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Simultaneous Determination of Caffeine, Catechin, Epicatechin, Chlorogenic and Caffeic Acid in *Cola nitida* Dried Nuts from Côte d'Ivoire Using HPLC

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Authors' contributions

This work was carried out in collaboration between all authors. Author YN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AC, MPB, EP, AA and GHB managed the analyses of the study. Author YN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: A simple high performance liquid chromatographic analysis (HPLC) for *Cola nitida* caffeine, catechin, epicatechin, chlorogenic and caffeic acid with a gradient system elution system was developed.
Study Design: Mature kola seeds were collected in October 2014-February 2015 in South of Côte d'Ivoire. Harvested kola nuts were transferred to the laboratory until used in the experiments.

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Place and Duration of Study: This study was carried out during the year 2016 at European institute of membranes, France.
Methodology: Kola nuts was extracted from mature kola seeds (*Cola nitida* Schott & Endl.) and the extract was obtained by infusion of kola nut powder in water-ethanol mixture at room temperature. The compounds were separated by of a C18 reversed-phase column with a gradient elution system of binary phase consisted of A (95/5, water-H_2O/methanol-MeOH + 0.1% trifluoroacetic acid-TFA) and B (100% acetonitrile-ACN + 0.1% trifluoroacetic acid-TFA) and an UV detector. All of these compounds were separated within 70 min. The validity of this method was confirmed by their quantitative measurement in kola samples.
Results: The limit of detection (LOD) and the limit of quantification (LOQ) of these compounds were within the range of 0.098 to 0.47 µg/mL and 0.3 to 1.45 µg/mL, respectively. All the analyses exhibited good linearity with correlations coefficients above 0.9974 and the accuracies for the analyses were 97 – 104%.
Conclusion: Using this analytical method, the bioactive compounds of kola nuts have been determined with satisfactory. The presence of these compounds (caffeine, catechin, epicatechin, cholrogenic acid and caffeic acid) in the extract justifies the industrial interest of kola nuts.

Keywords: Cola nitida; kola nuts; caffeine; polyphenol; HPLC; validation.

1. INTRODUCTION

Cola nitida plant species are endemic to West and Central Africa [1,2]. Nuts are used as masticatory when fresh, while the dried nuts are used for beverages and pharmaceutical purposes in Europe and North America [3,4]. Kola nut have become essential to the cultural and social well-being of the Ivorian people; no social or traditional ceremony is considered complete without the breaking of the kola nut [5]. The nuts are eaten for its tonic and stimulating effect [6-8]. There are also received great interest due to their aphrodisiac, medicinal and phamacological properties such as physical and intellectual stimulant, vomitting control, hunger and thirst conteracting effects [3,8-11].

These beneficial effects have been partly attributed to phenolic compounds, the anti-oxidative components of kola nuts [3,7,4,12]. The major constituents of *Cola nitida* polyphenols are catechins and epicatéchine and their polymerized products [11]. Also phenolic acids such as chlorogenic acid and caffeic acid are found in the extracts [6]. Kola nuts contain certain amount of caffeine, a plant alkaloid occuring also in some other popular beverages such as coffee and tea [13]. Caffeine also has attracted much scientific and public attention nowadays due to its stimulatory effects [11,13].

