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Optimization of Thin-layer Chromatography and High-Performance Liquid Chromatographic Method for *Piper guineense* Extracts

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In this study, a thin-layer chromatography (TLC) and a high performance liquid chromatographic (HPLC) methods were developed for the chemical profiling, qualitative and quantitative analysis of *P. guineense* extracts. To obtain a chromatogram with satisfactory resolution and favorable retention time, DryLab software was used to simulate and optimize a HPLC method for the analysis of *P. guineense* extracts. The aim was to achieve the best possible overall resolution while keeping the analysis time and solvent consumption to a minimum. With the optimized method, a total of 16 main components in the extract were separated with favorable resolution. Optimal TLC conditions were also developed using solvents of various solvent strength (S_T) and solvent selectivity (P_S) values. The mobile phase composition was systematically tested using various proportions of solvents differing in S_T and P_S values under the same experimental conditions. During the optimization, emphasis was set on achieving the best possible overall separation of the main components of the extracts (for example piperine). In addition, the effects of the developing chamber was tested using three types of unsaturated chamber conditions: horizontal chamber in sandwich configuration, horizontal chamber in non-sandwich configuration and twin-trough vertical chamber. During the study, a TLC method was developed, and the best mobile-phase composition giving favorable resolution of the bands was toluene: ethyl acetate (P_S 6-4 corresponding to 60:40 % v/v). The developing chamber conditions did not affect the TLC separation efficacy in the analysis of *P. guineense* extracts. The HPLC method was applied to determine the percentage content of piperine in *P. guineense.* The piperine content was 0.43 % w/w, linearity (0.997), interday precision (% relative standard deviation (RSD), 1.6), intraday precision (% RSD, 2.7 – 5.9), recovery (98.4%), limit of detection (0.001 µg /mL) and limit of quantification (0.003 µg /mL).

Keywords: DryLab simulation, Mobile-phase composition, TLC, HPLC, Piperine.

Piperine, a major alkaloid component in many pepper species, has interesting pharmacological activities, such as antidepressant effect [1] and effects on cognitive impairment [2]. Also West African guinea pepper, *Piper guineense* Schumach &Thonn, has been shown to contain piperine as its main component [3]. *P. guineense* is a tropical plant that is easily accessible in most parts of the world. It is a medicinal herb commonly cultivated for its seeds, fruits and leaves [4], its black berry fruits are sold in most local markets as flavoring agents [5]. It is also called Ashanti pepper.

Ethnopharmacologically, *P. guineense* has wide application in traditional medicine in the preparation of herbal formulations used in the treatment of various diseases [4]. Extracts from the plant have hypothermic, sedative and muscle relaxant properties [6]. The seeds are used in the preparation of Niprisan herbal formulation used to treat sickle-cell anaemia [4, 7]. The fruits and leaves of *P. guineense* are ground and combined with *other* herbs to prepare decoctions used for the treatment of epilepsy, convulsions and malaria [8]. Decoctions from the fruits and seeds are used for the treatment of venereal diseases, rheumatism, gastrointestinal diseases and respiratory diseases [9]. It is also used to treat stomach ache [10] and reportedly has oestrogenic and oxytocic activity [11].

Pharmacologically, *P. guineense* is rich in amides and alkaloids [3, 9]. The piperamide compounds from *P. guineense* and other *Piper* species have gained recognition in recent years for their strong efficacy as anticancer, anti-inflammatory, antimicrobial and antitumor agents in drug discovery [12, 13]. *Piper guineense* has bioactive compounds with significant radical scavenging activity [14]. Bioactive compounds and extracts from this plant inhibited the growth of Gram-positive and Gram-negative bacteria and could serve as a potential lead to new antimicrobial drugs [5, 9]. Amides and extracts from this plant have strong insecticidal properties and can be used as alternatives to synthetic insecticides, which have problems with high cost and toxicity [15]. It reportedly has antipsychotic properties and can be used in the management and treatment of psychotic disorders [6].

Due to the various therapeutic potentials of *P. guineense* extracts and the recent interest in its bioactive compounds, it is crucial to have a simple and easily available method for the analysis of its bioactive compounds for drug discovery.

DryLab and other computer simulation programs have provided rapid and optimal HPLC conditions for the separation of analytes, drugs and other pharmaceutical formulations [16]. The analysis, characterization and structural elucidation of bioactive compounds from plant extracts is a difficult task to accomplish, due to the presence of numerous compounds. The successful separation of these components is also challenging, due to the need for balance between resolution and analysis time. Optimization of these analytical methods is crucial to the successful extraction and isolation of bioactive compounds from their biological matrices [17].

