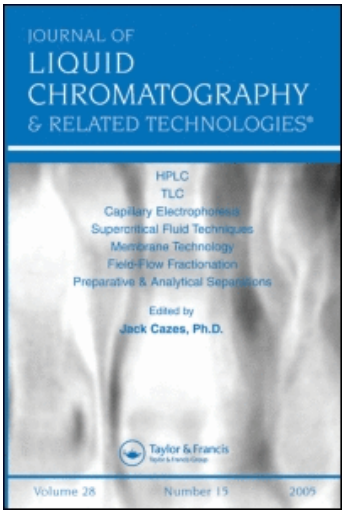

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LARGE VOLUME INJECTION OF HEXANE SOLUTIONS IN RPLC/UV TO ENHANCE ON SENSITIVITY OF THE ASSAY OF GINKGOLIC ACIDS IN *GINGKO BILOBA* STANDARDIZED EXTRACTS

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□ *The sensitivity of the compendial European Pharmacopoeia RPLC/UV method for assaying residual ginkgolic acids in Ginkgo biloba standardized extracts was essentially improved by operating modifications on the sample preparation procedure, including sample injection. The acidified methanol/water solution of the Ginkgo biloba standardized extract was extracted "in situ" with n-hexane (concentration factor of 20). From the organic layer, a relatively large volume (50 μL) of the hexane solution was directly loaded to the chromatographic column. A modified gradient elution profile was used in order to force the sample solvent to elute faster than analytes, without affecting peak symmetry. The separation is obtained in 22 minutes. As the amount of the target compounds loaded to column is higher, selective UV detection at 310 nm was possible. The method was validated according to specific operating guidelines for selectivity, linearity range, quantitation limits, precision, accuracy, and robustness. A limit of quantitation of 1 ppm (five times less than the accepted maximal content threshold for ginkgolic acids in standardized G. biloba extracts) was obtained. Confirmation of the target compounds through MS^2 detection and evaluation of the sensitivity afforded by such a detection system are also discussed.*

Keywords direct injection of n-hexane solutions, ginkgolic acids, improved sensitivity, liquid-liquid extraction, RPLC/UV, validation

INTRODUCTION

Ginkgolic acids (GA) and their structural related alkyl phenols (cardanols and cardols) are constituents of the products deriving from *G. biloba* vegetal materials.[1,2] Although these compounds have recognized molluscicidal, antimicrobial and antitumoral properties,[3] their contact allergenic, cytotoxic, mutagenic and neurotoxic potential was also

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discussed.[4–7] Therefore, the presence of such compounds in *G. biloba* extracts is considered as undesirable.[8,9] The recent monograph launched by the European Pharmacopoeia (EP) for standardized dry *G. biloba* extracts limits the amount of ginkgolic acids to 5 ppm.[10]

As ginkgolic acids contain an aromatic ring, their detection may be achieved by UV spectroscopy after liquid chromatography separation based on reversed phase (RPLC)[11,12] or argentation mechanisms.[13,14] Selective negative electrospray mass spectrometric detection (NI- ESI/MS) coupled to LC has also been successfully used for assaying residual ginkgolic acids in leaf extracts and phytopharmaceuticals.[15] A comparison between Evaporative Light Scattering and APCI/MS in detection of ginkgolic acid has been also reported.[16] The compendial method from the official EP monograph is based upon the conditions described in ref.[12]

According to the authors of the previously cited reference, liquid-liquid extraction (LLE) procedures using alkanes or alkyl acetates as extraction media for isolation and concentration of ginkgolic acids are labor intensive and lacks of reproducibility.

The approach used in the present work is based on the extraction of ginkgolic acids in n-hexane from strongly acidified methanol/water solutions of standardized *G. biloba* extracts. The concentration of the target compounds is controlled through the choice of a volumetric ratio between the aqueous layer and the extracting phase by 20:1. An aliquot from the organic layer was directly injected in the chromatographic column. Consequently, additional sample manipulation and the time consuming evaporation procedure are eliminated. Direct injection of analytes dissolved in solvents (*n*-alkanes) non-miscible with the mobile phases has been already reported.[17–19] Recently, studies were completed for other diluents, such as alkyl acetates and methyl *i*-butyl ketone.[20] The success of such kind of applications to avoid chromatographic peak symmetry distortion relates with a decreased retention of the analytes compared to the diluent. In the present study, analytes and diluent have similar increased apolar characteristics. Consequently, a step gradient elution profile was optimized in order to generate a delay between individual fronts of the target compounds and the front of the diluent. Thus, the method was validated for selectivity, linearity domains and quantitation limits, precision, accuracy, and robustness, according to guidances in place.[21,22]

EXPERIMENTAL

Reagents

All solvents were HPLC grade from Merck (Darmstadt, Germany). Formic acid (99%) 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. The certified reference substance (CRS) containing ginkgolic acids, batch 1 was obtained from European Pharmacopoeia, Strasburg, France. The content of the C17:1 ginkgolic acid in the CRS is declared as 0.35% by the European directorate for the Quality of Medicines & Healthcare (http://www.edqm.eu/en/Databases-10.html). Standardized Gingko biloba extracts from different commercial sources have been tested. No differentiation when applying the proposed sample preparation procedure was observed in relation to the commercial source of the standardized extract.

