated from the extract were identical in all respects with authentic specimens.

The Asian tree *Ginkgo biloba*, mentioned in the Chinese *Materia Medica* 5000 years ago,[1] has generated scientific interest for its reputed value in treatment of memory-related afflictions. Recent research has focused on the medicinal value of the leaves;[2] approximately 8000 tons of leaves are harvested globally to address the commercial demand for *G. biloba* products.[3] EGb 761,[4] a standardized extract of the leaves, contains 5−7% ginkgolides[5,6] and bilobalide (BB)[7] (terpene trilactones) (Figure 1) along with 22−24% flavonoids and less than 5 ppm ginkgolic acids (also known as anacardic acids).[4] This limit was imposed because alkylphenols such as ginkgolic acids have been reported to induce contact dermatitis;[8,9] cytotoxicity was also reported for these compounds.[10]

Extracts of *G. biloba* were reported to behave as an antiasthmatic,[11] scavenge radicals,[12] reduce cerebral insufficiency,[13] alleviate the symptoms of mild to moderate Alzheimer-type dementia,[13] and improve short-term memory in healthy subjects.[14,15] While numerous investigations have attempted to determine the effects of the individual constituents of *G. biloba* extracts on the central nervous system,[16] this aspect is still not well understood. Ginkgolide B (GB, BN 52021), a minor component of EGb 761, is a potent inhibitor of the platelet-activating factor (PAF) receptor.[17,18] While PAF has been linked to long-term potentiation (LTP)[19] and an increase of intracellular Ca^{2+},[20] the role of PAF in the CNS is not fully understood. BB, the predominant terpene trilactone present in the leaves, has been reported to strongly inhibit phospholipase 2 (PLA_2) activity in the brain and may contribute to the anti-ischaemic activity of EGb 761.[21]

An efficient and economical extraction of terpene trilactones is essential to secure the amounts of materials required for bioorganic and other studies. In contrast to conventional isolation procedures, which are tedious and lengthy,[22] the method described below concentrates the terpene trilactone in the leaves (ca. 0.2%) to 70% in three rapid steps of oxidation, extraction, and washing. The clear advantage, at least at the laboratory scale, is that the extraction is shortened from several days to a single day because of minimal emulsion formation in the EtOAc extraction process and that only one organic solvent is used. The resulting extracts are ideal as starting material for pure terpene trilactone preparation from leaves, for enriching terpene trilactone content in dietary supplements, and for providing low-flavonoid/high-terpene trilactone controls in medicinal studies.

Quantitative NMR analysis of the terpene trilactones in the extracts[23] has been performed by using the two olefinic protons of maleic acid as an internal standard and taking advantage of the 12-H signals, which in all terpene trilactones appear as well-separated singlets (Figure 2). In the following, DMSO was used instead of the solvent mixture originally described, and a small amount of acetic acid was added to avoid peak shifts in the 12-H region of the NMR.

During structural studies of the ginkgolides it was found that these cage compounds are extraordinarily stable despite the presence of multiple oxygen functions.[5] They are unaffected by boiling in HNO_3; alkali fusion of GA merely results in expulsion of C-10/C-11 to generate a hemiacetal, while treatment of GA in H_2SO_4 with $Na_2Cr_2O_7$ only converted the hydroxy lactone to its oxolactone. Utilizing this unique stability, we investigated various oxidation conditions and subsequent workup to destroy/remove components other than terpene trilactones in the leaves.

The process consists of boiling the terpene trilactone-containing material in water with an oxidizing agent, filtering, extracting the filtrate with EtOAc, washing the organic phase with basic solutions, and solvent removal. These extraction processes were first optimized with a *G. biloba* extract (Pharmanex Bioginkgo 27/7, 27% flavonoids/7% terpene trilactones) and then applied to leaves. Several oxidizing reagents such as H_2O_2, 3-chloroperbenzoic acid (MCPBA), and MnO_2 were tested for the extraction of terpene trilactones from Bioginkgo powder. The yields and percent contents of terpene trilactones of the extracts boiled with H_2O_2, MCPBA, and pure water were similar, the yield of terpene trilactones being ca. 55%. There was a significant decrease in the yield after MnO_2 treatment due to destruc-

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tion of BB (which is much less stable than the ginkgolides), but the amounts of ginkgolides also decreased, probably due to absorption onto solid MnO_2. Further investigations were focused on H_2O_2, as this was the only oxidizing agent that provided clear layer separation during extraction, while the other agents resulted in emulsions with lower recovery of terpene trilactones. Presumably the H_2O_2 treatment destroys the ingredient(s) that give rise to the tenacious emulsification in the EtOAc extraction step.