Several studies have been carried out to determinate simultaneous tea and coffee caffeine and polyphenols by High Performance Liquid Chromatography (HPLC) [14-17]. Yayabe et al. [15] and Wang et al. [14] presented simple analytical methods for caffeine and tea catechins, using an octadecylsilyl (ODS) column and an isocratic elution system (water/methanol/phosphoric acid). Nishtani and Sagesaka [18] had developed this HPLC method for the simultaneous determination of caffeine, eight tea catechins and other tea polyphenols using a C18 reversed phase column. In most of previous studies concerning kola nuts, caffeine and polyphenols have been analyzed by spectrophotometric methods [18,12,9,19-21]. But little research has been reported on the simultaneous determination of these bioactive compounds by HPLC. Recently, Niemenak et al. [7] presented a simple analytical method for simultaneous determination of kola nuts caffeine, catechin, epicatechin and theobromine using an octadecylsilyl (ODS) column and a binary mobile phase consisted of 2% acetic acid in water (A) and acetonitrile-water-concentrated acetic acid mixture. In this study, we have developped a precise gradient HPLC analytical method with an economical mobile phase for the simultaneous determination of caffeine, (+)-catechin, (-)-epicatechin, chlorogenic and caffeic acid in *Cola nitida* sample. This is the first report on the quantitative determination of catechins, acid phenol and caffeine in kola nuts from Côte d'Ivoire. We also performed a successful validation of this analytical procedure.

2. MATERIALS AND METHODS

2.1 Standards and Other Chemicals

All reagents used in this study were of pure analytical grade, unless otherwise specified,

were purchased from Sigma Aldrich (Germany): Caffeine, (+)-Catechin, (-)-Epicatechin, Chlorogenic and Caffeic acid, methanol, ethanol, acid trifluoroacetic (TFA).

2.2 Preparation of Standard Solutions

The standard solution containing about 50 ppm of each of five components (caffeine, catechin, epicatechin, chlorogenic acid, caffeic acid) was prepared with water-ethanol mixture (v/v,1/1) and used for the method validation and analysis of *Cola nitida* samples.

2.3 Kola Nut Samples

Kola nuts were extracted from mature kola seeds (*Cola nitida* Schott & Endl.) harvested from October to February 2015 in south of Côte d'Ivoire. Nuts were washed with distilled water, cut in smaller pieces and dried at room temperature (27±2°C) during two weeks. The dried sample was milled into powder using an electric blender and stored in plastic bags before their achievement at European institute of membranes (Montpellier, France). The extract was obtained by infusion of 1 g of kola nut powder (Ø < 100 µm) in 100 mL of EtOH 50% for 24h at room temperature as described by Nyamien et al. [21]. The extract obtained was filtered through sintered glass (40 -100 µm) before use as crude extract (CE).

2.4 Instrumentation and Chromatographic Conditions

The chromatographic analysis was performed on a liquid chromatographic system equiped with a Waters Alliance 2695 HPLC module (Milford, MA, USA) connected to a Waters 996 photodiode array (PDA) detector and column oven with a variable UV-vis detector. A quaternary pump, Waters 600 E, was used for high-pressure gradient elution. Data were collected, stored and analyzed using the EMPOWER software version 5.0 from Waters (Milford, MA, USA). Injections were made with an automatic injector Waters W717. The column used was a C18 Macherey-Nagel (250 mm x 4.6 mm – 5 µm Nucleodur - 100A °). 20 µL of sample are injected and the elution is carried out at a constant flow rate (0.8 ml / min) with a mobile binary phase consisted of A (95/5, H_2O/MeOH + 0.1% TFA) and B (100% ACN + 0.1% TFA). A gradient elution was performed by varying the proportion of solvent A to solvent B. The mobile phase composition started at 100% solvent A for 19 min, followed by a linear increase of solvent B to 6% for 31 min, 20% for 0.1 min and 100% for 9.9 min, and the bring mobile phase composition back to the initial conditions in 9.9 min for the next run. The washing cycle of the column is carried out after each injection. All the samples were filtered through 0.45 µm membranes (Fisher scientific) and the mobile phase was degassed before injection into HPLC. Absorption wavelength was selected at 274 nm. Peaks were considered to be chromatographically pure when there was exact coincidence of their corresponding UV spectra. Chromatographic peaks in the samples were identified by comparing their retention time and UV spectrum with those of the reference standards. Working standard solutions (0 to 50 ppm) were injected into the HPLC, and peak area responses were obtained. A standard graph for each component was prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the sample and corresponding standard graph.