Apparatus

Experiments were performed with an Agilent 1200 SL series LC/DAD (Agilent Technologies) system consisting of the following modules: degasser (G1379B), binary pump (G1312A), autosampler (G1367C), column thermostat (G1316B), and diode array detector SL series (G1315C). System control and data acquisition were made with the Agilent ChemStation version A 10.02. For the LC/ PI-ESI/MS/MS experiments the same 1200 SL series liquid chromatograph was coupled to an ESI ion source (G1948B), and a triple quadrupole mass spectrometric detector (G2571A). System control, data acquisition and interpretation were made with the Agilent Mass Hunter software version B 01.00. The operational parameters of the MS detector were: nebulizer pressure 60 psi; drying gas flow 12 L/min; drying gas temperature 300°C; capillary voltage 4000 V; fragmentor 140 V, dwell time 50 ms; resolution wide; collision energy 10 V, delta EMV 600 V. The choice of mass transitions and selection of ions is discussed in text.

Sample Preparation

500 mg of the sample (standardized dried *G. Biloba* extract) are dissolved in a 10 mL volumetric flask with 5 mL of methanol. After addition of 0.5 mL formic acid, completion to volume is made with water. After vortex mixing for homogenization, 0.5 mL of n-hexane is added in the volumetric flask. The liquid-liquid extraction is achieved in 1 minute, through mixing at the maximum vortex speed. Complete separation of the layers is obtained within 5 min without centrifugation. An aliquot of 0.25 mL from the hexane solution (upper layer) is withdrawn and transferred to an injection vial which is immediately capped.

Chromatographic Method

A Zorbax Eclipse XDB-C8 (P.N. 963967–906), 150 mm length, 4.6 mm internal diameter and 3.5 μm particle size (Agilent Technologies) was used. The column was thermostated at 35°C.

Gradient elution was applied, using aqueous 0.1% formic acid and acetonitrile as mobile phase components. The gradient composition profile starts at 70/30 ratio (v/v) between aqueous and organic constituents. After 5 minutes, a step gradient is made in 0.01 min. up to 20/80 (v/v) aqueous/organic solvent. This composition was kept constant for the following 20 minutes. The column is prepared for a new injection by a step jump back to 70/30 mixing ratio between aqueous and organic constituents, followed by an equilibration run time of 5 minutes. The flow rate was 1.5 mL/min and the injected volume was 50 μL. Detection is made at 310 nm.

Compendial Method (EP 04/2008:1827–Ginkgolic Acids)

The separation is achieved on a chromatographic column having 25 cm length and 4.6 mm internal diameter filled with octylsilyl silica gel of 5 μm particle size stationary phase, thermostated at 35°C. The method uses a binary gradient elution obtained by mixing acetonitrile and water additivated with 0.01% (v/v) trifluoroacetic acid at 1 mL/min flow rate. Injection volume is 50 μL. Detection is made at 210 nm. The separation takes 45 min (including column re-equilibration).

The assay is based on the comparison between the sum of the peak areas corresponding to C13:0, C15:1 and C17:1 ginkgolic acids in the chromatogram of the test solution (50 mg/mL standardized extract) with the peak area corresponding to C17:1 ginkgolic acid in the chromatogram of the reference solution obtained from the CRS (0.7 μg/mL C17:1 ginkgolic acid).

The acceptance limits for the system suitability test assessed on the reference solution chromatogram refer only to qualitative aspects (resolution between C13:0 and C15:1 not less than 2, and peak symmetry factors for peaks corresponding to C13:0, C15:1 C17:1 between 0.8 and 2.0).

By reproducing the compendial method, a signal to noise ratio for the peaks corresponding to C13:0, C15:1 and C17:1 ginkgolic acids in the chromatogram of the reference solution is about 2, 10 and 5, respectively (noise intensity was measured on the baseline between C15:1 and C17:1 peaks). According to the calculation relationship given in the monograph, the sum of the peak areas of ginkgolic acids C13:0, C15:1 and C17:1 in a sample situated at the maximum accepted threshold (5 μg/mL) is 35.7% from the peak area of C17:1 in the chromatogram of the reference solution.

