Determination of trace amounts of ginkgolic acids in *Ginkgo biloba* L. leaf extracts and phytopharmaceuticals by liquid chromatography–electrospray mass spectrometry

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Abstract

Ginkgolic acids (GAs) are toxic phenolic compounds present in the fruits and leaves of *Ginkgo biloba* L. (Ginkgoacae). Their maximum level in phytopharmaceuticals containing ginkgo extracts has been recently restricted to 5 µg/g by the Commission E of the former Federal German Health Authority. In order to detect ginkgolic acids at these low levels, a sensitive and selective analytical method, based on liquid chromatography–electrospray mass spectrometry (LC–ES-MS) has been developed. The three main phenolic acids (**1–3**) of the chloroform fruit extract were isolated and used as standards for quantification. In the LC–ES-MS negative ion mode, calibration curves with good linearities ($r=0.9973$, $n=6$) were obtained in the range of 0.5–10 µg/g for compounds **1**, **2** and between 0.1 and 7.5 µg/g ($r=0.9949$, $n=6$) for ginkgolic acid **3**. The detection limits at a S/N ratio of 3 were 0.1 (**3**) and 0.25 µg/g (**1**, **2**). Recoveries were around 101% at 5 µg/g for the substances detected in the leaf extracts. Good precision was achieved with relative standard deviations of less than 4% ($n=6$). The optimised method was applied to verify whether the amount of gingkolic acids was below 5 µg/g in a standardised leaf extract which is a constituent of a phytopreparation. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Ginkgo biloba (Ginkgoacae) is one of the oldest living tree species, dating back over 300 million years. In China, the ginkgo trees have been used for 5000 years to treat lung ailments such as asthma and bronchitis, and as a remedy for cardiovascular diseases [1]. In Europe, it is an important phytophar-maceutical with the leading sales position [2]. In 1998, *G. biloba* was the best selling medicinal herb in United States (US$90 million) [3].

The terpenes and the flavonoids present in leaf extracts are responsible for the pharmacological activity. In a synergistic combination, these substances appear to have beneficial effects in the treatment of cerebrovascular insufficiency and peripheral circulatory problems [4,5]. Recently, clinical studies stimulated interest in a *G. biloba* extract for improving symptoms of Alzheimer-type dementia [6,7]. The quality of commercial ginkgo preparations relies on both classes of components. However, most

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of these drugs are standardised on their flavonoid content only and the terpene composition is not taken into account. The total amount of flavonoid is usually estimated according to UV spectrophotometric dosage based on Pharmacopoeia guidelines. The quantification of the non-UV active ginkgolides requires more sophisticated methods such as gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography (HPLC) with UV detection but both need tedious sample preparation [8–10]. In our ongoing studies on the standardisation of phytopharmaceuticals, we have developed two other methods for the rapid detection of these constituents, one based on liquid chromatography–thermospray mass spectrometry (LC–TSP-MS) and the other on liquid chromatography–evaporative light scattering detection (LC–ELSD) [11,12].

Ginkgolic acids (GAs) and their related alkylphenols represent another category of constituents found in the fruit pods and in the leaves [13]. Besides molluscicidal and antimicrobial properties of these compounds, toxic effects such as allergy have been reported [12,13]. As crude leaf extracts are used for therapy, they have to satisfy criteria of security of use, and the level of these toxic constituents has to be carefully checked. In Germany, a monograph of the Commission E of the former German Health Authority (Bundegesundheitsamt, BGA) limited at 5 $\mu g/g$ the maximal permitted concentration of ginkgolic acids in commercial phytopreparations [14]. As ginkgolic acids possess an aromatic chromophore, various reversed-phase HPLC–UV analyses have been published [11,15]. These methods however are not very selective and required long separation times. The detection limits were not specified and according to the present study, UV detection did

not permit a valuable quantification below 5 $\mu g/g$. GC–MS represents another alternative for the determination of GAs and allowed their identification in mixtures. This method however required derivatisation of the compounds and its sensitivity could not be evaluated because no detection limits were determined [16].

