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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

Fengxia Deng, S. William Zito*

Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. Johns University, 8000 Utopia Parkway, Jamaica, NY 11439, USA

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Abstract

A gas chromatography–mass spectrometry (GC–MS) method was developed and validated for the simultaneous determination of seven major chemical markers (bilobalide, ginkgolides A, B, C, kaempferol, quercetin and isorhamnetin) in phytopharmaceuticals of *Ginkgo biloba* L. The intra-day relative standard deviations (RSD) and inter-day RSD's were based on the analysis of the standardized *Ginkgo biloba* L. extract on the same day and on the following 3 consecutive days. The intra-day RSD's ranged from 1.21% (bilobalide) to 6.20% (kaempferol). The inter-day RSD's ranged from 2.10% (bilobalide) to 10.42% (isorhamnetin). Mean recoveries ranged from a low of 63.0±5.3% (isorhamnetin) to a maximum of 103.5±6.0% (ginkgolide A). Calibration curves were linear in ranges between 2.73 and 36.36 μg/ml for the markers. Limits of detection ranged from a low of 0.5 μg/ml (bilobalide) to a high of 2.5 μg/ml (quercetin). The limits of quantitation were a low of 1.1 μg/ml (gingkolides A, B, C) to a high of 7.5 μg/ml (kaempferol). The method was applied to a standard extract (>6% total terpenoids and >24% total flavonoids) and six ginkgo capsule phytopharmaceuticals.

Keywords: *Ginkgo biloba* L.; Bilobalide; Ginkgolides; Flavonoids

1. Introduction

Ginkgo biloba L. leaf extract (GBE) is one of the top selling phytopharmaceuticals in the US and Europe [1]. Extracts from ginkgo leaves are widely used for treating cerebral insufficiency, fighting memory loss and as a potential drug for Alzheimer's disease [2]. Gingko is classified as a dietary supplement according to the Dietary Supplement Health and Education Act of 1994 (DSHEA). Under DSHEA, dietary supplements do not require review or approval by the FDA and this fact has lead to significant variations in pharmaceutical quality [3,4]. Ginkgolides, bilobalide, and flavonoids are the major chemical markers in ginkgo (Fig. 1) and may be the compounds responsible for the putative pharmaco-

*Corresponding author. Tel.: +1-718-990-1632; fax: +1-718-990-1877.
E-mail address: zitos@stjohns.edu (S.W. Zito).

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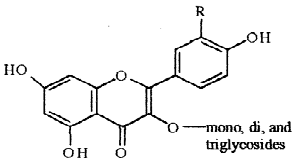
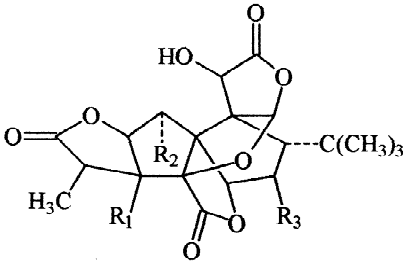
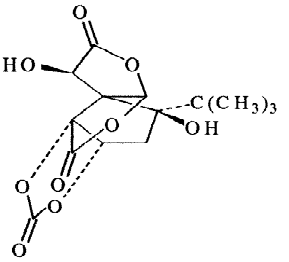


R	Flavonol glycoside
H	kaempferol derivatives
OH	quercetin derivatives
OCH_3	isorhamnetin derivatives





Bilobalide

R1	R2	R3	Ginkgolide
OH	H	H	A (GA)
OH	OH	H	B (GB)
OH	OH	OH	C (GC)

Fig. 1. The active constituents in ginko leaves.

logical action of ginkgo. Ginkgolides have been analyzed by gas chromatography–mass spectrometry (GC–MS), high-performance liquid chromatography (HPLC), LC–MS [4–8] and the flavonoids have been analyzed by HPLC [4–9]. However, these methods were not validated and required different extractions and separations for the flavonoid and terpenoid markers, making those methods time consuming and inefficient. This paper presents the development and validation of a simple one-step method for the simultaneous identification and quantification of the flavonoid and terpenoid chemical markers in *Ginkgo biloba* L. extract and commercial products.