Solutions

A reference stock solution of ginkgolic acids was obtained by dissolving 10 mg of the CRS to 10 mL with methanol. This is corresponding to 3.5 µg/mL ginkgolic acid C17:1. From the stock reference solution, serial dilutions were made according to data in Table 1. The first set of solutions is made in methanol and is injected as such. The second set of solutions is made in methanol/formic acid/water 10/1/9 (v/v/v) and is submitted to the sample preparation procedure (extraction in hexane). The third set of solutions is made in a methanol solution of 100 mg/mL of blank *G. biloba* extract/formic acid/water 10/1/9 (v/v/v) and is also submitted to the extraction procedure. The *G. biloba* blank extract was identified through the commercial investigated batches. This batch produced concentration results for C13:0, C15:1 and C17:1 below 0.1 µg/mL under LC-MS/MS analysis.

The system suitability proposed for the method is solution #1 from the first set. Same qualitative conditions as in the compendial method are imposed. Additionally, the signal to noise ratio calculated for the peak C17:1 should be higher than 10.

As reference solution, in the conditions of the proposed method, solution #4, set 2 (according to Table 1) has been chosen. Through calculations, the sum of the peak areas of ginkgolic acids in a test solution of a sample situated at the threshold limit (5 µg/mL) is 3.25 higher than the peak area of the C17:1 acid in the chromatogram of the reference solution.

TABLE 1 Solutions Used for Method Validation

Solution	#1	#2	#3	#4	#5	#6
Set			***1***			
Aliquot from the stock solution (mL)	0.9	1.3	2.2	4.4	5	5.5
Dilution to volume (mL)	10	10	10	10	10	10
Dilution solvent			methanol			
Concentration of C17:1 (µg/mL)	0.32	0.46	0.77	1.54	1.75	1.93
Total concentration of ginkgolic acids (C13:0 + C15:1 + C17:1 expressed as C17:1–µg/mL)	1	1.4	2.4	4.7	5.4	5.9
Set			***2 + 3***			
Aliquot from the stock solution (mL)	0.045	0.065	0.11	0.22	0.25	0.275
Dilution to volume	10	10	10	10	10	10
Dilution solvent	Set 2: methanol/formic acid/water 10/1/9 (v/v/v) Set 3: blank *G. biloba* extract in methanol 100 mg/mL/formic acid/water 10/1/9 (v/v/v)					
Extraction to volume (mL hexane)	0.5	0.5	0.5	0.5	0.5	0.5
Concentration of C17:1 (µg/mL)	0.32	0.46	0.77	1.54	1.75	1.93
Total concentration of ginkgolic acids (C13:0 + C15:1 + C17:1 expressed as C17:1–µg/mL)	1	1.4	2.4	4.7	5.4	5.9

RESULTS AND DISCUSSIONS

Liquid-Liquid Extraction and Concentration

Surprisingly, ginkgolic acids are poorly extracted in alkanes from aqueous environments. Our experiments showed that the extraction yields calculated for C13:0, C15:1 and C17:1 ginkgolic acids from the CRS material dissolved in water and extracted in n-hexane are not higher than 2%. This may be explained through formation of micelles stabilized in aqueous media by means of the dissociation of carboxyl and phenol moieties. To adversely influence dissociation of these acidic groups, the pH in the aqueous phase should be drastically reduced. Almost quantitative extraction yields are thus obtained. However, complete dissolution of standardized *G. biloba* extracts in acidified water cannot be achieved. The use of methanol as organic solvent for controlling quantitative dissolution of *G. biloba* extracts at 5% concentration level is necessary. The proportion of the organic solvent in the aqueous phase should be attentively fixed, as fully miscible ternary systems with hexane might be generated. We found that the mixture methanol/formic acid/water in volumetric proportion of 10/1/9 easily dissolves *G. biloba* standardized extracts (considering the 5% w/v concentration level) also allowing extraction in hexane.

Solutions made directly in hexane from the CRS material, submitted to concentration under nitrogen flow, produced inconsistent recovery data, after being retaken in methanol and submitted to the chromatographic analysis. This can be explained by vapor withdrawal of ginkgolic acids during the evaporation step. All these data confirm the opinion expressed in[12] that liquid-liquid extraction procedures applied for isolation and concentration of ginkgolic acids "are time consuming and lacks in reproducibility." The same opinion is expressed in ref.[23]

A large volumetric ratio between acidified methanol/water and hexane layers allows elimination of the evaporation step. The volumetric ratio was set at 20:1. Hexane was added directly ("in-situ") to the volumetric flask in which the solution in acidified methanol/water mixture of the *G. biloba* standardized extract was obtained. As the organic layer occupies the upper place, the withdrawal of an aliquot can be easily achieved immediately after extraction.

