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Simultaneous determination of terpene lactones and flavonoid aglycones in *Ginkgo biloba* by high-performance liquid chromatography with evaporative light scattering detection

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Abstract

A gradient high performance liquid chromatographic method with evaporative light scattering detection (ELSD) for the simultaneous determination of ginkgolide A, ginkgolide B, ginkgolide C, ginkgolide J, bilobalide, quercetin, kaempferol and isorhamnetin in *Ginkgo biloba* is described. Samples are analyzed by means of a reverse-phase column (Supelco Discovery C-18) using methanol (containing 0.05% TFA) and water (containing 5% methanol and 0.05% TFA) under gradient conditions as the mobile phase over 35 min. The evaporative light scattering detector (ELSD) used, is set at an evaporating temperature of 61 °C and compressed air pressure of 2.9 bar. The detection limits ($S/N > 3$) of the compounds tested are 20–35 ng on the column. The exponential linear calibration curves are observed for all the compounds tested with r^2 more than 0.998. The reproducibility of the method was evaluated by analyzing three sets of controls on 3 consecutive days with RSD% and relative errors (RE%) less than 17.26 and 14.67%. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Ginkgo biloba*; Ginkgolide A; Ginkgolide B; Ginkgolide C; Ginkgolide J; Bilobalide; Quercetin; Kaempferol; Isorhamnetin; High performance liquid chromatography; Evaporative light scattering detection

1. Introduction

The use of botanical dietary supplement has experienced a steady growth recently. Among the top 10 commercial products is the extract of the leaves of *Ginkgo biloba* L [1]. Numerous pharmacological and clinical studies have demonstrated that the extracts of *G. biloba* possess antioxidant, anti-ischemic, neuro-protective, cardiovascular and cerebrovascular activities, and have beneficial effects on cognitive deficits, including Alzheimer's-type and multi-infarct dementia, and peripheral vascular disease [1–4]. The biological

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active constituents of *G. biloba* extracts include ginkgolides, a group of unique diterpene lactones which selectively inhibit platelet-activating factor (PAF) [5], bilobalide, a neuro-protective sesquiterpene lactone [6] and flavonoids, which also make an important contribution to the benefits of *G. biloba* [7].

Commercial *G. biloba* products are usually standardized based on the content of terpene lactones and flavonoids [8]. Many investigations have been carried out on this issue. For flavonoids, high performance liquid chromatography with ultra-violet detection (HPLC–UV) is a very convenient approach and has been used for the determination of the total flavonoids in the dried leaves, standardized extracts ($\geq 24\%$) and finished drugs [9]. However, the *Ginkgo* terpene lactones are poor chromophores with very weak absorption in the 200–220 nm range. Even trace impurities interfere with the detection of these compounds by UV [10–12]. Alternative methodology is highly desirable. HPLC with infrared (IR) detection was proposed and has been used with considerable success [13,14]. But the sensitivity and baseline stability remain a problem. Gas chromatography with flame ionization detection (GC–FID) might be very reliable for the sensitivity and reproducibility [15,16], but it requires tedious purification and sample derivatization before the analysis. Multi-steps manipulations are time consuming and might introduce a source of error [15,16]. Gas chromatography with mass spectrometry (GC–MS), high performance liquid chromatography with mass spectrometry (HPLC–MS) and nuclear magnetic resonance spectroscopy (NMR) methods have one or more advantages in terms of simplicity, sensitivity and selectivity [16–19]. However, the instruments are expensive and might not be available in all QA/QC laboratories. The economic choice is HPLC with the relative inexpensive evaporative light scattering detection (ELSD).