The present paper describes a rapid qualitative and quantitative method for the analysis of three ginkgolic acids **1, 2** and **3** (Table 1) by LC–MS with electrospray ionisation (ESI) in ginkgo leaf extracts. The proposed method features enhanced selectivity and sensitivity compared to LC–UV. Its application for the analysis of a phytopharmaceutical is evaluated.
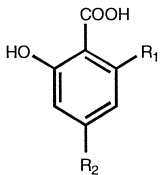
2. Experimental

2.1. Solvents and reagents

Chloroform, light petroleum, diethyl ether, formic acid and acetic acid were purchased from Fluka (Buchs, Switzerland). Acetonitrile (MeCN) and methanol (MeOH) were HPLC-grade (Maechler, Basle, Switzerland). Water was prepared by distillation on a Büchi (Flawil, Switzerland) Fontavapor 210 distillation instrument and passed through a 0.50-μm filter (Millipore, Bedford, MA, USA). Ammonium acetate and trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany).

2.2. Plant material

Ginkgo biloba fruits were obtained from Pharmaton SA (Lugano, Switzerland). They were sepa-

Table 1
Structures of the three ginkgolic acids (**1**, **2** and **3**) isolated

				M_r
	1	$R_1 = (CH_2)_7CH = CH(CH_2)_5CH_3$	$R_2 = OH$	362
	2	$R_1 = (CH_2)_7CH = CH(CH_2)_5CH_3$	$R_2 = H$	346
	3	$R_1 = (CH_2)_9CH = CH(CH_2)_5CH_3$	$R_2 = H$	374

rated into sarcotesta and nuts. Each part was lyophilised and extracted three times with chloroform for 24 h at room temperature. The leaves were collected near the Institut de Pharmacognosie et Phytochimie (Lausanne, Switzerland). The dry powder was extracted as described above.

2.3. Standards and samples

2.3.1. Isolation of ginkgolic acids

The crude chloroform extract (10.8 g) of *Ginkgo biloba* sarcotesta was subjected to silica gel open-column (750×65 mm I.D., 35–70 μm) with a step gradient of light petroleum–diethyl ether containing 1% formic acid (from 90:30 to 20:80, flow-rate: 8 ml/min) as mobile phase and yielded six fractions. The first fraction consisted of pure GA **1** (180 mg). Compounds **2** and **3** could not be resolved from this first fractionation and were isolated from fraction 2 by a combination of medium-pressure liquid chromatography (MPLC) and semi-preparative HPLC separations. Fraction 2 (7.9 g) was submitted to a LiChroPrep RP-18 column (MPLC, 45×14 mm I.D., 15–25 μm, Büchi, Flawil, Switzerland) with a water–MeCN step gradient (10:90 to 0:100, flow-rate: 15 ml/min) and yielded 1.4 g of compound **2**. Compound **3** (70 mg) issued from the same MPLC column was purified on a Nova-Pak RP-18 (PrepLCTM 100×40 mm I.D., Waters, Milford, MA, USA) semi-preparative HPLC (100% MeCN, flow-rate: 20 ml/min). The fractions in all steps of separation were collected automatically with a Retriever II collector (Isco, Mid-Glamorgan, UK). The three compounds were characterised by electron impact-mass spectrometry, desorption chemical ionisation-mass spectrometry and nuclear magnetic resonance (NMR) data [17–19].

2.3.2. Preparation of standard solutions

Pure reference compounds **1**, **2** and **3** (5 mg each, accurately weighted) were dissolved in MeOH in a 100-ml volumetric flask. These solutions were diluted to give standard solutions for the calibration curves in the range of 2.5–50 ng on-column ($n=6$) for **1**, **2** and between 0.5 and 37.5 ng on-column ($n=6$) for **3**. For LC–ES-MS, 5 μl were injected.

2.3.3. Extraction of GAs in the test extract

The standardized *Ginkgo biloba* extract GK 501 (Pharmaton, 1 g) was diluted in 50 ml of MeOH–water (50:50, v/v) and extracted three times with 20 ml of chloroform. The chloroform phases were combined, filtered through anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in 1 ml of MeOH. A 5-μl volume of this solution corresponding to 5 mg of the genuine extract was injected onto the column and analysed by LC–ES-MS in order to detect the ginkgolic acid levels. The extraction was repeated three times. Each sample was analysed twice.

