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Application of reverse-flow micellar electrokinetic chromatography for the simultaneous determination of flavonols and terpene trilactones in *Ginkgo biloba* dosage forms

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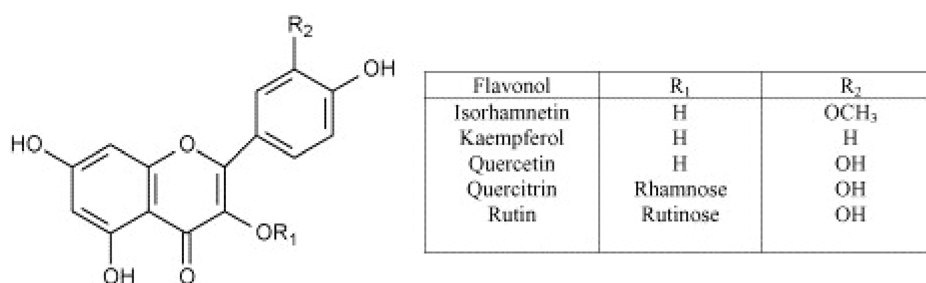
Abstract

A reverse-flow micellar electrokinetic chromatographic (RF-MEKC) method was developed for the simultaneous qualitative determination of 10 components consisting of the flavonol glycosides, rutin and quercitrin, the flavonol aglycones, isorhamnetin, kaempferol and quercetin, the terpene trilactones, ginkgolides A, B, C and J and the sesquiterpene, bilobalide. This method was used to fingerprint *Ginkgo biloba* solid oral dosage forms and validated for the quantitation of the marker compounds, rutin and quercetin in some commercial products. In addition to the usual variables, the influence of some essential background electrolyte (BGE) components such as sodium dodecyl sulphate (SDS) and <beta>-cyclodextrin concentrations were investigated. A polyimide fused-silica square capillary column (75 μm I.D. × 360 μm O.D.) with a total length of 60.0 cm and effective length of 45.0 cm was used for the separation. The final BGE consisted of 20 mM phosphoric acid, 40 mM SDS and 12 mM <beta>-cyclodextrin (pH 2.2) using reverse polarity with a voltage of −17.5 kV. Samples were injected electrokinetically at −5 kV for 3 s for the qualitative analysis and hydrodynamically at 20 mbar for 0.6 s for the quantitative assay. The total run time was 22 min and the limits of detection were 3.13 μg/ml and 1.88 μg/ml for rutin and quercetin, respectively. Fingerprint profiles of the solid oral dosage forms and the results of the quantitative analysis indicated that there were major discrepancies in the marker content between products and illustrates the value of this method for use as a procedure to assess product quality of commercially available *Ginkgo biloba* products.

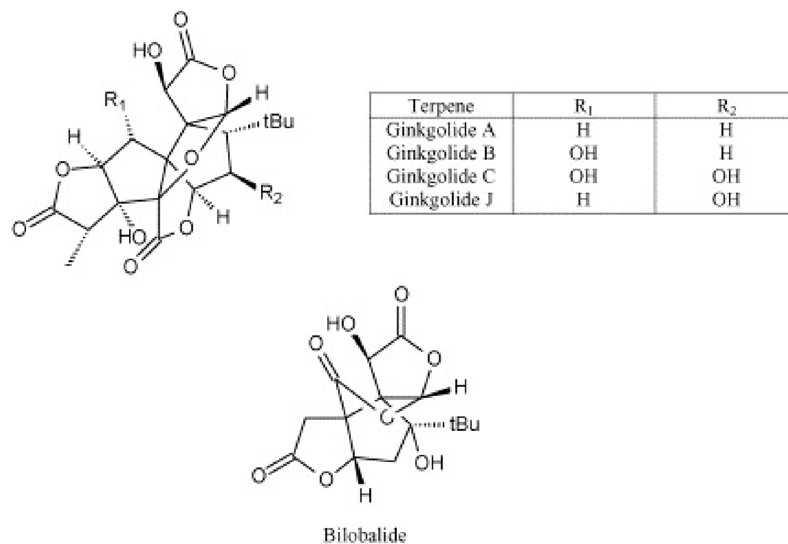
1. Introduction

Ginkgo biloba is currently one of the top selling complementary medicines in the USA [1], [2] and [3] and is the most frequently prescribed natural medicine in Germany [1]. Ginkgo leaf extracts are used to treat symptoms related to cerebral and peripheral insufficiency [4], including Alzheimer's disease [5] and its pharmacological activity is linked to the synergistic action of two distinctly separate classes of chemical compounds, the flavonoids (Fig. 1) and terpene trilactones (Fig. 2) [6]. The flavonoids are widely known for their antioxidant and free radical scavenging activity [7] and [8]. Ginkgolides A, B, C, J and M are potent but selective platelet activating factor (PAF) inhibitors [9] while bilobalide, a related sesquiterpene, exhibits neuroprotective properties [10], [11] and [12].



Flavonol	R_1	R_2
Isorhamnetin	H	OCH_3
Kaempferol	H	H
Quercetin	H	OH
Quercitrin	Rhamnose	OH
Rutin	Rutinose	OH

Fig. 1. The structures of selected flavonol marker compounds.



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

Fig. 2. The structures of ginkgolide A, B, C, J and bilobalide.