Injection of Samples Obtained in Diluents Non-Miscible with the Mobile Phase

HPLC injection of samples obtained in diluents non-miscible with the mobile phase is a new topic discussed only in very few publications.[17–20] Basically, such operation may be done without risking peak shape

distortion, only if the retention of the diluent is higher than the retention of the target compounds. As the diluent saturates a part of the stationary phase on injecting large volumes (up to 500 μL), retention of the analytes is reduced proportionally with the length of the column being blocked.

The present case sounds differently as ginkgolic acids have increased apolar character compared to hexane. Indeed, calculation of the logarithm of the partition coefficient between water and octanol (log P) through the fragment methodology[24] leads to values 3.29 for hexane, 8.69 for C13:0, 9.45 for C15:1 and 10.43 for C17:1 ginkgolic acids. Consequently, to overcome focusing phenomena on injection, a different approach should be used: the separation should be started at a mobile phase composition allowing the irreversible partition of the analytes in the stationary phase, while the diluent front moves slowly. After delaying analytes from the front of the diluent, a step gradient is applied to increase drastically the proportion of the organic modifier in the mobile phase. The fast change of the mobile phase composition refocuses analytes and diluent. The last is eluted from column, while the analytes are separated and detected. The key parameters for controlling the process are the mobile phase composition at the beginning of the chromatographic run, duration of the period for delaying analytes from the front of the diluent and the final composition used for eluting the target solutes. Some of the experimental results on which method optimization is based are shown in Table 2.

The composition of the first plateau does not seriously affect resolution between C13:0 and C15:1 ginkgolic acids and the retention of the last eluting compound, indicating that analytes are trapped in the stationary phase immediately after injection. The final composition of the gradient substantially influences retention and peak width. It has been found that the

TABLE 2 Influence of the Experimental Parameters on Quality Attributes of the Peaks Corresponding to the Target Compounds on their Injection in Hexane Solutions

Experimental Conditions						
Injection Volume (μL)	First Plateau (ACN) (%)	Second Plateau (ACN) (%)	Resolution C13:0/ C15:1	Absolute Retention Time for C17:1 (min)	Peak Symmetry C17:1	Baseline Peak Width for C17:1 (min)
25	30	80	2.5	19.7	1.16	0.55
50	30	80	2.1	19.0	1.05	0.79
100	30	80	1.3	18.0	2.56	0.97
50	30	75	2.9	27.3	1.25	0.85
50	30	85	1.6	18.4	1.20	0.70
50	20	70	3.1	28.7	1.72	1.38
50	20	75	3.0	24.2	1.31	0.84
50	20	80	2.1	19.0	1.39	0.63
50	40	80	2.1	18.9	1.56	0.59

volumetric ratio of 70/30 aqueous to organic components of the mobile phase, a composition plateau of 5 min and a final gradient composition of 20/80 aqueous/acetonitrile are representing an optimal compromise for elution conditions at an injected volume of 50 μL of hexane solutions.

Injection of crude hexane extracts does not cause column clogging. Any deterioration of the column performance was observed after more than 300 extracted samples being injected (also considering standardized *G. biloba* extracts from different commercial sources).

The application of the procedure on phytopreparations containing *G. biloba* extracts should be carefully considered. The success depends on content of the standardized extract in the preparation and on the ability of the methanol/formic acid/water mixture to selectively extract target compounds against existing excipients.

Selectivity of the Method

To confirm elution order of the target compounds, the separation method was monitored through PI-ESI/MS/MS detection. Negative ionization mode was also tested. Mobile phase additivation with ammonium formate was necessary to achieve highly and stable ionization yields. Deposits of the salt produced in the MS source imposed frequent cleaning operations. Contrarily to statements in ref.,[12] positive electrospray ionization was really efficient to observe the target compounds in the optimized chromatographic elution conditions. Molecular protonated ions are formed within the source. In MS/MS detection, the protonated molecular ion was considered as precursor. Water loss has been observed as main process to generate product ions through collisional induced dissociation (CID). CID also produces the neutral loss of C_{n-1} hydrocarbonate chain, the ion with $m/z = 151$ a.m.u. resulting invariably. Under the same collisional conditions (N_2 as collision gas, 10 V as collision potential), the later ion fragment losses water with formation of the ion with $m/z = 133$ a.m.u. Only the water loss from the precursor ion carries out the structural information about the parent target analyte. CID fragmentation schema is given in Figure 1.

