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Validation of an LC–MS/MS assay of terpene trilactones in *Ginkgo biloba* extracts and pharmaceutical formulations through standard addition method

Daniela Iuliana Sora[a,*], Venera Stefanescu[a], Victor David[b], Andrei Medvedovici[a,b]

[a] *Labormed Pharma S.A., Splaiul Independentei 319E, Bucharest 060044, Romania*
[b] *University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri 90, Bucharest 050663, Romania*

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ABSTRACT

A chromatographic column packed with 1.8 μm particle size octadecyl modified silicagel was used to separate terpene trilactones from *Ginkgo biloba* extracts/pharmaceutical formulations. Gradient elution was applied, using acidic methanol and water as mobile phase components (0.1% formic acid addition). No specific sample preparation is needed, except dissolution/extraction in methanol of the solid material. Baseline separation of bilobalide and ginkgolides A, B, C and J is obtained within 4 min. The gradient profile is needed to elute the remaining matrix from column. A separation cycle takes 7 min, including column re-equilibration. MS/MS detection with positive electrospray ionization and triple quadrupole mass analysis was used. Multiple reaction monitoring mode was applied for data acquisition, taking protonated molecular ions as precursors. In order to generate reproducible ionization conditions, the standard addition method was considered. The assay of terpene trilactones obtained under such conditions was validated according to guidances in place. Method intermediate reproducibility corresponds to a relative standard deviation of less than 10%. Accuracy, expressed in terms of absolute percent bias ranged from 90% to 110%. Spectral confirmation of target analytes was also included in the validation procedure.

1. Introduction

Ginkgo biloba is one of the most used medicinal plant, being commercialized as pharmaceutical oral solid dosage forms containing dried vegetal material or refined extracts, for the improvement of the peripheral and central blood circulation, arterial occlusive diseases, vertigo and against demential disorders (memory impairment and concentration difficulties) [1,2].

Among the pharmacologically most important compound classes contained in *Ginkgo* leaves/extracts, flavonol glycosides and terpene trilactones are considered responsible for the competitive inhibition of platelet activating factor, preventing thrombus formation, bronchoconstriction and suppression of allergic reactions [3]. The quality of extracts is commonly characterized through the content of flavonol glycosides and terpene trilactones, although other different classes of compounds may be representative, expressed as minimal or maximal thresholds (proanthocyanidins, catechins, non-flavonol glycosides, alkyl phenols, ginkgolic acids). Chemical analysis of *G. biloba* leaves and extracts has been reviewed in [4,5].

Terpene trilactones (trivially named as ginkgolides A, B, C, J, K, L, M and bilobalide and further abbreviated as G_A, G_B, G_C, G_J, G_K, G_L, G_M and B_B) are high-melting, non-volatile polar compounds (see Fig. 1). Liquid chromatography is therefore a method of choice for separation of such compounds, by using the reversed phase elution mechanism [6,7]. Gas chromatography may also be used, but requires derivatization as well as complex sample preparation procedures [8,9].

Terpene trilactones exhibit no specific absorption in the UV spectral domain and may be monitored only at low, non-selective wavelengths (190–220 nm) [7]. Consequently, UV detection is difficult and requires extensive clean-up procedures. As alternative, the refractive index detector (RID) has been indicated for detection of terpene trilactones [10,11], although response instability and the poor sensitivity represent major inherent drawbacks. Evaporative light scattering detection (ELSD) has gained in popularity over the last decades [5]. However, the narrow linearity intervals produced through ELSD response represent a major inconvenience. Structural information techniques have been also used for the assay of terpene trilactones. Nuclear magnetic resonance (NMR) has been used to quantitatively determine ginkgolides in dried leaves and commercially available dosage forms [12,13]. Mass spectrometric detection (MSD) was also considered. Positive ESI/MS was also used as detection for terpene trilactones in canine plasma [14] while negative APCI/MS/MS was applied for the same aim in rat plasma [15]. MS/MS detection using negative ion production through APCI or

* Corresponding author.
E-mail addresses: sora_dal@yahoo.com, iulia.sora@labormedpharma.ro (D.I. Sora).

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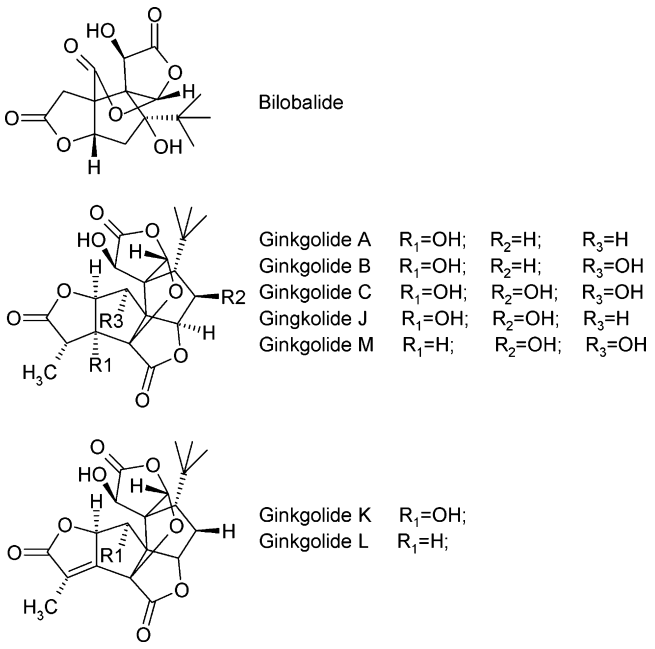

Fig. 1. Chemical structures of terpene trilactones from *Ginkgo* extracts.

ESI has been used for quantitation of terpene trilactones in plasma samples and standardized extracts [16–18]. Combined analysis of flavonol glycosides and terpene trilactones was recently made by HPLC/MS [19,20]. Capillary HPLC/MS and MS/MS were used to obtain a fingerprint profiles for more than 70 components from *G. biloba* containing nutritional supplements [21]. A similar approach, using capillary HPLC/MS with on-line column switching purification was used to assay flavonol glycosides and terpene trilactones in human urine [22].

European Pharmacopoeia introduced in the first supplement of its sixth edition [23], an official monograph for analytical characterization of *G. biloba* dry extracts, refined and quantified. Sample preparation procedure is time consuming and potentially induces variability on the final results. Detection is RID. A chromatographic run takes 25 min and quantitation is based on internal standard addition (benzyl alcohol).

The proposed method is based on HPLC/(+)ESI/MS/MS. No sample preparation is required excepting sample dissolution/extraction in methanol. To control the inherent variability of the mass spectrometric detector, the standard addition method has been chosen. The method was fully validated according to ICH guidelines [24,25] and may be successfully applied for assaying terpene trilactones in refined *G. biloba* dry extracts and quantified or pharmaceutical formulations containing such extracts.

