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VALIDATION OF CAPILLARY GAS CHROMATOGRAPHIC METHOD FOR DETERMINATION OF BILOBALIDE AND GINKGOLIDES A, B, C IN *GINKGO BILOBA* DRY AND LIQUID EXTRACTS

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Abstract: A method for identification and quantitative determination of ginkgolides A, B, and C and bilobalide in liquid and dry extracts of *Ginkgo biloba* extracts has been developed. Determinations made by employing capillary gas chromatography technique with FID detection were preceded by derivatization using BSTFA with TMCS addition at 120°C. Cholesterol was used as an internal standard. Validation of the method shows no interferences with concurrent constituents; average resolution (R), controlled for peaks of cholesterol and ginkgolide A was 1.53 (SD = 0.06). In the temperature program used (from 50°C to 300°C) the analyte retention times range from 11.2 min. (bilobalide) to 13.8 min. (ginkgolide C) and are of high repeatability of relative values (RRT): RSD = 0.05% ÷ 0.07% for ginkgolides. High correlation coefficients (r), detector signal linearity: from 0.99962 for ginkgolide C to 0.99985 for ginkgolide A were obtained within the concentration range under investigation. The method is of high sensitivity: limits of detection and limits of determination are 35 pg and 44 pg for bilobalide, respectively, while for ginkgolides (Gk) are: 78 pg and 92 pg for GkA, 57 pg and 68 pg for GkB, and 213 pg and 320 pg for GkC.

Keywords: capillary gas chromatography, drug analysis, tinctura Ginkgo biloba, bilobalide, ginkgolides A, B and C

Ginkgo leaves liquid and dry extracts belong to the group of herbal preparations used to symptomatic treat of mild to moderate dementia and peripheral arterial occlusive disease. It is helpful in neurosensory disturbances such as dizziness and tinnitus. Ginkgolides, especially ginkgolide B are known antagonists of platelet-activating factor PAF (1, 2).

The most characteristic and important constituents of ginkgo leaf are diterpene trilactones called ginkgolides A, B, C, J, and M, which differ in number and position of their hydroxyl groups, and the sesquiterpene trilactone bilobalide (Figure 1) (3, 4). Pharmaceutical dried leaves should contain not less than 0.1% of terpene lactones, calculated as the sum of bilobalide and ginkgolides A, B, and C. The leaf contains also flavonoids, long-chain alkylphenolic acids, alkylphenols and other constituents.

The ginkgolides and bilobalide were determined up to now by chromatographical and biological methods. Commonly used methods are: high-performance liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography (LC). Sometimes they are connected with mass spectrometry (MS). Another methods as thin- layer chromatography (TLC) and biological assays are also applied.

The HPLC method was used for determination of ginkgolides and bilobalide firstly (5). As an alternative, the GC assays were performed (6, 7). Although the HPLC analysis gave good results, the GC yielded better resolution and now it is the method of choice in the analysis of discussed compounds. From 1991, there were many publications concerning determination of Ginkgo biloba terpenes using GC (8–14). Some of them were coupled with the MS (6, 10, 12, 14) to improve compounds identification.

Simultaneously, many scientists still used HPLC for analysis.(15–17). Others performed LC-MS (18, 19) but only one fluorometric thin-layer chromatography assay (20) was reported.

The biological methods have been also applied. They used the ginkgolides PAF inhibitory potential and in consequence the reduction of platelet aggregation (8, 21). Both papers compare the results of investigation with chromatographic methods.

The aim of this paper is to optimize conditions for identification and determination of ginkgolides A, B, and C and bilobalide in dry and liquid extracts as well as in composite preparation using capillary gas chromatography and to perform full validation of the method. The method is intended for routine standardization assays of medicinal extracts from *Ginkgo biloba*.

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EXPERIMENTAL

Apparatus

TRACE GC gas chromatograph, produced by ThermoFinnigan (Rodano, Italy), equipped with a FID detector (3 pg C/s, linearity 10^6) and two injectors: split-splitless and cool on-column has been used. The split mode of split-splitless port was used. In temperature of 300°C, and split ratio 1:10, the split flow equals 17 mL/min. The temperature in oven was programmed from 50°C (for 2 min) up to 300°C (for 9.7 min) and the rate was 30°C min^{-1}. FID base body temperature was 325°C.