2. Experimental

2.1. Chemicals and samples

Standard reference compounds of ginkgolides A and B, bilobalide, kaempferol, and quercetin, as well as, squalane, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane, *N*,*N*-dimethylformamide (DMF) and ethyl acetate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ginkgolide C was a generous gift from Dr Jian Zhang (Key Laboratory of Ion Beam Bioengineering, Institute of Plasma Physics, Chinese Academy of Sciences, Hefei, China). Isorhamnetin

was purchased from Indofine Chemical Company (Somerville, NJ, USA). The standardized *Ginkgo biloba* L. extract was obtained from Spectrum. Inc., (New Brunswick, NJ, USA). Ginkgo products were purchased randomly from local pharmacy and health food stores in the New York area.

2.2. Standard stock and calibration solutions

Two composite stock solutions containing the seven chemical markers were prepared in ethyl acetate and stored at −20 °C in a freezer. Composite A contained, bilobalide (0.70 mg/ml), kaempferol (0.80 mg/ml), and quercetin (0.60 mg/ml). Composite B contained ginkgolide A (0.60 mg/ml), ginkgolide B (0.30 mg/ml), ginkgolide C (0.30 mg/ml), and isorhamnetin (0.15 mg/ml). Composite calibration solutions were prepared using appropriate aliquots of composite A and B and diluted with ethyl acetate. The final concentrations used for linearity determinations are listed in Table 1.

2.3. Sample preparation and derivatization

Samples of commercial ginkgo extract products were prepared by combining the contents of 10 capsules and taking one-half the weight of a single dose for analysis. A 40 mg sample of standardized *Ginkgo biloba* L. extract was also taken for analysis. Samples were weighed in a 15 ml centrifuge tube and hydrolyzed with 5 ml of 1 N HCL in 20% methanol. The mixture was then sonicated for 15 min and heated at 85 °C for 1 h in a heat block. The mixture was extracted with 5 ml ethyl acetate by vortexing for 1 min, sonicating for 5 min, and then centrifuging for 10 min. Then, 50 μl of the organic layer was taken for derivatization using 250 μl DMF containing 0.01% squalane as internal standard and 250 μl BSTFA containing 1% trimethylchlorosilane. The mixture was heated at 115 °C for 45 min, and 2 μl of the derivatized sample injected for GC–MS quantification.

2.4. GC–MS

The derivatized GBE sample, as well as derivatized composite calibration solutions were injected into a GC–MS system that consisted of an HP 5890 gas chromatograph coupled with an HP5971A mass spectrometer. Separations were accomplished with a fused-silica capillary column (25 m×0.20 mm) coated with 0.33 μm layer of cross-linked methyl siloxane [HP Ultra 1]. The injector was set at 275 °C and the detector at 290 °C. GC was performed in the splitless mode with a 1 min splitless-time. The oven temperature was initiated at 80 °C for 0.1 min, then increased to 245 °C at 25 °C/min, held for 25.5 min, and then raised to 270 °C by 60 °C/min and held for 8 min. The flow-rate of the carrier gas (helium) was maintained at 0.5 ml/min. The split vent was set at

Table 1
Linear calibration curves for the GC–MS analysis of the chemical markers in *Ginkgo biloba* L. extract

Constituents	$y = ax + b$ linear model[a]		Regression $(r^2)(n)$[b]	Concentrations (x) (μg/ml)				
	Slope (a±SD)	Intercept (b±SD)						
Bilobalide	0.026±0.001	−0.091±0.014	0.999(15)	6.36	12.73	19.09	25.46	31.82
Ginkgolide A	0.018±0.001	−0.051±0.005	0.999(15)	5.46	10.91	16.37	21.82	27.27
Ginkgolide B	0.026±0.001	−0.030±0.007	0.998(15)	2.73	5.45	8.18	10.91	13.64
Ginkgolide C	0.017±0.001	−0.010±0.003	0.999(15)	2.73	5.45	8.18	10.91	13.64
Kaempferol	0.033±0.002	−0.336±0.050	0.997(15)	7.27	14.54	21.82	29.09	36.36
Isorhamnetin	0.006±0.001	−0.019±0.003	0.991(15)	4.09	5.46	6.82	8.18	9.55
Quercetin	0.019±0.002	−0.166±0.032	0.991(15)	5.45	10.91	16.36	21.82	27.27