2. Experimental

2.1. Reagents

All solvents were HPLC grade from Merck (Darmstadt, Germany). Formic acid was extra pure grade. Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Ginkgolides were reference substances from ChromaDex™ LGC Standards GmbH Germany (B_B: batch 02274-822 – purity 99.7%; G_A: batch 07176-401 – purity 95.1%; G_B: batch 07181-502 – purity 82.8%; G_C: batch 07179-000 – purity 94.0%; G_J: batch 07188-201 – purity 91.3%). Evidence of the standards purity available from the producer was based on HPLC/MS, MS, NMR, GC/MS (for residual solvents), Karl-Fischer (for humidity) data. *Ginkgo* dry extract for peak identification, standard reference material, batch 1a, was obtained from European Pharmacopoeia, Strasburg, France. Tanakan 40 mg film coated tablets, containing EGB 761 (24% flavonol glycosides and 6% terpene trilactones) standardized *G. biloba* extract, batch T272, was from Beaufour Ipsen Pharma, France. Bilobil forte, capsules containing 80 mg *G. biloba* extract standardized at minimum 24% flavonol glycosides and minimum 4.8% terpene trilactones, was from KRKA d.d. (Novo Mesto, Slovenia), batch B49054. Gingium 120 mg coated tablets, batch 7N9312, containing *G. biloba* standardized extract (3.3–4.7% terpene trilactones) was from Hexal AG, Germany. Flavotan coated tablets (40, 80 and 120 mg) containing standardized *G. biloba* extract were produced by Labormed Pharma, Romania (batches 6060070782, 236010508, 336040508). All pharmaceutical formulations were obtained from the local market.

2.2. Apparatus

Experiments were performed with an Agilent 1200 SL series LC/MSD (Agilent Technologies) system consisting of the following modules: degasser (G1379B), binary pump (G1312A), thermostated autosampler (G1367C and G1330B, respectively), column thermostat (G1316B), AP-ESI standard interface (G1948B), and triple quadrupole mass spectrometric detector (G2571A). System control, data acquisition and interpretation were made with the Agilent Mass Hunter software version B 01.00.

2.3. Sample preparation

Standardized *G. biloba* dry extracts are dissolved in methanol to produce stock test solutions of 1 mg/mL extract. Pharmaceutical formulations based on standardized dry *G. biloba* extracts are sonicate in methanol (sonication for periods ranging from 5 to 30 min leads to similar results). Taking into account the declared content, the same nominal concentration is obtained. Extraction from vegetal dried materials was not considered in the present work. Centrifugation may be necessary to obtain a clear supernatant.

The stock solution used to produce standard additions contains each of the terpene trilactones (B_B, G_A, G_B, G_C and G_J) at 0.2 mg/mL level, in methanol (considering the declared purity).

Additions of $1 \div 6$ μg/mL from each terpene trilactones (1 unit increment) are produced by $50 \div 300$ μL aliquots from the stock solution of standards (50 μL increment) brought to a final volume of 10 mL. The stock test solution from *Ginkgo* extracts is diluted to half to produce sample solutions (#1–6) with standard additions, (0.5 mg/mL extract and $1 \div 6$ μg/mL from each terpene trilactones standard). Always, when making a dilution step, a mixture methanol/water 1:1 (v/v) was used for bringing to sign in the volumetric flask. For routine purposes, three replicates should be made and analyzed for each addition level. To obtain the linear regression parameters (slope and intercept), mean peak areas should be used. During the validation stages, the number of replicates made for each addition level was kept as mentioned, if not otherwise stated.

2.4. Chromatographic method

A Zorbax Eclipse XDB-C18 Rapid Resolution HT, 50 mm length, 4.6 mm internal diameter and 1.8 μm particle size (Agilent Technologies, cat. no. 927975-902), fitted with a Phenomenex Guard Cartridge C18, 4 mm × 2 mm (prod. no. AJO-4286) was used. The column was thermostated at 45 °C.

Gradient elution was applied, using 0.1% formic acid in water and methanol as mobile phase components. Mobile phase composition starts at 35% organic solvent. A fast gradient profile is produced, bringing the organic solvent to 80% in 5.5 min and then to 100% at

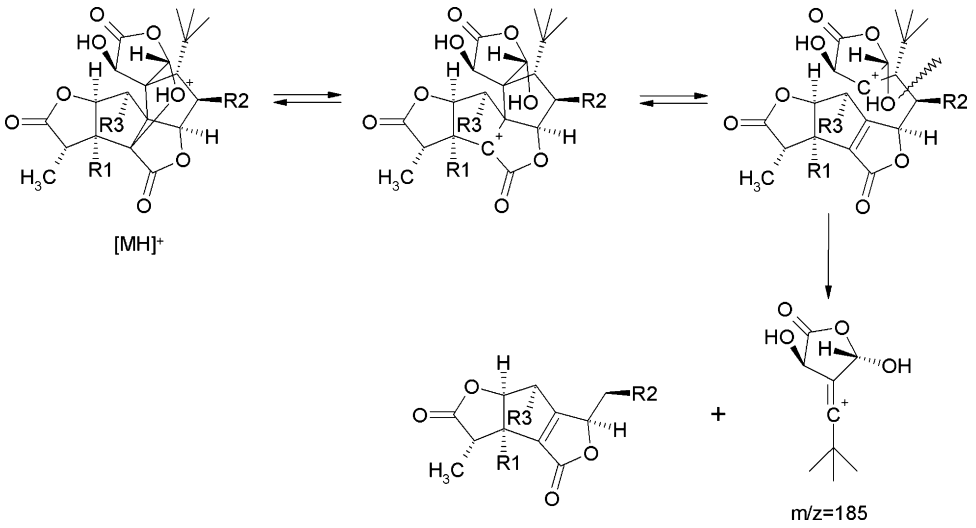

Fig. 2. Possible ionization pathway within the ESI source of ginkgolides A and B to produce m/z = 185 a.m.u. major ion.