Capillary column – 19091J-413, 30 m × 0.25 mm i.d., with the stationary phase HP-5, film thickness 0.25 µm (J&W Scientific, Folsom, USA) was used. Injections were made using Hamilton syringe model no. 701 (Bonaduz, Switzerland), of 10 µL in capacity and 50 mm in needle length. For data acquisition, chromatograms registration and calculations the software Chrom Card for TRACE v. 1.07 has been used.

CHEMICALS AND REAGENTS

Gases

As carrier gas, helium of purity class 5.0 (Linde Gaz, Poland) was used. It additionally passed through the filter OT3-2, R&D Oxygen/Moisture Trap, R&D Separations. The chromatograms were recorded at constant carrier gas flow of 1.7 mL/min (35 cm/s).

The following gases were delivered to the detector – synthetic air (350 mL/min), hydrogen (35 mL/min) and nitrogen (make-up gas, 33 mL/min) of purity class 5.0 (Linde Gaz, Poland).

Standard substances

Several standard substances were used for investigation: bilobalide, pure, (Chroma Dex™), (Blb); ginkgolide A, pure ≥ 95% (HPLC) (GkA), ginkgolide B, pure ≥ 95% (HPLC) (GkB), and ginkgolide C, pure ≥ 90% (HPLC) (GkC) (all from Buchs, Switzerland); and cholesterol, Polish Pharmacopoeia V, (Cefarm Wrocław, Poland).

Reagents necessary for derivatization were: N,O-bis(trimethylsilyl)trifluoroacetamide, pure ≥ 99% (GC), (Buchs, Switzerland) (BSTFA) and trimethylchlorosilane, pure ≥ 99% (GC), (Buchs, Switzerland) (TMCS).

Preparations

Tinctura Ginkgo bilobae (1:5), made of: *Ginkgo bilobae folium- 20 parts, Ethanolum 60°– 100 parts*, manufactured by Herbapol S.A.(Kraków, Poland) (TGB). Samples were taken from three different production series.

Dry extract from ginkgo biloba leaves (*Ginkgo bilobae extractum siccum*, 35-67:1, extractant – ethanol) (DE GB).

Mixture 1:1 (v/v) *Tinctura Ginkgo bilobae* (1:5) *cum Tinctura Crataegi* (1:5) (TGC).

Derivatization procedure

Silanization at 120°C was carried out using BSTFA as a basic reagent with 1% (v/v) TMCS catalyzing addition.

Standard solutions

Standards of Gk A, B and C and Blb: 2.30 mg GkA, 3.40 mg GkB, 1.60 mg GkC and 1.10 mg Blb we weighed up to 0.01 mg and put separately into 4 glass test tubes of 5 mL capacity. Then 0.5 mL BSTFA and 0.05 mL TMCS (7, 13) were added with a glass pipette to each test tube. After careful mixing, the test tubes were placed in a thermostat at 120°C. The samples were heated for 60 min, i.e. until an excess BSTFA evaporated. The drying of samples was completed on a water bath under lowered pressure. Dry residues (after cooling) were dissolved in 1.0 mL of methanol.

Preparation solutions

Liquid samples of TGB and TGC preparations: Prior to silanization, preparation volumes of 1,0 mL were evaporated and dried on a water bath under lowered pressure and then the silanization procedure described above was carried out. Then the samples were dissolved in 1,0 mL of methanol. The obtained suspensions were centrifuged in a test tube centrifuge for 5 min at 4000 rpm. Clear solutions were subjected to analysis employing CGC techniques.

For dry GB extract, the weighed amounts of 30.1, 50.7, and 70.7 mg were prepared with the accuracy of 0.1 mg and the procedure mentioned above was ended with centrifuging.

Standard solutions for determinations

For identification, one-component standard solutions of standard substances – 2.30 mg GkA, 3.40 mg GkB, 1.60 mg GkC and 1.10 mg Blb were weighed up to 0.01 mg and dissolved in 1,0 mL of methanol after derivatization. For cholesterol (IS), an appropriate weighed amount was directly dissolved in 1,0 mL of methanol, thus producing a 2.7 mg/mL solution.

Cholesterol do not undergo derivatization under conditions of any described investigation. The

peak of internal standard is appropriate for qualitative and quantitative interpretation.

