Analysis of Terpenelactones in *Ginkgo biloba* by High Performance Liquid Chromatography and Evaporative Light Scattering Detection

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A reversed phase HPLC method permitting the determination of 5 terpenelactones in *Ginkgo biloba*, without the need of any sample preparation is presented in this paper. The compounds were successfully separated within 25 min by using a C-12 column, an evaporative light scattering (ELS) detector and a mobile phase comprising of ammonium acetate buffer, methanol and isobutanol. All terpenelactones were detectable at concentrations as low as 20.3 μg/ml. The analysis of *G. biloba* market products showed remarkable variations in the lactone content, and more than 2 fold differences in the suggested daily doses of the total lactones, from 8.84 mg to 18.28 mg, respectively.

Key words *Ginkgo biloba*; HPLC; evaporative light scattering detection; ginkgolide; bilobalide

Ginkgo biloba L. (Ginkgoaceae), a 40 m high, monotypic tree with broad, fan shaped leaves, has been valued for many centuries in Asia. Chinese traditional medicine uses the leaves ("bai guo ye") and fruits ("baiguo") of this plant to treat a number of diseases such as asthma, tuberculosis and arteriosclerosis. In western medicine, the leaves and preparations thereof are utilized against demential disorders (memory impairment and concentration difficulties), arterial occlusive disease and vertigo.[1)] In the years 1998 and 1999 Ginkgo preparations were the top selling dietary supplements on the U.S.A. market, with more than 20% of the total sales in this segment.[2)]

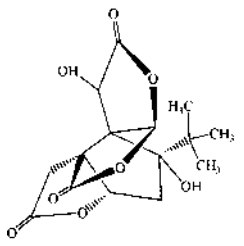
The monograph of the German Commission E lists terpenes and flavonoids as characteristic compounds of *G. biloba.* Terpenelactones such as bilobalide (**1**) and the ginkgolides A (**4**), B (**5**), C (**3**) and J (**2**) are not only structurally unique compounds but also pharmacologically active. They competitively inhibit the platelet-activating factor (PAF), thus preventing thrombus formation, bronchoconstriction and suppressing allergic reactions.[3—5)]

Several analytical methods for the analysis of terpenelactones in *G. biloba* by GC and HPLC (detection by UV, RI, ELS or MS) are reported in literature, but all methods require either complicated and time consuming sample preparation procedures or the derivatization of the compounds of interest.[6—14)] No method allows the injection of a crude *G. biloba* extract, resulting in the sensitive detection and baseline separation of **1**—**5**. In this paper we present an HPLC system permitting the separation of all main Ginkgo terpenelactones within 25 min, without the need of any sample preparation.

Results and Discussion

By carefully assessing all analytical parameters, a determination of the terpenelactones in a crude Ginkgo extract is possible. Initial experiments with a standard mixture of **1**—**5** showed that a separation is feasible only by a mobile phase consisting of water and methanol (for structures of the lactones see Fig. 1). Compounds **2**—**3** and **4**—**5** merge, if acetonitrile is used instead of methanol. When a crude Ginkgo extract is to be separated, the mobile phase has to be more refined, as **4** and **5** normally co-elute with other compounds of similar polarity. This overlapping can be avoided if a 9 : 1 mixture of methanol and isobutanol is used. Isobutanol is essential for this separation, and it cannot be replaced with isopropanol, ethanol or modifiers like MBE or THF. The right stationary phase was crucial for a successful separation as well, and best results were obtained with a Synergi Max-RP 80 Å from Phenomenex. For improved peak symmetry a 10 mM ammonium acetate buffer at pH 5.0 was used. Finally, ELS was chosen as detection method. This technique is based on light scattering and not absorption, thus allowing a more sensitive determination of the compounds **1**—**5**, as compared to UV detection (Fig. 2).