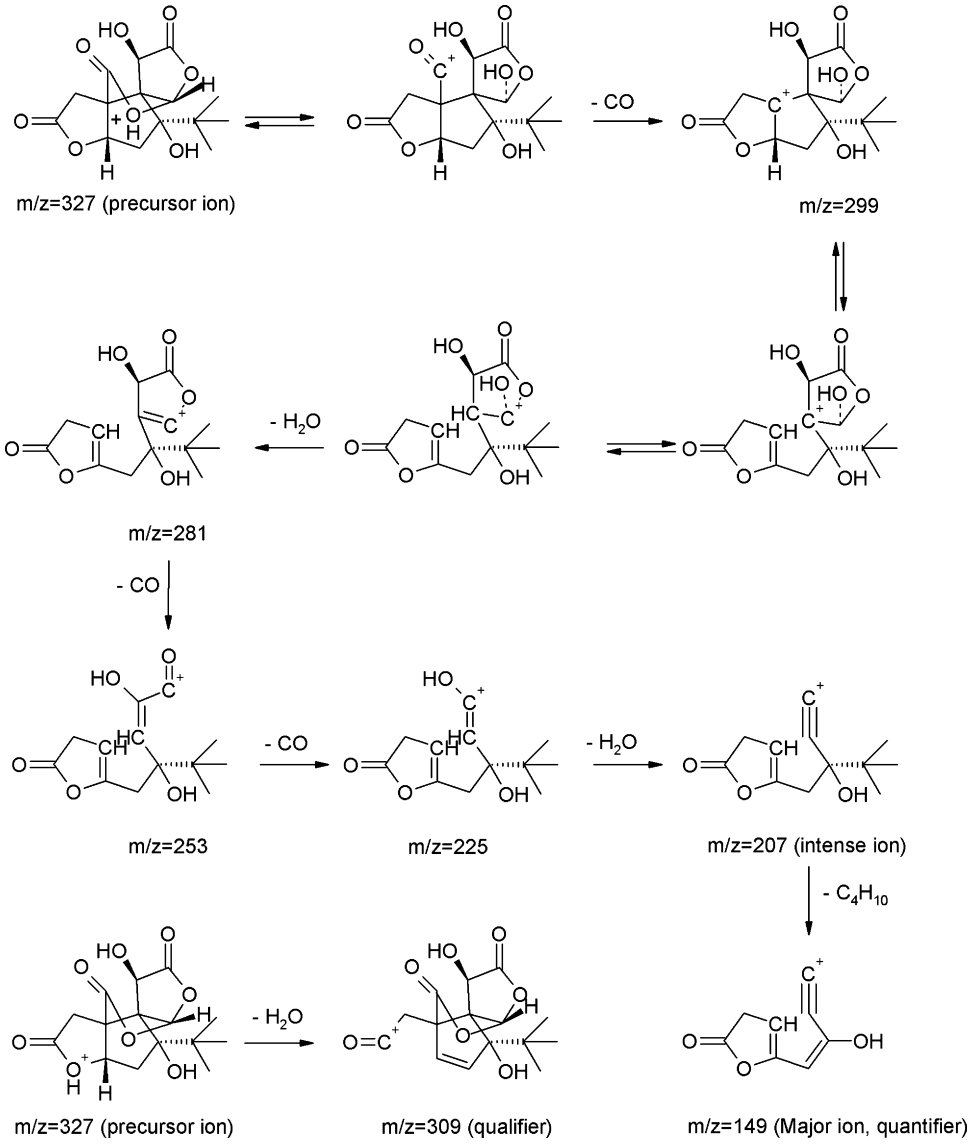

Fig. 3. Tentative CID pattern for bilobalide.

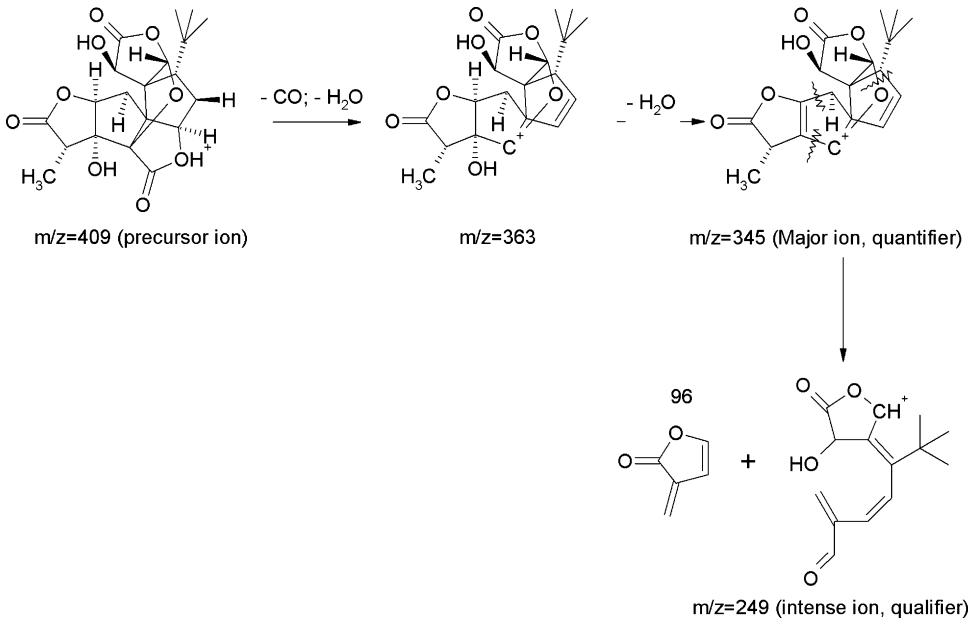

Fig. 4. Tentative CID ionization schema for ginkgolide A.

minute 6. Column re-equilibration takes 1 min after a step jump (0.01 min) to initial conditions. The flow rate was 0.8 mL/min and the injected volume was 5 μL. The gradient is used to eliminate from column sample residual matrix after each chromatographic run.

2.5. MS parameters

The optimized parameters controlling the ESI ion source were as following: drying gas (N_2) temperature: 300 °C; drying gas flow: 12 L/min; pressure of the nebulizing gas: 60 psi; capillary