The term “flavonoids” refers to a large group of structurally related compounds and includes flavone and flavonol glycosides, acylated flavonol glycosides, biflavonoids, flavane-3-ols and proanthocyanidins [13]. HPLC with UV detection is still the most common method of analysis of these compounds [14]. Various HPLC methods coupled to electrochemical [15] and evaporative light scattering detectors [16] have achieved a measure of success. In addition, mass spectrometry has attracted much attention, particularly for qualitative purposes [17] and [18] and, more recently quantitative methods have also been reported [19] and [20]. The flavonols are well suited for analysis by capillary electrophoresis (CE) since they are negatively ionised at high pH [21] and [22]. Morin et al. published two methods in 1993 on the separation of flavonol glycosides using borate buffer [23] and an anionic surfactant in micellar electrokinetic chromatography (MEKC) [24]. Morin and Dreux [22] also published a comprehensive paper on the separation mechanisms of commonly occurring natural compounds using CE, including some flavonoids and flavonol glycosides. That same year, Bjergegaard et al. [25] reported a method to separate kaempferol and quercetin glycosides using a cationic surfactant [25]. In 1994, Pietta et al. concentrated on optimising the separation of flavonols, including the effects of additives on resolution [26]. There have subsequently been a number of CE methods developed for the analysis of flavonol markers in various natural products which have one or more markers that are also present in *Ginkgo biloba* extracts [7], [21], [22], [23], [24], [25], [26], [27], [28], [29], [30], [31], [32], [33], [34] and [35]. These analyses were all performed under alkaline conditions and two MEKC papers describing only the determination of flavonols in standardised *Ginkgo biloba* leaf extracts have appeared in the scientific literature [36] and [37].

In contrast to the flavonols, the ginkgolides are unique chemical compounds with cage-like structures which are only present in *Ginkgo biloba* leaves. They have poor chromophores with maximum UV absorption at 220 nm and therefore little success has been achieved using HPLC with UV detection, particularly when analysing leaf extracts [9] and [38]. HPLC with refractive index detection (RI) [39] and [40] and, in particular, evaporative light scattering detection (ELSD) [2], [3], [16], [38], [41] and [42] have been shown to be more suitable detection techniques for these particular Ginkgo constituents [9]. Gas chromatography [43] and [44] and mass spectrometry have also recently been used [41], [45] and [46]. To our knowledge, only one method has previously been published for the analysis of ginkgolides A and B and bilobalide using CE [47].

Furthermore, relatively few methods have been published on the simultaneous determination of the flavonol aglycones together with terpene trilactones [16], [38] and [43] and the published literature is conspicuously absent on CE methods for such simultaneous determinations. Since CE is known to be extremely useful for fingerprinting complex matrices such as botanicals due to its versatility, high resolution and low cost [48], it was considered ideally suited for the routine QC analysis of complex multicomponent mixtures of compounds such as those contained in *Ginkgo biloba* and solid oral dosage forms thereof.

MEKC was selected for the CE analysis since it is capable of separating both neutral and charged analytes and therefore may accommodate different classes of chemical constituents present in a single sample. RF-MEKC differs from normal MEKC (N-MEKC) in that it is typically performed under acidic conditions where the effect of the electroosmotic flow (EOF) is considered negligible. A negative separation voltage is then applied at the injector end of the capillary to facilitate the migration of the anodic SDS micelles towards the detector [49]. To our knowledge, no such method for the determination of the flavonoids or ginkgolides is available in the literature.

A RF-MEKC method was then successfully developed for the simultaneous determination of 10 components consisting of two flavonol glycosides, rutin and quercitrin, the flavonol aglycones, quercetin, kaempferol and isorhamnetin, the terpene trilactones, ginkgolides A, B, C and J and also bilobalide. In addition, two further compounds, quinine and salicylic acid were included, the former as a micellar marker for the qualitative analysis and the latter as internal standard for use in the quantitative analysis, respectively. The glycosides, rutin and quercitrin were included in the assay to ascertain botanical authenticity. This method was then successfully applied to fingerprint some solid oral dosage forms of *Ginkgo biloba*. Moreover, since abnormal levels of aglycones compared to glycosides signifies degradation [13] and possible adulteration, the method was validated to quantify the amount of the glycoside, rutin and its aglycone, quercetin present in two commercial *Ginkgo biloba* products.

2. Experimental

2.1. Chemicals and reagents

Methanol (HPLC grade) and sodium hydroxide pellets were purchased from BDH chemicals (Poole, UK) and phosphoric acid (analytical grade) from Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS), <beta>-cyclodextrin, quinine hydrochloride, salicylic acid, rutin (85%), quercetin (85%), ginkgolide A (90%), ginkgolide B (90%) and bilobalide (95%) were purchased from Sigma (St. Louis, MO, USA). Quercitrin (99%) was supplied by Phytolab (Hamburg, Germany) and kaempferol (95%) and isorhamnetin (90%) from Indofine (New Jersey, USA). Ginkgolide J (95%) was purchased from Chromadex (Santa Ana, CA, USA). Ginkgolide C (95%) was a generous gift from Dr. Egon Koch of Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The purity of the flavonol reference standards were assumed on the basis of certificates of analysis provided by the suppliers. The peak purity of the terpene trilactones used as reference standards were checked chromatographically and confirmed by NMR spectroscopy. Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) and low protein binding Durapore filters were purchased from the same source. Six *Ginkgo biloba* products (Products A–F) were bought from a local pharmacy in Grahamstown, South Africa. Five of the products were solid oral dosage forms and one product contained pulverised leaf extract in a hard gelatine capsule.