Derivatization and chromatographic conditions used were as explained in Sections 2.3 and 2.4. Samples were prepared in triplicate as explained in Section 2.6.2.

[a] y = peak area ratio; x = concentration.

[b] n = the number of points in each cailibration curve, representing five different concentrations and determined three times each.

30 ml/min and the septum purge was set at 1 ml/min. The mass spectrometer was operated in the electron impact (EI) mode with an ionization energy of 70 eV. Identification was performed in the full scan mode in the range of 70–650 *m/z*. Quantification was done in the single-ion monitoring (SIM) mode using 299 *m/z* for bilobalide, 537 *m/z* for ginkgolideA, 625 *m/z* for ginkgolide B, 191 *m/z* for ginkgolide C, 559 *m/z* for kaempferol, 589 *m/z* for isorhamnetin and 647 *m/z* for quercetin.

2.5. Data analysis

MS ChemStation (ver. C02.06) was used for data sampling and integration of the chromatographs. Microsoft Excel (ver. 5.0) and GraphPad Prism (ver. 2.0) were used for statistical calculations. Data are expressed as means±SD (standard deviation of mean). Linear regression analysis using the least squares method was used to evaluate the calibration curve of each analyte as a function of its concentration.

2.6. Method validation

2.6.1. Standard and sample solution stability

The terpenoid and flavonoid content of the standard composite mixture solution was compared to freshly prepared reference composite mixture solutions immediately after the solutions had been prepared and following storage in a refrigerator for 24 h, 48 h, 7 days and 30 days.

2.6.2. Calibration curves

A series of standard composite mixture solutions were prepared in triplicate over the range of 30–150% of the corresponding nominal concentrations of the seven marker compounds. Nominal concentrations for the chemical markers are based on the extraction of 40 mg of standardized *Ginkgo biloba* L. extract and are 19 μg/ml for bilobalide, 16 μg/ml for ginkgolide A, 8 μg/ml for ginkgolide B, 8 μg/ml for ginkgolide C, 22 μg/ml for kaempferol, 7 μg/ml for isorhamnetin, and 17 μg/ml for quercetin. The calibration curves were based on the analysis of the standard composite mixture at five concentration levels (30, 70, 100, 130 and 150% of the respective nominal concentrations; see Table 1). The curves were constructed by plotting the peak area ratio for each compound against its corresponding concentration.

2.6.3. Precision and accuracy

To verify the precision of the proposed GC–MS method, intra- and inter-day precision of the assay of the GBE standard were obtained. Two concentration levels of the standardized extract were prepared and assayed on the same day and on the following 3 consecutive days.

The accuracy of the method was examined by using the standard addition method for recovery studies. Forty milligrams of standardized *Ginkgo biloba* L. extract was spiked with two different amounts of each marker compound (each solution made in triplicate). The following amounts were added: bilobalide (140/280 μg); ginkgolide A (120/240 μg); ginkgolide B (60/120 μg); ginkgolide C (60/120 μg); kaempferol (160/320 μg); isorhamnetin (30/60 μg) and quercetin (120/240 μg). The spiked samples were assayed using the method and the results expressed as mean recovery±SD.

3. Results and discussion

3.1. Hydrolysis of flavonoid glycosides and extraction of flavonoids and terpenoids

The influence of acid concentration, volume of methanol, and reaction time on flavonoid hydrolysis yield was studied using rutin, a quercetin glycoside. Methanol was found to enhance the solubility of the terpenoids and flavonoids and the optimum hydrolysis conditions were determined to be 1 N HCl with 20% methanol and heating at 85 °C for 1 h in a tightly capped centrifuge tube. Extraction with ethyl acetate gave high recoveries (>95%) for both terpenoids and flavonoids.

