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Determination of terpene trilactones in *Ginkgo biloba* solid oral dosage forms using HPLC with evaporative light scattering detection

M.-J. Dubber, I. Kanfer *

Faculty of Pharmacy, Division of Pharmaceutics, Rhodes University, Artillery Road, Grahamstown 6139, South Africa

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Abstract

A reversed phase high performance liquid chromatographic method with evaporative light scattering detection (RP-HPLC-ELSD) was developed for the quantitative determination of the terpene trilactones, ginkgolide A, B, C and J and the sesquiterpene, bilobalide in *Ginkgo biloba* solid oral dosage forms. Separation was achieved using a minibore Phenomenex Luna (5 μm) C_{18} column with dimensions 250 mm × 2.00 mm maintained at a temperature of 45 °C. A simple gradient method using a mobile phase of methanol:water and a flow rate of 350 μl/min facilitated baseline separation of the selected marker compounds within 14 min. The ELSD parameters affecting the detector response were optimized prior to the validation. The limits of detection and quantification were 31.25 and 62.50 ng, respectively. The percentage relative errors of the recovery ranged between −3.16 and +1.88 and both intra-day and inter-day percentage standard deviations were all better than 6%. This method was used to assay commercially available *Ginkgo biloba* products and proved to be suitable for the routine analysis of such products for quality control purposes.

Keywords: *Ginkgo biloba*; HPLC-ELSD; Ginkgolide A, B, C, J; Bilobalide; Routine analysis; Quality control

1. Introduction

Ginkgo biloba, often referred to as a "living fossil", is the oldest living tree specie on earth and is well known for its resistance to adverse growing conditions as well as its ornamental beauty [1]. Its use as a herbal preparation dates back to approximately 5000 years ago when ancient Chinese ingested brewed leaf extracts to treat cardiovascular and bronchial diseases [2]. Today, Ginkgo is one of the top selling herbal preparations in the US [1,3,4] and is the most common herbal preparation prescribed by practitioners in Germany [1]. Leaf extracts are marketed and sold as phytomedicines and used to alleviate symptoms related to cerebral insufficiency [5], Alzheimer's disease, depression, diabetic neuropathy, impotency, memory impairment, peripheral vascular disease, intermittent claudication, vertigo and tinnitus, amongst others [6]. Ginkgo's pharmacological activity is attributed to the synergism of two distinctly different chemical classes, the flavonoids and terpene lactones. The flavonoids are ubiquitous in the plant kingdom and have been extensively studied, most notably for their antioxidant and free radical scavenging activity [7]. On the other hand, ginkgolides A, B, C, J and M are found exclusively in *Ginkgo biloba*. Their action involves the inhibition of platelet activating factor (PAF) [8] while studies have shown that bilobalide, a related sesquiterpene compound has neuro-protective effects [9,10].

The ginkgolide structures, including bilobalide are shown in Fig. 1. The ginkgolides are non-volatile compounds and polar in nature and are therefore suitably analyzed by reverse phase high performance liquid chromatography (RP-HPLC). However, they have poor chromophores and can only be monitored at low, non-selective wavelengths (190–220 nm) [8]. This makes UV detection difficult, especially when analyzing crude Ginkgo extracts and extensive clean-up procedures are usually required prior to the analysis of leaf extracts and dosage forms [8,11,12]. Refractive index (R.I.) detection has been used successfully [13,14] but unstable baseline problems and low sensitivity [12,13] detract from its selectivity. In contrast, mass spectrometry (MS) has proved to be a valuable detection method due to its sensitivity and tandem mass spectrometry (MS–MS) facilitates exclusive analysis of selected peaks of interest with unequivocal peak identification which is particularly useful when analyzing crude extracts such as botanicals [11,15]. Analysis of the

* Corresponding author. Tel.: +27 46 603 8381; fax: +27 46 636 1205.
E-mail addresses: i.kanfer@ru.ac.za, bri@ru.ac.za (I. Kanfer).