In order to validate the method, linearity, limit of detection, peak purity, accuracy and precision were determined. The calibration curves for all five compounds were linear from 500 to 31.2 μg/ml (see Table 1 for exact data); the limit of detection for the terpenelactones was found to be 20.3 μg/ml or lower. Peak purity of **1**—**5** was confirmed by studying the PDA and ELS-data; no indications of impurities could be found. Accuracy of the method was confirmed by performing a recovery experiment. Sample NPC-GB-2 was spiked with a known amount of the standard compounds, extracted and analyzed. The recovery rates were between



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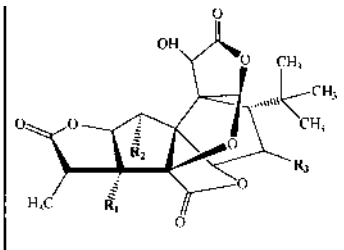

	R_1	R_2	R_3
2	OH	H	OH
3	OH	OH	OH
4	OH	H	H
5	OH	OH	H

Fig. 1. Structures of Bilobalide (**1**), Ginkgolides J (**2**), C (**3**), A (**4**) and B (**5**)

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98.26% for **2** and 100.10% for **1**. Finally, all standards and samples were injected three times. A standard deviation of max. 3.07% confirmed the precision of the method.

Prior to the analysis of several market products the efficiency of our extraction procedure was verified. One sample (NPC-GB-1) was repeatedly extracted with 3 ml of methanol, and each extract analyzed separately. After the second repetition no terpenelactones were detectable in the extracts anymore, therefore a three-fold extraction should guarantee an exhaustive procedure.

Nine different Ginkgo products, all claiming the content of 60 mg standardized extract per unit (1 capsule or tablet), were finally analyzed. Fig. 3 shows the separation of three samples (NPC-GB-1, NPC-GB-2 and NPC-GB-4), and in Table 2 the quantitative results of the study are combined; the data includes the percentage (g/100 g) of each individual terpenelactone in a product as well as the total daily intake of these compounds (these values allow a direct comparison of the products). As it is evident from the results, the extracts were of rather heterogeneous nature (see Fig. 4 for the relative composition of **1**—**5** in the products). Whereas **2** was al-

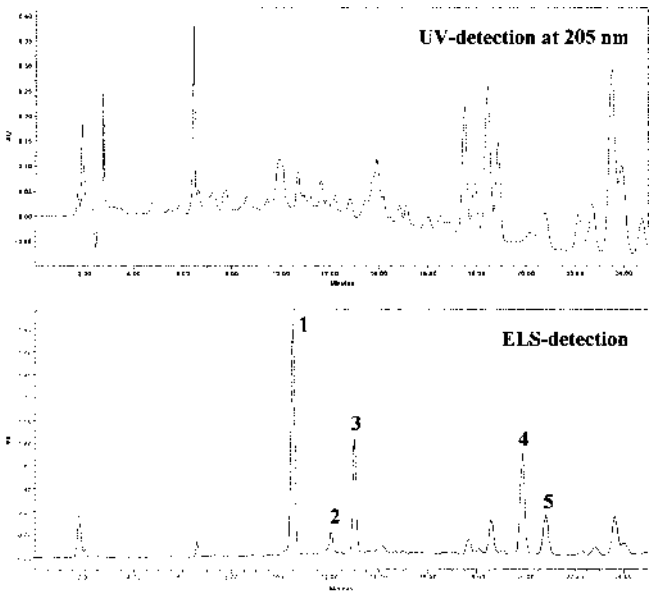

Fig. 2. Analysis of Terpenelactones **1**—**5** in a Crude *G. biloba* Extract

Sample NPC-GB-5; column: Synergi Max-RP 80 Å, 4 μm particle size, 150×4.6 mm; mobile phase: 10 mM ammonium acetate buffer, pH 5.0 (A), methanol/isobutanol=9/1 (B); from 90A/10B to 80A/20B in 10 min, then in 15 min to 75A/25B; flow rate: 1.0 ml/min; detection: UV at 205 nm and ELS; ELS parameters: 45 °C, 2.4 bar N_2, gain 9; injected sample volume: 10 μl; temperature ambient; peak assignment according to Fig. 1.

