

Development and Validation of a Gas Chromatographic Method for Identification and Quantification of Terpene Trilactones in *Ginkgo biloba* L. Extract and Pharmaceutical Preparations

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Abstract: A gas chromatographic method was developed and validated for the determination of bilobalide and ginkgolides A, B, C and J in *Ginkgo biloba* L. The *Ginkgo* terpene trilactones have been dissolved in 57% of pure ethanol in purified water and separated by extraction with a mixture of toluene/ethyl acetate. After evaporation of the solvent with nitrogen and derivatization, these chemical markers have been quantified using GC-FID. The intra-day RSD was ranged from 1.13% for bilobalide to 2.34% in case of ginkgolides. The inter-day RSD was ranged from 2.54% and 3.68% for, bilobalide and ginkgolides respectively. Mean recoveries were $99.78\% \pm 0.82\%$ and $99.48\% \pm 0.86\%$ for ginkgolides and bilobalide, respectively. Based on chromatograms, the response of the terpene trilactones was linearly dependent on the terpene concentration, i.e. linear calibration curve was found in the range of the method and was applied to analyze standard extract (containing 5-7% total terpenoids and 22-25% total flavonoids) as working standard and for the analysis of six *Ginkgo* pharmaceutical products offered on the market.

Keywords: *Ginkgo biloba*, bilobalide, ginkgolides, gas chromatography.

1. INTRODUCTION

The extracts of *Ginkgo biloba* L. (GBE) are the most frequently sold phytopharmacological products in America and Europe and largely used as a supplement of lunching in Japan, China as well in the United States [1-3]. The flavonoid glycosides and terpene trilactones presented in *Ginkgo* are considered as interesting molecules from a pharmaceutical point of view. The ginkgolides A, B, C and J and the bilobalide are the names of terpenes in GBE. The first ones were regarded as diterpenes and the bilobalide as sesquiterpene (C₁₅ closed terpene). Structures of ginkgolides A, B, and C were determined by two Japanese groups in the sixties of past century [4-6] whereas the structures of the bilobalide [7] and ginkgolide J [8] were discovered few years later. They are highly oxidizable compounds, especially in the presence of air's oxygen.

The interest of the analysis of ginkgolides had increased in the eighties because of the need to explain their exact therapeutic action and the increase in the cost of the extracts of *Ginkgo*.

Some clinical studies showed the effect of *Ginkgo* in the improvement of peripheral and cerebrovascular circulation

[9,10] and treatment of the neurosensory problems [11]. In addition, it was noticed, that a significant improvement of the skin blood perfusion and blood's viscosity and elasticity fall [12], and consequently, the increase in the transport of oxygen and glucose towards the various bodies and with the brain particularly [13,14].

The extracts of *Ginkgos*' sheets were largely used for the treatment of the cerebral insufficiency by modulation of the cerebral energy metabolism and inhibition of the acetylcholinesterase, thanks to terpenes trilactones [15,16]. The GBE were also used to fight with variety of neurological energy disorders of the Alzheimer disease passing through the depression, lack of attention and temporary loss of the memory [17-19]. GBE is categorized, like vitamins C, B and beta-carotene, as the diet supplementary agents with antioxidant and free anti-radicals properties [20,21]. The extracts of the *Ginkgos*' sheets are also largely applied to treat the cerebral vascular ischemia [22]. Indeed, these extracts can correct the cerebral damage caused by an ischemia by improving blood circulation and increasing the cerebral blood flow rich in glucose and oxygen [14]. The ginkgolides' structures, including bilobalide are shown in Fig. (1).

Various analytical techniques were used to quantify or identify the terpene trilactones in the standardized extracts and the products of *Ginkgo*. The ginkgolides are non-volatile compounds and polar in nature. Therefore, they were suitably analyzed by reverse phase high performance liquid

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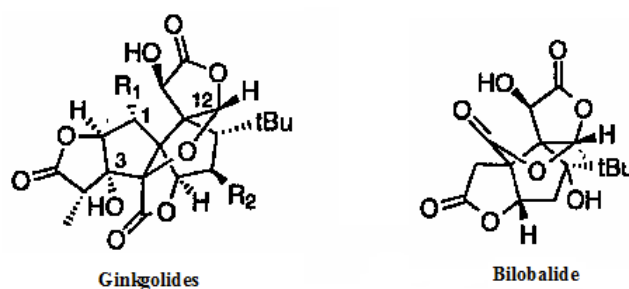