To check the efficiency of the extraction, GK 501 (1 g) was spiked with 250 ng of a ginkgolic acid, extracted three times with 20 ml of chloroform and treated as described above. Each sample was analysed twice. The procedure was applied for samples spiked with **2** and **3**.

2.4. Instruments

2.4.1. HPLC analyses

The isolation was followed by HPLC. A Hewlett-Packard (Palo Alto, CA, USA) Model 1100 liquid chromatograph equipped with a photodiode array high-speed spectrophotometric detector was used in combination with a HP Chemstation software. The pre-column (8×4 mm I.D.; Macherey–Nagel, Oensingen, Switzerland) as well as the analytical column (125×4 mm I.D.; Macherey–Nagel) were packed with 300-5 Nucleosil RP-4 particles (4 μm). The mobile phase consisted of water–acetonitrile both containing 0.5% acetic acid. The gradient employed was 50% to 100% acetonitrile in 6 min with 4 min at 100% MeCN. The flow-rate was 1 ml/min. The UV chromatograms were recorded at 310 and 214 nm.

2.4.2. Liquid chromatography–electrospray mass spectrometry analyses

The LC separations were achieved using the HPLC conditions described above. 1/10 of the total flow-rate was split into the source with a high-pressure micro-splitter (Upchurch Scientific, WA, USA). The split ratio was adjusted with the aid of a micrometric valve directly mounted on the splitter. LC–ES-MS was performed with a Finnigan MAT ion trap mass spectrometer (LCQ), equipped with a

Finnigan electrospray source. The stainless steel capillary temperature was set to 200°C. The pressure of the nebulizing gas (nitrogen) was 88 p.s.i. (1 p.s.i.=6894.76 Pa). The ES-MS system was operating at 3.5 kV in the negative ion mode. In order to reduce fragmentation, no source-induced dissociation (SID) was used. Full scan spectra were acquired between m/z 150 and 800.

2.4.3. Liquid chromatography–thermospray mass spectrometry analyses

The thermospray ionisation conditions were optimised on a Finnigan MAT TSQ-700 triple quadrupole instrument equipped with a TSP 2 interface. The source block and the vaporizer temperatures were 280°C and 100°C, respectively. The aerosol temperature varied with the gradient. The electron-multiplier voltage was 1400 V, dynode −15 kV. The filament and the discharge were off. Post-column addition of buffer (0.5 M ammonium acetate) was performed with a Waters 590-MS programmable HPLC pump (0.2 ml/min) by means of a tee junction (Waters). Full scan spectra between m/z 150 and 900 were obtained in the positive ion mode.

2.4.4. Liquid chromatography–atmospheric pressure chemical ionisation mass spectrometry analyses

The Finnigan MAT LCQ, equipped with a Finnigan atmospheric chemical ionisation (APCI) source was used for the detection. The parameters were set as followed: discharge current 5.0 μA, vaporiser 450°C, capillary temperature 150°C. The buffer (acetic acid) was added post-column by the syringe pump of the LCQ (5 μl/min) to yield a final concentration of 1% in the mobile phase. Full scan spectra between m/z 150 and 900 were obtained in the positive ion mode.

3. Results and discussion

3.1. Isolation of the ginkgolic acid standards

In order to develop an LC–MS method for the quantification of trace amounts of GAs in the gingko leaves, the reference compounds have been isolated as they were not commercially available. The isolation was performed on a chloroform fruit extract as it

has been reported that the fruits contain large amounts of GAs [10]. Three main GAs (**1**, **2** and **3**) were obtained in a pure form by successive column chromatography (CC) on SiO_2 and MPLC on RP-18 separations. The structures of **1**, **2** and **3** (Table 1) were established on the basis of MS, ^{1}H- and ^{13}C-NMR data which were in good agreement with literature values [17–19].

3.2. Method development

The optimisation of the LC–MS method required an evaluation of GA ionisation with different LC–MS techniques and the development of a fast and robust LC chromatographic separation which was compatible with the LC–MS ionisation conditions.

The efficiency of LC–MS ionisation was checked by comparing three different techniques: TSP, APCI and ESI. The most abundant compound (**2**) was used as reference. In each case loop injections of **2** (0.1 μg) were performed under different ionisation conditions and with different buffers.