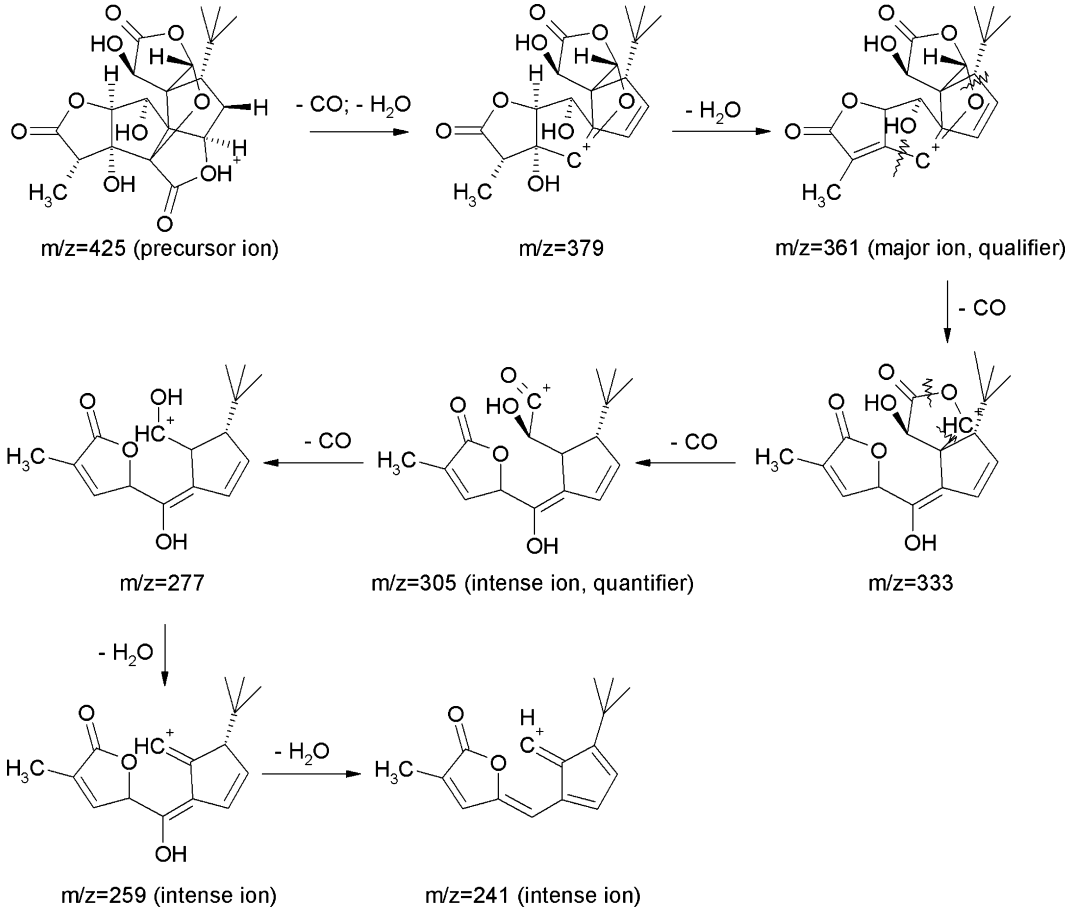

Fig. 5. Tentative CID ionization schema for ginkgolide B.

voltage: 4000 V. The fragmentor potential was set at 140 V. Collisional induced dissociation (CID) was carried out at 25 V for $G_{A–J}$ and at 20 V for B_B, using N_2 as collision gas.

Transitions being monitored for each of the compounds are: for B_B – *m*/*z* 327 a.m.u. to *m*/*z* 149 a.m.u. for quantitation (quantifier transition) and to *m*/*z* 309 a.m.u. for spectral confirmation (qualifier transition); for G_A – *m*/*z* 409 a.m.u. to *m*/*z* 249 a.m.u. for quantitation and to *m*/*z* 345 a.m.u. for spectral confirmation; for G_B – *m*/*z* 425 a.m.u. to *m*/*z* 305 a.m.u. for quantitation and to *m*/*z* 361 a.m.u. for spectral confirmation; for G_C – *m*/*z* 441 a.m.u. to *m*/*z* 325 a.m.u. for quantitation and to *m*/*z* 177 a.m.u. for spectral confirmation; for G_J – *m*/*z* 425 a.m.u. to *m*/*z* 309 a.m.u. for quantitation and to *m*/*z* 235 a.m.u. for spectral confirmation. Isolation window for the precursor ions was set to unit. The spectral confirmation factor results by rationing the intensities of the qualifier signal to the quantifier one, and then multiplying by 100.

2.6. Ionization patterns

According to literature data, negative ion mode is preferred for quantitation of terpene trilactones [20,26]. Our experimental trials lead to conclusion that reproducible ionization yields were obtained in the negative mode when using ammonium formiate in the mobile phase. However, on use of the ammonium formiate

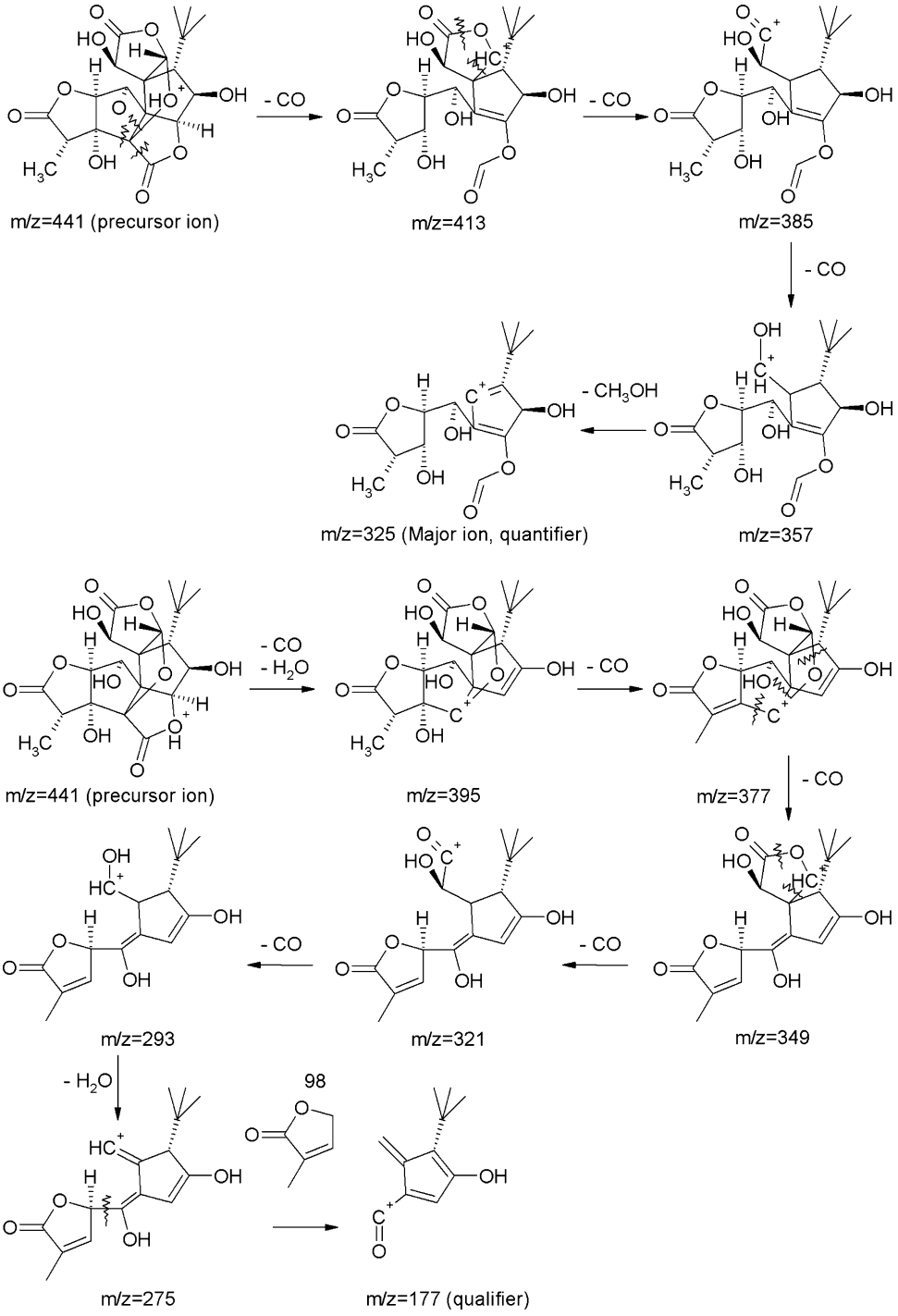

Fig. 6. Tentative CID ionization schema for ginkgolide C.