Generally, the ELSD process involves nebulization, evaporation and detection. In the first step, the chromatographic effluent is nebulized by a stream of pressurized nitrogen or air into droplets, from which the solvent can be easily evaporated. In the nebulization chamber, a narrow droplet size distribution is created by eliminating the larger droplets, which condense on the sides of the glass walls of the chamber and flow outside through a siphon-overflow. Next, the droplets are carried by the nebulizing gas toward the evaporator, where evaporation occurs and more volatile mobile phase is converted to gas and the analytes remain as particles. Finally, the solute particles emerging from the evaporator enter the light cell where they are directed toward a polychromatic light beam. The light, scattered by the analyte particles of non-volatile material, is measured by a photomultiplier or a photodiode. The intensity (peak area) of the signal is related to the concentration of the solute in the effluent. Camponovo et al. first introduced the HPLC–ELSD to the determination of ginkgolides and bilobalide in *G. biloba* product [16]. Compared with GC–FID, the simplification of sample preparation could be successfully achieved by employing three-steps of liquid–liquid extraction [16]. Unfortunately, the reported sensitivity (1100 ng) is poor when compared with that obtained with GC (55–105 ng) [16]. LC–MS (240–310 ng) [16], or even HPLC–IR (500–1000 ng) [14]. Recently, the ELSD has enjoyed renewed interest as an approach in the analysis of herbal products in part because of the dramatic improvements in the design of the instruments, which have made the ELSD more sensitive than before [20]. In 1996, Strode et al. reported a supercritical fluid chromatographic (SFC) method with ELSD for the determination of terpene lactones in *G. biloba* with detection limits of around 20 ng of bilobalide, ginkgolide A and B, and around 40 ng of ginkgolide C [21]. The high sensitivity of SFC–ELSD is of considerable interest. However, it is HPLC, not SFC, that is the most preferred and convenient approach for the routine QA/QC of herbal products today. This prompted us to conduct the current research to explore the application of HPLC–ELSD for the determination of terpene lactones in *G. biloba* products. On the other hand, the possibility of using HPLC–ELSD to assay flavonoid aglycones as well in *G. biloba* products might also be of interest because up to now there has been no literature regarding this issue.

In the present paper, a quantitative HPLC–ELSD method is described for the determination of terpene lactones (ginkgolide A, B, C, J, bilobalide) and flavonoid aglycones (quercetin, kaempferol and isorhamnetin) in *G. biloba* products in a single run with the sensitivity similar with that of reported for SFC–ELSD.

2. Experimental

2.1. Reagents and chemicals

Methanol (HPLC grade) was purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Deionized water was obtained with an in-house Nano-pure® water system (Barnstead, Newton, MA, USA). Ginkgolide A, B, C, J, bilobalide, quercetin, kaempferol and isorhamnetin were obtained from Herbal Standard, Inc. (St. Louis, MO, USA). *G. biloba* capsules were purchased in a local pharmacy, Chicago, USA.

2.2. Chromatographic conditions

A Waters 2690 Alliance HPLC system (Milford, MA, USA), equipped with an on-line degasser and an autosampler, was used for solvent delivery. The measurements were carried out on a Supelco Discovery RP-18 column (250 × 4.6 mm, 5 μm particle size, col # 24855-08, bonded phase lot # 3651, silica lot # PS 183, Supelco, Bellefonte, PA, USA) protected by a Waters Delta-Pak C_{18} guard column (Waters Technology Ireland, Ltd., Wexford, Ireland) and set at 20 °C. The solvents used for separation were water (containing 5% methanol and 0.05% TFA), solvent A; and methanol (containing 0.05% TFA), solvent B. Solvent gradient conditions are reported in Table 1. All injections were 10 μl in volume. The column effluent was directed to a Sedex 75 evaporative light scattering detector (ELSD) (Cedex 94141, Alfortville, France). Nebulization of the eluent in the ELSD was provided by a stream of pressured air at 2.9 bar. The nebulization was performed at room temperature, and the nebulized effluents were evaporated at 61 °C. The detector output was interfaced, using a SATIN box, to the Waters Millennium 2000® chromatographic manager system (Waters, Milford, MA, USA) loaded on a Compaq 6400X/10000/CDS computer (Houston, TX, USA) for data handling and chromatogram generation.

Prior to each run, the HPLC–UV–ELSD system was allowed to warm up for 20–30 min and the pumps were primed using the protocol suggested by the manufacturer. Using freshly prepared mobile phase, the baseline was monitored until stable before the samples were run.

2.3. Preparation of standard solutions

In a clean, dry 10-ml volumetric flask, reference standard (ginkgolide A, B and C, bilobalide, quercetin, kaempferol and isorhamnetin, 2 mg each, ginkgolide J, 3.5 mg, Fig. 1) was accurately weighed and dissolved in methanol to make a stock solution. Working standard solutions for calibration were prepared by diluting the stock solution with methanol in appropriate quantities.