With TSP or APCI, ions were only observed in the positive ion mode. However, due to the relatively high temperature used, the protonated molecule [M+ H]$^+$ of **2** was not observed and the only ion present corresponded to the decarboxylation of the acid. The sensitivity of these two techniques was rather weak. In APCI, no significant improvement was observed by changing the buffer system (1% acetic acid to 1% formic acid). With electrospray, the ionisation of **2** in the negative ion mode provided intense deprotonated molecules while the positive ion mode failed to give any useful MS response. The good results obtained in negative ES-MS were easily explainable by the soft ionisation provided by this technique for thermolabile constituents and by the prompt deprotonation of these acidic molecules. In order to enhance the sensitivity of the ES detection, different solutions of ammonium hydroxide (1, 7, 14%) were added in the mobile phase with the aid of a syringe pump (flow 3 μl/min). The best response was obtained with the addition of a 14% solution, which gave a final pH of 9 for the eluent. This alkaline buffer promoted the formation of the GA anions in the liquid phase before ionisation [20].

In order to evaluate the separation of GAs, an artificial mixture of the three isolated compounds was used (injection of 0.1 μg each on the column).

The separation was first performed on a C_{18} reversed-phase by applying an MeCN–water gradient and post-column addition of a 14% ammonium hydroxide solution (3 µl/min) for LC–ES-MS detection. Under these conditions, a sensitive detection was obtained, but an important tailing of the different GAs was noticed. The addition of 0.05% TFA in the mobile phase significantly improved the LC resolution but the peaks eluted at 100% MeCN, which was not satisfactory. Moreover, this buffer suppressed the LC–MS ionisation even if the pH was increased to 8 by post-column addition of NH_3. It is indeed known that TFA ion may mask the charge on the analyte at the droplet surface and that consequently no evaporation/desorption of that ion could take place [20].

Based on these considerations, the separation of the GAs was finally undertaken on a reversed-phase C_4 column using an MeCN–water gradient containing 0.5% acetic acid. In this case, a good resolution was obtained and elution took place before 100% MeCN. With acetic acid, no ion suppression was observed and intense $[M-H]^-$ ions were recorded for the three different GAs. No significant enhancement of the ionisation was observed by post-column addition of ammonium hydroxide. This method was finally retained for the analysis of the different extracts and for quantification purposes.

Under the optimised conditions, the first analyses were performed in full scan mode (m/z 150–800) to estimate qualitatively the content of the chloroform extract of the fruits and leaves of *G. biloba*. The analysis of the chloroform fruits extract showed only three peaks at m/z 361, 345, 373 corresponding to the isolated acids **1**, **2** and **3**, respectively. In the chloroform leaf extract, only **2** and **3** were present (Fig. 1). No trace of other constituents was observed. This first analysis, demonstrated that the concentration of ginkgolic acids in the fruit and leaf extracts was several orders of magnitude higher than the limit
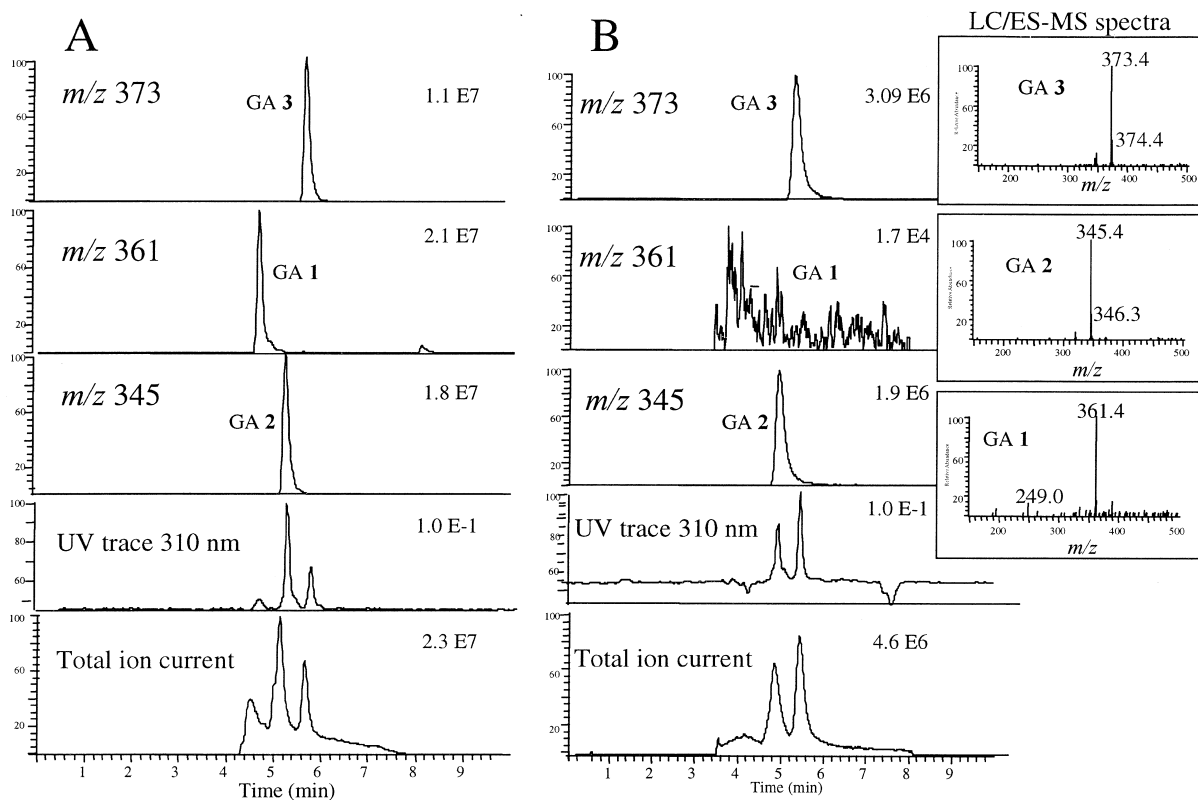