The aim here was to develop a TLC method for the chemical profiling and qualitative analysis of various *P. guineense* seed and leaf extracts. During the optimization, emphasis was set on achieving the best possible overall separation of the main components of the extracts. Also it was aimed at optimizing an HPLC method for the analysis of *P. guineense* extracts, using DryLab software to achieve the best possible overall resolution for the piperamides, while keeping the analysis time and solvent consumption to a minimum. This is the first report on optimized TLC conditions with optimum mobile-phase composition and effect of developing chamber conditions for the analysis of *P. guineense*

extracts. To develop a rapid and robust HPLC method and to enhance separation efficiency, a critical-resolution map and peak-matching functions in DryLab software were also used to develop an HPLC method for the analysis of *P. guineense* extracts.

HPLC conditions: DryLab software simulation made it possible and easy to develop an optimum condition for the extracts of *P. guineense*. The method development began with two initial experiments of a 30 min and 60 min gradient at 255 nm. Acetonitrile was chosen as the organic eluent, because it is miscible with water and often maintains a low pressure drop for the instrument. Moreover, it transmits in a low UV region with water as a mobile phase and can be easily used for extracts with poor UV chromophores, provided the detector is set above 220 nm. It is a frequently used eluent in reversed-phased chromatography. Data from the two initial experimental runs were entered into DryLab. Parameters such as the dwell volume, gradient conditions, column length, column diameter and flow rate were also entered into DryLab. Figure 3a and Figure 3b show the chromatogram of the initial experiment (supplementary data). To maintain constant peak areas, equal volumes of the sample were injected during the two initial experimental runs. The optimum gradient run was predicted, based on the data. Using a resolution map, peak-tracking and peak-matching functions, adjustments were made, runs were predicted and a new chromatogram was simulated with emphasis on analysis time, solvent consumption and optimum resolution of the peaks. The use of DryLab software allowed the optimization of the HPLC method in a minimum number of runs, and the simulated separation was very similar to the actual experimental separation. The best possible overall resolution for piperine was achieved while keeping the analysis time and solvent consumption to a minimum. The simulated separation conditions were experimentally confirmed (Figure 4) (supplementary data). The optimized method was a 19 min binary gradient with the proportion of organic solvent increasing from 39% to 80.4%, flow rate of 1 mL/min and injection volume of 20µL. This condition is the best separation condition, with the shortest run time and good peak shape. With the optimized method, a total of 16 major components in the extracts were separated with favorable resolution. The compounds were identified in the extracts by comparing the retention time and UV spectra with that of the reference standards and also with previous literature. The UV spectra of the major components in the *P. guineense* extracts were taken, based on the chromatogram of the optimized 19 min run. The piperamide peaks identified were piperylin (**1**), 4, 5-dihyropiperlonguminin (**2**), piperlonguminin (**3**), 4, 5-dihydropiperine (**4**) and piperine **(5**). The overall resolution and the resolution of the critical peak pairs with the optimized method were better than or equal to previously published method [3], albeit at the cost of a slightly longer analysis time. The optimized method will be used to analyze extracts and fractions produced from *P. guineense*. The HPLC method used in these analyses provided satisfactory overall resolution for most of the main piperamides of *P. guineense*. The method is simple, sensitive and accurate.

TLC composition: The optimal TLC conditions for the separation of *P. guineense* extracts were achieved by systematically testing solvents of various S_T and P_S values according to [18]. Table 1 shows the group, S_T and P_S values of individually selected solvents. The solvent combinations tested during the method development (Table 2) were all chosen, because they had been previously used in the TLC analyses of extracts of various *Piper* species. A review of the literature indicated that bicomponent solvent mixtures containing ethyl acetate as the other solvent appeared to be the most widely used, but that acetone had also been applied for the task. Most of the TLC methods were developed for other *Piper* species, and only one fairly recent study on the extract of *P. guineense* with a TLC method was reviewed [14]. In this study, the solvent combination used was *n*-hexane-ethyl acetate-methanol (60:40:1). The optimized TLC condition enhanced TLC separation compared with a published method on the extracts of this species [14]. The TLC separations obtained, using the solvent combinations tested are presented in Figure 1. It can be seen that the combination of toluene and ethyl acetate in various proportions resulted in the highest number of separated bands with the best overall resolution. The difference between toluene-ethyl acetate (6:4) and (55:45) was very subtle, but (6:4) was chosen for the TLC analyses of the *P. guineense* extracts. This solvent mixture has not been previously used for the analysis of *P. guineense*, but was applied for the analysis of Indian long pepper (*Piper longum* L.) root extract [19]. As observed from the result, the precoated TLC plates that serve as the reaction carriers revealed the influence of the mobile phase and the effect of their various proportions on the TLC separation of the *P. guineense* extracts.

Table 1: Selected solvents for the TLC mobile-phase optimization of *Piper guineense* extracts.