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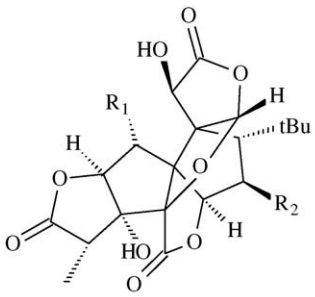
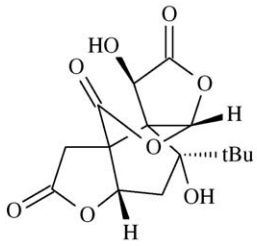


Terpene	R_1	R_2
Ginkgolide A	H	H
Ginkgolide B	OH	H
Ginkgolide C	OH	OH
Ginkgolide J	H	OH



Bilobalide

Fig. 1. The structures of ginkgolide A, B, C, J and bilobalide.

ginkgolides using gas chromatography (GC) has also been investigated [16,17] but this analysis method requires complex sample preparation as well as derivatization. Both GC and MS detectors require highly trained personnel for operation and the exorbitant costs, low availability and maintenance of such equipment makes these detection methods currently impractical for routine analyses [8,12,15]. On the other hand, the relatively inexpensive evaporative light scattering detection (ELSD) system has gained popularity for the analysis of non-volatile compounds that possess poor chromophores. Detection involves the nebulization of the LC effluent, followed by the evaporation of the more volatile solvent and then a silicon photodiode measures the scattering of light caused by the remaining solute particles [12]. The light scattering response is determined by the solute size and concentration [12,18].

Recently, a number of papers have been published using HPLC with ELSD detection for the determination of the ginkgolides in complex matrices such as pharmaceutical dosage forms, beverage, snack and dietary supplement products [4,12,19,20] and one paper described the assay of a Ginkgo extract in an injectable formulation [18]. Ganzera et al. [19] quantified ginkgolides A, B, C and J as well as the amount of bilobalide present in nine commercial products with a total analysis time of 25 min using a gradient program with a buffered mobile phase of 10 mM ammonium acetate, methanol and isobutanol. The method validation was described very briefly with a linear range of 31.2–500 μg/ml and LOD values of 20.3 μg/ml (203 ng on-column). The LOQ values were not disclosed and although excellent recoveries were reported (99.31–100.10%), the experiment was performed once only at the lower calibration range. Details regarding the formulation of the commercial products were not provided.

Li and Fitzloff [12] published a comprehensive method for the determination of both ginkgolides and flavonol aglycones, quercetin, kaempferol and isorhamnetin using a gradient method of methanol, water and trifluoroacetic acid (TFA). The final analysis time was 50 min, including the equilibration period however, all four ginkgolides, including bilobalide eluted within 16 min and this method has potential for application to the analysis of ginkgolides. The reproducibility of the method as shown by the analysis of three sets of three controls over a 3 day period was relatively poor with a relative error (%R.E.) and relative standard deviation (%R.S.D.) of 14.67% and 17.26% being recorded on the third day, respectively. Recovery studies were not reported. In addition, only a single product was analyzed resulting in no available comparative data and since formulations containing natural products are not normally required to be standardised, the robustness of the method was not sufficiently vindicated.

Herring [4] recently published a method separating four ginkgolides and bilobalide within 14 min using a gradient method of water, methanol and TFA. The LOD was relatively high at 125 ng on-column and whilst the method showed acceptable reproducibility, no recovery studies were performed which is a critical determinant of method accuracy.

Tang et al. [18] assayed the content of ginkgolides A, B and C, together with bilobalide in a Ginkgo injection for quality control purposes. In addition, separation of the relevant peaks was compared using two columns, a Diamonsil and YWG. Baseline separation was obtained on the Diamonsil column using a simple isocratic method of methanol and water but the run time was 41 min. The analysis time was halved using the YWG column however separation between bilobalide and ginkgolide C was compromised by an interfering peak which was speculated to be ginkgolide J but confirmation was circumvented due to a lack of the reference standard. Moreover, the calibration range was from 75 to 800 μg/ml, the limits of detection and quantification were not given and solid phase extraction was used for sample clean-up.