For internal standard calibration, a five-component standard solution was prepared by mixing the specified volumes of one-component solutions, namely 400 µL of Blb, 200 µL of GkA, 100 µL of GkB, 200 µL of GkC, and 100 µL of cholesterol (IS) solutions to obtain a five-component methanol standard solution (stdBlbGkABCchol(IS)) of the following concentrations: 0.44 mg/mL Blb, 0.46 mg/mL GkA, 0.34 mg/mL GkB, 0.32 mg/mL GkC and 0.27 mg/mL cholesterol (IS). This solution was also used for checking linearity and precision and establishing limits of determination and detection, as well as a basic solution for determination of recovery of the newly developed method.

For determining the recovery three solutions were made:

C1. Equal amounts of 100 µL of stdBlbGkABCchol(IS) and TGB solutions were taken.

C2. 100 µL of TGB solution was taken and 10 µL of cholesterol solution and 90 µL of methanol were added.

C3. 100 µL of stdBlbGkABCchol(IS) was taken and 10 µL of cholesterol solution and 90 µL of methanol were added.

Preparation of solutions for determinations

Preparation solutions – 1.000 mL of TGB or TGC was taken and dissolved in 1.000 mL of methanol after derivatization.

Chromatographic analyses were made instantly after solutions were prepared.

Analysis

Calibration for quantitative analysis of derivatization reaction

For samples of TGB and DE GB preparations some calibration tests were performed to check quantitatively how does the derivatization reaction proceed: various volumes of TGB, namely 1.000 mL, 1.750 mL, 2.500 mL and various weighed amounts of DE GB, i.e. 30.1 mg, 50.7 mg, and 70.7 mg were taken. Methanol (1.000 mL) extracts of derivatization products were analyzed chromatographically under specified conditions. It was found that quantitative analysis should be carried out on 1.0 mL TGB tincture samples.

Effect of parameters changes on the results of measurements

The effects were investigated after establishing chromatographic analysis conditions. Such factors as the method of determination, i.e. preparation of samples, analysis duration, mobile phase temperature and pressure etc. were taken into account.

Both standard and preparation solutions were delivered into the column at fixed carrier gas flow rate or pressure and using the chromatograph oven isothermal or programmable mode within the temperature range from 40°C to 325°C. The injection temperature was varied in the range 200-325°C and thermostat temperature rise was (5-40°C/min). Volumes from 0.5 to 2.0 µL were injected. The measurements were made in cycles and each of them was commenced after checking system tightness and zero-line stability under the specified conditions. Retention times, peak areas and heights were recorded at the same time to establish the conditions of measurement.

The obtained results of parameters evaluation are listed in Table 1, whereas examples of chromatograms are presented in Figure 2.

Selectivity and specificity of the method

To determine an effect of matrix components on the results of determination, comparative analysis was carried out for appropriate standard solutions as well as TGB and TGC preparation solutions. The peaks of analytes were identified by comparing the absolute (t_R) and relative (RRT) retention times and peak areas (A) and heights (H) were recorded (Figure 2).

These chromatograms show peaks of retention times corresponding to Blb (approx. 11.2 min), GkA (approx. 13.1 min), GkB (approx. 13.6 min), and GkC (approx. 13.8 min). Additional peaks of retention times below 11 min recorded from preparation chromatograms (Figures 3, 4) do not affect the results of determination.

The location of peaks in chromatograms defined by retention times, t_R and relative values, RRT (Table 1) is repeatable, thus enabling their identification.

To calculate RRT for ginkgolides and Blb, the retention time of cholesterol as the internal standard was used as the reference; ± 0.5% criterion was used. The relative retention times (RRT) were 0.858 for Blb, 1.006 for GkA, 1.044 for GkB, and 1.057 for GkC relative to cholesterol and computed means values RSD% did not exceed 0.2% for Blb and 0.1% for ginkgolides.

The selectivity of the method was checked by establishing optimal separation conditions for ginkgolides and Blb (Figure 2). The separation conditions were controlled by computing resolution R for IS and GkA peaks. The criterion R ≥ 1.0 was

Table 1. Validation parameters.