Table 1. Calibration Data for Compounds **1**—**5**, Including Correlation Coefficient (R^2), Regression Equation and Limit of Detection (LOD)

Compounds	R^2	Regression equation[a)]	LOD (μg/ml)
1	0.9990	$y=1.54X+0.080$	10.0
2	0.9988	$y=1.61X-0.047$	14.8
3	0.9993	$y=1.59X-0.022$	12.2
4	0.9993	$y=1.63X-0.086$	19.8
5	0.9994	$y=1.52X-0.025$	20.3

a) y reflects the peak area, X the amount of compound in μg/ml; the detector response is logarithmic.

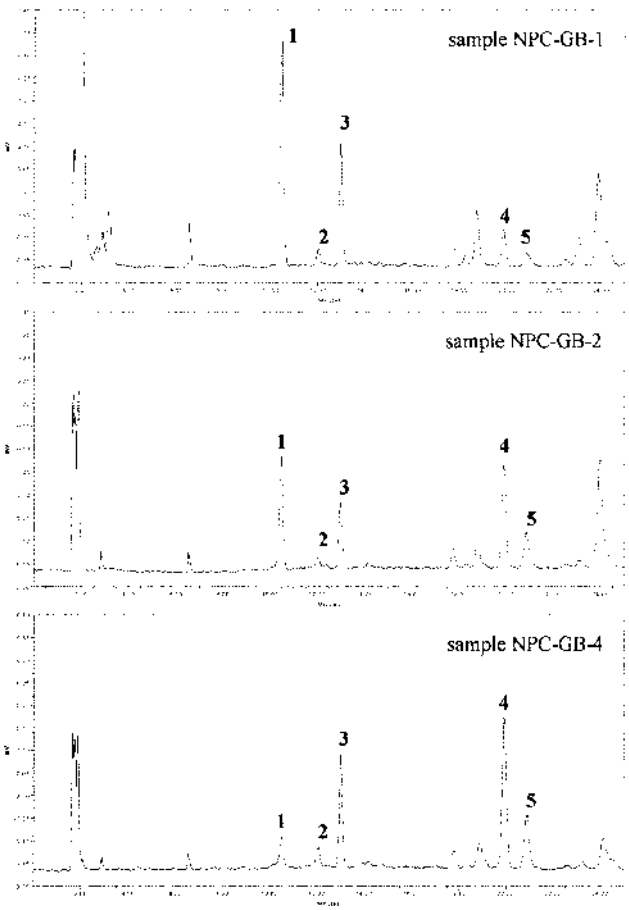

Fig. 3. Comparison of Different *G. biloba* Market Products

NPC-GB-1, NPC-GB-2 and NPC-GB-4; separation conditions same as in Fig. 1, for peak assignment see Fig. 1.

Table 2. Analysis of Different *G. biloba* Market Products (Values in g/100 g) and the Suggested Daily Dose of the Terpenelactones **1**—**5** in mg[a)]

Sample	**1**	**2**	**3**	**4**	**5**	**1**—**5**/d
NPC-GB-1	0.39 (1.14)	0.07 (1.21)	0.27 (1.08)	0.16 (0.87)	0.10 (0.18)	9.24
NPC-GB-2	0.32 (1.27)	0.07 (3.07)	0.24 (2.07)	0.37 (0.30)	0.24 (1.21)	9.39
NPC-GB-3	0.42 (0.44)	0.06 (0.45)	0.22 (1.00)	0.16 (2.42)	0.08 (0.52)	8.84
NPC-GB-4	0.13 (1.72)	0.11 (2.24)	0.34 (1.23)	0.46 (0.94)	0.32 (1.07)	15.42
NPC-GB-5	1.16 (0.15)	0.21 (1.35)	0.67 (0.81)	0.73 (0.86)	0.49 (1.15)	15.13
NPC-GB-6	0.48 (0.46)	0.05 (0.68)	0.23 (0.63)	0.20 (1.85)	0.11 (1.29)	9.23
NPC-GB-7	0.28 (0.69)	0.05 (0.91)	0.18 (0.26)	0.14 (0.48)	0.07 (1.00)	8.91
NPC-GB-8	0.42 (0.56)	0.10 (1.15)	0.35 (0.33)	0.57 (0.43)	0.42 (0.87)	18.28
NPC-GB-9	0.06 (0.40)	0.08 (0.82)	0.30 (0.19)	0.39 (0.16)	0.30 (0.32)	10.98