Lang and Wai [20] published a method which focused on the extraction procedures required for accurate quantification of

terpene trilactones in beverages, snacks and capsules containing *Ginkgo biloba*. The three analyzed capsules were extracted with 5% KH_2PO_4, followed by a liquid–liquid extraction with sodium chloride and a mixture of ethylacetate and THF. The terpenes were separated within 30 min on a Phenomenex C_{18} (250 mm × 4.6 mm I.D.) column using a gradient of water and a mixture of methanol and isobutanol. Reference to a previous study [21] was provided concerning the recovery data of the capsules (95–102%) with RDSs of <7.4% performed at a single concentration level using ginkgolide A, B, C and bilobalide only. The LOD was 40 ng on column load for all reference standards but the LOQ was not provided.

Our paper describes an accurate, precise, rapid and reproducible RP-HPLC-ELSD method which was fully validated according to USP standards for the determination of ginkgolide A, B, C and J, including bilobalide using a simple gradient of methanol and water as mobile phase. Baseline separation of the reference standards was achieved within 14 min using a Phenomenex Luna (5 μm) C_{18} minibore column with dimensions 250 mm × 2.00 mm I.D. which requires lower solvent flow rates than conventional columns and thereby contributed to reduced analysis costs through a reduction in solvent consumption. Sample clean-up involved an inexpensive liquid–liquid extraction procedure. This method was successfully applied for the quantitative analysis of the selected terpene marker compounds in various commercially available *Ginkgo biloba* capsules (formulated as soft and hard gelatine capsules) and tablets and is particularly suitable for the routine analysis and quality control of such products.

2. Experimental

2.1. Chemicals and materials

Methanol was supplied by Romil (The Source, Waterbeach, Cambridge, GB) and ethyl acetate by BDH chemicals (Poole, UK). Ginkgolide A (90%), ginkgolide B (90%) and bilobalide (95%) was purchased from Sigma (Missouri, USA) and ginkgolide J (95%) was obtained from Chromadex (Santa Ana, CA, USA). Ginkgolide C (90%) was a generous gift from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The peak purity of the terpene trilactones used as reference standards were checked chromatographically and confirmed by NMR spectroscopy. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA) and all samples were filtered using low protein binding durapore (PVDF) membranes purchased from the latter source. Five *Ginkgo biloba* commercial products (Products A–E) were purchased from a local pharmacy in Grahamstown, South Africa. Three of the products were formulated in tablet form, one product contained liquid Ginkgo extract in a soft gelatine capsule and one product contained pulverized Ginkgo leaf extract in a hard gelatine capsule.

2.2. Instrumentation and chromatographic conditions

All experiments were performed using a Waters 2690 Separations Module equipped with an on-line degasser system and column heater for the HPLC analysis (Milford, MA, USA). An Alltech ELSD 2000 detector system was supplied by the same source and Empower® Software was used for the processing and integration of data. The ginkgolides were separated on a Phenomenex Luna (5 μm) C_{18} column with dimensions 250 mm × 2.00 mm which was maintained at a temperature of 45 °C. The mobile phase consisted of methanol:water in proportions of 30:70 for the first 6 min, after which the methanol was increased in linearly to 70:30 for the remainder of the analysis time. The flow rate of the mobile phase was 350 μl/min and the total run time was 16 min. The ELSD gas flow and drift tube temperature were optimized prior to the analysis and were set at 1.5 l/min and 117.5 °C, respectively.

2.3. Preparation of standard solutions

The stock solution for the calibration curve was prepared by weighing 5 mg of each reference standard into a 25 ml volumetric flask, dissolving the contents in methanol and then filling it to volume with the same solvent. In order to obtain the final concentration range of 12.5–100 μg/ml, appropriate concentrations of the stock solution were pipetted into 10 ml volumetric flasks and then filled to volume with methanol.