Table 1
Solvent gradient conditions

Final time	Flow rate (ml/min)	A [water (containing 5% methanol and 0.05% TFA)]%	B [methanol (containing 0.05% TFA)]%	
0	1.0	75	25	Start gradient
35	1.0	25	75	End gradient
36	1.0	10	90	Wash out
42	1.0	10	90	
43	1.0	75	25	Equilibration before next run
50	1.0	75	25	

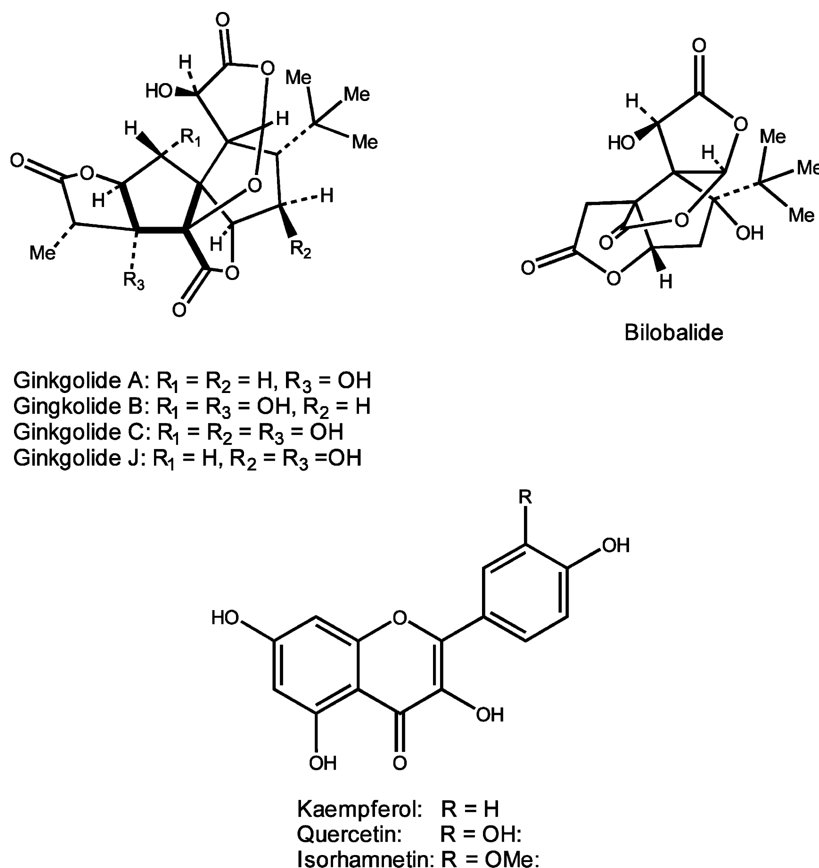

Fig. 1. Structure of ginkgolide A, B, C, J, bilobalide, quercetin, kaempferol and isorhamnetin.

Three sets of controls were also prepared so as to lie in the lowest, middle and highest regions of the calibration curve. All working solutions were stored at − 20 °C and brought to room temperature before use.

2.4. Preparation of sample solution

The contents of a *G. biloba* capsule were exactly weighed (ca. 0.5 g) into a PTFE-capped 20-ml sample vial. Methanol (18 ml) was added, and the mixture was shaken for a while and then sonicated at room temperature for 60 min. After cooling, the mixture was filtered through filter paper (Whatman # 1) into a 250-ml round-bottom flask, and the residue was returned to the sample vial. Another 18 ml of methanol was added and the mixture was sonicated at room temperature for 30 min. The extract was filtered through filter paper (Whatman # 1) into the same round-bottom flask. The combined methanol extracts were evaporated under reduced pressure at 45 °C. The residue was suspended in 20 ml of hot water and extracted with acetyl acetate (3×20 ml) by liquid–liquid extraction. The combined acetyl acetate extract was evaporated under vacuum at 45 °C and the resulting residue was re-dissolved and transferred with methanol to a 10-ml volumetric flask and made up to the volume with methanol. The sample

solution was centrifuged before 10 μl of the supernatant was subjected to HPLC–ELSD analysis.