The concentration of H_2O_2 was varied to determine the optimal concentration. After washing, the total amount of extract decreased with higher percentages of H_2O_2, but without washings the amount of extract increased. Good separation of layers was found for 5% and 10% H_2O_2, whereas 1% H_2O_2 gave an emulsion and 3% H_2O_2 required more time for separation. The highest recoveries of terpene trilactones after washing were found with 3% and 5% H_2O_2. After extraction, H_2O_2 was detected in the organic layer (by the color change on filter paper impregnated with potassium iodide), while with 10% H_2O_2, the excess peroxide gave rise to an exothermic reaction upon workup. Therefore 5% H_2O_2 was used in all further investigations. With respect to the boiling time, 1 h was found to be optimal.

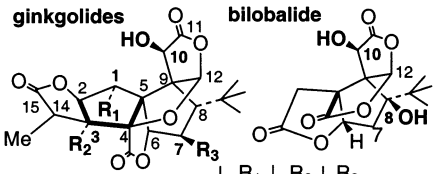
The Bioginkgo powder as well as leaves gave rise to acidic water (pH 3) after boiling. Since peroxide oxidation should work better under acidic conditions, oxidation with 1% of several acids and 5% H_2O_2 was tested. The results were either no change or decreased recovery of terpene trilactones. Thus the use of aqueous H_2O_2 without additional acid was found to be optimal.

After boiling with peroxide, all mixtures were extracted thrice with EtOAc. With respect to the number of extractions, three turned out to be sufficient. In the case of leaf extractions, a precipitate present in the organic layer had to be removed by filtration. Washing of the EtOAc layer with basic solutions was critical for high terpene trilactone content. Base was used to neutralize organic acids and to destroy peroxide excess; however, the lactone rings could open under such treatment, resulting in increased water solubility. For GB it was reported that lactones start opening at pH 6.5.[24]As expected, aqueous Na_2CO_3 (pH 10.4) and NaSH (pH 10.8) resulted in lower recovery yields of terpene trilactones, with BB and ginkgolide C (GC) no longer being detected in the extract. However, washing with saturated solutions of $NaHCO_3$ and $Na_2S_2O_3$ (both pH 8.3) and Na_2SO_3 (pH 9.3) led to consistent extraction yields and high terpene trilactone content. Further workup yielded extracts containing 50−70% terpene trilactones. In contrast to basic solutions, use of aqueous NH_4Cl (pH 5) was not effective in removing impurities.

Since the lactones of terpene trilactones are closed at pH 3, the presence of any terpene trilactone in the discarded aqueous layer after washing with Na_2SO_3 (pH 9.3) was tested after acidification of the combined water layers with 1 N HCl; no terpene trilactones were detected by TLC. Using the same procedure for the water layers after washing with Na_2CO_3 (pH 10.4), GC and decomposition products of BB were found.

A typical extract prepared from Bioginkgo 27/7 is shown in Figure 3A. Small quantities of pure terpene trilactones were obtained from such extracts with semipreparative reversed-phase HPLC;[25] larger quantities can be purified by a number of different procedures.[25,26]

After optimization, the protocol was applied to dried leaves. The total terpene trilactone contents were similar



	R_1	R_2	R_3
Ginkgolide A (GA)	H	OH	H
Ginkgolide B (GB)	OH	OH	H
Ginkgolide C (GC)	OH	OH	OH
Ginkgolide J (GJ)	H	OH	OH
Ginkgolide M (GM)	OH	H	OH

Figure 1. Ginkgolides A, B, C, J, M and bilobalide.

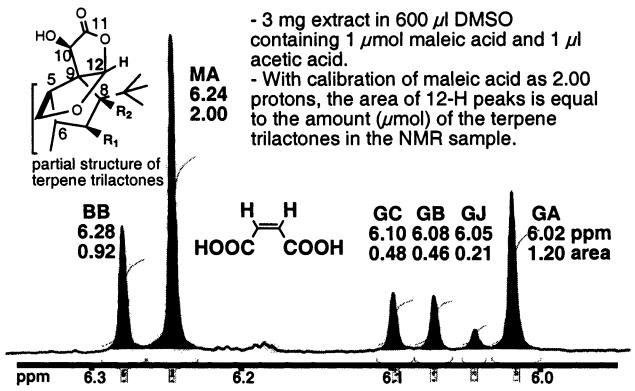


Figure 2. Quantitative ^1H NMR of TTLs based on 12-H signals.

for the treated Bioginkgo 27/7 powder (65%), the yellow leaves (65%), and the green leaves (70%) (Figure 3).