Fig. (1). Chemical structures of the terpenes trilactones in *Ginkgo biloba*. GA : $R_1 = R_2 = H$; GB : $R_1 = OH, R_2 = H$; GC : $R_1 = R_2 = OH$; GJ : $R_1 = H, R_2 = OH$.

chromatography (RP-HPLC). However, they have poor chromophores and can only be monitored at low and non selective wavelengths (190–220 nm). This makes UV detection difficult, interfered with trace impurities especially when analyzing crude *Ginkgo* extracts and extensive clean-up procedures that are usually required as prior to analyze leaf extracts and dosage forms [23-26]. In spite of this detection difficulty, an Egyptian group validated and published a RP-HPLC method using UV detection at 220 nm [27]. A better approach for the HPLC detection was refractive index (RI) detection but unstable baseline problems and low sensitivity detract from its selectivity [28,29]. Mass spectrometry (MS) coupled to LC or GC (LC-MS, GC-MS) was used and has proved a good detection due to its sensitivity and low limits of detection and quantification [3,23,30-34]. The terpene trilactones have been analyzed by HPLC by using evaporative light scattering detection (ELSD) [25,35,36] or by gas chromatography by using flame ionization detection (GC-FID) [29,30]. Reverse-flow micellar electrokinetic chromatography (RF-MEKC) was applied by Dubber and Kanfer [37] and NMR by Van Beek *et al.* [38]. A new method, using LC-atmospheric pressure chemical ionization (APCI)-MS in the negative ionization has been used to analyse ginkgolides and bilobalide in food products [39,40]. However, the methods using instruments like mass spectrometry MS, NMR, APCI-MS and RF-MEKC are very expensive and increase analysis cost and might be not available in routine QC laboratories. HPLC-ELSD due to the simplification of sample preparation and its inexpensive detection and GC-FID due to its availability and its inexpensive cost were the economic choices for routine QC laboratories. However, the ELSD process needs nitrogen to make nebulization and an oven to evaporate the solvent or the mobile phase. In the present paper, a quantitative GC-FID method is described for the determination of terpene trilactones (ginkgolides A, B, C, J and bilobalide) in *Ginkgo biloba* extracts and products by using a simple purification and sample derivatization before the analysis and a fast run, only 13 minutes for one injection as compared to those in literature [34].

2. MATERIALS AND METHODS

2.1. Reagents

The used reagents were of analytical grade without previous purification. Standard reference compounds of ginkgolide A, ginkgolide B, bilobalide (terpene trilactones) and standardized *Ginkgo biloba* L. extract (raw material)

were purchased from Indena (Italy), squalane and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMSC) and N,N-dimethylformamide (DMF) were purchased from Sigma (Germany), hexane was purchased from Carlo Erba (Italy), pure ethanol was purchased from Riedel-de-Haën (Germany) and toluene were purchased from Scharlau (Spain). Water was purified by osmosis system. *Ginkgo* products were provided by OPALIA laboratories and purchased from a local pharmacy in Hammam-Lif area (Tunisia).

2.2. Standard Stock and Calibration Solutions

Ginkgo biloba stock solution was prepared from standardized *Ginkgo biloba* L. extract by dissolution 57% of pure ethanol in purified water and was stored in refrigerator (-4°C) for stability. The solution contained 40 mg/mL of *Ginkgo biloba* L. extract. Appropriate aliquots were taken from the stock solution and used in the extraction step. The quantities of *Ginkgo biloba* in the aliquots were used for linearity determination.

2.3. Sample Preparation and Derivatization

A 500 mg of standardized *Ginkgo biloba* L. extract was taken for the analysis (standard stock solution). Samples of *Ginkgo biloba* extract products were prepared by taking 2 mL of the liquid forms in a dry and clean tube tests. Capsules' samples could be prepared by combining the contents of 15 capsules and taking the equivalent of 500 mg of GBE from the mixture for analysis. Tablets' samples were prepared by crushing in a mortar 15 tablets and taking the equivalent of 500 mg of GBE. Samples were dissolved in 12.5 mL of 57% of pure ethanol in purified water, sonicated for 20 minutes and vortexed every 5 minutes. A 2 mL of the clear solution were transferred into a dry tube tests in which one adds 500 μ L of squalane (1 mg/mL in hexane) as internal standard. The mixture was extracted with 4 mL of a mixture of toluene and ethyl acetate (7/3) by vortexing for 1 minute. Then, 2 mL of the organic phase was transferred into a 10 mL head space vial and the solvent was evaporated by a nitrogen flow (a temperature between 40 and 50°C can be used for evaporating). To the residue, one was added 400 μ L of dimethylformamide and 400 μ L of mixture BSTFA/TMSC (99/1) which had been heated on the head space's four or another heat block at 100°C for 1 hour. After cooling, a 2 μ L of the derivatized sample was injected for GC-FID identification and quantification. The preparation time was estimated to one hour and the derivatized time was estimated to one hour and 25 minutes.

