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13. ABSTRACT *(Maximum 200 words)*

A rapid capillary electrophoresis (CE) method for the quantification for four biologically active biflavanones present in three different traditional "African medicinal preparations from the seeds of Garcinia kola was developed. The four biflavanones of interest (GB1, GB2 and GB1-glycoside and kolaflavanone) were quantified in a traditional tea preparation and two commercially available ethanolic formulations. The optimum separation conditions consisted of a 100 mM borate, pH 9.5 running buffer, which gave baseline resolution of all four components in less than 12 minutes. Linear calibration ranges for each component were between 2.5 and 1000 ug/ml. Limits of detection for the biflavanones quantified in this study were between 3 and 6 ug/ml. the "fingerprint" of the biflavanones in the aqueous tea and two ethanolic formulations was found to be similar, however concentrations of the four biflavanones were up to 50 fold higher in the ethanolic preparations. The major component in all three formulations was GB1.

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Capillary electrophoresis; Garcinia kola; kolaviron; biflavanone

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raumacline (**2**) seems to have been performed already during the 1950 s but at the time a complete structure determination was not attainable [18]. The search for the appropriate enzymes for the raumacline (**2**) biosynthesis from ajmaline (**3**) pointed to a rather specific process catalyzed by membrane-bound peroxidase and NADPH-dependent reductase which reduced an opened dialdehyde intermediate of ajmaline (**3**) conversion [5], [19] (Fig. **2a**). Deducing the biosynthetical pathway of the new compound **1** from vomilenine (**4**), we can suppose the formation of a structurally similar dialdehyde intermediate after deacetylation of **4** causing destabilization of the C-17 hydroxylated indolenine skeleton followed by cleavage of the C7-C17 bond. Further reduction of a ring-opened (*chano*) form of the formed sarpagan alkaloid would result in formation of the putative deoxy-derivative of **1** (Fig. **2b**). The hydroxylation of the indole moiety may be a late step of this biosynthetic pathway. However, final studies on the appropriate enzymes involved in the biosynthesis of **1** must be performed to clarify the biosynthetic process in detail.

The isolation of the new monoterpenoid indole alkaloid **1** from *R. serpentina* hairy root culture indicates its high potential in the production of new putatively pharmacologically-active substances. In addition, the here described *in vitro* plant system might be an important source for rare alkaloids which can be used as substrates for the detection of novel enzymes involved in the biosynthesis of ajmaline structurally related compounds, including the rare raumacline alkaloids. Such alkaloids might also be of interest for *in vivo* NMR monitoring of alkaloid formation by *R. serpentina* cell suspension cultures. Examples of successful applications of the alkaloids isolated from hairy roots of *R. serpentina* were displayed in our recent publications [20], [21].

Acknowledgements

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Capillary Electrophoresis Determination of Biflavanones from *Garcinia kola* in Three Traditional African Medicinal Formulations

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Original Paper

Abstract

A rapid capillary electrophoresis (CE) method for the quantification of four biologically active biflavanones present in three different traditional African medicinal preparations from the seeds of *Garcinia kola* was developed. The four biflavanones of interest (GB1, GB2 and GB1-glycoside and kolaflavanone) were quantified in a traditional tea preparation, and two commercially available ethanolic formulations. The optimum separation conditions consisted of a 100 mM borate, pH 9.5 running buffer, which gave baseline resolution of all four components in less than 12 minutes. Linear calibration ranges for each component were between 2.5 and 1000 μg/mL. Limits of detection for the biflavanones quantified in this study were between 3 and 6 μg/mL. The "fingerprint" of the biflavanones in the aqueous tea and two ethanolic formulations was found to be similar, however concentrations of the four biflavanones were up to 50 fold higher in the ethanolic preparations. The major component in all three formulations was GB1.

Key words
Capillary electrophoresis · *Garcinia kola* · kolaviron · biflavanone

Introduction

The seeds of *Garcinia kola* enjoy a folk reputation in African traditional medicine, and they have been used in many herbal preparations either singly or in combination with other plants. Among these are a tea prepared from a blend of *G. kola* and *Combretum micratum* and two ethanolic formulations consisting of blends of kolaviron and other natural products. *G. kola* contains a complex mixture of biflavonoids [1], [2], [3], prenylated benzophenones [4] and xanthone [5]. Kolaviron, is a mixture of C-3/C-8 linked biflavonoids found in *G. kola* and has been shown to have hepato-protective activity [6], [7], bronchodilator effect [8], and antidiabetic activity [9]. A number of products derived from *G. kola* have been manufactured and marketed as dietary supplements or phytomedicines. Like many dietary supplements, *Garcinia* products are susceptible to chemical variability due to growth, harvest, drying, and storage conditions. Therefore suitable analytical methods are needed for the growing demand of dietary supplements including those containing *G. kola* to ensure their quality, safety and efficacy.