2.2. Instrumentation and methods

All experiments were performed using a PrinCE electrophoresis system Model 0500-002 (Lauerlabs, Emmen, The Netherlands) and Linear UVIS 200 detector (Linear Instruments Corporation, Reno, NV, USA). A polyimide fused-silica square capillary column (75 μm I.D. × 360 μm O.D.) with a total length of 60.0 cm and effective length of 45.0 cm was used for the separation (Polymicro Technologies, Phoenix, AZ, USA). The capillary was conditioned at the start of each day with 15 min 1 M sodium hydroxide, 15 min 0.1 M sodium hydroxide, 15 min water and then 40 min 0.1 M sodium hydroxide. The capillary was flushed between consecutive injections with 10 min 1 M sodium hydroxide, 5 min water and 5 min BGE. The BGE (pH 2.2) was prepared by adding 25 mM phosphoric acid to water which was then filtered through a 45 μm membrane (Type HVLP, Millipore Corporation, Bedford, USA) before adding the SDS and <beta>-cyclodextrin. The polarity was reversed (−17.5 kV) and samples were injected electrokinetically at −5 kV for 3 s for qualitative analysis and hydrodynamically at 20 mbar for 0.6 s for the quantitative assays. The measured current was approximately −80 μA throughout the analysis and the flavonols were monitored at a wavelength of 250 nm and the ginkgolides at 190 nm.

2.3. Preparation of standard solutions

For qualitative analysis a standard mixture containing all selected marker compounds was prepared from two separate solutions of the flavonols (125 μg/ml of each of rutin, quercetin, quercitrin and kaempferol and 62.5 μg/ml of isorhamnetin) and ginkgolides (1 mg/ml solution of all the ginkgolides, including bilobalide) using equal portions (50:50) of a methanol–electrolyte solution (10 mM phosphoric acid, 40 mM SDS, pH 2.2). For the validation of the quantitative assay, 200 μg/ml rutin and 100 μg/ml quercetin stock solutions were prepared with the methanol–electrolyte solution (50:50) and then diluted to provide the calibrator ranges of 12–84 μg/ml and 6–42 μg/ml, respectively. A quinine hydrochloride stock solution (1.25 mg/ml) was prepared in the methanol–electrolyte solution (50:50) which was used as the micellar marker for both qualitative and quantitative analyses and a 1 mg/ml salicylic acid stock solution was also prepared in the same solvent and was used as the I.S. in the quantitative assay. Volumes were corrected for the addition of the I.S. and the micellar marker.

2.4. Sample solutions

2.4.1. Solid oral dosage forms

A minimum of 20 tablets of each solid oral dosage form (Products A–E) were weighed and then powdered using a mortar and pestle. A mass of powder equivalent to one tablet was then weighed and transferred into a Kimax tube before dispersion with 20 ml of methanol. The mixture was sonicated for 30 min and then manually agitated to ensure re-dispersion before sonication was continued for a further 30 min. The extract was centrifuged at 350 × *g* for 10 min and the supernatant decanted into a 50 ml Kimax tube. Twenty millilitres of fresh methanol was then added to the remaining residue, the contents re-dispersed and sonicated for 30 min followed by centrifugation and decantation of the supernatant into the same 50 ml Kimax tube which was then evaporated to reduce the volume. The above procedure was then repeated and the combined extracts were evaporated to dryness using nitrogen before reconstitution with appropriate volumes of a methanol–electrolyte solution (50:50). All samples were filtered through 0.45 μm Durapore filter membranes before injecting. Fingerprint profiles were assessed for Products A–D whereas the rutin and quercetin content in Product E were quantitatively determined.

2.4.2. Hard gelatine capsule

Quantitative analysis of Product F was undertaken to determine the content of rutin and quercetin. Forty capsules of this product were individually emptied and weighed and transferred to a mortar and pestle for mixing. A mass of powder equivalent to the contents of a single capsule was extracted according to the procedure described in Section 2.4.1.

2.5. Method validation for the quantitative assay

A calibration curve for each standard was constructed on each day of the validation by analysing a mixture containing both rutin and quercetin at five different concentration levels and the peak height of analyte/I.S. ratios were plotted against the concentration for each reference standard in order to obtain linear calibration responses. The precision and accuracy of the assay were assessed by spiking aliquots of powdered material equivalent to the content of one capsule of Product E chosen as the matrix for spiking, with low and high concentrations of rutin and quercetin. This process was performed in triplicate on each day of the validation. Product E contained a significant amount of rutin and therefore recovery of rutin at the lowest level was not assessed. The limits of detection (LODs) and limits of quantification (LOQs) were determined by serial dilution of the lowest calibrator concentration and established at a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively.

3. Results and discussion

RF-MEKC, as opposed to the more commonly used CE and CZE methods, is a relatively uncommon method of analysis, with few published papers on the conditions of separation in the absence of the influence of the EOF. This is particularly the case with respect to its application for the quantitative determination of the quality of medicines (both orthodox and complementary medicines such as *Ginkgo biloba*).