2.4. GC-FID Conditions

The derivatized samples and derivatized calibration solutions were injected into a GC-FID system that consisted of an HP 5890 gas chromatograph with flame ionization detection (FID). Separations were accomplished with a fused-silica capillary column (10 m × 0.53 mm) coated with 0.88 μm layer of cross-linked methyl siloxane (HP-1). The injection and detection temperatures were 280°C and 300°C respectively. The injection mode is split with ratio of 2: 1. The flow-rate of the carrier gas (nitrogen) was maintained at 3 mL/min. The oven temperature was initialised at 250°C for 2 minutes, and then increased to 300°C at 5°C/min, had been holding for 1 minute. The total analysis time was 13 minutes. The quantification was performed with flame ionisation detection with flows 400, 45 and 25 mL/min successively for compressed air, hydrogen and nitrogen as make-up.

2.5. Calculations

The recoveries' percentages (*R%*) of bilobalide, ginkgolides or sum of terpenes content were determined by the formula (1):

$$R\% = \frac{AE}{AS} \times \frac{AIS}{AIE} \times \frac{ME}{MS} \times 100 \quad (1)$$

where AE (AS) is the peak area of bilobalide, sum of the ginkgolides or sum of terpenes in the sample (standard) chromatogram. AIE (AIS) is the peak area of internal standard (squalane) in the sample (standard) chromatogram. ME (MS) is the mass in mg of *Ginkgo biloba* in the sample (standard) preparation. The bilobalide, ginkgolides or sum of terpenes content in the analysed extract were determined by the formula (2):

$$mg \text{ (terpene)}/100 \text{ ml} = \frac{\% \text{ (terpene)}}{100} \times \frac{4}{100} \times Ta \times 1000 \quad (2)$$

where %(terpene) is the percentage of bilobalide, ginkgolides or sum of terpenes and Ta is the assay of bilobalide, ginkgolides or sum of terpenes in the raw material (GBE) in %.

2.6. Method Validation

2.6.1. Standard and Sample Solutions Stability

The terpenoid content of the standard and sample solutions were compared to freshly prepared reference solutions, prepared and stored in the refrigerator for 24 hours. The terpenoid content has not been changed during 24h.

2.6.2. Calibration Curves

Aliquots of the standard stock solution were taken and transferred to the test tubes. The contents of *Ginkgo biloba* L. extracts were 64, 72, 80, 88 and 96 mg by tube corresponding to 60, 80, 100, 120 and 140% of theoretical quantity of *Ginkgo biloba*. Calibration curves were based on the sum of responses of ginkgolides, the responses of bilobalide and the sum of responses of all terpene trilactones. The calibration lines were constructed by plotting the areas of ginkgolides and bilobalide against their corresponding concentration.

2.6.3. Precision and Accuracy

The results of precision and accuracy determination were obtained from the recoveries of the ratios of found quantities (*Ginkgo biloba* in the presence of liquid forms matrix) to the introduced quantities (*Ginkgo biloba* only). The precision of the proposed method was verified by calculation of the repeatability's RSD and intermediate precision's RSD of six preparations done successively during one day and the following 3 consecutive days. The accuracy was determined by calculation of the mean recoveries ± SD of five levels of concentrations.