Fig. 1. LC–ES-MS analysis (full scan mode m/z 150–800) of the fruit (A) and the leaf (B) extracts of *Ginkgo biloba* L. The spectra were expanded between m/z 150 and 500.

of 5 μg/g required in the phytopharmaceuticals. The determination of these concentrations was not evaluated. At this level of concentration the quantification could be performed by simple LC–UV.

3.3. Quantification

For the quantification of trace amounts of GAs in phytopharmaceuticals, enriched samples were obtained by liquid–liquid partition. The standardized *G. biloba* extract, GK 501 which is a constituent of the phytopreparation Gincosan® was dissolved in MeOH and water and extracted three times with chloroform. The enriched chloroform extract (5 μl) corresponding to 5 mg of the original extract, was injected for LC–MS analysis. With this type of sample enrichment, a level of 5 μg/g corresponded to 25 ng of pure standards on column.

Calibration curves were established by injecting six samples of each isolated standard (**1**–**3**) in the 2.5–50 ng range (0.5–10 μg/g). Quantification of the three ginkgolic acids were carried out using selective ion monitoring (SIM) detection of their respective $[M-H]^-$ ions. MS–MS experiments were tried on the LCQ but generated an unfavourable fragmentation where many ions were observed with rather low intensities. In order to avoid source contamination and as no compound of interest was eluting before 3.5 min, the divert valve was used for diverting the flow in the source between 3.5 and 8 min.

Excellent calibration curves were obtained for **1** ($y=-207\,395+1.45146\cdot10^{6}x$), **2** ($y=-520\,307+1.70034\cdot10^{6}x$) within the range of 0.5–10 μg/g (linearity: $r=0.9973$, $n=6$, triple injection for each concentration). Detection limits were 0.25 μg/g (S/N 3) for both compounds. The calibration curve of **3** ($y=-360\,326+2.63212\cdot10^{6}x$) was within the range of 0.5–37.5 ng (0.1–7.5 μg/g, linearity: $r=0.9949$, $n=6$). In this case the detection limit (S/N 3) reached 0.1 μg/g. The analysis of GK 501 extract under these conditions revealed the presence of two GAs (**2**, **3**) in trace amounts (Table 2). The recovery of the method was estimated by spiking the same extract with 5 μg/g of **2** or **3** (Fig. 2). Table 2 summarises all the results obtained. UV detection (310 nm) was performed in parallel to LC–MS, the detection limits in this case were also evaluated; they were inferior to those of LC–MS and did not permit