2.4. Sample preparation and extraction

2.4.1. Solid oral dosage forms

A minimum of 20 tablets of Products A–C were individually weighed and pulverized using a mortar and pestle. An aliquot of powder equivalent to the mass of a single tablet was then weighed and carefully transferred to a 20 ml Kimax tube. Twenty millilitres of methanol was added, the powder was well dispersed and the mixture was sonicated in an ultrasonic bath for 30 min. The contents were then manually agitated to ensure adequate re-dispersion before sonication was continued for a further 30 min. The extract was then centrifuged at 350 × *g* for 10 min followed by decantation of the supernatant into a 50 ml Kimax tube. An additional 20 ml of fresh methanol was added to the remaining residue; the contents were re-dispersed and sonicated for 30 min followed by centrifugation and decantation of the supernatant into the same 50 ml Kimax tube. The above procedure was repeated once more. The combined extracts were evaporated to dryness using nitrogen before re-suspension in 20 ml hot water and liquid–liquid extractions were then performed in triplicate (3 × 20 ml) with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness using nitrogen. The samples were reconstituted in 18 ml of methanol, decanted into appropriate volumetric flasks and then filled to volume. All samples were filtered through 0.45 μm PVDF membranes before injecting 5 μl onto the column.

2.4.2. Soft gelatine capsule

A single weighed capsule of Product D was sliced longitudinally and the contents were carefully squeezed out into a 20 ml Kimax tube. The remaining gelatine coat was then sliced into two halves and added to the contents. Twenty millilitres of

methanol was added and the capsule was extracted as described in Section 2.4.1 above.

2.4.3. Hard gelatine capsule

A minimum of 20 capsules of Product E were individually emptied and weighed before ensuring thorough mixing using a mortar and pestle. Twenty millilitres of methanol was added to a mass of powder equivalent to the contents of one capsule and extracted as previously described in Section 2.4.1.

3. Results and discussion

3.1. Method development

A previous in-house investigation on the separation of the ginkgolides proved that methanol:water was the most suitable mobile phase. The addition of acetonitrile, even in small quantities compromised the resolution between ginkgolides A and B which is consistent with the findings by Tang et al. [18]. The incorporation of phosphoric acid to the aqueous component of the mobile phase gave no results. Subsequently, methanol and water were selected as the mobile phase components.

All four ginkgolides, including bilobalide were well separated within 19 min using an isocratic method of methanol:water (30:70) at a column temperature of 45 °C and flow rate of 300 μl. When the proportions of the mobile phase were adjusted to 35:65 methanol:water, ginkgolides C, J and bilobalide co-eluted. A gradient method was proposed to decrease the analysis time and increase sensitivity by changing the ratios of methanol:water from 30:70 after bilobalide, ginkgolide J and C had eluted by linearly increasing the percentage methanol to 40:60 over the remaining 12 min of the 20 min run time. The last peak eluted at 17 min and subsequently, the following gradient involved changing methanol:water from 30:70 at 7 min to a linear change of 50:50 over 20 min which decreased the run time by 1 min only. Slight changes in the gradient profile ensued until changing the gradient from 30:70 methanol:water at 6 min to 30:70 over the following 10 min resulted in an analysis time of under 16 min. The flow rate was then slightly increased to 350 μl/min and using the previous gradient all selected marker compound eluted within 14 min. Fig. 2 shows the elution orders of the reference standards using this method. Both the isocratic and gradient method gave suitable chromatograms for analysis but the gradient method was selected due to the enhanced sensitivity and the improved peak shape of ginkgolides A and B caused by the higher organic composition of the mobile phase.