Chromatograms depicted in Figure 2a are obtained from a single MS detection process, with extraction of ion chromatograms according to the mass to charge ratio of the molecular protonated ions. In the CRS material dilution, other ginkgolic acids and related compounds could be observed under such MS detection conditions (ginkgolic acids C13:1, C15:0; C17:2, and cardanol). The MS/MS detection, when monitoring m/z transitions: 321.5 to 303.3 a.m.u for C13:0; 347.5 to 329.4 a.m.u. for C15:1 and 375.6 to 357.3 a.m.u for C17:1 also confirms the elution order

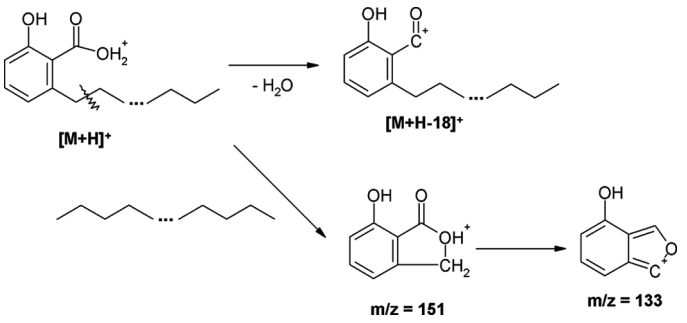

FIGURE 1 Tentative CID fragmentation pattern of ginkgolic acids.

and retention times from Figure 2b (the small shifts in retention time are produced by differences in the void volumes of connection tubings from the liquid chromatograph fitted with DAD and PI-ESI/MS/MS, respectively). Figure 2B corresponds to sample #3 (set 3, according to data in Table 1). The sensitivity obtained through MS/MS detection is at least one order of magnitude higher than the UV one. The selection of a G. biloba standardized extract (blank) used for evaluation of the matrix effect, containing less than 0.1 µg/mL ginkgolic acids C13:0, C15:1 and C17:1 was achieved by means of MS/MS detection.

Figure 2d corresponds to solution #1, set 3 (see Table 1). Figure 2C represents the UV trace monitored at 210 nm from the same sample. One can observe the effective gain in selectivity obtained through monitoring at a higher UV wavelength. It is worthwhile to note that residual matrix brought by the *G. biloba* standardized extract on the sample preparation procedure is not affecting the selectivity of the separation and the peak symmetry of the target compounds.

Linearity, Quantitation Limits, Recoveries

The concentration interval for the linearity study ranged from 1 to 5.9 µg/mL sum of ginkgolic acids (expressed as C17:1). As the leaflet and material safety data sheet of the CRS material are not providing any information about the individual content of C13:0 and C15:1, we assumed that their molar absorption coefficients at 310 nm are identical to the C17:1 one. From determinations carried out on the unextracted CRS material, the average amount ratios C13:0/C15:1/C17:1 are 1/5.4/3.1. Three parallel linearity studies were made. First linearity trial was made on unextracted solutions #1 – #6 from the first set. The second and third ones were made on extracted solutions #1 – #6 from sets 2 and 3 (extracted samples with and without matrix). Comparison between the regression slopes resulting