additive, the cleaning of the source was frequently needed, owing to accumulation of crystals. Because sensitivity was not a major concern for our study, the positive ion mode was preferred. Ionization within the ESI source leads to protonated $[M+H]^+$ ions for all compounds. Adducts with sodium, ammonium, potassium ions and dimer + sodium ion are also produced. Among the molecular ion and adducts, the sodium adduct is the most intense. In the case of G_A and G_B, the major ion produced within the ESI source is characterized by m/z = 185 a.m.u. This may be produced through the ionization route depicted in Fig. 2, although it is difficult to explain why G_C and G_J do not follow the same pattern.

Isolation of sodium adducts as precursor ions does not generate through CID any ionic product fragments. B_B produces the largest number of product ions having relative intensities higher than 50% through CID of the protonated molecular ion. Two main collisional dissociation patterns may be considered. One route is initiated through the cleavage of the substituted cyclopentane ring, following the opening of the out-of-plane protonated lactame cycle, generating product ions with m/z of 217, 173, 135 and 111 a.m.u. The other route (Fig. 3) refers to successive losses of neutrals as CO, water and *i*-butane, leading to product ions having mass to charge ratios of 309, 207 and 149 a.m.u.

Protonated molecular ions of $G_{A,B,C,J}$ generate MS/MS spectra characterized by a large number of product ions with reduced intensities compared to the major fragment. Tentative CID patterns for G_A, G_B and G_C are given in Figs. 4–6. It seems that protonation within the source is positioned on the oxygen atom of the tetrahydrofuran ring. In the CID stage, this ring is initially opened, followed by the cleavage of the cyclopentane one. Product ions are formed due to "erosion" of the resulting structures, by successive losses of small neutral molecules as CO, H_2O, CO_2 and CH_3OH.

Trials of making identification of ginkgolides K, L and M in the analyzed standardized extracts fail (when monitoring protonated molecular ions and molecular adducts with Na, K and ammonium ions under single stage MS and selected ion monitoring mode). As G_M is isolated only from roots [5], this is a confirmation that the analyzed standard leaf extracts were not adulterated. G_K and G_L were reported in ref. [27], but the confirmation of the finding was still not made by other authors.

2.7. Calculation of the results

The concentration of the target compound in the sample solution (μg/mL) results by considering the absolute value of the ratio A/B, where A and B are the intercept and the slope of the linear regression computed from data obtained with the solutions resulting after the standard addition. The percentage x% (w/w) of each terpene trilactone in the standardized *G. biloba* extract is calculated according to the following relationship:

$$x\% = \frac{\left|A/B\right| \times V_1 \times V_2}{10 \times m_E \times V} \quad (1)$$

where m_E is the quantity (mg) of the dry standardized *G. biloba* extract (or its equivalent in a pharmaceutical formulation) used to produce the stock solution, V_1 is the volume (mL) of the stock solution, V_2 is the volume (mL) of the solution with addition, and V is the aliquot (mL) from the stock solution used to obtain the solution with addition. As the concentration of the stock solution was deliberately set to 1 mg/mL ($V_2/m_E = 1$) and the concentration of the extract in the solution with addition is halved with respect to the concentration of the stock solution ($V_1/V = 0.5$), the percentage x% (w/w) of each terpene trilactone in the standardized *G. biloba* extract may be found by rationing the computed concentration in the sample solution to 5.

2.8. Methodology for validation

Validation was carried out according to available guidelines [24,25] and refers to the following aspects:

(a) *Selectivity*: Selectivity was evaluated taking into consideration the structural confirmation factors obtained for each of the terpene trilactone standards analyzed as individual solutions, as mixture, as spikes to a *G. biloba* standardized extract, in *G. biloba* dry extract for peak identification (standard reference substance from Eur. Ph.), and in extracts from the pharmaceutical formulations Tanakan, Bilobil forte, Gingium and Flavotan. The normal variation interval for each structural confirmation factor corresponds to a relative standard deviation of 10% calculated from values resulting during analysis of individual terpene trilactone standard materials. The recovered structural confirmation factors confirm the selectivity of the method.

(b) *Linearity*: The standard addition method imposes a rigorous choice of analyte concentrations being added to the real sample. These should be low enough in order to produce no alteration of the linear relationship between the detector response and the real concentration levels of target compounds within the analyzed samples. At the same time, the sensitivity of the method should be high enough in order to differentiate between the quantities of standards added to the real sample. Linearity was studied for an extended concentration interval (up to 30 μg/mL) on a mixture of terpene trilactone standards. Seven concentration levels were considered (1, 2, 5, 10, 20, 25, 30 μg/mL) each level being analyzed for six replicates (including the procedure for preparation of solutions). As the matrix appearing in the ionization source may have a tremendous influence on

Table 1
Operational parameters considered for evaluation of the robustness of the method.

Operational parameter	Method reference conditions	Variation	Observed influence on	Measured indicators
Ion source parameters				
Dry gas temperature	300 °C	±5 °C; ±50 °C	Detection	F, PA
Dry gas flow	12 L/min	±1 L/min; ±0.5 L/min	Detection	F, PA
Nebulizer pressure	60 psi	−5 psi; −10 psi; −20 psi	Detection	F, PA
Capillary voltage	4000 V	±50 V; ±500 V	Detection	F, PA
Chromatographic separation parameters				
Column temperature	45 °C	±2.5 °C	Separation	RT
Mobile phase flow	0.8 mL/min	±0.05 mL/min; ±0.1 mL/min	Separation and detection	RT, F
Mobile phase composition (starting value)	35% solvent B	±0.7%; ±2.5%	Separation	RT
Mobile phase composition (gradient)	8% solvent B/min	±1%/min	Separation	RT, F
HCOOH concentration	0.1% HCOOH	±0.05%	Separation and detection	RT, F, PA
Column batch	USWDY04740	USWDY04722	Separation	RT

RT, retention time; PA, peak area value; F, structural confirmation factor.