A. Retention time, t_R (min)					
	Blb	IS	GkA	GkB	GkC
Mean, n = 80	11.203	13.052	13.134	13.627	13.790
SD	0.034	0.055	0.055	0.062	0.064
RSD%	0.31	0.42	0.42	0.45	0.47
μ, 95%	0.007	0.011	0.011	0.012	0.013
B. Relative retention time, RRT					
	Blb	IS	GkA	GkB	GkC
Mean, n = 80	0.858	1	1.006	1.044	1.057
SD	0.002	-	0.001	0.001	0.001
RSD%	0.18	-	0.05	0.07	0.07
μ, 95%	0.00035	-	0.00012	0.00015	0.00016

C. Peak area, A * 10^6 (0.1 μV*s)/ peak height, H * 10^4 (μV)				
	Blb	GkA	GkB	GkC
Mean, n = 80	1.06/ 6.76	1.41/ 6.67	1.89/ 8.02	0.80/ 3.25
SD	0.12/ 0.31	0.04/ 0.36	0.13/ 0.25	0.01/ 0.22
RSD%	10.90/ 4.50	2.90/ 5.40	6.60/ 3.10	1.60/ 6.70
μ, 95%	0.09/ 0.24	0.03/ 0.29	0.10/ 0.20	0.01/ 0.18
D. Recovery				
	Blb	GkA	GkB	GkC
	c (mg mL^{-1}), n = 3			
C1 (fortific. + sample)	0.382	0.304	0.154	0.073
C2 (sample)	0.258	0.199	0.056	0.014
C3 (fortification)	0.133	0.114	0.094	0.060
Recovery % = (C1 – C2) / C3 * 100%	93.6	92.9	104.2	99.5

Blb – bilobalide; GkA, GkB, GkC – ginkgolides A, B, and C; IS – internal standard (cholesterol)
SD – standard deviation, RSD – relative standard deviation, μ, 95% – confidence interval 95%

Table 2. Results of quantitative GC analyses of dry extract – DE GB.

	Blb	GkA	GkB	GkC	Blb	GkA	GkB	GkC
$m_{DE\ GB}$ (mg)	c (mg mL^{-1} of methanol), n=3				mg/g DE GB			
70.7	0.769	0.824	0.203	0.460	10.90	11.70	2.87	6.51
50.7	0.554	0.534	0.135	0.263	10.90	10.50	2.67	5.18
30.1	0.286	0.277	0.068	0.121	9.50	9.20	2.27	4.01

Blb – bilobalide; GkA, GkB, GkC – ginkgolides A, B, and C

taken into account. The average resolution for IS and GkA peaks derived from chromatograms recorded during standard solution split injection was 1.53 (n = 50, SD = 0.07, RSD = 4.88%).

The results described above are shown in Table 1.

Precision

The precision is defined as the degree of consistence between measurements repeated many times. The precision has been expressed by absolute (SD) and relative (RSD%) standard deviation, while assuming that the result of individual determination should be within the range x = x ± 2SD (Tables 2, 3, and 4).

Accuracy

The accuracy of the method was described by the results of recovery % (22) obtained for standard solutions containing the specified amount of the preparation with 100% of constituents under investigation (Table 1).

Linearity

The linearity of the peak area vs. analyte concentration relationship was examined using the standard solution stdBlbGkABCchol(IS) and the split function of the injector used. The relationship was checked for the following analyte concentrations: 0.046-0.92 mg/mL GkA, 0.034-0.68 mg/mL GkB, 0.016-0.32 mg/mL GkC and 0.044-0.88 mg/mL Blb.

Table 3. Results of quantitative GC analyses of liquid extracts TGB.

Sample concentrations, c (mg mL^{-1})					
Batch	n=9	Blb	GkA	GkB	GkC
A	Mean	0.305	0.232	0.068	0.023
	SD	0.041	0.021	0.005	0.002
	RSD%	13.6	9.0	7.7	10.1
	μ, 95%	0.027	0.014	0.003	0.002
B	Mean	0.330	0.275	0.081	0.057
	SD	0.026	0.014	0.004	0.014
	RSD%	8.0	4.9	5.0	24.4
	μ, 95%	0.017	0.009	0.003	0.009
C	Mean	0.585	0.409	0.121	0.068
	SD	0.045	0.032	0.006	0.006
	RSD%	7.8	7.8	4.7	8.2
	μ, 95%	0.030	0.021	0.004	0.004

Blb – bilobalide; GkA, GkB, GkC – ginkgolides A, B, and C;
SD – standard deviation, RSD – relative standard deviation, μ, 95% – confidence interval 95%

Table 4. Results of quantitative GC analyses of the TGC preparation.