a) Relative standard deviations are given in parentheses ($n=3$).

ways a minor compound, the most dominant lactones were either **1** or **4**, usually followed by **3**. These variations are either based on different manufacturing (extraction) procedures or seasonal/geographical variations of the terpene content in the leaf. The importance of the latter factor has been alluded to in a previous publication, and will be studied as an ongoing project by our group.[11] Although the extracts were of diverse composition, the suggested total intake of **1**—**5** was more uniform, ranging from 8.84 to 18.28 mg/d (2-fold variation).

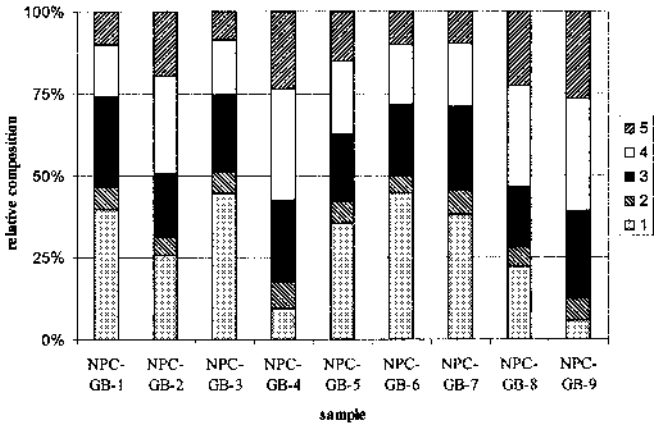

Fig. 4. Relative Composition of the Terpenelactones **1**—**5** in Nine Different Market Products, Each Containing Standardized *G. biloba* Extract

Additionally, our system was successfully applied for the LC-MS analysis of a *G. biloba* extract (Fig. 5). The terpenelactones **1**—**5** were readily assigned in sample NPC-GB-5 with their molecular peak at $[M-H]^-$, if detection was performed in negative ESI mode, with source voltage set to 3.0 kV, ionization voltage to 50.0 V, probe temperature to 350 °C and sheath gas (N_2) to 60 psi.

In conclusion, the method presented in this paper facilitates the analysis of terpenelactones in *G. biloba* significantly. Because not only is no sample preparation required, but also by the use of an ELS detector, a sensitive detection and baseline separation of the compounds is feasible within 25 min. The accuracy of the results obtained, in combination with high throughput possibilities should be desirable for scientific and commercial applications.