3. RESULTS AND DISCUSSION

3.1. Solubility and Extraction of Terpene Trilactones

The 57% of pure ethanol in purified water was used to improve the solubility of terpene trilactones. This solvent, which is used in producing liquid pharmaceutical forms of *Ginkgo*, showed good solubility of terpenes. In the same case, pure methanol and 50% methanol in purified water were used to verify the solubility of terpenes and they gave the same results but methanol was toxic to the environment and health. The solvent used in this method was the best choice to dissolve *Ginkgo biloba* extracts as compared to other solvents in the literature like 1N HCl in 20% methanol [34] or 10% NaH₂PO₄ (m/v) with controlling pH and temperature [30]. A mixture of toluene and ethyl acetate (7/3) was used to enhance the extraction of terpenes from *Ginkgo biloba* L. extract. It gave high recoveries (> 98%) and constant retention times as compared to other solvent extraction reported in the literature [30,34]. The advantage of this mixed solvent was that it would give good separation without centrifugation of the two solutions' phases if we analyzed liquid forms. There would be a problem of phases' separation if we used ethyl acetate only [41] or in the mixture with tetrahydrofuran [30] to the extraction of terpenes from *Ginkgo biloba* in liquid forms.

3.2. Derivatization Reaction of Terpenes

The derivatization reaction of terpenes trilactones was based on a direct addition of the reagent BSTFA/TMSC (99/1) in the presence of DMF to the dried residue obtained with a nitrogen's flow at a temperature of 100°C in 1 hour in a block four (a four of head space apparatus can be used). This temperature was used less than other temperatures as 120°C [30] or 115°C [34] used before to avoid degradation of some compounds. The time of the derivatization reaction was selected to be 1 hour which was optimized and was less than many other periods [41]. The use of DMF in the reaction was better than the use of pyridine. Indeed, currently the pyridine is evaluated as a possible carcinogenic agent and causes troubles for sexual activity [41,42]. The mixture of BSTFA/TMSC (99/1) was the derivatization reagent more frequently used by researchers. Then, it was selected in this proposed method. These conditions gave good repeatability of responses and retention times of analytes.

3.3. Identification of Terpene Trilactones

Fig. (2) shows a typical separation of bilobalide, ginkgolides A, J, B, C and the internal standard squalane in 13 minutes only. As compared to other chromatograms

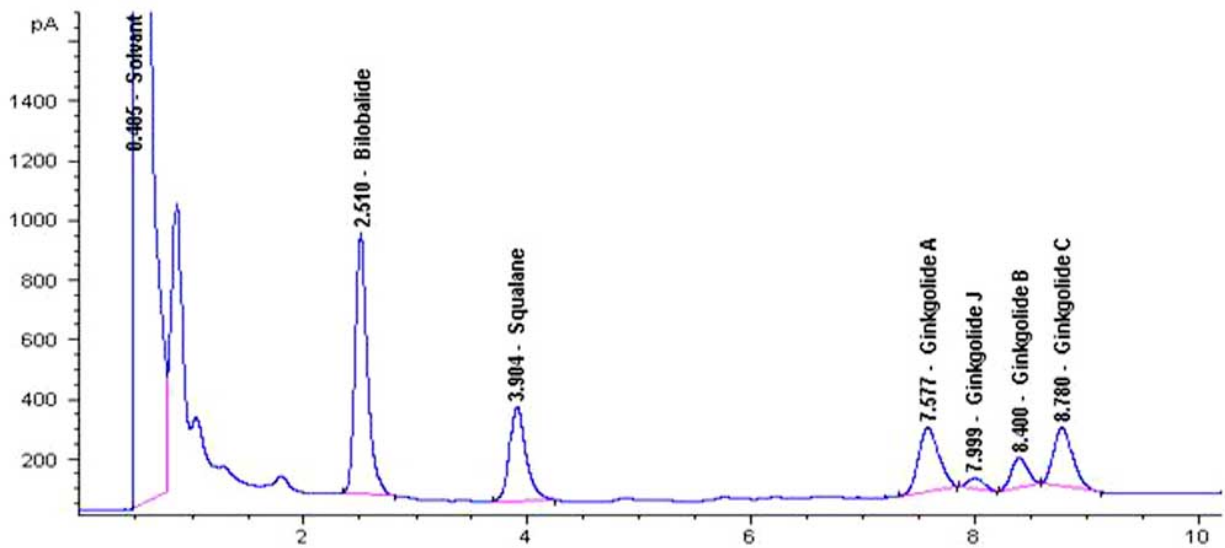


Fig. (2). A typical GC-FID chromatogram of a *Ginkgo biloba* L. extract.