Group	Selected solvents	Individual strengths (S_i)	Selectivity values (S_v)
I	*n*-hexane, cyclohexane	0.1	0.001
II	methanol	5.1	2.18
VI	ethyl acetate	4.4	1.48
	acetone	5.1	1.52
VII	toluene	2.4	0.89

Table 2: Solvent combinations tested for TLC optimization of *P. guineense* extracts.

Solvent combination	Proportions (v/v)
A. *n*-Hexane – ethyl acetate	5:3
B. *n*-Hexane – acetone	2:3
C. Cyclohexane – ethyl acetate	5:3
D. *n*-Hexane – ethyl acetate – Methanol	60:40:1
E. Toluene – ethyl acetate	7:3
F. Toluene – ethyl acetate	6:4
G. Toluene – ethyl acetate	1:1
H. Toluene – ethyl acetate	55:45
I. Toluene – ethyl acetate	65:35

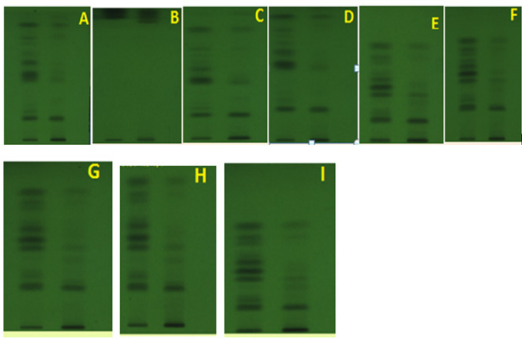

Figure 1: Thin-layer chromatographic separation of *Piper guineense* seed and leaf extracts with the solvent combinations tested. For key to plate numbering, see Table 2.

Developing chamber conditions: The effect of the developing chamber type on the separation efficiency was tested by comparing an unsaturated twin-trough chamber with the horizontal developing chamber in the tank and sandwich configurations (Figure 2). This was done with the mobile phase giving the best resolution of the bands toluene-ethyl acetate (P_S 6:4 v/v). Different volumes of 3, 4 and 5µL of the seed and leaf extracts were applied on HPTLC precoated silica gel 60 F_{254} plates. In the figure, plate A is a twin-trough vertical chamber, plate B a horizontal chamber in nonsandwich configuration and plate C a horizontal chamber in sandwich configuration. The chamber type did not markedly affect the separation efficacy, and the choice of chamber type was therefore made, based on other criteria in the analysis of *P. guineense* extracts.

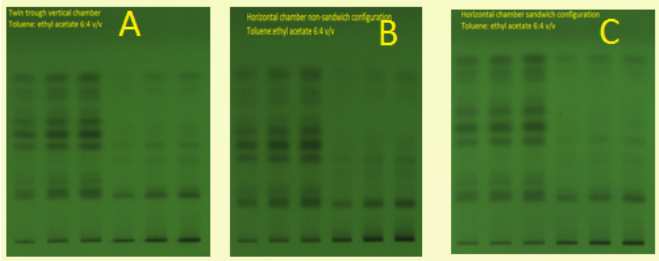

Figure 2: Thin-layer chromatographic separation of *Piper guineense* seed and leaf extracts with the optimum solvent combination toluene-ethyl acetate (6:4) in an unsaturated twin-trough chamber (A), horizontal developing chamber in nonsandwich configuration (B) and horizontal developing chamber in sandwich configuration (C).

Validation of the HPLC method

Method validation was carried out using a piperine standard solution.

Linearity: 1 mg/mL concentration of piperine was prepared in methanol for the calibration curve, the standard was diluted to appropriate concentrations to access the sensitivity and accuracy of the method. Triplicate injection of 20 µL of the standard solution was analyzed with HPLC. The calibration curve was linear over a range of concentrations; the linearity was favorable and fell within the concentration range of 2–12 µg/mL. The R^2 with respect to peak area was 0.997. The slope and intercept were 75 267 and 354.84, respectively.

Table 3: Linearity for reference alkaloids (n = 6).

Standard	Linear range[a] (µg/mL)	Calibrations equations[b]	R^2	RSD (%)
Piperine	2 – 12	y =75267x + 354.84	0.997	0.05 – 0.47

[a]Calibration curve represents six data points with three replicates per data point.
[b]y represents the peak area and x the concentrations (µg/mL).
R^2 = coefficient of determination, RSD = relative standard deviation.

Limit of detection and limit of quantification: The values obtained from the LOD (0.001 µg/mL) and LOQ (0.003 µg/mL) revealed that the method has favorable sensitivity. The LOD was evaluated and calculated to be the lowest concentration of piperine that could be detected. From the calibration curve, these parameters were calculated as the relative standard deviation (RSD) of the response and slope (S). Thus

LOD = 3.3.SD/S, where SD is the standard deviation and S the slope of the calibration curve.