Recently, capillary electrophoresis (CE) has proven to be a useful technique for the separation of a variety of natural products, including flavanoids and their glycosides from various sources [10], [11], [12], [13], [14], [15], [16], [17]. The use of buffers containing components which can interact with the analytes to form complexes such borate [11], [12], [13] and micellar forming surfactants such as SDS [13], [15] and cetyltrimethylammonium bromide (CTAB) [16] have been used to enhance selectivity and provide an electrophoretic separation mechanism when the analytes do not bear a native charge. CE methods for the analysis of the constituents in traditional Chinese herbal remedies have re-

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cently been reported [14] and are efficient methods for both fingerprinting and quantifying components of these herbal preparations. In this work we report a CE method for the quantitative analysis of biologically active biflavanones present in three traditional African medicinal preparations from the seeds of *Garcinia kola*.

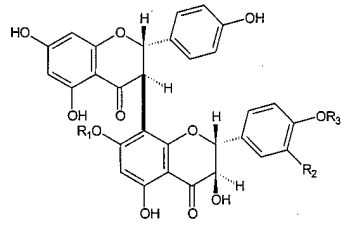
Materials and Methods

Chemicals

Ethanol and d_6-acetone were purchased from Aldrich (Milwaukee, WI, USA). Sodium hydroxide was purchased from Hewlett Packard (Waldbronn, Germany). Boric acid was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Healers' Botanika™ Hangover Tonic (tonic) and Healers' Botanika™ Streptol (streptol) was purchased from Axxon Biopharm™ (Silver Spring, MD, USA). Water was purified with a Millipore™ system (Milford, MA). Tea bags were prepared by enclosing about 2 grams of ground *Garcinia kola* seed in a sachet.

Seeds of *G. kola* were brought at a local market at Orba Nsukka, Enugu State, Nigeria in October 1999 and the sample was authenticated by Dr. J.C. Okafor of the Forestry Department Enugu. Nigeria. Voucher specimens ICEDD991002, have been deposited in the Department of Pharmacognosy, the University of Nigeria, and Bioresources Development and Conservation Programme (BDCP) Herbaria. The reference standards used for the quantitative determination were purified in our laboratory from kolaviron extract of *G. kola* seeds according to the technique reported by Iwu and Igboko [3].

Working up the organic extract from 10.0 g of *G. kola* yielded four major compounds in sufficient amounts and purity (>98 %) to be used as standards. Purity of the isolated standards was determined from HPLC and CE analysis of each purified compound. The compounds were identified by comparison of their spectral data and R_f values with those of the authentic isolates as 3″,3‴,4′,5,5″,7,7″-heptahydroxy-4‴-methoxy-3,8″-biflavanone (kolaflavanone); 3″,4′,4‴,5,5″,7,7″-heptahydroxy-3,8″-biflavanone (GB1); 7″-*O*-alpha-D-glucopyranosyloxy-3″,4′,4‴,5,5″,7-hexahydroxy-3,8″-biflavanone. (GB1 glucoside) and 3″,3‴,4′,4‴,5,5″,7,7‴-octahydroxy-3,8″-biflavanone (GB2). The structures of these compounds are shown in Fig. **1** showing relative configurations based on NMR data.



	R_1	R_2	R_3
Kolaflavanone	H	H	CH_3
GB1	H	H	H
GB1-7″-O-glucoside	Glc	H	H
GB2	H	OH	H

Fig. **1** Structures of biflavanones from *Garcinia kola*.

Instrumentation

All CE experiments were performed on a Hewlett Packard 3DCE System (Hewlett Packard, Waldbronn, Germany). All separations were performed in a fused-silica capillary (Polymicro Technology, Phoenix AZ, USA) having a total length of 63.5 cm with an effective length of 55 cm and an internal diameter of 50 μm at a constant temperature of 25 °C. The applied voltage was 28.5 kV and the current was 45 μA. Samples were injected hydrodynamically with 50 millibar of positive pressure applied for 2 seconds. Mesityl oxide or a system peak was used to measure electroosmotic flow (EOF). UV spectra were obtained from 200–600 nm for each component with the CE diode array detector, quantification was performed at a wavelength of 325 nm.