Table 2
Summary of the results obtained for the quantification of the standardized *Ginkgo biloba* extract GK 501[a]

	Ginkgolic acids		
	1	**2**	**3**
Precision			
% RSD at 5 μg/g (Mean=6)	3.5	3.2	3.6
Recovery of the method			
Non-spiked extract:			
Quantity detected (μg/g) (Mean=6)	–	0.50±0.016	0.17±0.006
Spiked extract with 5 μg/g:			
Quantity detected (μg/g) (Mean=6)	–	5.40±0.171	5.30±0.188
% Recovery (Mean=6)	–	101±1.1	102±3.2

[a] The recovery of the method was calculated for a 5 μg/g spiking with ginkgolic acids **2** and **3** (25 ng of each).

quantification of the three GAs below 5 μg/g (limit of detection obtained by UV: 10 μg/g).

4. Conclusion

A simple and reliable LC–ES-MS method has been developed for the qualitative and quantitative analysis of ginkgolic acids in ginkgo leaf extracts. The proposed method is rapid, selective and sensitive, allowing the quantification of the GAs below 1.5 μg/g. This fulfils the requirement for the control of ginkgolic acid content in phytopreparations found on the market.

In our particular example, the GA levels found in the standardized *G. biloba* extract GK 501 were below 5 μg/g, in accordance with regulations. This method will now be used in a broad survey of various commercial ginkgo preparations in order to examine the general situation concerning the safety of these phytopharmaceuticals.
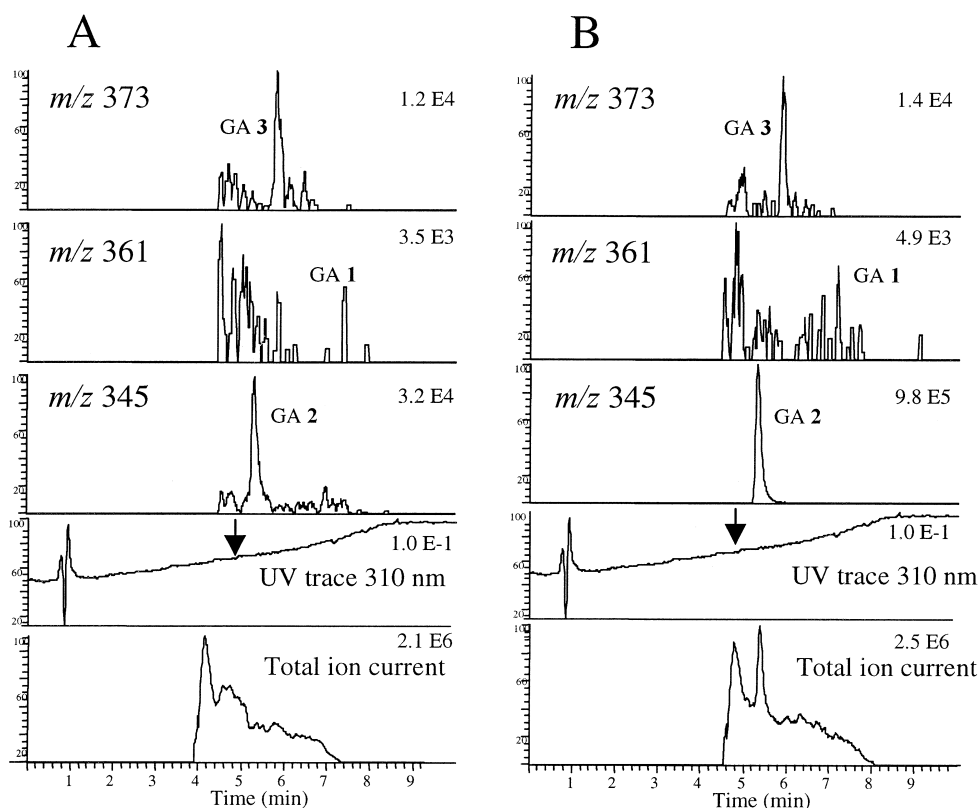
Acknowledgements

Fig. 2. LC–ES-MS (SIM mode) analysis of the standardized extract GK 501 without spiking (A) and spiked (B) with **2** at 5 μg/g (25 ng). The arrows indicate the expected retention time for ginkgolic acid **1**.

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