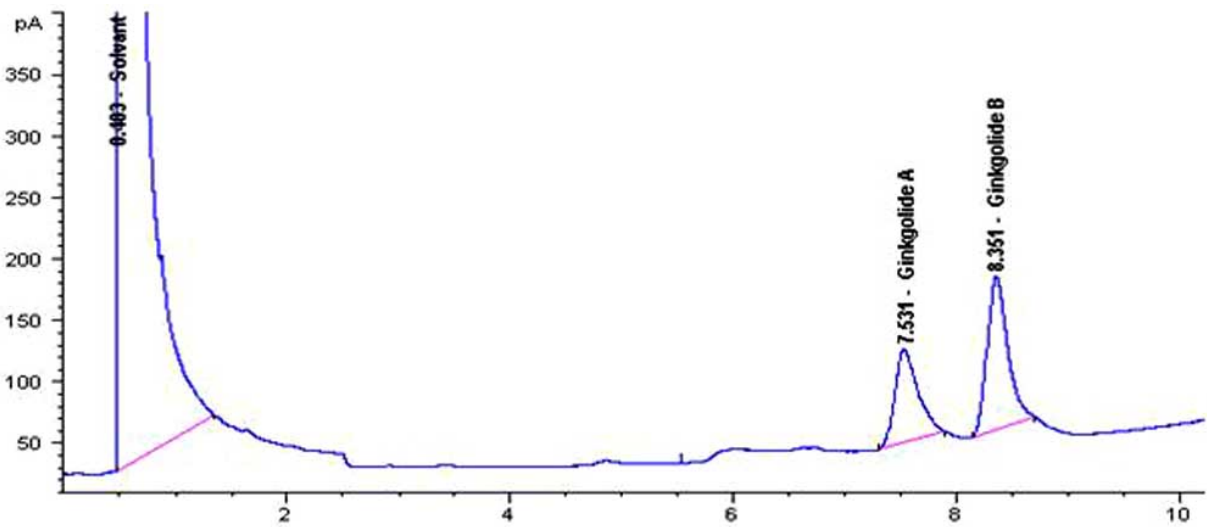


Fig. (3). A GC-FID chromatogram of ginkgolides A and B.

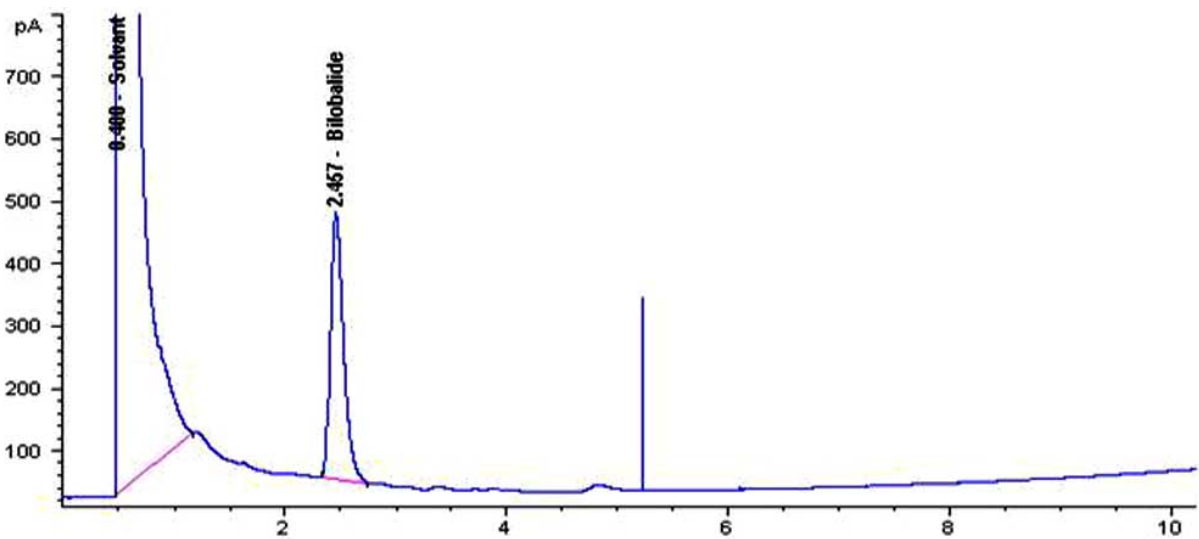


Fig. (4). A GC-FID chromatogram of bilobalide.