All ^{1}H-NMR data were collected using a Bruker DRX-600 spectrometer. Acetone-d_6 was used as the solvent for the standard sample. The degraded sample was dissolved in CE background electrolyte with the addition of 10 % D_2O. The two-dimensional WATERGATE-TOCSY experiment employed a modified MLEV-17 (18–20) spin-lock sequence for a total mixing time of 80 ms, including the 2.5 ms trim pulses at the beginning and the end of the spin-lock sequence.

Procedures

The experimental details concerning the extraction and isolation procedures of *G. kola* biflavonoids have been described [3], [21]. Identity of the purified fractions was confirmed by TLC and mass spectrometric analysis. Standard solutions at a concentration of 1 mg/mL of GB1, GB1-glucoside, GB2, and kolaflavone (KF) were prepared by dissolving the pure components in 50/50 ethanol and water. The standard solutions were ultrasonicated for 5 minutes prior to serial dilution. Tonic and streptol samples were diluted 10 fold and 20 fold, respectively, with 50/50 ethanol and water prior to analysis in order to bring the most abundant component within the calibration range. A tea bag containing 2.15 g of ground *Garcinia kola* seed was placed into 110 mL of water at 90 °C. The tea was then steep covered for 10 minutes. The tea bag was squeezed gently and removed. The tea was centrifuged at 10,000 rpm for 5 minutes to remove particulates. Recovery of biflavonoids from the aqueous extraction was estimated to be about 4.9 % of the total present in the seeds. All solutions were stable for several days, but were prepared fresh daily.

Results and Discussion

Analytical conditions

Borate buffers at high pH have been shown to be superior for the separation of mono-flavones and their glycosides [11], [12]. The high pH is necessary to ionize the acidic phenolic groups, and the ability of borate to complex with diols on both the aglycone and the attached sugars in the glycosides can further enhance resolution through differential complexation. Optimum conditions for the separation of the four major components quantified in this study and eleven other unidentified components present in the herbal preparations were 100 mM borate buffer at pH 9.5. A representative electropherogram of the tonic run under these conditions is shown in Fig. **2**. Under these conditions all of the compounds had a negative charge and the elution order of the major components (KF, GB1, GB1-glucoside, and GB2) can be explained by the ability of these components to complex with the

borate buffer. Kolaflavanone lacks the diol needed for effective complexation and elutes first due to the lower negative charge density. The diol at the 3‴ and 4‴ position in GB 2 allows for strong borate complexation resulting in a longer elution time. Since GB1 and GB1-glucoside both lack this diol, they also have decreased ability to complex with borate and exhibit lower elution times compared to GB2. The elution of GB1-glucoside after both KF and GB1 is likely due to borate complexation with diols present in the sugar moiety. Although *cis*-1,2-diols are optimum for the formation of borate sugar complexes [12], the glucoside must have some ability to form a complex based on its elution after GB1. At pH 9.5 ionization of sugar hydroxy groups would not be a factor affecting the electrophoretic mobility.

The CE assay was very reproducible in terms of analysis times as long as a simple rinsing procedure was followed. Table **1** summarizes the reproducibility of the analysis times for each component for nine successive injections. Relative standard deviations for the migration times were about 1%. When a relative migration time (ratio of analyte migration time to neutral marker migration time) was used to correct for slight differences in electroosmotic flow the RSD fell to about 0.5 %. These numbers are comparable to other CE methods for flavanoids reported in the literature [14].

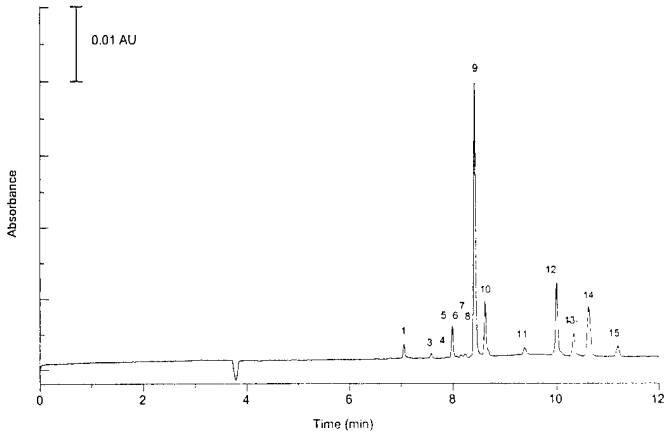


Fig. **2** Electropherogram showing the separation of biflavanone components in the tonic preparation. Run conditions: 100 mM borate buffer pH 9.5, 100 mBar sec injection, E = 447 V/cm, λ = 325 nm. See Table **4** for peak identification.