Sample concentrations, c (mg mL^{-1})					
Sample No.	n=3	Blb	GkA	GkB	GkC
1	Mean	0.186	0.102	0.038	0.034
	SD	0.005	0.003	0.003	0.005
	RSD%	2.460	3.234	5.754	4.919
	μ, 95%	0.004	0.003	0.002	0.004
2	Mean	0.182	0.110	0.041	0.025
	SD	0.008	0.007	0.005	0.003
	RSD%	4.158	6.104	9.346	6.407
	μ, 95%	0.009	0.008	0.005	0.003
3	Mean	0.179	0.097	0.037	0.031
	SD	0.006	0.003	0.001	0.002
	RSD%	3.247	2.811	3.295	4.029
	μ, 95%	0.007	0.003	0.001	0.003

Blb – bilobalide; GkA, GkB, GkC – ginkgolides A, B, and C;
SD – standard deviation, RSD – relative standard deviation, μ, 95% – confidence interval 95%

The results were obtained by using the linear regression method.

For all the analytes the high correlation coefficients r and detector signal linearity were found: 0.99985 for GkA, 0.99974 for GkB, 0.99962 for GkC and 0.99976 for Blb.

Limit of detection and limit of determination

Determinations were made by recording chromatograms with an appropriate split for the solution prepared by dissolving the standard solution stdBlbGkABCchol(IS) with methanol (1:100) and analyzing the ratio of detector signal for the sample containing the specified weight of the substance to the baseline noise.

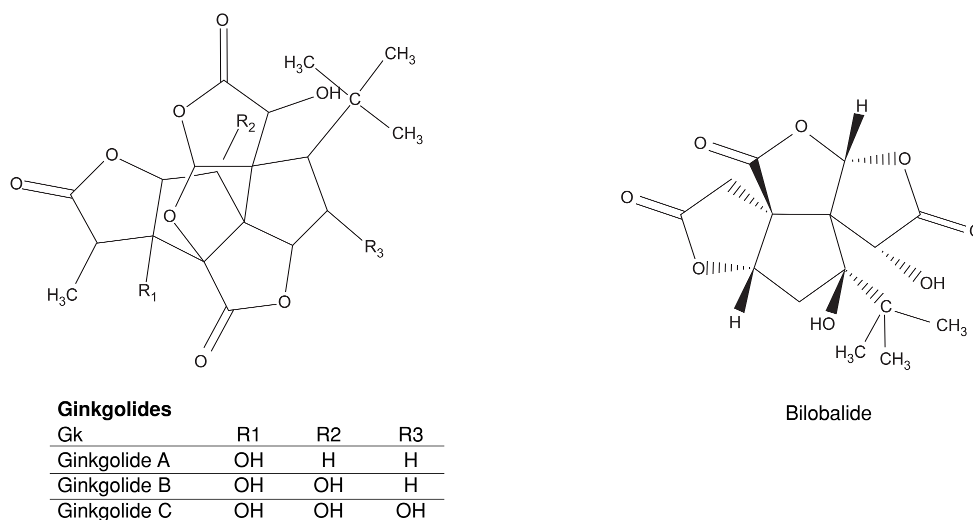
The weights of the substance for which the ratio of detector signal to the baseline noise was ≥ 3 and ≥ 6 were used as a limit of detection and limit of determination, correspondingly.

The limits of detection and determination for analytes converted into concentrations in the standard solution under examination were 35 pg and 44 pg, respectively, for bilobalide, while for ginkgolides (Gk) were: 78 pg and 92 pg (GkA), 57 pg and 68 pg (GkB) and 213 pg and 320 pg (GkC).

As the result, the basic parameters of measurement and the way of quantitative analysis were established.

Quantitative analysis conditions

Samples were injected with a 1.0 μL syringe (“empty needle” technique with injected volume control before and after on-column injection) into the capillary column through a split-splitless injector



Gk	R1	R2	R3
Ginkgolide A	OH	H	H
Ginkgolide B	OH	OH	H
Ginkgolide C	OH	OH	OH

Figure 1. Structures of ginkgolides A, B, C, and bilobalide.