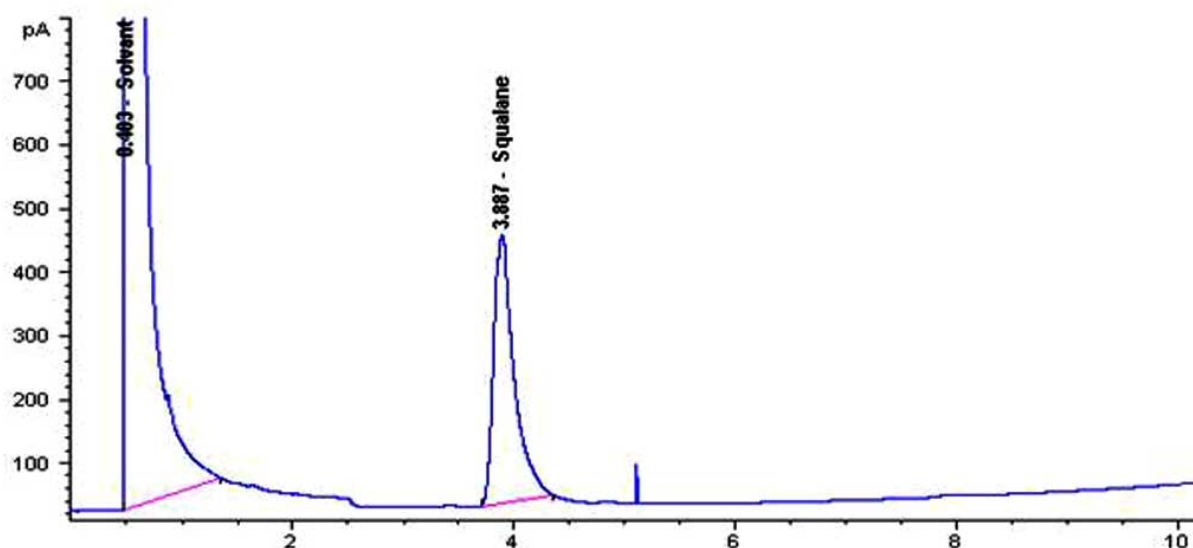


Fig. (5). A GC-FID chromatogram of squalane.

obtained before, the separations were longer (55 or 30 minutes) [42,43]. The terpene trilactones were identified by comparing in first step the retention times of the sample peaks to reference peaks (ginkgolide A, ginkgolide B and bilobalide) (Figs. 3-5) and in second step to the peaks in chromatogram obtained by Lang *et al.* [30] (ginkgolide J and ginkgolide C which were not available in our laboratory). The responses were better and higher than those obtained before by GC-FID [30] and the resolutions between peaks were above 1. The retention times of peaks were stable and the standard deviation coefficient of six successive injections was less than 0.5%.

3.4. Method Validation

3.4.1. Linearity and Interferences

The linearity of the method was verified by calculation of the coefficient of regression of the calibration curves. Therefore, R^2 were above 0.9979 for all calibration curves. The calculation of t-Student value of comparison of calibration curves of *Ginkgo biloba* only and in the presence of the matrix of the liquid form shows that there are no effects of matrix on the response of terpenes. The formulas (3) and (4) were used in this test:

$$t_s = \frac{a_{11} - a_{12}}{\sqrt{S_{a11}^2 - S_{a12}^2}} \langle t(0.05;26) \quad (3)$$

$$t_0 = \frac{a_{01} - a_{02}}{\sqrt{S_{a01}^2 + S_{a02}^2}} \langle t(0.05;26) \quad (4)$$

where t_s and t_0 are successively t-Student slope and t-Student zero value. a_{1i} and S_{a1i} are the slopes and standard deviation of slopes, a_{0i} and S_{a0i} are zero value and standard deviation of zero value. The results are presented in the Table 1.

3.4.2. Precision and Accuracy

The intra-day RSD's were 1.13% for bilobalide, 2.34% for ginkgolides and 1.54% for sum of terpenes. The inter-day RSD's were 2.54% for bilobalide, 3.68% for ginkgolides and 3.00% for sum of terpenes. The criterion of accuracy was verified by calculation of recoveries and accuracy's interval of ratios of found quantities (*Ginkgo biloba* in the presence of liquid forms matrix) on the introduced quantities (*Ginkgo biloba* only). Mean recoveries were 99.78 ± 0.82 % for ginkgolides and 99.48 ± 0.86 % for bilobalide. The recoveries obtained with the proposed method were better than obtained by other method reported before [30,42,43].