3.1. Method development

3.1.1. The effect of SDS on effective mobility

Although the addition of SDS is unnecessary for the separation of flavonols, the terpene trilactones are fairly neutral and the addition of negatively charged micelles was essential for the detection and separation of both classes of compounds simultaneously.

The effect of SDS on the effective mobility of the ginkgolides and flavonols was assessed by changing the concentration of SDS in the BGE while keeping the molarity of phosphoric acid (25 mM) and <beta>-cyclodextrin (10 mM) constant. The mobilities of both the ginkgolides and flavonols increased with increasing concentrations of SDS from 20 to 80 mM. In RF-MEKC an inverse relationship exists between mobility and micellar concentration [50] which explains the decrease in run time (or increased mobility) with higher concentrations of SDS. At 80 mM SDS, baseline resolution between the flavonol aglycones was not achieved and ginkgolides A and B co-eluted at 20 mM SDS, hence 40 mM SDS was selected as the optimal micellar concentration.

3.1.2. The effect of <beta>-cyclodextrin on effective mobility

Initially the BGE consisted of phosphoric acid and SDS only and the flavonol aglycones, isorhamnetin, kaempferol and quercetin co-migrated. Increasing concentrations of <beta>-cyclodextrin in the BGE (6 mM, 12 mM and 24 mM) improved the resolution between the flavonol aglycones present in the sample solution by changing the apparent distribution co-efficient of the more hydrophobic flavonol aglycones which prevented them from being completely incorporated into the core of the anionic SDS micelles [51]. Although the effective mobilities of all compounds decreased with increasing <beta>-cyclodextrin concentrations the resolution between ginkgolides A and B remained unaltered. Resolution between bilobalide and ginkgolide C decreased slightly. However, ginkgolide J was not detected at a concentration of 24 mM <beta>-cyclodextrin due to increased background noise which can be attributed to the production of joule heat as well as the lengthened analysis time. <Beta>-cyclodextrin concentration had minimal effect on the resolution of the ginkgolides as these analytes are probably too large to be incorporated into the cavity of <beta>-cyclodextrin molecular structure. A <beta>-cyclodextrin concentration of 12 mM was thus established as being optimal for the simultaneous separation of all the relevant compounds.

3.1.3. The effect of pH and sample loading on ginkgolides A and B

Sample loading had minimal effect on the resolution of the flavonols and ginkgolides C and J as well as bilobalide in the reference standard mixture however, it affected the resolution between ginkgolides A and B significantly and sample loading time was therefore optimized using these two compounds only. When the sample was injected at −5 kV for 6 s or longer, ginkgolide A and B co-migrated. This was problematic since the ginkgolides lack strong chromophores and hence require extracts to be concentrated and/or higher sample loads to be injected to achieve an appropriate response. However, smaller sample loading was investigated and it was found that good resolution was obtained at −5 kV for 3 s with acceptable sensitivity. A square capillary with a larger internal diameter (75 μm) was used which increased the path length in the detector cell and thus enhanced the response. The pH of the BGE after preparation was found to be 2.2 and was adjusted to values between 1.8 and 4.5 but minimal effect on the resolution between ginkgolides A and B was observed. The ginkgolides are fairly neutral molecules and are therefore minimally affected by changes in the pH of the BGE and a final pH of 2.2 was thus selected.

3.2. Qualitative analysis of *Ginkgo biloba* commercial products

Fig. 3 shows the separation of all the relevant Ginkgo components as well as quinine, the micellar marker compound, within 22 min. Whilst analysing the solid oral dosage forms, the voltage was changed from −17.5 kV to −20 kV after 11 min to decrease the analysis time to less than 20 min. Fig. 4 shows the fingerprints of four commercial *Ginkgo biloba* products (A, B, C and D). The migration orders of the selected flavonols and ginkgolides were reversed in contrast to the elution orders observed in both reversed phase HPLC (RP-HPLC) and N-MEKC. In RF-MEKC, the acidic conditions suppress the EOF rendering its effect negligible. The application of a negative separation voltage at the injection end of the capillary causes the anionic SDS micelles to be attracted to the anode, now at the detector end. Highly hydrophobic analytes such as quinine which partition exclusively into the micelle phase will therefore reach the detection window ahead of the other compounds [49], [50] and [51]. Quinine therefore migrated the fastest, followed by the selected marker compounds in decreasing order of hydrophobicity [49] and [50]. The flavonols thus migrated ahead of the ginkgolides with the aglycones isorhamnetin, kaempferol and quercetin reaching the detector before the more hydrophilic glycosides, quercitrin and rutin. Ginkgolides B and A migrated soon after rutin followed by bilobalide, ginkgolide C and then ginkgolide J, respectively.