2.5 Validation of the Method

The validation of this analytical method was performed according to the ICH guidelines (ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology (Recommended for Adoption at Step 4 of the ICH Process on 6 November 1996 by the ICH Sterering Committee)).

Detection limits, quantification limits and linearity were evaluated using standard samples. The linearity of the assay was performed with an eight point calibration curve from 0.66 to 50 $\mu g.mL^{-1}$, and each point was run at least three times. These solutions were prepared by diluting stock analytes solution in water/ethanol mixture at room temperature. The limit of detection (LOD) and the limit of quantification (LOQ) based on the standard deviation of the response and the slopes were determined.

Specificity, accuracy and repeatability were evaluated with crude cola extract solution. To demonstrate specificity, we performed peak purity test using photo-diode-array analysis and confirmed that each chromatographic peak of the five compounds was attributable to a single component (data not shown). In order to verify their accuracy, we carried out recovery test, adding known amounts (0-25 µg/mL) of the standard sample to a preparation of kola extract.

To test the precision of the assay method, the intraday and interday variability at three assay concentration of each compound were evaluated for three replicates over five successive days.

To evaluate robustness, stability of analytical solutions was performed. The experiment showed stability when the standards of every compound were prepared with ethanol/water mixture and kept in a refrigerator.

2.6 Statistical Analysis

Results are presented as mean±standard deviation (Mean±SD) of more than three independent experiments. Statistical analysis was performed using Microsoft Excel 2007.

3. RESULTS AND DISCUSSION

3.1 Validation of the Method

Table 1 shows the results of the linear regression analysis of the calibration curve data. It can be concluded that all the analyte exhibited good linearity over the evaluated range with significant correlation coefficients (r > 0.9974). In all cases, the slopes of the calibration graph confirm a good precision of the experimentation with a low relative standard deviation (RSD < 1.79% for all cases). The standard calibration technique for water/ethanol mixture could be used for the determination of caffeine, catechin, epicatechin, chlorogenic and caffeic acid in this work.

The lowest concentration that can be quantified (LOQ) with acceptable accuracy and precision were 0.42, 0.78, 1.45, 0.4 and 0.3 µg/mL for caffeine, catechin, epicatechin, chlorogenic and caffeic acid, respectively. Furthermore, the limit of detection (LOD) were 0.13, 0.26, 0.47, 0.13 and 0.098 µg/mL for caffeine, catechin, epicatechin, chlorogenic and caffeic acid. These values of LOQ were sufficiently sensitive to evaluate the studied analytes in the *Cola nitida* samples.

The recovery rates of all compounds were in the range of 97 to 104% with relative low standard deviation (RSD < 2.19%, n=5).

The data is listed in Table 3. These results (RSD < 2.1% for all cases) confirm that good precision can be obtained with this operation condition.

After the standard solution was kept for two months, no significance decrease of each compound was observed.

Table 1. Results of validation of the analytical method evaluated with standard samples

Compounds	Equation	r [a]	CV[b] slope (%)	Detection limit (µg/mL)	Quantification limit (µg/mL)
Caffeine	y= 138854 x	0.9999	0.7	0.13	0.42
Catechin	y= 33193 x	0.9974	1.44	0.26	0.78
Epicatechin	y= 34096 x	0.9979	1.45	0.47	1.45
Chlorogenic acid	y= 55035 x	0.9977	1.79	0.13	0.4
Caffeic acid	y= 76440 x	0.9974	1.39	0.098	0.3

[a]: linearity was expressed as the correlation coefficient of each calibration curve, which was determined by eight calibration points with three experiments.
CV[b]: coefficient of variation of the slope (n = 3)
y= peak area; x, concentration of each compound
r[a], correlation coefficient.