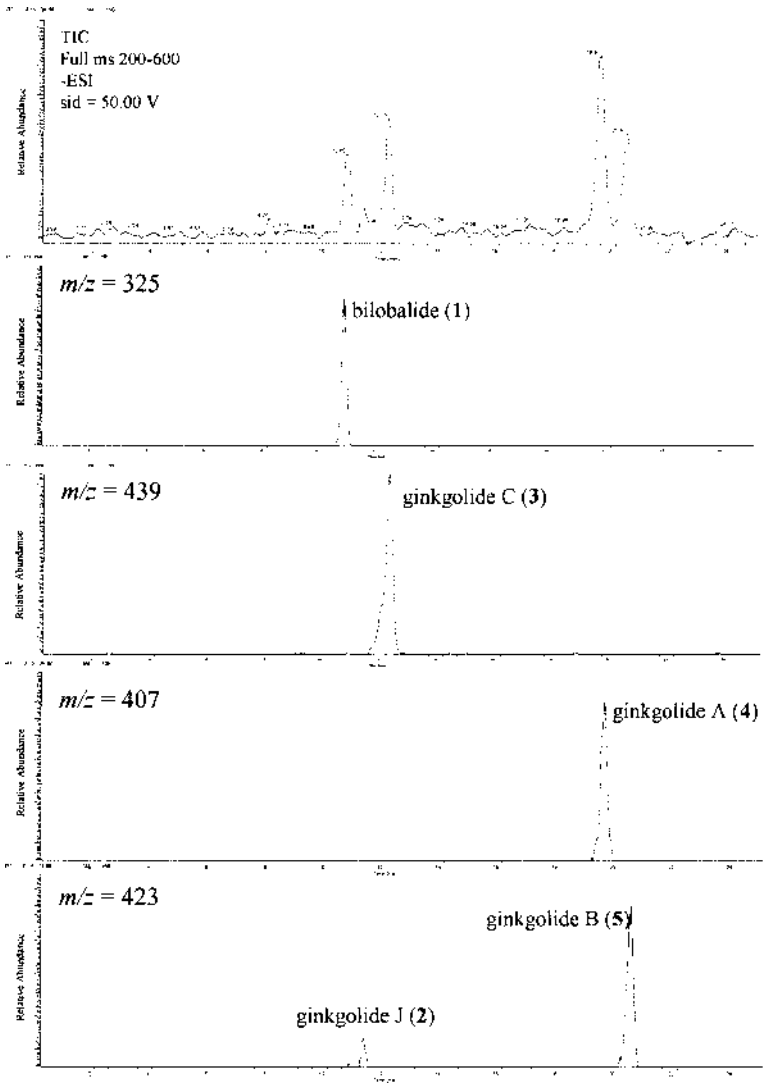

Fig. 5. LC-MS Analysis of a *G. biloba* Extract (sample NPC-GB-5), Separated under Optimized Conditions

MS-parameters: ESI-mode, ionization voltage: 50 V, source voltage: 3.0 kV, probe temperature 350 °C. Assignment of peaks according to Fig. 1.

Experimental

Materials For the isolation of standard compounds **1**—**5** a commercial *G. biloba* extract, purchased from Nutra Source (1300 Industrial Road, San Carlos, CA, U.S.A.; Lot No. UNGB-000506) was used. Identity and purity of the isolated compounds were confirmed by chromatographic (TLC, HPLC) and spectroscopic (IR, 1D- and 2D-NMR, HRESIMS) methods. The market products analyzed in this study (NPC-GB-1 to NPC-GB-9) were purchased in supermarkets in Mississippi and California. Voucher specimens of all samples are deposited at the NCNPR/University of Mississippi.

Solvents and reagents used were of HPLC grade and were purchased from Baxter (isobutanol; Muskegon, MI, U.S.A.) and Fisher Scientific (methanol, water, glacial acetic acid and ammonium acetate; Fair Lawn, NJ, U.S.A.).

Sample Preparation Samples (500.0 mg) were extracted three times with 3 ml of methanol by sonication for 10 min. After centrifugation at 3000 rpm for 10 min, the supernatants were combined in a 10 ml volumetric flask and adjusted to the final volume with methanol. Prior to use, all samples were filtered through a nylon membrane filter (0.45 μm pore size) from Gelman (Ann Arbor, MI, U.S.A.). Every sample solution was injected in triplicate; relative standard deviations were below 3.07% for all experiments.

For recovery experiments, one sample (NPC-GB-2) was spiked with 1.00 ml of the standard stock solution, and extracted by the above-mentioned procedure. The recovery rates obtained were 100.10% for **1**, 98.26% for **2**, 99.37% for **3**, 98.33% for **4** and 99.31% for **5**.