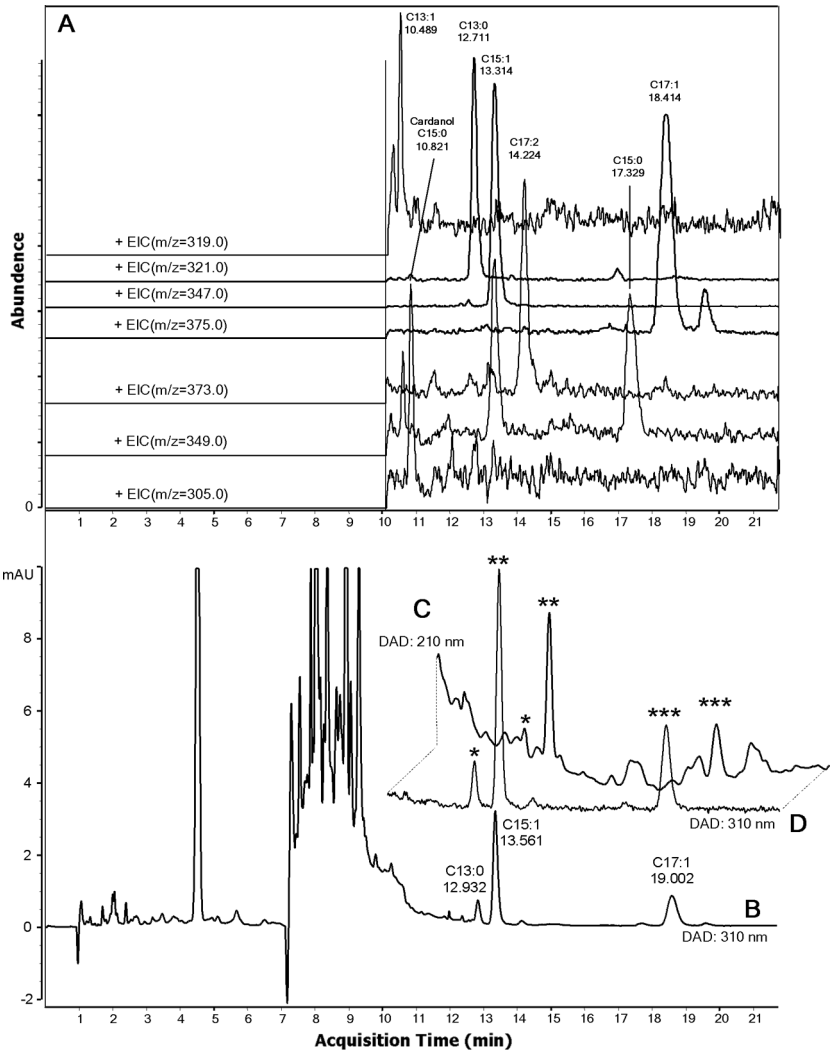

FIGURE 2 **A**–Identification of ginkgolic acids and related compounds from the extracted CRS material through PI-ESI/MS detection (extracted ion chromatograms for m/z values corresponding to protonated molecular ions of the target compounds); **B**–UV monitored chromatogram (310 nm) from sample #3, set 2; **C**–UV monitored chromatogram (210 nm) of sample #1, set 3; **D**–UV monitored chromatogram (310 nm) from the same sample as in **C**.

from the first two linearity studies emphasizes the extraction yield in hexane without matrix interference. Comparison between the last two linearity trials deals with the influence of the matrix on extraction. Comparison between the first and the third linearity trials evaluates overall recoveries of the analytes through the LLE sample preparation procedure. Results are given in Table 3.

TABLE 3 Regression Data from Linearity Studies Carried out on Ginkgolic Acids C13:0, C15:1 and C17:1. Evaluation of Extraction and Matrix Effects on the Recoveries of the Target Analytes

	Standard Solutions in Methanol (No Extraction)				Extracted Solutions without Matrix				Extracted Solutions with Matrix				Extraction Yields			
Parameter	C13:0	C15:1	C17:1	Sum	C13:0	C15:1	C17:1	Sum	C13:0	C15:1	C17:1	Sum	C13:0	C15:1	C17:1	Sum
Slope	14.3025	14.1027	14.6189	14.2922	14.4295	14.5998	13.7783	14.3138	13.5684	13.9207	13.6218	13.7861	Comparison between trials 2 and 1			
Intercept	−0.1276	−0.3572	−0.7417	−1.2266	−0.1237	−1.0488	−1.1454	−2.3180	−0.1943	0.7498	−0.2859	0.2695	94.9	98.7	93.2	96.5
R^2	0.9994	0.9996	0.9993	0.9996	0.9995	0.9998	0.9998	0.9999	0.9989	0.9987	0.9980	0.9986	Comparison between trials 3 and 2			
s_B	0.2538	0.1969	0.2649	0.2117	0.2345	0.1400	0.1440	0.1095	0.3248	0.3565	0.4324	0.3683	100.9	103.5	94.3	100.2
s_A	0.1059	0.4438	0.3427	0.8390	0.0979	0.3155	0.1862	0.4340	0.1355	0.8032	0.5594	1.4601	Comparison between trials 3 and 1			
LOQ(*)	0.07	0.30	0.22	0.56	0.06	0.21	0.13	0.30	0.09	0.52	0.36	0.96	94.0	95.3	98.9	96.3
LOQ (**)	0.08	0.34	0.29	0.67	0.08	0.29	0.22	0.47	0.11	0.52	0.43	1.04				

(*) LOQ calculated according to the relationship $[2 \times t \times (s_A + s_B \times C_m)]/(B + 2 \times t \times s_B)$, where B is the slope of the linear regression equation, s_A and s_B are the standard deviations of the intercept and of the slope, C_m is the mean value of the concentrations set, and t is the Student coefficient chosen for n-2 degrees of freedom (n = number of concentration levels) and for a level of confidence P% = 95% (bilateral) [25].

(**) LOQ calculated according to the relationship $(10 \times s_A - A)/B$, where A is the intercept, B the slope and s_A the standard deviation of the intercept.

Recoveries of ginkgolic acids C13:0, C15:1 and C17:1 are in the 90–110% interval. The limits of quantitation (LOQ) computed by means of two different approaches are similar. The LOQ for the total content of ginkgolic acids expressed as C17:1 with UV detection (310 nm) is placed at the 1 μg/mL level. This falls in good agreement with the observed signal to noise ratios measured in the resulting chromatograms.