2.5. Optimization of ELSD parameters

In order to obtain minimum noise and maximum detection signal in ELSD, three basic parameters, nebulizer gas flow rate (pressure), evaporating temperature and gain were varied to optimize the detection of compounds tested. In-home compressed air was subjected to two-steps of filtration before entering the detector to minimize extraneous particles from being introduced via the gas.

2.6. Efficiency of liquid–liquid extraction

In order to evaluate the efficiency of liquid–liquid extraction, methanolic standard (0.2 mg each) solution was evaporated under vacuum, and the resulting residue was suspended in 20 ml of hot water and extracted with acetyl acetate (3×20 ml). The combined acetyl acetate extract was evaporated under vacuum at 45 °C and the residue was dissolved in methanol (4×2 ml) and transferred into a 10-ml volumetric flask and made up to volume with metflhanol. Triplicate
test samples were prepared and analyzed using the current HPLC–ELSD method. The extraction efficiency was calculated by comparing measured amounts of standard in the test samples with actual amounts in the standard mixture solution.

2.7. Reproducibility

The precision and accuracy of the method were assessed by within and between run validations. The variation was evaluated by injecting three sets of controls on 3 consecutive days. By substituting the peak-area into the calibration curve equation from the same run the measured concentrations were obtained. By comparing calculated and theoretical concentrations, the relative errors (RE%) were obtained. The coefficient of variance (RSD%) was calculated by comparing the measured concentrations.

3. Results and discussion

3.1. Chromatography

Several combinations of methanol and water were evaluated for use as the mobile phase in order to improve the resolution and sensitivity. After trying several types of gradients and varying duration, an optimum solvent system was found as described under Section 2. In ELSD, under fixed chromatographic conditions, nebulizing gas flow rate (pressure) and evaporating temperature are the major instrumental adjustments available for maximizing detector response efficiency. In our case, ca. 2.9 bar was the lowest gas pressure that could be used while still enabling proper nebulizer operation. The best results were obtained with an evaporating temperature of 61 °C, this is lower than that reported by Camponovo et al. [16]. Under established chromatographic conditions, standard mixture solutions were injected into the HPLC–ELSD system. As shown in Fig. 2, the baseline separation was achieved with the compounds eluting in the order of bilobalide, ginkgolide J, C, A, B, quercetin, kaempferol and isorhamnetin, which was similar to that reported by van Beek et al. [14] and Camponovo et al. [16].

3.2. Limit of detection

As shown in Fig. 3, the detection limit ($S/N > 3$) of the described method was observed for 20–35 ng of compounds tested on the column in the current assay, which is much better than that (1100 ng) reported by Camponovo et al. [16].

3.3. Calibration

In ELSD, second-order polynomial calibrations (peak area against amount) were observed in the range of 70–3500 ng for ginkgolide J and 40–2000 ng for rest of the compounds tested on column. After log-transformation, the data provided a linear function for these compounds following the equation: $Y = a + bX$ with Y being the log value of the peak area, X the log value

of sample amount, a the intercept and b the slope. This was consistent with the previous results that ELSD provided, an exponential linear response for ginkgolide A, B, C, J and bilobalide with a regression coefficient more than 0.998.

3.4. The efficiency of liquid–liquid extraction

Three sets of standard mixture solutions were subjected to liquid–liquid extraction as described in Section 2. The extraction efficiency was observed to be 96.93, 98.54, 99.19, 96.82, 94.71, 102.0, 94.82 and 95.71% for bilobalide, ginkgolide J, C, A, B, quercetin, kaempferol and isorhamnetin, respectively. It could be inferred that 94–100% of the terpene lactones and flavonoid aglycones were extracted from the methanolic extract of *G. biloba* products, under the described conditions.

3.5. Reproducibility

The reproducibility of the method was evaluated by analyzing three sets of three controls on 3 consecutive days ($n = 3$) and calculating the RSD% and RE%. During the period of collecting the reproducibility data, the HPLC–ELSD system was run non-stop for 3 days. As shown in the Table 2, the RSDs (%) and REs (%) were less than 5.60 and 9.10%, respectively, in days 1 and 2. However, the large RSD (17.26%) and RE (14.67%) were observed on day 3, Although the controls were made on each day. The large RSDs and REs on the 3rd day might mean that there is instrument fatigue after 48 h of operation. We believe the output of the light source or detector response stability needs to be improved. These large RSDs are not observed when shorter periods of continuous operation are utilized [20].