Table 2
Structural confirmation factors (according to definition in Section 2.5) for terpene lactones (values in table result from averaging of three experimental results).

Compound	Structural confirmation factors												
	Individual solutions of terpene lactone standards	Minimum allowed (RSD = 10%)	Maximum allowed (RSD = 10%)	Mixture of terpene lactone standards	Spikes of standards on standardized extract	*Ginkgo* dry extract for peak identification	Flavotan	Tanakan	Bilobil	Gingium	Mean (columns 5–11)	Standard deviation	Relative standard deviation (RSD%)
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
B_B	15.7	12.6	18.2	15.8	15.4	15.2	15.3	16.1	14.9	16.2	15.6	0.5	3.1
G_A	90.3	72.2	104.7	92.8	87.9	95.1	88.8	93.1	86.5	89.3	90.5	3.2	3.5
G_B	68.3	54.6	79.2	73.6	67.8	65.9	68.9	69.9	67.1	65.9	68.4	2.7	4.0
G_C	40.2	32.2	46.6	40.5	40.4	39.9	40.8	41.1	39.8	39.5	40.3	0.6	1.4
G_J	34.3	27.4	39.8	35.6	34.3	35.4	34.7	35.3	33.7	32.8	34.5	1.0	3.0

the ionization yields, the linearity study was repeated on solutions representing spikes of terpene trilactone standards in a solution of *Ginkgo* standardized extract (having a concentration of 500 μg/mL reported to the dried material). The added concentrations interval was limited up to 10 μg/mL of each terpene trilactone standard. Six different levels were produced, corresponding to added concentrations of 1, 2, 4, 6, 8 and 10 μg/mL from each terpene trilactone standard. The sample with null addition was also quantified and the resulting peak area for each terpene trilactone was subtracted from values resulting in samples with known added levels. These corrected data were then used to calculate linear regression parameters. Once again, six replicates were considered, including sample preparation. Low limits of quantitation (LLOQs) were calculated for both situations (mixture of standard compounds and spikes of standard compounds to standardized *Ginkgo* extract solution). Evaluation of LLOQs was necessary to decide on addition levels to be used during the regular application of the method for routine purposes. Calculation of LLOQ was made in two ways: (a) according to the well known formula $(5 \times s_A - A)/B$, where s_A is the standard variation of the intercept, A is the intercept and B the slope of the linear response function; (b) according to relationship $[t \times (s_A + s_B \times C_m)]/(B + 2 \times t \times s_B)$, where t is the Student coefficient chosen for $n-2$ degrees of freedom (n = number of concentration levels) and for a level of confidence $P\% = 90\%$ and C_m is the average of the concentrations used during experiments [28].

(c) *Precision*: Precision was evaluated through *repeatability* and *intermediate reproducibility*. Repeatability was evaluated for solutions of a *G. biloba* standardized extract (500 μg/mL) to which additions of 1, 6 and 10 μg/mL for each of the terpene lactone standards were made. Six replicates were analyzed at each concentration level (null addition was also considered). All determinations were made in a single experimental session (within a day). The procedure evaluates together the variability of the sample preparation operations and the variability of the MS/MS detection on short term (intra day). Intermediate reproducibility was emphasized in the same manner, but replicates were analyzed in experimental sessions delayed one to another by a minimum 24 h interval. This leads to evaluation of sample preparation and long term variability of the mass spectrometric detector (inter day). Precision was expressed as relative standard deviations calculated for peak areas of target compounds at a given addition level and as relative standard deviation of the computed initial concentration of terpene lactones in the *Ginkgo* standardized extract by using the standard addition method applied for three concentration levels (1, 6 and 10 μg/mL).

(d) *Accuracy*: Due to the fact that a *Ginkgo* extract free of terpene trilactones (as a blank matrix) is not commercially available and taking into account the influence of the matrix on the ionization yield affecting quantitative estimation, accuracy should be treated differently compared to a common approach. Consequently, a standardized *Ginkgo* extract was tested for terpene trilactones content according to the standard addition method herein presented (standard additions of 1–6 μg/mL of each terpene trilactones with increments of 1 μg/mL; resulting concentrations will be referred as initial values). To a methanol solution from the previously characterized standardized *Ginkgo* extract, three different additions of a standard terpene trilactones solution were made, in order to generate 500 μg/mL of matrix and initial concentration values +2, 4 and 6 μg/mL from each of the terpene trilactones. These samples further referred as accuracy samples were again submitted to the standard addition method for assay of terpene trilactones (6 addition levels, 1–6 μg/mL from each terpene trilactone, with increment of

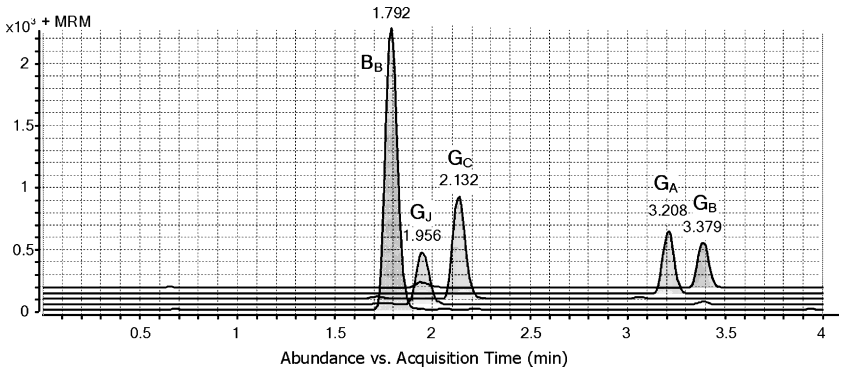

Fig. 7. Typical chromatogram obtained for separation of terpene trilactones from a dry standardized *Ginkgo biloba* extract, refined and quantified (conditions are given in Section 2).

1 μg/mL). Experimental values obtained from these accuracy samples will be compared to the "true" values, corresponding to initial values + added known concentration values, expressed in terms of recovery (%).

(e) *Stability*: The stability is assessed using the stock solution of a standardized *Ginkgo* extract (1000 μg/mL) and a stock solution of terpene trilactone standards (200 μg/mL each terpene trilactone), both made in methanol and kept frozen at −20 °C. Initially and at one month interval (over a period of three months) the two solutions are removed from the freezer and are thawed unassisted to room temperature. The standard addition method is then applied (for only four addition levels, 1, 2, 4 and 6 μg/mL, respectively). The assay for each terpene trilactone in the standardized *Ginkgo* extract is compared to the one obtained at the initial test point. Stability of both solutions is observed through calculation of the % recovery against the initial test point.