Table 1. Linearity Results of Calibration Curves of Terpenes

		Regression Equation	R^2	ts calc.	to calc.
Bilobalide	GLE only	$y = 69.70x - 161.2$	0.9986	1.172	1.781
	GLE + M	$y = 68.29x - 58.2$	0.9979		
Ginkgolides	GLE only	$y = 63.67x - 10.2$	0.9984	1.631	1.769
	GLE + M	$y = 65.78x - 230.2$	0.9980		
Terpenes	GLE only	$y = 133.37x - 171.4$	0.9989	0.593	1.335
	GLE + M	$y = 134.07x - 288.4$	0.9999		

$t(0.05; 26) = 2.056$; M: matrix; GLE: *Ginkgo biloba* Leaf Extract.

Table 2. Results of Terpene Recoveries in Pharmaceutical Products

	Product 1*	Product 2*	Product 3*	Product 4*	Product 5*	Product 6*
Ginkgolides	97.73 ± 1.13%	98.55 ± 1.25%	100.54 ± 2.18%	103.03 ± 2.55%	101.49 ± 1.58%	99.24 ± 2.41%
Bilobalide	98.35 ± 1.27%	99.12 ± 1.54%	99.62 ± 2.46%	102.38 ± 2.64%	102.05 ± 1.93%	99.44 ± 2.13%
Sum of terpenes	98.03 ± 1.08%	99.63 ± 1.15%	100.18 ± 2.23%	102.75 ± 2.48%	101.88 ± 1.43%	99.57 ± 2.10%

*: These were products produced in OPALIA Pharma and other pharmaceutical industries. The concentrations of terpenes were 2.667 µg/µL.

3.4.3. Limits of Detection (LOD) and Quantification (LOQ)

The LOD was determined by the formula $3S_b/\text{slope}$ which S_b is the standard deviation of six measurements of the blank, whereas LOQ was determined by the formula $3S_b/\text{slope}$. LOD and LOQ were successively 0.4 µg/mL and 1.3 µg/mL.

3.5. Analysis of Samples

The proposed and validated method was applied to the analysis of six pharmaceutical samples containing *Ginkgo biloba*. In Table 2 the assay results were presented characterizing recoveries and content of bilobalide, ginkgolides and sum of terpenes.

These results showed, that obtained recoveries were between 97 and 103%, and complied with specifications which were between 90 and 110%, and the contents were all complied with specifications which were between 101 and 150 mg/100 mL for ginkgolides, in range of 94 and 141 mg/mL in case of bilobalide, and in range from 180 to 308 mg/mL for sum of terpenes.

CONCLUSIONS

Based on the above results, we consider that the developed and validated method could be applied for the determination of terpenes in *Ginkgo biloba* L. extract and in its pharmaceutical formulations with adequate linearity, reproducibility and recovery. Previously published methods for the determination of such terpenes needed to use an expensive laboratory equipments and materials, and also included a time-consuming extraction and derivatization steps. The proposed here method is an effective and simple and enables rapid determination of terpenes in GBE. This procedure presents a good linearity, accuracy, precision and no interference between excipients and other terpenes, as well as indicate sufficiently low LOD and LOQ. This method provides significant cost reduction and labor time advantages for routine quality control procedures performed daily in pharmaceutical laboratories and would be applied for more specific and high-throughput determination of terpenes in oral pharmaceutical forms (capsules or tablets) of *Ginkgo biloba* L. extract by further optimizing a little the preparation of the solutions by using an ultrasonic bath.

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ABBREVIATIONS

APCI-MS = Atmospheric pressure chemical ionization–mass spectrometry

BSTFA	=	N,O-bis(trimethylsilyl)trifluoroacetamide
DMF	=	N,N-dimethylformamide
GBE	=	Ginkgo biloba extract
GC-FID	=	Gas chromatography-flame ionization detector
LC-MS	=	Liquid chromatography – mass spectrometry
LOD	=	Limit of detection
LOQ	=	Limit of quantification
NMR	=	Nuclear magnetic resonance
RF-MEKC	=	Reverse flow micellar electrokinetic chromatography
RP-HPLC	=	Reversed-phase high performance liquid chromatography
RSD	=	Relative standard deviation
QC	=	Quality control
TMSC	=	Trimethylchlorosilane

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