The most important parameters affecting the ELSD signal response is the nebulizer gas flow rate and drift tube temperature. The gas flow rate influences the droplet size of the column effluent before evaporation occurs. Higher flow rates result in the formation of smaller aerosol droplets and less scattering of light with subsequent lower sensitivity but a more stable baseline. On the other hand, lower gas flow rates are associated with larger droplet formation, augmented light scattering and therefore a higher response but the baseline is compromised. It is therefore pertinent to optimize this parameter to ensure that the optimal signal to noise ratio (*S*/*N*) is achieved [22]. The gas flow

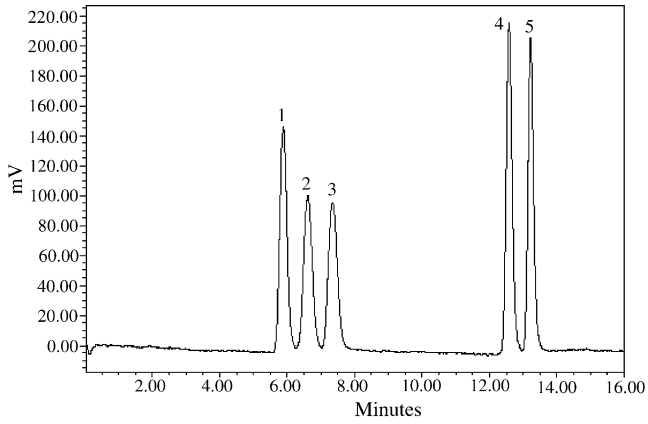


Fig. 2. HPLC-ELSD chromatogram of the reference standards. Peak 1: Bilobalide, peak 2: ginkgolide J, peak 3: ginkgolide C, peak 4: ginkgolide A, peak 5: ginkgolide B.

was therefore investigated over the range of 0.7–3.5 l/min in increments of 0.2 l/min. The sensitivity was highest at 0.7 l/min as predicted and nebulizer gas flow rates higher than 2.2 l/min obviously compromised the sensitivity. As a result, 1.5 l/min was chosen since it exhibited the highest *S*/*N* ratio.

The drift tube temperature facilitates the evaporation of the nebulized aerosol so that the light scattering response of the non-volatile solute can exclusively be determined. Mobile phases with a high aqueous content require higher drift tube temperatures for evaporation than those comprised predominantly of organic components. Similarly, optimal sensitivity of non-volatile solutes requires higher drift tube temperatures than semi-volatile solutes [22]. It was therefore predicted that a relatively high drift tube temperature would be required for adequate evaporation since the ginkgolides are non-volatile and the mobile phase consisted substantially of water. The effect of temperature on sensitivity was determined over the range of 70–120 °C in increments of 10 °C until 110 °C and thereafter increased by increments of 2.5 °C. A drift tube temperature of 117.5 °C was finally selected based on the evaluation of the *S*/*N* ratio.

Tablet extraction efficiency was also investigated using various solvents frequently used in ginkgolide extraction procedures [8]. A mass of powder equivalent to the mass of a single tablet of Product C which contained sufficient quantities of the relevant marker compounds was extracted exclusively with methanol (3 × 20 ml). The same procedure was followed using ethyl acetate (3 × 20 ml) as extraction solvent and finally the method of Li and Fitzloff [12] was investigated by using methanol as the primary extraction solvent followed by a liquid–liquid extraction using hot water and ethyl acetate as described in Section 2.4.1. The various extraction procedures were also performed on reference standards. It was found that although methanol proved to be an excellent extraction solvent it was unselective and interfering compounds co-eluted with the peaks of interest. When ethyl acetate was used as the primary extraction solvent, its lower polarity ensured little interference but the polar ginkgolides were not as effectively extracted as with methanol. It was concluded that the method described by Li and Fitzoff [12] was the most

Table 1
Linearity and range

Constituents	$y = ax + b$ linear model	Regression, R^2	Concentrations, x (μg/ml)				
Ginkgolide A	$y = 1.45x + 3.52$	0.9989	12.68	25.35	50.70	81.12	101.40
Ginkgolide B	$y = 1.45x + 3.48$	0.9986	12.68	25.35	50.70	81.12	101.40
Ginkgolide C	$y = 1.38x + 3.47$	0.9988	12.60	25.20	50.40	80.64	100.80
Ginkgolide J	$y = 1.34x + 3.52$	0.9942	12.75	25.50	51.00	81.60	102.00
Bilobalide	$y = 1.43x + 3.45$	0.9974	12.93	25.85	51.70	82.72	103.40

suitable and efficient extraction method since it extracted the most amounts of ginkgolides with minimal interference.