3.2. The derivatization reaction

Direct derivatization without a drying step simplified the procedure and improved the flavonoid recovery since drying resulted in an approximate 10% loss of compound. The derivatization reaction was optimized against reagents, temperature, and

reaction time. Van Beek noted in his recent review [5] that derivatization with BSTFA containing 1% trimethylchlorosilane at 120 °C for 45 min gave the highest yield for ginkgolides. However, in our case the flavonoids began to decompose at 120 °C, therefore, we lowered the temperature to 115 °C. This minor modification of the published procedure optimized the flavonoid yields and didn't significantly affect the ginkgolide yields. The optimized conditions for derivatization were to combine 50 μl of the sample in ethyl acetate with 250 μl DMF containing 0.01% squalane and 250 μl of BSTFA containing 1% trimethylchlorosilane in DMF (1:1) and to heat at 115 °C for 45 min in a tightly capped vial.

3.3. Chemical marker identification

Fig. 2 shows a typical separation of bilobalide, ginkgolides A, B, C, kaempferol, isorhamnetin and quercetin. The chemical markers were identified by comparing their retention times and mass spectra to that of purchased standards. Ginkgolide C was not available commercially and was confirmed by comparison to mass spectral data in the literature [4,5,10]. The molecular ions were absent in their EI mass spectra and the major ions came from the loss of a methyl group from the molecular ion. The extent of fragmentation of ginkgolides was higher than that observed for the flavonoids, therefore the abundance of the ionic species of high m/z was low. The mass spectral pattern of these TMS derivatives is demonstrated by the mass spectra of kaempferol and ginkgolide A (Fig. 3).

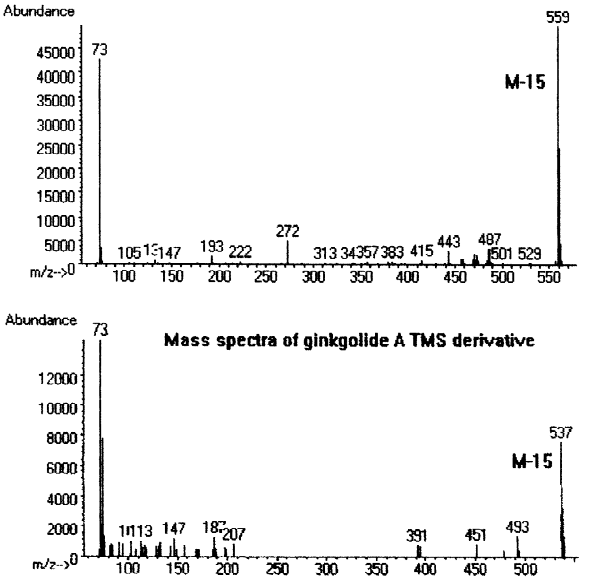


Fig. 3. Mass spectrum of kaempferol TMS derivative.

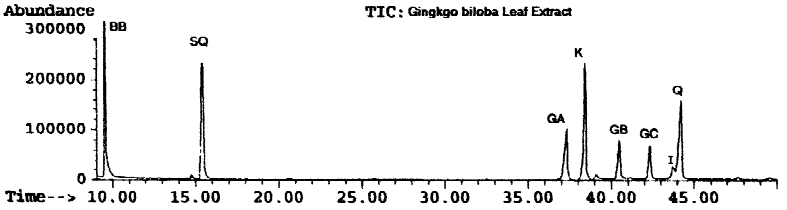
3.4. Method validation

3.4.1. Standard and sample solution stability

Standard and sample solutions were stable in ethyl acetate for at least 1 month. After 1 month storage in a −20 °C freezer, recoveries of 97±2% for bilobalide, 102±3% for total ginkgolides and 95±4% for total flavonoids were determined.