3.6. Sample analysis

As shown in Fig. 4, three sets of samples were analyzed according to the method described above. Again, a stable baseline was observed and the peaks of bilobalide, ginkgolide A, B, J and C, kaempferol and isorhamnetin were well separated. Besides diterpene lactone peaks, there are some other peaks observed in the chromatogram (Fig. 4). Based on the analysis of a *Ginkgo* chemical library, these peaks are most likely flavonoids, as they did not show in the chromatogram of the blank sample and their peak area did not increase when the sample was spiked with diterpene lactones (ginkgolide A, B, C, J and bilobalide) standard solutions. Near the quercetin peak, a big

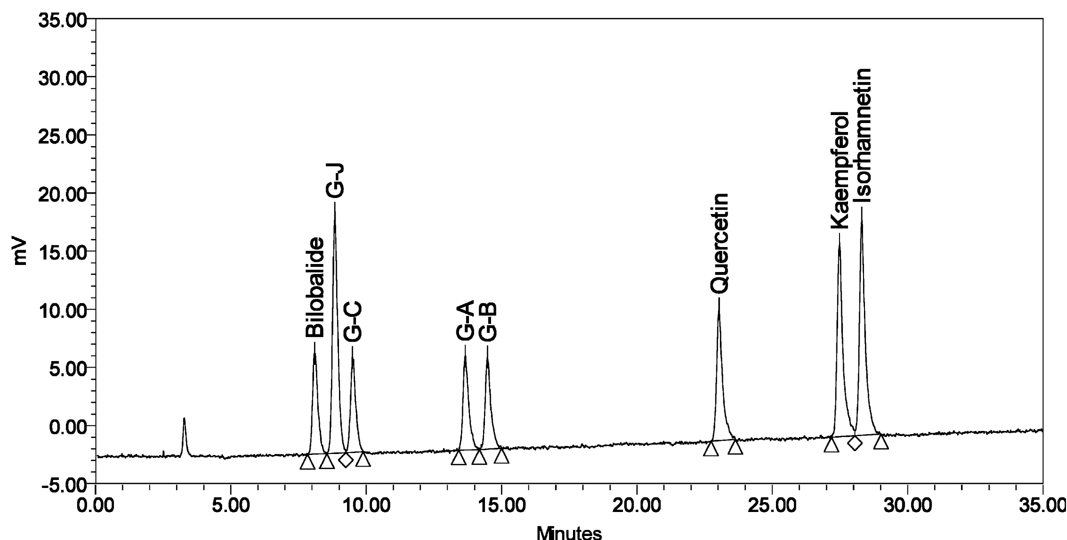

Fig. 2. A typical HPLC–ELSD chromatogram of standard mixture with 200 ng of ginkgolide A, B, C, bilobalide, quercetin, kaempferol and isorhamnetin and 350 ng of ginkgolide J on column.