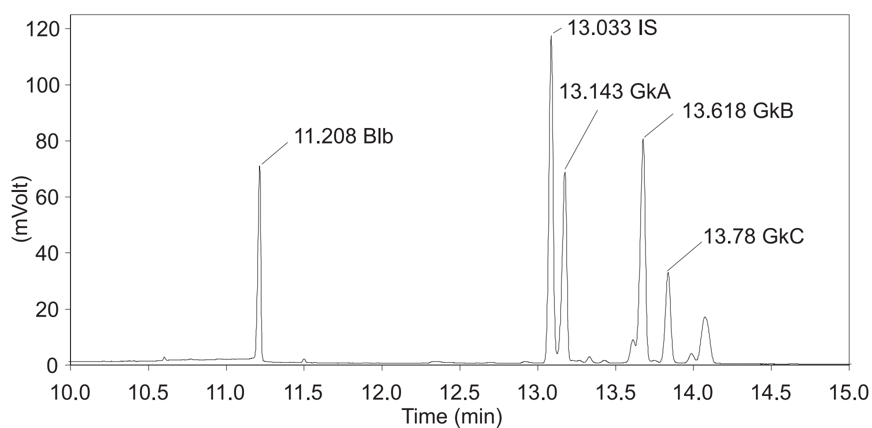


Figure 2. Chromatogram of standard solution (stdBlbGkABCchol(IS)).

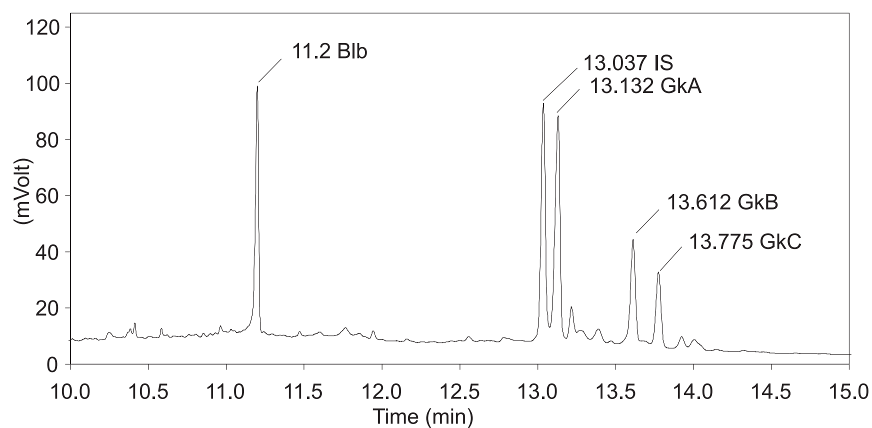


Figure 3. Results of analyses of 30.1 mg of dry extract DE GB.