3.4.2. Limits of detection (LOD) and quantitation (LOQ)

The LOD was estimated as $3S_b$/slope of the



BB=bilobalide; SQ=squalane (IS); GA=ginkgolide A; K=kaempferol; GB=ginkgolide B; GC=ginkgolide C; I=isorhamnetin; Q=quercetin.

Fig. 2. A typical GC–MS chromatogram a *Ginko biloba* L. extract.

calibration curve where S_b was the standard deviation of the blank measurements ($n=10$). The actual LOD was then determined by the analysis of samples of known concentrations and visually found to be 0.5 μg/ml for bilobalide, ginkgolides A, B and C, 2.5 μg/ml for kaempferol and quercetin, and 1 μg/ml for isorhamnetin. Similarly, the LOQ was estimated as $10S_b$/slope of the calibration curve where S_b was the standard deviation of the blank measurements ($n=10$). The actual LOQ was then determined by the analysis of samples of known concentrations that gave reproducible results within 20% of the actual concentration. The LOQ was determined to be 1 μg/ml for bilobalide, 2.5 μg/ml for ginkgolides A, B and C, 7.5 μg/ml for kaempferol, 2.5 μg/ml for isorhamnetin and 5 μg/ml for quercetin.

3.4.3. Linearity and range

Under the experimental conditions described in Sections 2.3 and 2.4, linear calibration curves were obtained over the entire range of concentrations studied. Regression analysis of the peak area ratios (y) vs. concentration (x) for each ginkgolide and flavonoid were carried out and reported in Table 1. They demonstrated acceptable linearity with r^2 between 0.991 and 0.999. A t-test was performed on the y-intercepts and yielded t-values greater (negative) than the t-value (2.14) found in the t-table at the 95% confidence limit in all cases, indicating that the y-intercept was significantly different from zero.

3.4.4. Precision and accuracy

Table 2 shows that the relative standard deviation (RSD) for intra-day measurements for bilobalide was not higher than 2.0%, was not higher than 4.0% for the ginkgolides, and was not higher than 7.8% for the flavonoids. The inter-day RSD for bilobalide was not higher than 3.3%, was not higher than 5.3% for the gingkolides, and was not higher than 10.4% for the flavonoids.

The criterion of accuracy is the relationship between the amounts of added standards and the amounts detected by the GC–MS assay. As shown in Table 3, the recovery values are expressed as the percentage of assayed concentration relative to the calculated concentration. They are 80±6% for bilobalide, 103±5% for ginkgolide A, 97±7% for ginkgolide B, 96±8% for ginkgolide C, 89±5% for kaempferol, 63±5% for isorhamnetin and 91±6% for quercetin. Isorhamnetin showed lower recovery, probably due to its minor content in the extracts or perhaps to its lower polarity with respect to the other analytes. Other investigators using HPLC analysis have also reported low recovery of isorhamnetin [9].

Table 2
Intra-day and inter-day reproducibility of the GC–MS analysis of the chemical markers in *Ginkgo biloba* L.

Constituents	Mean (μg/ml)	Intra-day RSD[a] (%)	Inter-day RSD (%)
Bilobalide	5.52	1.21	3.30
	8.82	2.00	2.10
Ginkgolide A	13.39	3.35	4.85
	18.18	4.00	5.26
Ginkgolide B	5.41	3.14	3.68
	9.09	3.50	2.90
Ginkgolide C	3.96	1.46	4.57
	9.09	2.30	4.20
Kaempferol	24.61	5.28	8.75
	36.36	6.20	9.21
Isorhamnetin	3.29	4.47	10.02
	4.55	4.90	10.42
Quercetin	23.11	6.18	9.35
	27.27	7.82	9.83

Derivatization and chromatographic conditions used were as explained in Sections 2.3 and 2.4. Samples were prepared in triplicate as explained in Section 2.6.3.

[a] RSD = SD/Mean*100.