(f) *Robustness*: Robustness was evaluated taking into consideration the operational parameters controlling the chromatographic separation and the ionization within the MS source. The impact of the variation of the operational parameters was estimated with reference to quantitative (peak area values) and qualitative indicators (absolute retention times and structural confirmation factors). The selected operational parameters used for evaluation of the robustness are presented in Table 1. For illustration of the robustness, a sample solution containing 500 μg/mL of standardized *Ginkgo* extract with an addition of 2 μg/mL from each terpene trilactone was used. Three replicates are run for each of the conditions described in Table 1. Relative standard deviation is then calculated for each of the mean values of indicators at all particular values of the respective operational parameter. The following thresholds are considered as normal for the robustness indicators: RSD% less than or equal to 10% for peak area values and spectral confirmation factors; RSD% less than or equal to 5% for the absolute retention time values.

3. Results and discussions

A typical separation of terpene trilactones is given in Fig. 7. To eliminate possible interferences induced by less selective mass transitions, baseline chromatographic separation between target compounds was obtained.

As it can be observed from Table 2, the structural confirmation factors are not affected by the matrix reaching the MS source. Although a variation of 10% as relative standard deviation for structural confirmation factors is commonly accepted in mass spectrometry, it seems that the presence of the matrix provided by the *Ginkgo* standardized extracts or by the residual co-extracted pharmaceutical formulations is producing less effect in terms of variability. Method selectivity may consequently be sustained.

However, as it can be observed from Table 3, the matrix reaching the ion source generates an increase by a factor of about 2.5 the ionization yields of the target compounds (slopes of the linear regressions are 2.2 ÷ 2.9 times higher when matrix is present). Although better fittings may be found through weighing by $1/x$ or $1/x^2$ over the extended concentration interval in absence of the matrix, the true linear fittings produce correlation factors (R^2) higher than 0.996. Relative standard deviations of slopes (obtained without or with matrix) are within 5%. Such variability is increased in the presence of the matrix for the first three eluting compounds, while matrix effects stabilize response for the two last eluting ones. Roughly speaking, the two alternatives for calculating LLOQs produce similar results, and it may be considered that an increment of 1 μg/mL for the standard addition should be enough to produce significant increases in terms of detector response.

Data characterizing the precision of the standard addition method are presented in Table 4. One can observe that the standardized *Ginkgo* extract used during experiments corresponds to conditions for terpene trilactones in the monograph of the European Pharmacopoeia (2.6 ÷ 3.2% bilobalide and 2.8 ÷ 3.4% sum of ginkgolides A, B and C). The inter day variability of the mass spectrometer may extend the relative standard deviation of the resulting computed values in the 15% interval, if considering the ginkgolide J, which is present at the lower amount. However, it is worthwhile to note that for the precision procedure only three standard additions were made (not six, as recommended in the routine method).

Data resulting from the accuracy study are presented in Table 5. As expected for the MS/MS detection, biases from theoretical values, expressed as percentage, fall in the ±10% interval.

Stability of the solutions (samples and stock standard mixture) was considered together, the solvent being methanol. The long-term storage was made at −20 °C. Stability data are given in Table 6. One can conclude that solutions are stable for at least three months.

The ion source parameters may strongly affect ionization yields and consequently, the structural confirmation factors and peak areas. It was observed that none of the ion source operational parameters is affecting the variation of the structural confirmation factors. However, only variations of ±5 °C for the drying gas temperature, of ±0.5 L/min for the drying gas flow, up to −5 psi for the nebulizer gas pressure and of ±50 V for the capillary voltage produce variations of peak area values within the accepted RSD% threshold.

Variations of the flow rate (±0.1 mL/min), of the mobile phase gradient formation (±1%/min) and of the formic acid concentration in the constituents of the mobile phase (±0.05%) are not bringing the variability of the structural confirmation factor outside the 10% RSD threshold. Peak area values are not affected by changes of the formic acid concentration in the mobile phase. Absolute retention is not affected by the studied variations of the column

Table 3
Results from the linearity study carried out on terpene lactones mixtures with and without matrix.

Analyte	Mean slope (Counts × s × mL × μg^{-1})	Mean intercept (Counts × s)	Correlation factor	Standard deviation for B	Relative standard deviation for B	Standard deviation for A	Low limit of quantitation[a] (μg × mL^{-1})	Low limit of quantitation[b] (μg × mL^{-1})
	B	*A*	R^2	s_B	RSD%	s_A	LLOQ[a]	LLOQ[b]
B_B[c]	933	559	0.9985	15	1.6	141	0.5	0.7
B_B[d]	2066	85	0.9987	83	4.0	527	1.3	0.8
G_A[c]	599	349	0.9987	13	2.1	66	0.3	0.7
G_A[d]	1468	136	0.9986	52	3.6	373	1.2	0.8
G_B[c]	675	651	0.9971	18	2.6	112	0.4	0.9
G_B[d]	1773	328	0.9984	52	2.9	143	0.3	0.4
G_C[c]	1238	710	0.9990	31	2.5	162	0.4	0.9
G_C[d]	3067	323	0.9980	36	1.2	376	0.6	0.4
G_J[c]	1591	144	0.9998	44	2.8	302	0.9	1.0
G_J[d]	4601	110	0.9988	73	1.6	195	0.2	0.2

[a] LLOQ calculated according to relationship: $LLOQ = (5 \times s_A - A)/B$.

[b] LLOQ calculated according to relationship: $LLOQ = [t \times (s_A + s_B \times C_m)]/(B + 2 \times t \times s_B)$, where C_m is the mean concentration and $t(n-2, P\%)$ is the Student coefficient for $n-2$ degrees of freedom and a probability of 90% (bilateral); $t(5, 90\%) = 2.015$; $t(4, 90\%) = 2.132$ [28].

[c] Linearity made on dilutions from a mixture of standard terpene lactones in methanol (7 concentration levels, 6 replicates per concentration level).

[d] Linearity made on spikes of terpene lactones to standardized *Ginkgo* extract (6 concentration levels, 6 replicates per concentration level, peak areas corrected by substraction of values resulting after injection of the standardized *Ginkgo* extract with no addition of terpene lactones standards).

Table 4
Data obtained from the precision procedure applied for assaying terpene trilactones in *Ginkgo biloba* extracts through the standard addition method.