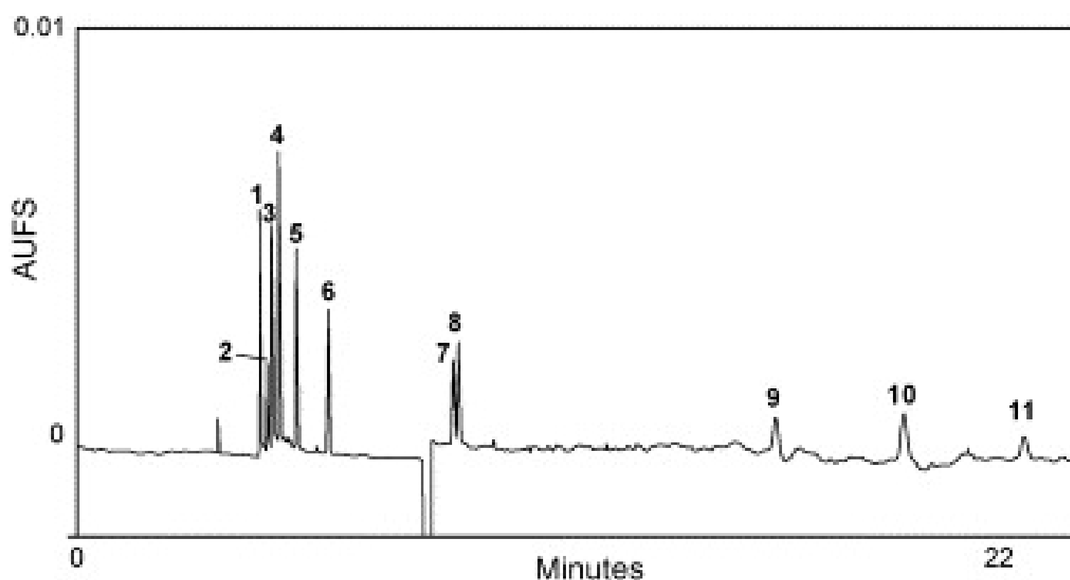


Fig. 3. Separation of the reference standards of the selected marker compounds. Sample electrolyte: 50:50 methanol–electrolyte (10 mM phosphoric acid, 40 mM SDS, pH 2.2); BGE: 40 mM SDS and 25 mM phosphoric acid (pH 2.2); 12 mM <beta>-cyclodextrin; electrokinetic injection: −5 kV for 3 s; voltage: −17.5 kV (0–8.2 min), −20 kV (8.2–24 min); detection λ: 250 nm for 8.2 min and then 190 nm; labeled peaks: 1 = quinine, 2 = isorhamnetin, 3 = kaempferol, 4 = quercetin, 5 = quercitrin, 6 = rutin, 7 = ginkgolide B, 8 = ginkgolide A, 9 = bilobalide, 10 = ginkgolide C, 11 = ginkgolide J.