3.2. Linearity and range

Linear calibration curves were drawn using five concentration points on each of the 3 days and plotting the double logarithm of both *x*- and *y*-axis values. The ranges and correlation coefficients are presented in Table 1. The respective ranges were substantially lower than those described in the previous papers cited above.

3.3. Precision and accuracy

The precision and accuracy of the method was determined by spiking powdered tablet material from Product B with amounts corresponding to low, medium and high concentrations of each reference standard. Table 2 depicts the results of this investigation. Since insufficient reference material was available, the recovery study for ginkgolide J was performed on the second day of the validation only. The precision is indicated by the intra-day and inter-day percentage relative standard deviations (%R.S.D.) which were well below 5.0% except for the intra-day %R.S.D. for the medium recovery level of ginkgolide C on the first day (5.37%). The accuracy is represented by the percentage relative error which ranged between +1.88 and −3.16 over all 3 days for ginkgolide A, B, C and bilobalide and +0.13 and −1.52 for ginkgolide J on the second day. These results reflect excellent method accuracy and precision. Moreover, the reproducibility of the method is better than the results obtained by Li and Fitzloff [12], Lang and Wai [20], and Herring [4] while Ganzera et al. [3] did not report reproducibility data.

3.4. Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were determined by serial dilution of the stock solution containing all reference standards according to a signal to noise ratio of 3:1 and 10:1, respectively. The LOD of the ginkgolides was 31.25 ng and the LOQ was 62.50 ng. The LOD correlated with the range of LODs given by Li and Fitzoff [12] for the ginkgolides but were better than those proposed by Herring [4], Ganzera et al. [19] and Lang and Wai [20]. No LOQ values were given by the previously mentioned authors [4,12,19], and Tang et al. [18] did not report either LOD or LOQ values.

3.5. Sample analysis

Fig. 3 shows the superimposed HPLC-ELSD chromatograms of the five analyzed commercial *Ginkgo biloba* products and Table 3 indicates the content of the present marker compounds. All five marker compound were quantifiable in each product

Table 2
Recovery of the ginkgolides and bilobalide

Constituent	Mean spiking level (μg/500 mg tablet)	Intra-day %R.S.D. ($n = 3$)			Inter-day %R.S.D. ($n = 9$)	Mean recovery (%) ± S.D. ($n = 9$)	%R.E. (%)
		Day 1	Day 2	Day 3			
Bilobalide	101.00	1.99	0.33	0.78	1.56	101.88 ± 1.58	+1.88
	403.80	3.61	1.82	0.93	1.08	99.36 ± 1.07	−0.64
	1245.40	0.47	1.00	1.46	2.98	100.36 ± 2.99	+0.36
Ginkgolide A	101.00	1.46	0.70	0.14	1.77	101.53 ± 1.80	+1.53
	403.60	4.82	1.87	0.30	1.27	100.24 ± 1.28	+0.24
	1245.00	0.62	1.52	1.18	2.26	100.97 ± 2.28	+0.97
Ginkgolide B	403.00	0.54	1.98	0.94	0.49	101.16 ± 0.50	+1.16
	806.20	4.20	1.32	0.26	1.16	99.61 ± 1.16	−0.39
	1612.20	0.90	0.86	1.85	0.88	100.09 ± 0.88	+0.09
Ginkgolide C	400.80	0.44	2.77	1.63	1.46	97.94 ± 1.43	−2.06
	801.60	5.37	1.95	1.69	0.50	96.84 ± 0.49	−3.16
	1603.20	0.68	1.36	1.28	4.04	98.26 ± 3.97	−1.74
Ginkgolide J (Day 2)	288.40	–	1.34	–	–	100.13 ± 0.31 ($n = 3$)	+0.13
	576.80	–	1.56	–	–	99.23 ± 0.58 ($n = 3$)	−0.77
	1153.60	–	1.57	–	–	98.48 ± 1.03 ($n = 3$)	−1.52