The content of terpene trilactones in leaves depends on the season and other factors such as tree age.[27] As described for leaves and for EGb761 (26% flavonoids, 6% terpene trilactone), BB was always the major component of the terpene trilactone content (ca. 50%).[28,29] The procedures described above led to minimal BB destruction and high recovery yield and maintained a natural distribution pattern.

These leaf extracts possessed a significant amount of the undesired ginkgolic acids. The ginkgolic acids were removed by reversed-phase chromatography.[30] The contents of the two predominant ginkgolic acids found in *G. biloba* leaves were measured by HPLC using PDA detection; in each case measured, the individual ginkgolic acids were present in amounts lower than the limit of quantitation (10 ppm).

Experimental Section

General Experimental Procedures. ^1H and ^{13}C spectra were acquired on either a 300 or 400 MHz Bruker NMR using standard pulse sequences and parameters; further details are described below. Negative ion FABMS data were determined using a JMS-HX110 tandem mass spectrometer (JEOL, Tokyo Japan) instrument using a Xe beam (6 kV) with a 10 kV acceleration voltage and *m*-nitrobenzyl alcohol (NBA) matrix. Analytical HPLC measurements were performed on a Waters 996 PDA system with the Millenium Version 2.15.01 software package. Concentration graphs and linear regression were determined with Microsoft Excel 2000. Semipreparative HPLC isolations were achieved on a LC-908 (Japan Analytical Industry Co., Ltd.) instrument equipped with internal refractive index and ultraviolet detectors and a recorder.

Extraction Material. Bioginkgo 27/7 brand *G. biloba* extract was generously donated by Pharmanex (Provo, UT).

Plant Material. The leaves were collected from a female *G. biloba* tree in Morningside Park, New York, NY. The yellow fallen leaves were collected from the ground in November 2000,

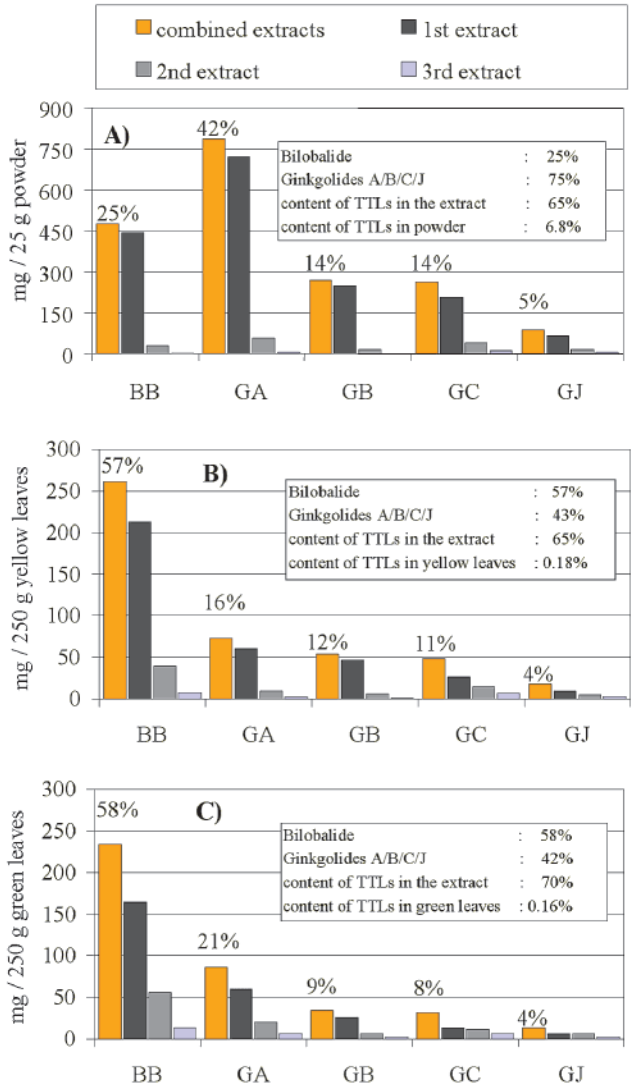


Figure 3. Amounts and distribution pattern of TTLs prepared from (A) 25 g of Bioginkgo powder; (B) 250 g of dried leaves; and (C) 250 g of green leaves.

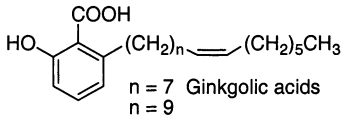


Figure 4. Ginkgolic acids.

while green leaves were collected from the tree in June 2001. They were frozen in liquid nitrogen, crushed, and lyophilized until constant weight. The moisture content was 59% in the yellow leaves and 73% in green leaves. Further samples of these leaves are stored in our laboratory.