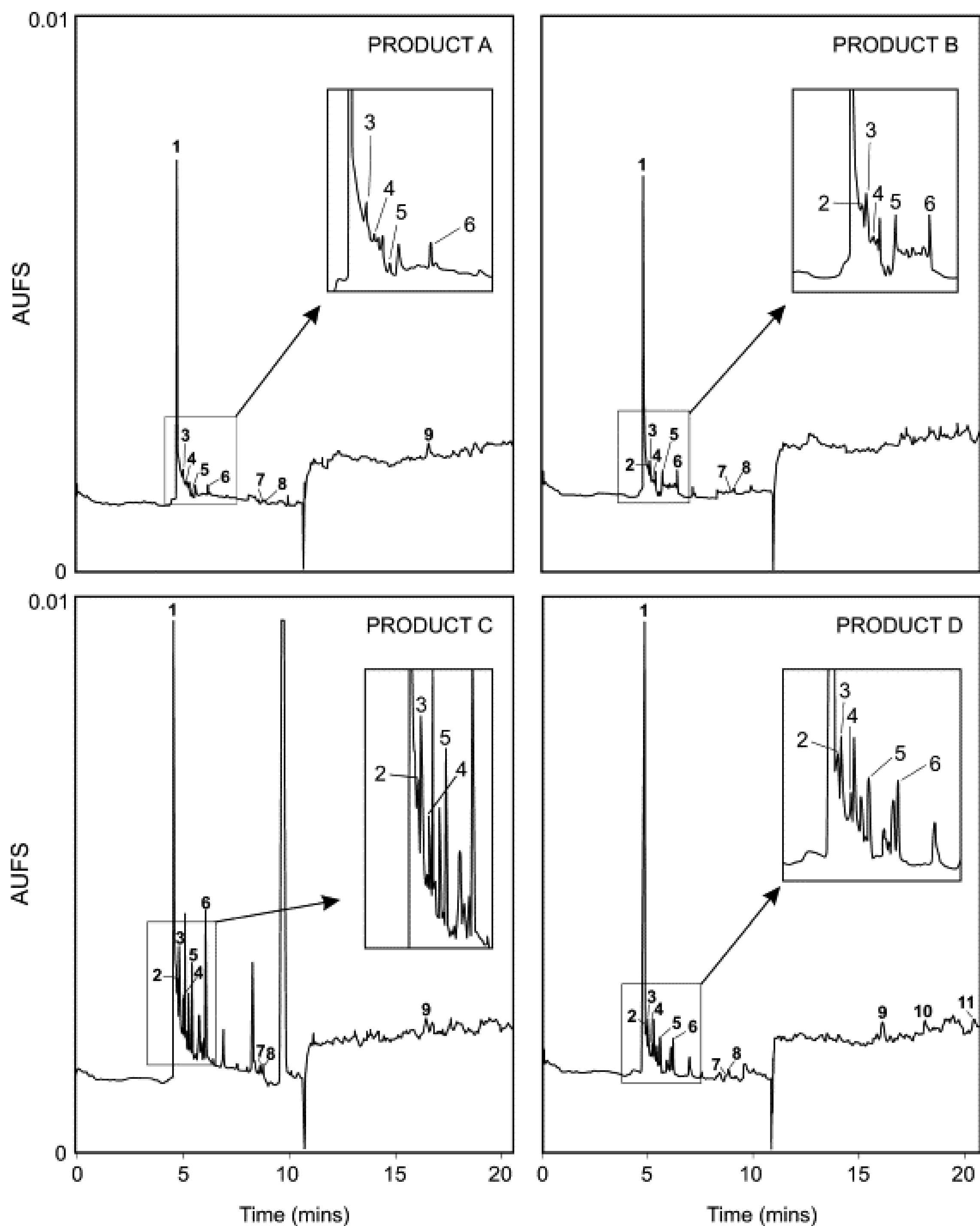


Fig. 4. Electropherograms of Products A, B, C and D. Sample electrolyte: 50:50 methanol–electrolyte (10 mM phosphoric acid, 40 mM SDS, pH 2.2); BGE: 40 mM SDS and 25 mM phosphoric acid (pH 2.2); 12 mM <beta>-cyclodextrin; electrokinetic injection: −5 kV for 3 s; voltage: −17.5 kV (0–11 min), −20 kV (11–19 min); detection λ: 250 nm for 8.2 min and then 190 nm; labeled peaks: 1 = quinine, 2 = isorhamnetin, 3 = kaempferol, 4 = quercetin, 5 = quercitrin, 6 = rutin, 7 = ginkgolide B, 8 = ginkgolide A, 9 = bilobalide, 10 = ginkgolide C, 11 = ginkgolide J.

Although both the ginkgolides and flavonols are not considered to be highly hydrophobic, this mode of MEKC is particularly suitable for the analysis of *Ginkgo biloba* extracts since if N-MEKC was used as described by Oehrle [47], the terpene trilactones would elute first. This is problematic particularly when using UV detection as the ginkgolides have poor chromophores and require to be monitored at low wavelengths and therefore interference with excipients and other water-soluble compounds present in extracts would be inevitable. RF-MEKC facilitated slower migration of the hydrophilic components which facilitated maximal separation. Moreover, as previously stated, bilobalide is unstable above pH 7 [9]. The flavonols, on the other hand, have UV maxima at higher and more selective wavelengths in the UV range, minimising the effects of interfering compounds.

The fingerprints of the commercial Products A, B, C and D, (Fig. 4) indicated that large discrepancies occurred in both the flavonol and ginkgolide marker content. Products A and B exhibited similar profiles with less peaks present overall (identified and unidentified) in both the flavonol and ginkgolide migration windows compared to Products C and D. This is of particular importance since up to 33 flavonoids have been identified in leaf extracts [13]. Product D revealed the presence of all selected marker compounds while Product C seemed to have the highest content of flavonols but had less terpene trilactones than Product D. The fingerprinting of natural products is becoming a widely accepted tool to assess the quality of complex mixtures of compounds present in herbal preparations [52]. Since the pharmacological activity of natural products used as medicines is usually attributed to the synergistic action of multiple and sometimes “unknown” components [53], fingerprinting facilitates the comparison of products based on chemical profiling while the content of proposed active constituents can simultaneously be assessed.

3.3. Method validation

Calibration curves for rutin and quercetin were constructed on each day of the validation. The response profile was linear for both compounds within the ranges of 12–84 μg/ml for rutin and 6–42 μg/ml for quercetin while the coefficients of determination (R^2) varied between 0.9915 and 0.9989. Five concentrations were used to construct the calibration curve on the first day and three on the subsequent days. The LODs and LOQs were determined by means of serial dilution of the reference standards. The LOD for rutin was found to be 3.13 μg/ml and for quercetin, 1.88 μg/ml and the LOQ for rutin and quercetin were 6.25 μg/ml and 3.75 μg/ml, respectively. The accuracy and precision of the method was determined using quality control (QC) samples as well as performing a recovery study. Two QC samples corresponding to low and high calibration concentrations of each reference standard were injected in triplicate midway through the analysis on each day. The accuracy and precision of the method can be gleaned from Table 1 where percentage relative error (%RE) values were less than 5% except for day 2 where the values for rutin QC 2 and quercetin QC 1 were +6.6 and −6.3, respectively. Intra-day percentage relative standard deviation (%RSD) values ranged form 0.9 to 8.5 for triplicate injections while inter-day results showed similar consistency. In addition, one product was selected (Product F) and spiked with low and high concentrations of reference standards to assess the recovery of the method. On each of 3 days, Product F was assayed in triplicate to determine the contributions of rutin and quercetin to the overall recoveries. It was found that Product F contained a quantifiable amount of rutin near the lower recovery level and was therefore spiked with amounts which corresponded to medium and high concentrations of the calibration range. Table 2 depicts the results of this experiment and the precision is indicated by the intra-day and inter-day %RSDs. Rutin had a higher overall recovery than quercetin which was expected since rutin has a sugar moiety present in position R_1 (Fig. 1) which enhances its polarity and therefore its solubility. Lower recoveries for quercetin at higher spiking levels have also previously been documented [19].