Linearity and limits of detection

Table **2** summarizes the calibration data and other assay parameters obtained for the four biflavanones. Corrected peak areas (peak area/ analysis time) were used in all quantitative analysis. Calibration curves were linear over the 2.5 – 1000 μg/mL concentration range examined. Limits of detection, defined as a signal to noise ratio of three, ranged from 1.9 to 3.1 μg/mL. Limits of quantitation, defined as a signal to noise ratio of five, ranged from 3.16 to 5.16 μg/mL.

Quantitative and qualitative determination of biflavanones in herbal preparations

Table **3** summarizes the quantitative data obtained for the four biologically active biflavanones. The streptol preparation contained the highest levels of all four biflavonoids, with GB1 and GB2 the major components. The tonic contained slightly lower levels of these components, but the relative concentrations were similar. Clearly, the organic content (40% ethanol) in the two commercial tinctures significantly enhances the levels of all the biflavonoids compared to the aqueous tea preparation.

Concentrations of the major components are at least a 100-fold greater in the ethanolic formulations. Levels of the major components followed the same relative trend in the tea, with GB1 and GB2 the major components.

In addition to the four major components quantified in this study, several other unidentified components were resolved under optimum conditions, providing a detailed "fingerprint" of each formulation. A comparison of the relative analysis times of the minor peaks found in all three preparations revealed that 15 unique components (including the four major components quan-

Table **1** Reproducibility of analysis times for biflavonoids (n = 9)

Analytes	*Avg. μ_{ep} ($cm^2/V\,sec$) × 10^4*	*Avg. migration Time (min) (S.D.)*	*% RSD*	*Avg. rel. migration time (min) (S.D.)*	*% RSD*
KF	−2.85	8.13 (0.08)	0.96	2.13 (0.01)	0.51
GB1	−2.98	8.57 (0.09)	1.00	2.25 (0.01)	0.58
GB1-g	−3.04	8.78 (0.09)	1.01	2.31 (0.01)	0.56
GB2	−3.37	10.24 (0.11)	1.10	2.69 (0.01)	0.43

Table **2** Linearity and Assay parameters for the four biflavonoids

Analytes	*Concentration range (μg/mL)*	*Slope*	*Intercept*	*R^2*	*LOQ (S/N = 5) (μg/mL)*	*LOD (S/N = 3) (μg/mL)*
KF	2.5 – 1000	0.029	0.132	0.9998	5.16	3.10
GB1	2.5 – 1000	0.042	−0.121	0.9998	3.16	1.90
GB1-g	2.5 – 1000	0.042	−0.086	0.9999	4.18	2.51
GB2	2.5 – 1000	0.039	−0.185	0.9997	4.59	2.76

tified) were resolved in the assay. These components are listed in Table **4**, along with the percent of the total corrected peak area for each component found in each of the three formulations. Although no quantitative information is available for the minor components resolved in this study due to a lack of suitable standards, a comparison of the peak profiles and relative area percents should be useful for "fingerprinting" each of the preparations and for assessing quality and batch reproducibility of the herbal products. The assay could also prove useful in component analysis of crude extracts of the plant materials to assess quality prior to formulation. Previously, others have shown CE methods for flavonoids to be useful in analyzing levels of flavones in crude extracts from plants and to determine concentrations of specific components as well as for general fingerprinting of the plant species for phytotaxonomic comparisons [22]. This assay has already proven useful in our lab for the qualitative analysis of crude extracts for following the fractionation and purification process used to isolate standards of the major components.

Isomerization of GB1-glucoside in borate buffer

In the course of developing the method some samples were diluted in running buffer prior to analysis. Interestingly, in the case of GB1-glucoside, a noticeable shoulder appears on the peak after one hour in the borate buffer, indicating degradation to a second component. After 18 hours at room temperature in borate running buffer (100 mM, pH 9.5) the ratio of the two components was nearly equal (Fig. **3**). No stability problem was observed in either ethanol : water or in a non-complexing buffer such as phosphate at a similar pH and concentration (data not shown). Because borate buffers are reported in the literature for the separations of flavonoid glycosides, we undertook a series of NMR experiments to elucidate the structure of this degradation product. Table **5** summarizes the results of NMR experiments on both GB1-glucoside standard in d_6-acetone and a degraded sample left in borate buffer for over 24 hours. The stereochemistry of the anomeric protons of the glucosides of GB1 were assigned by analogy to the $^1H/^2H$ coupling constants and chemical shifts of known glucoside systems [23], [24]. The α-stereochemistry yields a coupling constant in the range between 3 and 5 Hz while the β-stereochemistry yields a coupling constant about 8 Hz. Overall, the NMR data strongly suggest a mixture of the α and β forms of the glycoside are present after treatment with buffer. The β epimer has a slightly longer elution time and probably forms a more stable complex with the borate background electrolyte than the α epimer resulting in partial resolution of the two peaks in the CE assay. The lack of isomerization observed in non-complexing buffer suggests that an interaction with borate may catalyze the conversion from the α to β forms.