General Extraction of Terpene Trilactones. Bioginkgo 27/7 extract (25 g) or lyophilized leaves (250 g) were boiled for 1 h in 0.5 L (extract) or 2 L (leaves) of 5% aqueous H_2O_2 (or other oxidation reagents in water). After passing (extract = RT, leaves = boiling) through a Buchner funnel without filter paper, the remaining solution was extracted up to three times with EtOAc (250/125/75 mL). The organic layer was washed with a saturated aqueous salt solution ($NaHCO_3$/$Na_2S_2O_3$, $NaHCO_3$/Na_2SO_3, or Na_2SO_3) followed by water and 80% aqueous NaCl (saturated aqueous solution diluted to 80%). After drying over Na_2SO_4, solvent was removed to yield an amorphous yellow powder with 50−60% terpene trilactone content.

For further purification these extracts were dissolved in EtOAc and passed through an activated charcoal column. Concentration gave an off-white amorphous powder with 60−70% content of terpene trilactones. A more rigorous procedure involved dissolving the extracts in MeOH, adding activated charcoal, and filtering over Celite 545.

TLC Analysis of Terpene Trilactones. Thin-layer chromatography of terpene trilactones was performed as previously reported.[31]

HPLC Separation of Terpene Trilactones. A 1 × 25 cm 5 μm YMC ODS-AM column eluted with a 1 mL/min H_2O/MeOH/THF (7:2:1) solvent system was used to separate the terpene trilactones; monitoring was performed with a refractive index detector.[22]

Removal of Ginkgolic Acids. Ginkgolic acids were removed from the extracts by reversed-phased chromatography using polymeric resins such as Dianon HP-20[30] or Amberlite XAD-16 with a step gradient of increasing methanol (30−90%) in water. Terpene trilactone-containing fractions (40−80%, as determined by NMR or TLC) were combined, and the solvent was removed to afford the final products. WP C18 silica was also employed (twice) with aqueous 60% methanol successfully to remove ginkgolic acids.

Extraction of Ginkgolic Acids. Standards were isolated from hexane-extracted *G. biloba* leaves that were subjected to chromatography with silica gel (1% MeOH in $CHCl_3$), RP-18 silica gel (0−100% MeOH, retaining the 100% MeOH fraction), and semipreparative HPLC (1 × 25 cm 5 μm YMC ODS-AM column, H_2O/MeOH/AcOH (100:10:1) solvent system, monitored at 215 μm). Spectroscopic analyses of these compounds (^{1}H NMR, ^{13}C NMR, and negative mode FABMS) provided results similar to those previously reported.[32]

Quantitation of Ginkgolic Acids. Ginkgolic acid standards (3, 5, 25, 50, 250 μg) in MeOH (5 μL) were injected onto a 4.6 × 150 mm Phenomenex Luna 5 μm C18(2) column with 0.1% TFA in a MeCN solvent system (flow rate = 1 mL/min) and detection at 215 nm; concentration graphs showing good linearity ($R^2 \geq 0.99$). Multiple injections determined the level of quantitation to be 10 ppm. Samples were prepared by dissolving 1−2 mg of extract in 100 μL of MeOH; 20 μL injections were then analyzed in a similar fashion.