To avoid consideration of the extraction yields, it is recommended that for routine applications, the reference solution to be processed through applying the extraction procedure in hexane (from a solution of the CRS material).

The signal to noise ratios calculated for ginkgolic acids C13:0, C15:1 and C17:1 in the chromatogram of a sample extracted in hexane having the same concentration as the reference solution from the compendial method are 29, 147 and 33, respectively.

Precision

Precision was evaluated on samples obtained through extraction of the CRS material. The considered concentration levels (as sum of ginkgolic acids) were 1 and 4.7 μg/mL, respectively. Six replicates from the same prepared samples were used for evaluation of repeatability. Six different samples at each of the concentration levels, prepared by different analysts during different experimental sessions (in between delayed by minimum 24 hours) were assumed for intermediate precision. The relative standard deviation (RSD%) for the sum of the peak areas at 1 μg/mL level was found 3.4% when considering repeatability. The mean recovery was 101.0% (minimum value of 98.6%; maximum value of 107.0%). The RSD% at 4.7 μg/mL level was found 3.6%. The mean recovery was 97.8% (minimum value of 95.9%; maximum value of 103.3%). When considering the intermediate precision, the RSD% of the sum of the peak areas at 1 μg/mL level was 2.4%. A mean recovery of 98.9% was found (minimum value of 95.5%; maximum value of 102.4%). At 4.7 μg/mL concentration level, the RSD% of the sum of the peak areas was 1.8%. A mean recovery of 98.1% (minimum value of 96.1%; maximum value of 100.6%) was determined.

Accuracy

Accuracy was evaluated at four different concentration levels (1, 3.9, 4.7 and 5.9 μg/mL sum of ginkgolic acids expressed as C17:1). The accuracy procedure was carried out by considering the matrix effect (the blank *G. biloba* standardized extract). Three replicates were independently processed

TABLE 4 Experimental Data Obtained from the Accuracy Evaluation Procedure

Total Concentration of Ginkgolic Acids (μg/mL)	Peak Areas Corresponding to				Experimental Determined Concentration (μg/mL)	Recovered (%)	Mean Recovery (%)
	C13:0	C15:1	C17:1	Sum			
1 μg/mL	1.3	8.2	4.4	13.9	0.99	98.9	98.9
	1.3	7.8	4.3	13.4	0.95	95.2	
	1.4	8.3	4.7	14.4	1.02	102.5	
3.9 μg/mL	5.4	30.5	17.3	53.2	3.84	98.4	97.4
	5.4	29.8	17.0	52.2	3.77	96.6	
	5.4	30.1	17.0	52.5	3.79	97.1	
4.7 μg/mL	6.2	36.5	20.6	63.3	4.57	97.3	99.3
	6.4	37.7	21.5	65.6	4.74	100.8	
	6.2	37.3	21.4	64.9	4.69	99.7	
5.9 μg/mL	8.5	45.3	25.6	79.4	5.74	97.3	97.4
	8.5	44.9	25.4	78.8	5.70	96.5	
	8.4	45.8	26.1	80.3	5.81	98.4	

at each concentration level. Experimental results are given in Table 4. The method was found as accurate.

Robustness

Both operational parameters controlling sample preparation (the liquid-liquid extraction) and chromatographic separation have been considered. Robustness procedure was carried out on samples having a total concentration of ginkgolic acids of 4.7 μg/mL expressed as C17:1 (solution #4, set 3 from Table 1). While the influence of the parameters involved by the liquid-liquid extraction step were considered only from the quantitative point of view (recovery), the impact of the operational parameters of the chromatographic method were also assessed qualitatively (retention of solutes and resolution between the critical pair C13:0/C15:1).

The robustness of the method when considering the operational parameters controlling sample preparation procedure is illustrated in Table 5.

Robustness of the method according to operational parameters controlling the chromatographic separation is given in Table 6.

In agreement with data given in Tables 5 and 6, the method behaves robustly with respect to the operational parameters controlling both sample preparation and separation steps.

The stability of the prepared samples submitted to the chromatographic separation was checked during 24 hours period in capped vials and was found satisfactory.