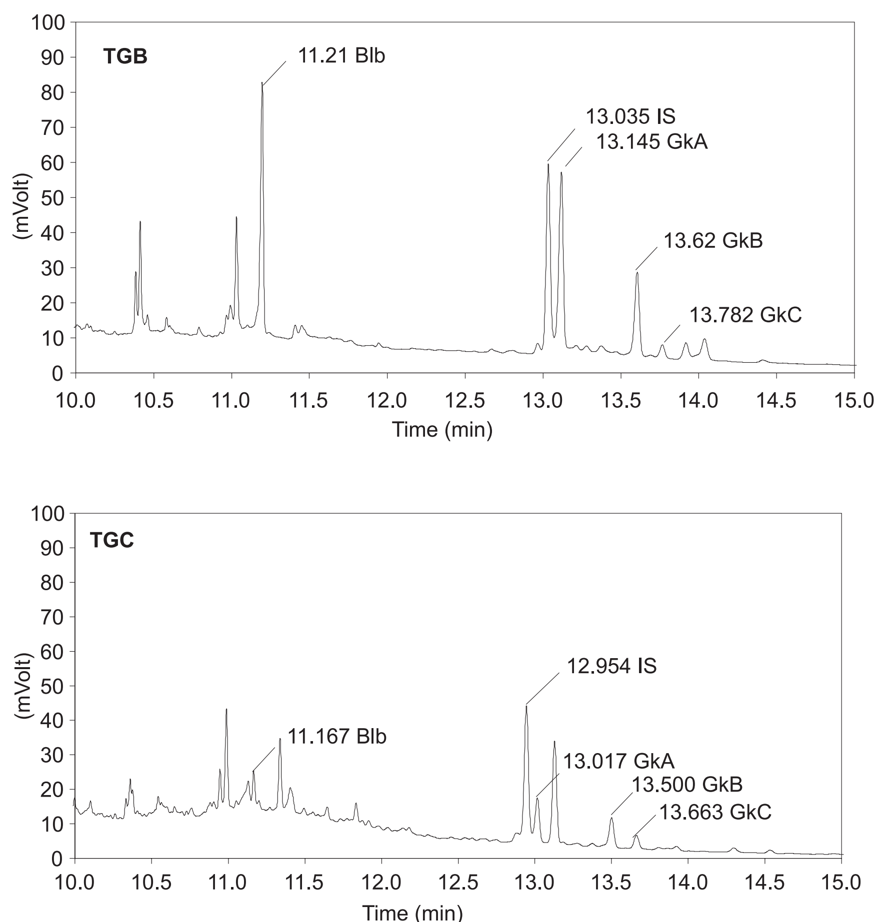


Figure 4. Comparison of chromatograms of analyzed liquid extracts: TGB and TGC.

at specified split ratio 1:10, at the temperature of 300°C. The flow rate of carrier gas (helium) through the column was constant at 1.7 mL/min (35 cm/s). The temperature in oven was 50°C through 2.0 min and then raised at the rate of 30°/min up to 300°C. FID detector had the temperature of 325°C. Gases: hydrogen, air, and nitrogen flew through the detector with speed; 35 mL/min, 350 mL/min, and 33 mL/min, respectively.

The internal standard method was used for calculation purposes based of integrated peak areas with multilevel calibration based on detector response factor RF. The analyte concentrations were determined by analyzing the relationship between areas of their peaks and those of the internal standard. The results are presented in Tables 2, 3 and 4.

RESULTS AND DISCUSSION

The method for identification and qualitative determination of ginkgolides A, B, and C, as well as bilobalide in liquid and dry extracts and also complex preparations of ginkgo leaves using capillary GC was developed and validated. Due to similar volatility of ginkgolides under investigation and the presence of water in liquid extracts, in the first stage the sample derivatization conditions were defined. The derivatization process has been described quantitatively when weighed amounts of 30-70 mg of dry DE GB extract and TGB tincture at amount not exceeding 1.0 mL were used in the silanization reaction.

There are similar absolute retention times for ginkgolides, but the differences that occur are sufficient to identify peaks originated from individual ginkgolides. The peak identification is facilitated by high repeatability of relative retention times.

The time of analysis of this newly developed method is shorter than in former assays (below 15 min), because of an innovative temperature program. The program starts at low temperature (50°C) and the further fast heating was used till the

isotherm at the maximum temperature (300°C) is achieved. This led to well developed peaks which have low retention time values comparing to the hitherto described methods. The peak of internal standard – cholesterol, that has not been subjected to derivatization in contradiction to previous methods – is placed in chromatograms in the close vicinity of ginkgolide peaks. The controlled resolution for the internal standard and ginkgolide A is 1.53 in average. The good separation of individual constituents of mixtures under investigation allows identification and qualitative analysis of analytes, as confirmed by the results obtained for liquid TGC extracts.

The newly developed method is of high sensitivity: the limits of detection and determination are 35 pg and 44 pg for bilobalide, 78 pg and 92 pg for GkA, 57 pg and 68 pg for GkB, and 213 pg and 320 pg for GkC.

When analyzing both tinctures and dry extract no effects of the matrix on the results were found, thus indicating a good selectivity of the method (Figures 2, 3). A wide linearity range guarantees that good results are obtained even if individual product series differ in constituent content. There are satisfactory recovery and precision of the method. In CGC quantitative analyses collected in Tables 2 and 3, a good repeatability of the results has been achieved.

CONCLUSION

One can conclude that the developed method enables easy qualitative and quantitative analyses of ginkgolides A, B, and C, as well as bilobalide, both in dry and liquid (simple and complex) extracts of *Ginkgo biloba* leaves and in composite preparations. The method can be used in routine standardization assays of materials and phytopharmaceuticals containing ginkgolides and bilobalide. Full validation shows that the results are accurate and precise so the method can by widely applied.

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