Table 2. Results of validation of the analytical method evaluated with *Cola nitida* extract

Compounds	25 µg/mL		10 µg/mL		2 µg/mL	
	Recovery (%)	CV[a] %	Recovery (%)	CV[a] %	Recovery (%)	CV[a] %
Caffeine	101	0.9	99.7	0.8	98.6	1.8
Catechin	104	1.94	103.3	1.2	99.1	1.65
Epicatechin	97	2.19	100.1	1.01	98.2	1.19
Chlorogenic acid	102	1.7	99.1	1.1	97.4	1.45
Caffeic acid	101	1.68	98.7	1.03	100.2	1.01

[a]: coefficient of variation of 5 measurements

Table 3. Results of the precision study of the analyzed compounds

	Within-day (n=3)		Between-day (n=3)	
	Concentration mean± SD (µg/mL)	CV[a]%	Concentration mean± SD (µg/mL)	CV[a]%
Caffeine	2.00±0.011	1.8	2.00±0.011	1.6
	5.00±0.011	1.2	5.00±0.011	1.2
	10.00±0.003	0.8	10.00±0.003	1.2
Catechin	10.00±0.001	1.2	10.00±0.001	1.3
	20.00±0.001	1.6	20.00±0.001	1.4
	50±0.002	2.1	50±0.002	1.8
Epicatechin	10.00±0.003	1.01	10.00±0.003	1.6
	20.00±0.011	0.93	20.00±0.011	1.2
	50.00±0.015	1.3	50.00±0.015	1.4
Chlorogenic acid	10.00±0.002	1.1	10.00±0.002	0.98
	20.00±0.005	0.98	20.00±0.005	1.2
	50.00±0.001	1.5	50.00±0.001	1.4
Caffeic acid	10.00±0.001	1.03	10.00±0.001	1.1
	20.00±0.021	1.2	20.00±0.021	1.3
	50.00±0.011	1.5	50.00±0.011	1.5