TABLE 5 Influence of the Operational Parameters Controlling Sample Preparation on the Results of the Method

Operational Parameter	Variation of the Operational Parameter (*)	Mean Peak Areas (n = 3) Corresponding to				Experimental Concentration (Sum of Ginkgolic Acids Expressed as C17:1) (μg/mL)	Recovery (%)
		C13:0	C15:1	C17:1	Sum		
Volumetric ratio between MeOH:	9.8 : 9.2	6.6	36.7	20.5	63.8	4.61	98.0
H_2O in the solution of the	**10 : 9**	6.8	38.4	21.9	67.1	4.85	103.1
standardized extract	10.2 : 8.8	6.1	37.4	21.5	65	4.70	99.9
Extraction time (min.)	0.5	6.2	37.2	20.2	63.6	4.59	97.7
	1	6.8	38.4	21.9	67.1	4.85	103.1
	1.5	6.4	36.7	20.4	63.5	4.59	97.6
Volume of formic acid added	0.4	6.6	37.3	21.1	65	4.70	99.9
during dissolution of the	**0.5**	6.8	38.4	21.9	67.1	4.85	103.1
standardized dry extract (mL)	0.6	6.5	37.1	20.8	64.4	4.65	99.0

(*) bolded parameters correspond to nominal values considered in the Experimental section, paragraph 2.3.

TABLE 6 Robustness of the Method According to Operational Parameters Controlling Chromatographic Elution

Compound	Retention Time (min)			Resolution $R_S^{C13:0/C15:1}$			Ginkgolic Acids Recovery (%)		
Column temperature (%C)	***32.5***	***35****	***37.5***	***32.5***	***35****	***37.5***	***32.5***	***35****	***37.5***
C13:0	13.393	12.932	12.697	2.05	2.07	2.10	100.6	99.8	100.1
C15:1	14.071	13.561	13.302						
C17:1	19.969	19.002	18.435						
Flow rate (mL/min)	***1.4***	***1.5****	***1.6***	***1.4***	***1.5****	***1.6***	***1.4***	***1.5****	***1.6***
C13:0	13.554	12.932	12.476	2.11	2.07	2.09	101.2	99.8	104.6
C15:1	14.240	13.561	13.070						
C17:1	20.117	19.002	18.203						
Mobile phase composition on the 1st plateau (% organic component)	***29***	***30****	***31***	***29***	***30****	***31***	***29***	***30****	***31***
C13:0	13.214	12.932	12.675	2.13	2.07	1.99	97.8	99.8	98.3
C15:1	14.121	13.561	13.232						
C17:1	19.419	19.002	18.837						
Mobile phase composition on the 2nd plateau (% organic component)	***79***	***80****	***81***	***79***	***80****	***81***	***79***	***80****	***81***
C13:0	13.511	12.932	12.456	2.31	2.07	2.01	104.8	99.8	105.0
C15:1	14.223	13.561	13.004						
C17:1	20.337	19.002	17.882						
Column batch	**#1***	**#2**	**#3**	**#1***	**#2**	**#3**	**#1***	**#2**	**#3**
C13:0	12.932	11.987	11.633	2.07	2.56	2.18	99.8	102.9	101.8
C15:1	13.561	12.532	12.154						
C17:1	19.002	17.009	16.338						

(*) operational parameters used in the method according to data presented in the Experimental section.

CONCLUSIONS

For improving sensitivity on assaying ginkgolic acids from standardized extracts of *G. biloba*, a LLE sample preparation procedure has been proposed. Extraction is made in hexane from a phase containing methanol/concentrated formic acid/water (10/1/9). An aliquot from the hexane layer (50 μL) can be directly injected onto the chromatographic column. The use of water-immiscible solvents as diluents in RPLC has received attention only recently. The few literature reports deal only with compounds having less retention compared to the non-miscible diluent, in the elution conditions. In the present situation, analytes have an increased apolar character, compared to the solvent. A step gradient elution profile was optimized to delay the solutes from the diluent front, allowing conservation of good selectivity and peak shape symmetry. This application relating to non-miscible diluent injection was fully validated according to actual guidances. Identity of the target compounds was confirmed via PI-ESI and MS or MS/MS detection. PI-ESI achieves satisfactory ionization yields and allows, in the MS/MS mode, at least one order of magnitude a higher sensitivity compared to UV detection. Other ginkgolic acids and related compounds (cardanol) have been identified by means of MS detection in the CRS material delivered by the European Pharmacopoeia.

By using the more selective 310 nm wavelength (operation supported due to the concentration of the sample by a factor of 20 through the LLE procedure), a quantitation limit at the 1 μg/mL concentration level was obtained (concentration is addressed as sum of ginkgolic acids C13:0, C15:1 and C17:1 and expressed as C17:1). Such quantitation limit represents 20% from the maximum threshold limit accepted for ginkgolic acids in standardized dry extracts, according to the EP compendial specification limits. The proposed method was found precise (relative standard deviations of the sum of peak areas corresponding to target analytes is placed below 5%), accurate (recoveries are between 90 and 110%) and robust with respect to operational parameters controlling both extraction and chromatographic separation steps.

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