3.5. Sample analysis

The method was applied to the analysis of GBE and six commercially available phytopharmaceuticals

Table 3
Recoveries of terpenoids and flavonoids from *Ginkgo biloba* extract

Constituents	Added (μg)	Mean recovery±SD[a] (%)
Bilobalide	140.0/280.0	79.8±6.0
Ginkgolide A	120.0/240.0	103.5±5.2
Ginkgolide B	60.0/120.0	97.1±7.4
Ginkgolide C	60.0/120.0	95.9±7.8
Kaempferol	160.0/320.0	88.7±4.8
Isorhamnetin	30.0/60.0	63.0±5.3
Quercetin	120.0/240.0	90.9±6.2

Derivatization and chromatographic conditions used were as explained in Sections 2.3 and 2.4.

[a] SD is the standard deviation of the mean recovery of triplicate sample analyses for each added amount.

Table 4
Content of terpenoids and flavonoids in some *Gingko biloba* L. commercial products

Sample	BB%	GA%	GB%	GC%	K%	I%	Q%	ETT[a]%	TT[b]%±SD	ETF[c]%	TF[d]%±SD
1	0.034	0.281	0.204	0.030	1.600	0.028	0.511	>0.60	0.55±0.04	>2.45	2.38±0.42
2	1.360	1.078	0.496	0.600	0.900	0.061	1.057	>0.85	3.53±0.04	>3.40	5.12±0.65
3	0.677	0.559	0.356	0.360	0.793	0.062	0.922	>0.97	1.95±0.15	>4.01	4.51±0.58
4	0.316	0.925	0.549	0.501	1.098	0.090	1.255	>0.60	2.29±0.19	>2.43	6.20±0.83
5	0.763	0.881	0.577	0.450	0.715	0.072	1.140	>0.89	2.67±0.15	>3.57	4.82±0.51
6	0.087	0.246	0.230	0.200	0.234	0.015	0.323	>1.88	0.76±0.06	>7.5	1.45±0.18
GBE[e]	1.048	2.576	1.434	1.600	5.021	0.414	4.714	>6.00	6.65±0.10	>24.0	25.80±0.29

[a] ETT, expected total terpenoids determined from the label.
[b] TT, determined total terpenoids±SD; TT=(BB+GA+GB+GC).
[c] ETF, expected total flavonoid glycosides.
[d] TF, determined total flavonoid glycosides±SD; TF=(K+I+Q)*2.54 [6].
[e] GBE, standardized *Gingko biloba* L. leaf extract (>6% total terpenoids and >24% total flavonoid glycosides).

of ginkgo. Table 4 contains the assay results expressed as percent of the seven chemical markers as well as the total flavonoid glycosides and total terpenoids per capsule. In addition, Table 4 shows the expected total flavonoid glycoside and terpenoid content per capsule based upon the information found on the product label. Sample 6 contained less total flavonoid glycosides (1.45% vs. >7.5%) and terpenoids (0.76% vs. >1.88%) than would have been expected from the label claim. Interestingly, samples 2–5 were found to have significantly greater amounts of total flavonoid glycosides and terpenoids than would be expected from their labeled amount of GBE standardized extract. The higher content is most likely due to the fact that these capsules contained powdered leaf along with the GBE or to the fact that the minimal values (6% and 24%) were used for the determination of the total ginkgolides and flavonoid glycosides.

These results support previous reports of inconsistent content of commercial *Ginkgo biloba* L. products [3–5,9]. Indeed, it was precisely that fact that stimulated the use of a standardized extract in the preparation of gingko products as well as the inclusion of the term standardized on the bottle labels. However, our results show that even with the use of a standardized extract, there is still a great deal of variation in the content of the chemical markers in each capsule. This is most likely due to the fact that standard GBE sets a minimal amount for the total ginkgolides (>6%) and flavonoids (>24%) and doesn't indicate the actual concentrations. A better way to label these products would be to determine the actual amounts per capsule. Therefore, the efficient, reliable and validated analytical method presented in this report provides a new analytical assay that can be used to insure that the labeling accurately represents the content per dose of commercial *Gingko biloba* L. phytopharmaceuticals.

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