Compound	Concentration (μg/mL) found for replicate						Mean concentration (μg/mL)	RSD%	Mean concentration (%, w/w) in the initial *Ginkgo biloba* standardized extract	Minimum RSD% for measured peak area values	Maximum RSD% for measured peak area values
	1st	2nd	3rd	4th	5th	6th					
B_B (R)	14.9	15.7	15.7	15.0	15.0	15.0	15.2	2.5	3.0	0.5	1.6
B_B (I.R.)	15.8	15.5	14.8	14.7	15.2	14.1	15.0	4.1	3.0	1.4	5.1
G_A (R)	6.8	7.5	7.5	7.2	7.2	7.6	7.3	4.1	1.5	1.0	2.1
G_A (I.R.)	6.7	6.2	5.9	6.4	5.9	6.3	6.2	4.9	1.2	1.7	4.8
G_B (R)	4.3	4.1	3.8	3.9	4.1	4.5	4.1	6.2	0.8	0.9	2.6
G_B (I.R.)	4.2	3.8	3.6	3.6	3.2	3.3	3.6	10.0	0.7	1.7	4.6
G_C (R)	5.3	5.1	5.2	5.1	5.2	5.1	5.2	1.6	1.0	0.9	1.9
G_C (I.R.)	5.6	5.2	4.7	4.9	4.7	4.3	4.9	9.2	1.0	2.0	8.3
G_J (R)	2.2	2.2	2.2	2.2	2.3	2.3	2.2	2.3	0.5	0.5	2.0
G_J (I.R.)	2.2	2.1	2.0	1.8	1.8	1.5	1.9	13.3	0.4	1.0	6.4

(R) Repeatability procedure. (I.R.) Intermediate reproducibility procedure.

Table 5
Determination of the accuracy of the standard addition method used to assay terpene trilactones in standardized *Ginkgo biloba* extracts.

Compound	Initial concentration determined in the standardized *Ginkgo* dry extract (%, w/w)	"True" concentration values in Accuracy sample # (μg/mL)			Found concentration values in Accuracy sample # (μg/mL)			Recovery of the target compound in Accuracy sample # (%)		
		1	2	3	1	2	3	1	2	3
B_B	3.20	18.0	20.0	22.0	19.2	20.9	23.7	100.7	104.5	107.7
G_A	1.28	8.4	10.4	12.4	8.5	10.1	13.3	101.2	97.1	107.3
G_B	0.71	5.6	7.6	9.6	5.9	7.7	10.4	105.4	101.3	108.3
G_C	0.94	6.7	8.7	10.7	7.0	8.8	10.8	104.5	101.1	100.9
G_J	0.42	4.1	6.1	8.1	4.1	5.9	7.7	100.0	96.7	95.1

Note: Six standard additions, equivalent to 1 ÷ 6 μg/mL, increment of 1 μg/mL, were made for initial and accuracy samples; three replicates were made at each addition level; the mean peak area values are always used for calculation of A, B and resulting concentration value.

Table 6
Stability of terpene trilactones, as solution in methanol, at −20 °C, in the presence of the matrix (*Ginkgo* extract).

Compound	Initial	1st month		2nd month		3rd month	
	Concentration (μg/mL)	Concentration (μg/mL)	Recovery (%)	Concentration (μg/mL)	Recovery (%)	Concentration (μg/mL)	Recovery (%)
B_B	15.1	15.5	102.6	16.1	106.6	15.5	102.6
G_A	6.7	6.1	91.0	7.35	109.7	6.05	90.3
G_B	3.3	3.5	106.1	3.5	106.1	3.5	106.1
G_C	4.8	5.1	106.3	5.0	104.2	4.9	102.1
G_J	2.2	2.1	95.5	2.3	104.5	2.1	95.5

Table 7
Agreement between data obtained for the assay of terpene trilactones in *Ginkgo biloba* dry standardized extracts refined and quantified and commercial pharmaceutical products containing standardized extracts, resulting after application of the compendial method (HPLC/RID) and the standard addition assay by HPLC/MS^2.

Sample	Batch #	B_B (%)			$G_A + G_B + G_C$ (%)		
		RID	MS^2	Recovery	RID	MS^2	Recovery
	067487-000	3.0	3.1	103.3	2.8	3.0	107.1
	067497-000	3.2	3.1	96.9	2.8	3.0	107.1
Ginkgo biloba dry standardized extract,	067498-000	3.2	3.1	96.9	3.1	3.2	103.2
refined and quantified	067500-000	3.1	3.0	96.8	2.9	3.0	103.4
	067513-000	3.2	3.1	96.9	3.2	2.9	90.6
	067514-000	3.1	2.9	93.5	3.1	2.9	93.5
Flavotan 120 mg[a]	236_04_02_09	2.7	2.9	107.4	3.2	3.0	93.8
Flavotan 120 mg[a]	236_04_02_09	3.0	2.8	93.3	3.1	3.0	96.8
Bilobil 80 mg[a]	B49054	2.9	3.0	103.4	3.3	3.2	97.0
Gingium 80 mg[a]	7B6387	3.1	3.1	100.0	3.1	3.0	96.8
Gingium 120 mg[a]	7N9312	3.1	3.2	103.2	2.9	3.0	103.4
Tanakan 40 mg[a]	T272	3.0	3.1	103.3	3.1	2.9	93.5

[a] Reported percentage values are given with respect to the assayed content of the standardized extract.

temperature, mobile phase gradient, acidic additive in the solvents and chromatographic column production batch. However, only ±0.05 mL/min flow rate variation and ±0.7% variation of the starting composition of the mobile phase may induce variability of retention time within the accepted RSD% threshold.

The variation intervals of the operational parameters found to not influence the quality attributes of the method are within the limits granted by the producer of the chromatographic and detection equipments, if appropriate qualification is made. Consequently, the method should be considered robust with respect to the investigated operational parameters.

As the method is routinely performed in our laboratory for quality characterization of the *G. biloba* standardized dry extracts, refined and quantified, a comparison with the compendial HPLC/RID method was made. Some of the results are enlisted in Table 7.

4. Conclusions

An LC-(+)ESI/MS/MS method was used to assay terpene trilactones (bilobalide and ginkgolides A, B, C, and J) in *G. biloba* standardized extracts or in pharmaceutical formulations containing such extracts. No special sample preparation is needed; a simple dissolution/extraction in methanol should be applied. The quantitation is based on the standard addition method. Standard addition principle make possible that the sample matrix reaching the MS source is always the same. Its impact on ionization yields of the analytes is thus reproducible, allowing a minimal sample preparation procedure. The chromatographic method, based on a fast gradient elution, achieves baseline separation of the analytes within 4 min. A separation cycle takes 7 min, column re-equilibration included. The method was fully validated, according to guidelines in force, for selectivity, linearity, precision, accuracy and robustness. Accuracy (expressed as % bias) and precision (expressed as % RSD) are within 10%. Due to the standard addition method, the inherent variability of the MS/MS detector may be controlled in order to produce accuracy and precision needed for pharmaceutical quality control purposes. The ESI of terpene trilactones, as well as CID ionization patterns were also discussed. Structural confirmation of the target compounds is possible and should be considered as robust against the operational parameters of the method.

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