Table 1
Accuracy and precision (QC samples)

QC samples	Day 1				Day 2				Day 3				Inter-day accuracy ($n=9$)			
	Rutin ($n=3$)		Quercetin ($n=3$)		Rutin ($n=3$)		Quercetin ($n=3$)		Rutin ($n=3$)		Quercetin ($n=3$)		Rutin		Quercetin	
	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2
Theoretical concentration (μg/ml)	33.6	58.7	17.0	29.7	34.0	59.4	16.9	29.5	33.4	58.4	16.8	29.5	33.6	58.9	16.9	29.6
Calculated concentration (μg/ml)	32.4	55.9	16.9	28.5	34.4	63.4	15.8	30.6	34.2	58.2	16.8	29.4	33.7	59.1	16.5	29.5
RSD (%)	8.5	3.1	4.7	2.4	1.6	4.4	2.6	0.9	8.4	3.8	2.7	2.3	3.3	5.8	3.5	3.8
RE (%)	−3.6	−4.9	−0.4	−3.9	+1.1	+6.6	−6.3	+3.7	+2.6	−0.3	−0.2	−0.4	+0.1	+0.5	−2.3	−0.2

Table 2
Accuracy and precision (recovery assay)

Constituent	Mean spiking level (μg/350 mg capsule)	Intra-day RSD (%, $n=3$)			Inter-day RSD (%, $n=9$)	Mean recovery (%) ± SD ($n=9$)
		Day 1	Day 2	Day 3		
Rutin	508	3.4	6.9	10.1	8.2	97 ± 8.0
	1016	6.5	5.2	3.6	5.7	92 ± 5.2
Quercetin	334	3.8	3.0	4.7	5.9	98 ± 5.8
	669	9.5	5.8	7.5	2.3	83 ± 1.9

3.4. The specificity of the method

The specificity of the method was determined by comparing the relative migration times of rutin and quercetin to the incorporated micellar marker, quinine. In addition, on day 3 of the validation, Products E and F were spiked with reference standards to confirm identification of the relevant peaks of interest since slight changes in elution times could possibly result in erroneous peak identification.

3.5. Analysis of commercial products

Standardised *Ginkgo biloba* extracts are required to contain 22–27% flavonol glycosides [54]. Due to the lack of commercially available reference standards for all the flavonol components, conventional QC analysis of the flavonols in *Ginkgo biloba* plant extracts or dosage forms involves hydrolysis of the flavonol glycosides to their corresponding flavonol aglycones, quercetin, kaempferol and isorhamnetin. The flavonol glycoside content is then back-calculated using molecular weight conversion factors. Although this method is widely accepted, the flavonol glycoside content is often exaggerated as many flavonols have lower molecular weights than the adopted standard value and the contribution of the inherent aglycones is not included in the assay [8], [9] and [55]. In addition, the flavonol glycosides may serve as excellent QC indicators since a decrease in the ratio of flavonol glycosides to aglycones signifies degradation, possibly due to rigorous extraction procedures and/or incorrect storage [13], [19] and [56]. A recent article has suggested that manufacturers may adulterate Ginkgo extracts with rutin to claim a higher total flavonol content which is difficult to detect when using the acid hydrolysis method [57]. The analysis of intact flavonol glycosides is also a good indication of the source of raw material used in extracts [9] and [57]. Moreover, another recent publication which investigated the pharmacological effect of rutin on the antidepressant activity of St John's Wort found that rutin was required above threshold concentrations to decrease the immobility time of rats in the forced swimming test (FST). The authors hypothesised that rutin influenced the bioavailability of other chemical compounds present in the extract and advised that *Hypericum* extracts should routinely be analysed for sufficient rutin content [58]. These reports allude to the potential importance of rutin in the QC of natural products. In addition, the controversy as to whether the flavonol glycosides are absorbed in their glycosidic form or exclusively hydrolysed in the small intestine prior to absorption is still predominantly unresolved [59], [60] and [61].

As a result, two flavonol markers were selected for quantification, the flavonol glycoside rutin and it corresponding aglycone, quercetin. Two commercial products were chosen for the quantitative analysis of quercetin and rutin, Products E and F. The electropherograms of these products are shown in Fig. 5. The total run time was 10 min and the intra-day and inter-day RSDs were all less than 9%. Product E contained 2395.25 μg of rutin and 271.50 μg of quercetin per tablet while Product F contained sufficient rutin for quantification (273.99 μg) but no quercetin was detected. There was an 11-fold difference in the amount of rutin present between the products. On the other hand, the high ratio of rutin to quercetin within products reflects appropriate storage conditions. From these results it can be concluded however that there are major disparities in the flavonol marker content of these two products and that Product E may be considered to be of superior quality when compared to Product F. Although only some flavonols were determined by Pietta and Mauri [36], their method was applied to a single *Ginkgo biloba* extract dissolved in methanol and emphasis was more on the advantages of CE above that of HPLC analysis. No attempt was made to determine the three main aglycones, quercetin, kaempferol and isorhamnetin. Also, with regards to the CE analysis of terpene trilactones in *Ginkgo biloba*, an un-informative MEKC-UV method was published by Oehrle [47] for the determination of only ginkgolides A and B and bilobalide whereas ginkgolides C and J were not measured or even identified/separated. Furthermore, since bilobalide is unstable and degrades above pH 7, Oehrle's method is thus not suitable for the comprehensive analysis of all the relevant ginkgolides and bilobalide found in *Ginkgo biloba.*