[a] *: coefficient of variation (n=3)*

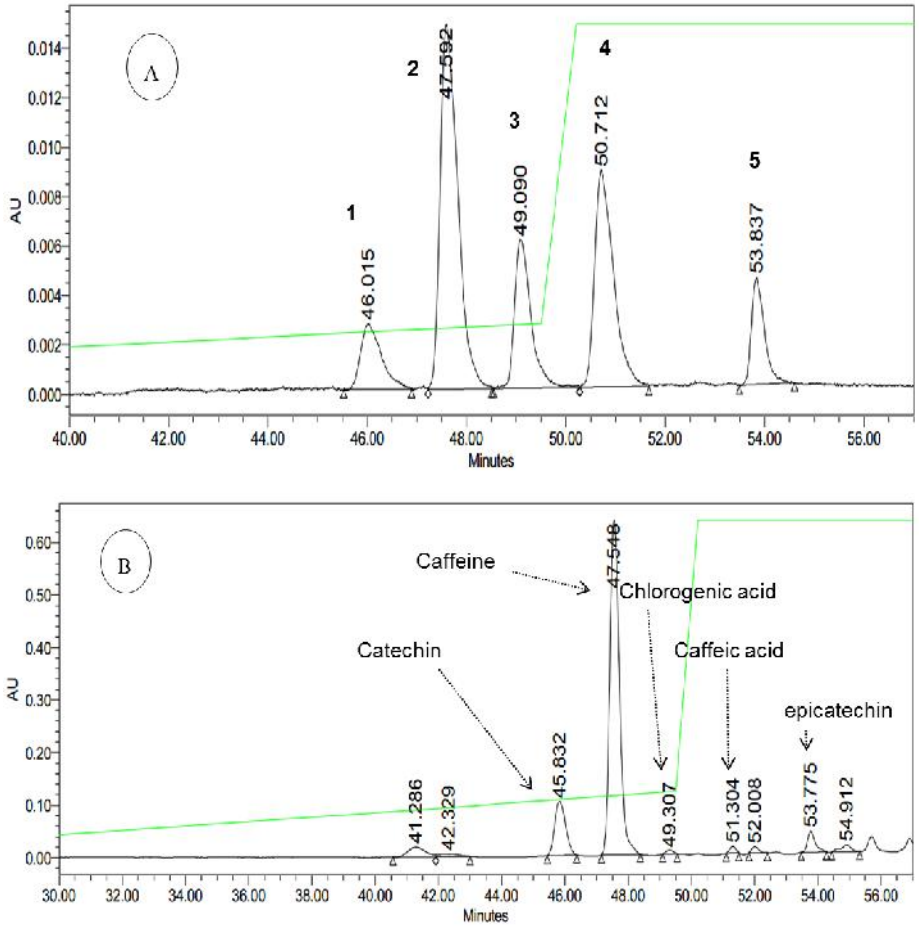

Fig. 1. Typical chromatogram obtained for: (A) EtOH 50% standard solution of catechin (1), caffeine (2), chlorogenic acid (3), caffeic acid (4) and epicatechin (5) ; (B) *Cola nitida* extract

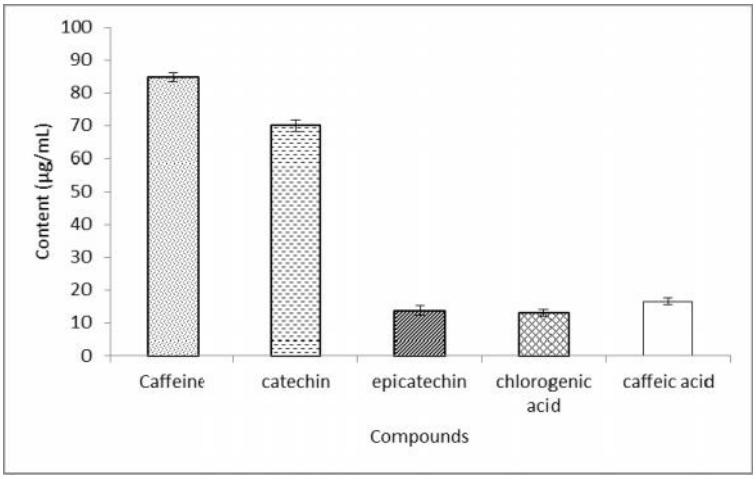

Fig. 2. Caffeine, catechin, epicatechin, chlorogenic and caffeic acid content in kola nut extract

3.2 Application of the Proposed Method

Fig. 1 shows the representative chromatographic separative chromatograms obtained with the proposed method for the analysis of the analytes solution and kola nut with analytes. It can be concluded that the method used allows good separation and identification of the compounds analyzed from their different retention times (Rt). Catechin, caffeine, chlorogenic acid, caffeic acid and epicatechin retention time were 46.015, 47.592, 49.090, 50.712 and 53.837 min, respectively (Fig. 1A). Comparing these figures on the basis of the different retention times, each compound has been identified during the analysis of the kola nut extract (Fig.1B).

The order of elution of the various compounds is substantially the same as that observed during the work of Niemenak et al. [7]. However, during the work of these authors, no compound is observed between the peak of caffeine and that of the epicatechin on the chromatogram. This could be explained by the variation in the operating conditions used in this study.

Fig. 2 shows the different contents of the compounds analyzed by the proposed HPLC method in *Cola nitida* sample extracted by water/ethanol mixture. It can see that the caffeine (84.83 µg/mL) is present in higher proportion in the kola extract, followed by catechin (69.90 µg/mL), caffeic acid (16.41 µg/mL), epicatechin (13.59 µg/mL) and chlorogenic acid (12.94 µg/mL). Thus, caffeine (alkaloid) and catechin (phenolic compound) are the major compounds of kola nuts extract. Our results agree with those reported by littérature [3,7,22].

4. CONCLUSION

Using this analytical method, caffeine, catechin, epicatechin, cholrogenic acid and caffeic acid could be determined simultaneously. A good repeatability of the results was etablished. This method offers a promising way to investigate the chemical properties and beneficial health effect of kola nuts. Futhermore, this method is simple, sensitive and accurate and can be applied to all kinds of kola nut products.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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