Table 2
The reproducibility of the method

Constituent	Spiked amount (μg ml^{-1})	Day-1			Day-2			Day-3		
		Measured amount (mean ± SD, $n = 3$, μg ml^{-1})	STDEV (%)	RE (%)	Measured amount (mean ± SD, $n = 3$, μg ml^{-1})	STDEV (%)	RE (%)	Measured amount (mean ± SD, $n = 3$, μg ml^{-1})	STDEV (%)	RE (%)
Bilobalide	10.0	9.48 ± 0.53	5.58	−5.32	9.42 ± 0.32	3.42	−5.85	9.78 ± 0.78	7.95	−2.32
	80.07	74.93 ± 0.77	1.02	−6.41	73.05 ± 1.03	1.41	−8.77	70.60 ± 0.77	1.09	−11.83
	180.15	182.34 ± 2.01	1.10	1.21	184.65 ± 1.90	1.03	2.49	191.88 ± 2.84	1.48	6.51
Ginkgolide J	17.86	17.31 ± 0.27	1.55	−3.07	17.14 ± 0.80	4.65	−4.03	17.29 ± 1.16	6.72	−3.18
	142.88	142.92 ± 1.33	0.93	0.03	139.08 ± 1.63	1.17	−2.66	130.09 ± 1.97	1.51	−8.95
	321.48	317.04 ± 3.87	1.22	−1.38	317.65 ± 3.86	1.22	−1.19	332.09 ± 2.32	0.70	3.30
Ginkgolide C	10.5	10.49 ± 0.31	2.95	−0.14	9.80 ± 0.49	5.02	−6.64	10.15 ± 0.78	7.69	−3.38
	84.0	78.78 ± 0.75	0.95	−6.21	79.03 ± 1.52	1.92	−5.92	73.47 ± 1.85	2.52	−12.54
	189.0	191.91 ± 0.61	0.32	1.54	190.76 ± 0.55	0.29	0.93	199.80 ± 1.86	0.93	5.72
Ginkgolide A	10.05	9.59 ± 0.49	5.11	−4.58	9.59 ± 0.43	4.45	−4.62	10.0 ± 1.16	11.56	−0.55
	80.43	74.87 ± 1.56	2.08	−6.92	74.24 ± 0.35	0.46	−7.70	69.43 ± 1.48	2.14	−13.68
	180.97	186.68 ± 5.16	2.76	3.16	186.10 ± 2.53	1.36	2.83	194.36 ± 1.91	0.98	7.40
Ginkgolide B	9.94	9.15 ± 0.51	5.60	−7.86	10.04 ± 0.55	5.47	1.09	9.77 ± 0.55	5.64	−1.68
	79.49	72.98 ± 1.79	2.46	−8.19	73.69 ± 0.85	1.15	−7.30	67.83 ± 1.86	2.75	−14.67
	178.85	187.18 ± 3.36	1.79	4.66	179.91 ± 4.28	2.38	0.59	194.13 ± 3.19	1.64	8.55
Quercetin	10.8	10.67 ± 0.08	0.80	−1.22	9.94 ± 0.26	2.58	−7.92	10.12 ± 1.75	17.26	−6.34
	86.4	81.35 ± 1.88	2.31	−5.85	78.54 ± 0.57	0.72	−9.10	74.07 ± 1.73	2.34	−14.27
	194.4	198.41 ± 5.11	2.57	2.06	205.56 ± 6.57	3.20	5.74	210.30 ± 1.46	0.69	8.18
Kaempferol	10.22	10.91 ± 1.78	16.32	6.71	9.82 ± 0.34	3.44	−3.91	10.16 ± 1.12	11.07	−0.63
	81.78	77.48 ± 2.64	3.41	−5.25	85.37 ± 9.05	10.60	4.40	71.69 ± 2.43	3.39	−12.34
	184.0	185.60 ± 1.14	0.61	0.87	180.65 ± 7.07	3.92	−1.82	197.04 ± 1.50	0.76	7.09
Isorhamnetin	10.17	10.12 ± 0.60	5.91	−0.44	9.98 ± 0.48	4.85	−1.84	10.72 ± 1.32	12.32	5.44
	81.32	83.71 ± 1.32	1.58	2.94	88.67 ± 12.13	13.68	9.04	71.36 ± 2.51	3.52	−12.25
	182.97	174.01 ± 2.23	1.28	−4.90	175.66 ± 8.12	4.62	−3.99	191.19 ± 1.27	0.66	4.49