Table **3** Levels (μg/mL) of biflavanones found in three different herbal preparations (n = 3)

Biflavanone	*Tea*	*Streptol*	*Tonic*
	Concentration (μg/mL) (S.D.)/% RSD		
KF	5.06 (0.14) /2.77	861 (48)/5.57	582 (14)/2.41
GB1	58.84 (0.86)/1.46	5593 (91)/1.63	3688 (79)/2.14
GB1-glucoside	6.46 (0.22)/3.41	820 (28)/3.41	735 (7.8)/1.06
GB2	29.68 (0.36)/1.21	1935 (40)/2.07	1165 (65)/5.58

Table **4** Component analysis (% total peak area) of herbal preparations (u = unidentified component, * = not detected)

Peak/identity	*Migration time (min)*	*Tea*	*Streptol*	*Tonic*
1/u	7.06	*	*	2.64
2/u	7.40	1.87	*	*
3/u	7.58	*	*	0.94
4/u	7.89	2.58	0.42	0.53
5/KF	7.99	7.05	6.60	5.67
6/u	8.06	*	0.36	0.48
7/u	8.15	*	0.25	0.78
8/u	8.23	*	1.04	0.87
9/GB1	8.41	57.57	55.45	46.10
10/GB1-g	8.61	5.12	9.27	10.05
11/u	9.38	*	0.95	1.60
12/GB2	10.00	23.23	16.25	12.29
13/u	10.33	2.58	3.92	4.51
14/u	10.63	*	4.74	11.52
15/u	11.20	*	0.75	2.00

Conclusions

This study has successfully demonstrated that CE can serve as a direct, robust and rapid method for both the quantitative and qualitative analysis of the biflavonoid constituents in different herbal formulations derived from *G. kola*. The distribution of the biflavonoids (GB1, GB2, GB1-glucoside and KF) in the three formulations was found to be qualitatively and quantitatively different. The commercially formulated tinctures contained more components and much higher concentrations of the four biflavanones quantified in this study. The unique and powerful capabilities of CE including high resolution and short analysis times, make it a powerful analytical tool in the quality control of these herbal products and other flavonoid-containing products such as *Gingko biloba* proprietary products.

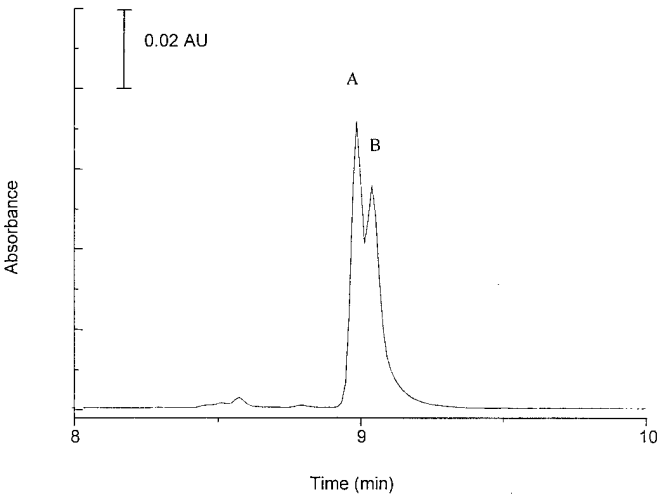


Fig. **3** Electropherogram of GB1-glucoside, α-epimer (**A**) dissolved in 100 mM, pH 9.5 borate after 18 hrs at room temperature. Electropherogram shows the formation of the β-epimer (**B**). Run conditions as in Fig. **2**.

Table **5** Summary of the chemical shift and coupling constant data for glucosyl protons showing α to β isomerization

Compound	*Chemical shift for anomeric proton*	*Stereochemistry coupling constant*	*Chemical shifts of remaining protons*
GB1-glucoside in acetone	5.60 ppm	α 5 Hz	2.94 ,2.74, 2.70 ppm
GB1-glucoside (borate buffer)[a]	5.34 ppm	α 4Hz	2.91, 2.87, 2.65 ppm
GB1-glucoside (borate buffer)[a]	5.19 ppm	β >7Hz	4.28, 3.09, 3.04, 2.75, 2.72 ppm

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