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References and Notes

(1) Deng, Q. *Drug Use Perspective* **1988**, *1*, 57−58.
(2) Del Tredici, P. In *Medicinal Aromatic Plants−Ind. Profiles, Vol. 12 (Ginkgo biloba)*; van Beek, T. A., Ed.; Harwood: Amsterdam, 2000; pp 7−23.
(3) Schmid, W. *Nature* **1997**, *386*, 755.
(4) Drieu, K.; Jaggy, H. In *Mediciant Aromatic Plants−Ind. Profiles, Vol. 12 (Ginkgo biloba)*; van Beek, T. A., Ed.; Harwood: Amsterdam, 2000; pp 267−277.
(5) Maruyama, M.; Terahara, A.; Nakadaira, Y.; Woods, M. C.; Takagi, Y.; Nakanishi, K. *Tetrahedron Lett.* **1967**, *4*, 315−320. See also: Nakanishi, K. *Pure Appl. Chem.* **1967**, *14*, 89−113.
(6) Weinges, K.; Rümmler, M.; Schick, H. *Liebigs Ann. Chem.* **1987**, 521−526.
(7) Nakanishi, K.; Habaguchi, K.; Nakadaira, Y.; Woods, M. C.; Maruyama, M.; Major, R. T.; Alauddin, M.; Patel, A. R.; Weinges, K.; Bäher, W. *J. Am. Chem. Soc.* **1971**, *93*, 3544−3546.
(8) Hill, G. A.; Mattacotti, V.; Graham, W. D. *J. Am. Chem. Soc.* **1934**, *56*, 2736−2738.
(9) Lepoittevin, J. P.; Benezra, C.; Asakawa, Y. *Arch. Dermatol. Res.* **1989**, *281*, 227−230.
(10) Siegers, C. P. *Phytomedicine* **1999**, *6*, 281−283.
(11) Mahmoud, F.; Abul, H.; Onadeko, B.; Khadadah, M.; Hainea, D.; Morgan, G. *Jpn. J. Pharmacol.* **2000**, *83*, 241−245.
(12) Louajri, A.; Harraga, S.; Godot, V.; Toubin, G.; Kantelip, J. P. *Biol. Pharm. Bull.* **2001**, *24*, 710−712.
(13) Schulz, J.; Halama, P.; Hoerr, R. In *Medicinal Aromatic Plants−Ind. Profiles, Vol. 12 (Ginkgo biloba)*; van Beek, T. A., Ed.; Harwood Academic Publishers: Amsterdam, 2000; pp 345−370.
(14) Polich, J.; Gloria, R. *Hum. Psychopharmacol. Clin. Exp.* **2001**, *16*, 409−416.
(15) Kennedy, D. O.; Scholey, A. B.; Wesnes, K. A. *Psychopharmacology* **2000**, *151*, 416−423.

(16) DeFeudis, F. V. *Ginkgo biloba extract (Egb 761): From Chemistry to the Clinic*; Ullstein Medical: Wiesbaden, 1998.
(17) Braquet, P.; Etienne, A.; Touvay, C.; Bourgain, R. H.; Lefort, J.; Vargaftig, B. B. *Lancet* **1985**, *1*, 1501.
(18) Ishii, S.; Shimizu, T. *Prog. Lipid Res.* **2000**, *39*, 41−82.
(19) Kato, K.; Clark, G. D.; Bazan, N. G.; Zorumski, C. F. *Nature* **1994**, *367*, 175−179.
(20) Bito, H.; Nakamura, M.; Honda, Z.; Izumi, T.; Iwatsubo, T.; Seyama, Y.; Oguram A.; Kudo, Y.; Shimizu, T. *Neuron* **1992**, *9*, 285−294.
(21) Klein, J.; Chatterjee, S. S.; Löffelholz, K. *Brain Res.* **1997**, *755*, 347−350.
(22) O'Reilly, J. In *Medicinal Aromatic Plants−Ind. Profiles, Vol. 12 (Ginkgo biloba)*; van Beek, T. A., Ed.; Harwood: Amsterdam, 2000; pp 99−108.
(23) van Beek, T. A.; Lelyveld, G. P. *Phytochem. Anal.* **1993**, *4*, 261−268.
(24) Zekri, O.; Boudeville, P.; Genay, P.; Perly, B.; Braquet, P.; Jouenne, P.; Burgot, J.-L. *Anal. Chem.* **1996**, *68*, 2598−2604.
(25) Teng, B. P. In *Ginkgolides−Chemistry, Biology, Pharmacology and Clinical Perspectives*; Braquet, P., Ed.; J. R. Prous Science Publishers: Barcelona, 1988; pp 37−48.
(26) van Beek, T. A.; Lelyveld, G. P. *J. Nat. Prod.* **1997**, *60*, 735−738.
(27) Laurain, D. In *Medicinal Aromat. Plants−Ind. Profiles, Vol. 12 (Ginkgo biloba)*; van Beek, T. A., Ed.; Harwood: Amsterdam, 2000; pp 63−77.
(28) van Beek, T. A.; Lelyveld, G. P. *Planta Med.* **1992**, *58*, 413−416.
(29) Sticher, O. *Planta Med.* **1993**, *59*, 2−11.
(30) Chang, M.; Yu, Z.; Cooper, R.; Zhang, D. C. PCT Int. Appl. WO 99/26643, June 3, 1999.
(31) van Beek, T. A.; Lelyveld, G. P. *Phytochem. Anal.* **1993**, *4*, 109−114.
(32) Itokawa, H.; Totsuka, N.; Nakahara, K.; Takeya, K.; Lepoitteven, J.-P.; Asakawa, Y. *Chem. Pharm. Bull.* **1987**, *35*, 3016−3020.

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