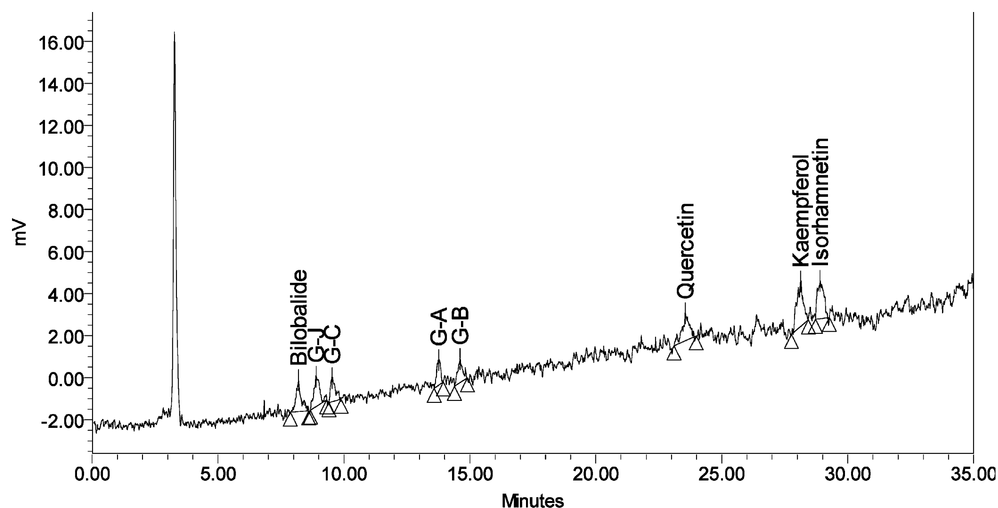

Fig. 3. A typical HPLC–ELSD chromatogram of standard mixture with minimum detectable concentration of 20 ng of ginkgolide A, B, C, bilobalide, quercetin, kaempferol and isorhamnetin and 35 ng of ginkgolide J on column.

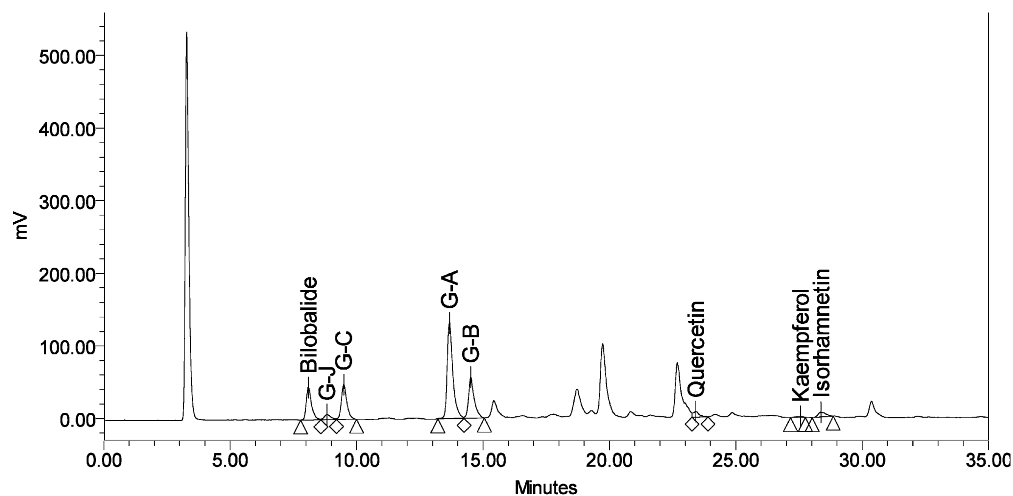

Fig. 4. A typical HPLC–ELSD chromatogram of acetyl acetate extract (10 μl) of *G. biloba* commercial product.

peak was observed which made an accurate integration of quercetin impossible. The average content of ginkgolide A, B, C, J, bilobalide, quercetin, kaempferol and isorhamnetin in a *G. biloba* commercial product was found to be 0.24, 0.07, 0.15, 0.40, 0.16, 0.07, 0.01 and 0.01% (w/w), respectively.

4. Conclusions

A HPLC method has been developed for the detection and quantitation of terpene lactones and flavonoid aglycones of *G. biloba* using an ELSD. With this method, ginkgolide A, B, C, J, bilobalide, quercetin, kaempferol and isorham-

netin were successfully quantitated, using calibration curves, with detection limit of 20–35 ng on the column. Although HPLC–ELSD might not be better than GC in sensitivity and reproducibility, the sample preparation in HPLC–ELSD is much simpler. Compared with HPLC–UV, HPLC–ELSD has a big advantage in terms of sensitivity and selectivity. The HPLC–ELSD method was found to be rapid, relatively inexpensive and straightforward. Moreover, the high efficiency of liquid–liquid extraction makes it very applicable for the analysis of any *G. biloba* product of interest.

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