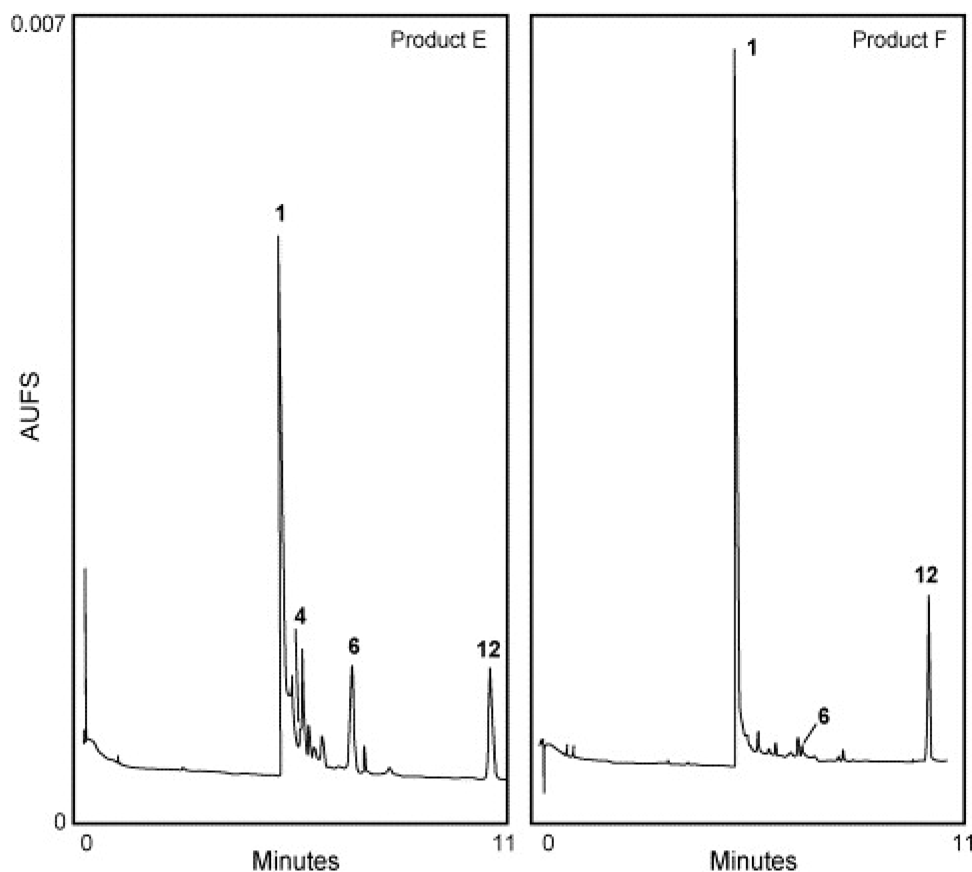


Fig. 5. The chemical profiles of Products E and F. Sample electrolyte: 50:50 methanol–electrolyte (10 mM phosphoric acid, 40 mM SDS, pH 2.2); BGE: 40 mM SDS, 25 mM phosphoric acid (pH 2.2); 12 mM <beta>-cyclodextrin; hydrodynamic injection: 20 mbar for 0.6 s; voltage: −17.5 kV; detection λ: 250 nm; labeled peaks: 1 = quinine, 4 = quercetin, 6 = rutin, 12 = salicylic acid.

4. Conclusions

To our knowledge, no papers have previously been published on the simultaneous determination of both ginkgolides and flavonols using CE. The novelty in this paper therefore lies in the application of a RF-MEKC procedure for the simultaneous qualitative determination of a mixture of multicomponents, two flavonol glycosides, rutin and quercitrin, three flavonol aglycones, isorhamnetin, kaempferol and quercetin, four terpene trilactones, ginkgolides A, B, C and J and one sesquiterpene, bilobalide in *Ginkgo biloba* solid oral dosage forms.

Furthermore, this RF-MEKC method was found to be particularly useful for the analysis of *Ginkgo biloba* solid oral dosage forms since this method was developed under acidic conditions in which both the flavonols and terpene trilactones are chemically stable. Importantly, RF-MEKC facilitated the UV-detection of the terpene trilactones (which possess poor chromophores) in extracts since slower migration of these hydrophilic components resulted in maximal separation and reduced interference which was facilitated by using an electrophoretic system which provided a reverse-flow.

Also, by using RF-MEKC we provide a novel QC approach by including the simultaneous determination of intact flavonol glycosides, rutin and quercitrin, together with the normal aglycones, quercetin, kaempferol and isorhamnetin in the assay in order to ascertain botanical authenticity and detect adulteration as well as to determine appropriate extraction and storage conditions.

Quantification of the flavonol glycoside, rutin and flavonol aglycone, quercetin in two dosage forms also indicated major disparities in the flavonol marker content. The results of this study re-iterate that effective QC criteria need to be implemented to ensure consistent product quality and this method shows great potential for the routine QC analysis of *Ginkgo biloba* solid oral dosage forms.

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