Calibration Each standard compound (5.00 mg) was dissolved in 10.00 ml of methanol (stock solution); further calibration levels were prepared by diluting the stock solution with methanol. Within the range of concentrations injected (31.2 to 500.0 μg/ml) the detector response was linear (see Table 1 for regression equation, correlation coefficient and limit of detection). The response of an ELS detector is a function of the mass and follows an exponential relationship (log of response *versus* log of concentration is linear).[15)]

Analytical Methods HPLC experiments were performed on a Waters Alliance 2690 HPLC system, equipped with a 996 photodiode array detector (Waters, Milford, MA, U.S.A.) and a Sedex 55 ELS detector (SEDERE, Alfortville, France). A Synergi Max RP 80 Å column (150×4.6 mm, 4 μm particle size) from Phenomenex (Torrance, CA, U.S.A.) was used as stationary phase. The mobile phase consisted of 10 mM ammonium acetate buffer, adjusted to pH 5.0 with glacial acetic acid (A), and a 9 : 1 mixture of methanol and isobutanol (B). Analysis was performed using the following gradient elution: in 10 min from 90A/10B to 80A/20B, then to 75A/25B in 15 min. Each run was followed by a 5 min wash with 100B and an equilibration period of 10 min. All separations were performed at ambient temperature, with flow rate and detection wavelength set to 1.0 ml/min and 205 nm, respectively. The ELS detector was set up to a probe temperature of 45 °C, at gain 9 and the nebulizer gas (N_2) adjusted to 2.4 bar. Ten microliters of sample were injected and peaks were assigned by spiking the samples with standard compounds, and comparison of the UV-spectra (if applicable) and retention times.

LC-MS analysis was performed on a Finnigan AQA system (Thermoquest, San Jose, CA, U.S.A.) and a Finnigan HPLC system (AS3000 autosampler, P4000 pump and UV6000LP detector). Best results could be obtained in negative ESI mode, with ionization voltage set to 50 V, source voltage to 3.0 kV, probe temperature to 350 °C and sheath gas set to 60 psi.

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References

1) Leung A. Y., Foster S., "Encyclopedia of Common Natural Ingredients Used in Food, Drugs and Cosmetics," John Wiley & Sons, New York, 1996.
2) Blumenthal M., *Herbalgram*, **47**, 64—65 (1999).
3) Schulz V., Hänsel R., Tyler V. E., "Rational Phytotherapy," Springer, Berlin-Heidelberg-New York, 1998.
4) Vesper J., Hansgen K. D., *Phytomedicine*, **1**, 9—16 (1994).
5) Newall C. A., Anderson L. A., Phillipson J. D., "Herbal Medicines," The Pharmaceutical Press, London, 1996.
6) Mauri P., Pietta P., *J. Pharm. Biomed. Anal.*, **23**, 61—68 (2000).
7) Hasler A., Meier B., *Pharm. Pharmacol. Let.*, **2**, 187—190 (1992).
8) Camponovo F., Wolfender J. L., Maillard M., Potterat O., Hostettmann K., *Phytochem. Anal.*, **6**, 141—148 (1995).
9) Mauri P., Migliazza B., Pietta P., *Mass. Spectrom.*, **34**, 1361—1367 (1999).
10) Wang H. F., Ju X. R., *Sepu*, **18**, 394—397 (2000).
11) Van Beek T. A., Scheeren H. A., Rantio T., Melger W. C., Lelyveld G. P., *J. Chrom.*, **543**, 375—387 (1991).
12) Thompson J., Strode B., Taylor L. T., Van Beek T. A., *J. Chrom.*, **738**, 115—122 (1996).
13) Zhang J., Pan J., Xie H., Yang Z., Hu X., Yang K., *Fenxi Huaxue*, **28**, 53—56 (2000).
14) Pietta P. G., Mauri P. L., Rava A., Sabbatini G., *J. Chrom.*, **549**, 367—373 (1991).
15) SEDERE-Sedex 55 manual, 1995.