Table 3
Content in dosage forms

Product ($n=3$)	Bilobalide (μg/tablet/capsule)	Ginkgolide A (μg/tablet/capsule)	Ginkgolide B (μg/tablet/capsule)	Ginkgolide C (μg/tablet/capsule)	Ginkgolide J (μg/tablet/capsule)
Product A	723.75 ± 15.19	1432.0 ± 32.10	809.50 ± 24.36	657.0 ± 25.93	298.75 ± 15.91
Product B	314.70 ± 14.75	304.30 ± 2.52	176.0 ± 6.98	183.0 ± 2.65	110.34 ± 8.09
Product C	1358.25 ± 27.03	1590.75 ± 25.03	1062.0 ± 12.14	788.75 ± 14.51	292.0 ± 15.60
Product D	1258.50 ± 30.55	3445.0 ± 82.61	1910.0 ± 73.99	1547.5 ± 68.01	641.5 ± 35.47
Product E	584.00 ± 6.98	353.30 ± 3.30	219.30 ± 5.31	252.0 ± 6.38	93.70 ± 2.05

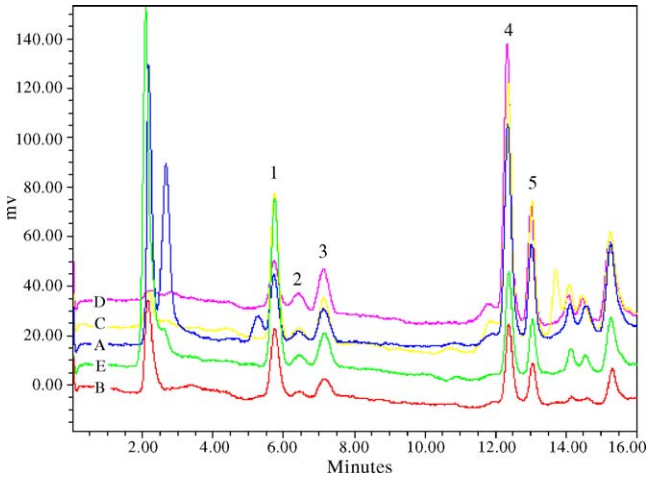


Fig. 3. Superimposed HPLC-ELSD chromatograms of Products A–E. Peak 1: Bilobalide, peak 2: ginkgolide J, peak 3: ginkgolide C, peak 4: ginkgolide A, peak 5: ginkgolide B. A: Product A, B: Product B, C: Product C, D: Product D, E: Product E.

although between product content differed remarkably. From these results it can be concluded that Product C is of superior quality to the other brands and since Product B contains consistently lower amounts of all reference standards, this product may be considered of lower quality.

4. Conclusion

The ginkgolides are unique chemical compounds found exclusively in *Ginkgo biloba* preparations and are therefore frequently used as marker compounds for QC purposes. For the analysis of these markers, ELSD detection provides advantages above using UV detection in terms of sensitivity and selectivity and is relatively inexpensive and easily operable compared to GC and MS equipment. Where it is obvious that pertinent information relating to method validation is lacking in one or more areas of the RP-HPLC-ELSD methods published to date, a comprehensively validated method was developed for the determination of ginkgolides A, B, C and J as well as bilobalide in commercially available *Ginkgo biloba* tablets and capsules. This method demonstrated the necessary speed, accuracy, precision, sensitivity and reproducibility to be considered for routine QC analysis of such products. Furthermore, the inexpensive sample clean-up procedure, the use of methanol and water as the mobile phase and the lower solvent flow rate afforded by the employment of a minibore column offers definite economic advantages for high sample throughput. The tablet/capsule assays revealed that although quantifiable amounts of all marker compounds were present, there are major disparities in the actual terpene content in the commercial products analyzed. These results emphasise the need for the implementation of effective QC criteria to ensure consistent product quality, safety and efficacy.

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