LOQ = 10.SD/S, where SD is the standard deviation and S the slope of the calibration curve.

Table 4: Interday and intraday precision of piperine alkaloid (standard). RSD (%) = relative standard deviation.

Intraday precision Day 1	Intraday precision Day 2	Intraday precision Day 3	Interday precision
n = 6 RSD (5.9)	n = 6 RSD (3.9)	n = 6 RSD (2.7)	n = 18 RSD (1.6)

Table 5: Extraction recoveries with pure piperine (standard). RSD = relative standard deviation.

Added (µg/mL)	Found (µg/mL)	Recovery (%)	RSD (%)
50.0	54.1	98.4	5.8

Precision and recoveries: The precision and accuracy of the method were accessed by carrying out recovery studies. The interday and intraday precisions were within the range of 1.6 – 5.9%; this range was satisfactory and acceptable and revealed that the method was reproducible. The precision was determined by injecting six replicates of the standard solution on the same day and on 3 consecutive days. The measure of precision was expressed as RSD. For recoveries, a total of 500 mg of *P. guineense* extract was spiked with piperine standard solution at high and low concentrations. The mixture was extracted and a stock solution was prepared for the calibration curve. The following equation was used to calculate the recovery.

Recovery (%) = (amount detected) / (amount spiked) X 100%

Method application: The method developed was applied for the quantification and rapid estimation of piperine from *P. guineense*. The results show that piperine, which is one of the bioactive compounds isolated from *P. guineense*, constituted 0.43% w/w of the seed of *P. guineense.* It became necessary to quantify the piperine from *P. guineense*, due to its diverse biological and therapeutic potentials in recent pharmacological studies [2]. The piperine content (0.43% w/w) in *P. guineense* was compared with the contents previously estimated in Javanese long pepper (*Piper chaba* W. Hunter) (1.12% w/w), black pepper (*Piper nigrum* L.) (3.56% w/w) and *Piper longum* (0.0011% w/w) [20]. The results revealed that although piperine is present in *P. guineense*, it is more abundant in *P. nigrum* and *P. chaba*.

With the DryLab simulation program, a rapid HPLC method was developed and an improved choice of mobile-phase was obtained for the analysis of *P. guineense* extracts. The method can be used not only to analyze extracts and fractions produced from *P. guineense*, but also as a quality-control tool by pharmaceutical industries to explore piperine and other active compounds in *P. guineense* for drug discovery and for other therapeutic purposes. The optimized method is accurate, precise, sensitive and suitable for the analysis of extracts and fractions produced from *P. guineense*.

Experimental

Plant material: The seeds and leaves of *P. guineense* were collected from a rural village in Imo State, South eastern Nigeria and were authenticated at the Department of Crop Science of the Federal University of Technology, Owerri, Nigeria. Voucher specimens are deposited at the Department of Crop Science of the same university.

Extraction: The plant materials were powdered with a grinder. In all, 1.0 g each of dried powdered seeds and leaves was extracted twice by sonication with 25 mL of methanol for 10 min. The extracts were filtered with whatman filter paper (Whatman GE Healthcare, Chicago, IL, USA) into a flask of known weight, evaporated with a rotary evaporator at 40°C, freeze-dried and weighed**.**

Chemicals: The solvents used were HPLC grades of acetonitrile purchased from Merck KGaA (Darmstadt, Germany), toluene (Merck), ethyl acetate (Merck), *n*-hexane (Merck), acetone (Merck), cyclohexane (Sigma-Aldrich Corp. (Merck), St. Louis, MO, USA) and methanol (Merck) . Ultrapure water was obtained, using a Milli-Q water system (Merck Millipore, Billerica, MA, USA). Analytical grade piperine standard (≥ 97.0% purity) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium). TLC aluminium sheets coated with 20 x 20 cm silica gel 60 F_{254} (Merck, Germany) and 10 cm × 10 cm glass silica gel 60 F_{254} HPTLC plates (Merck, Germany).

TLC analysis: A total of 100 mg of the sample were dissolved in 1 mL of methanol (stock solution), and a 5 mg/mL concentration was prepared from the stock solution for the TLC analysis. A CAMAG linomat IV TLC spotter (CAMAG AG, Muttenz, Switzerland) was used to apply the extracts on the plates. TLC aluminium sheets coated with 20 x 20 cm silica gel 60 F_{254} (Merck), cut to 4 cm × 10 cm, were used for testing the various mobile-phase compositions.

A CAMAG twin-trough vertical chamber and CAMAG horizontal chamber were used. A CAMAG REPROSTAR 3 was used to view the plates at 254 nm. Aliquots of 5 µL of the sample solutions were applied on the plate. The application speed was 6 sec/µL, plate width 40 mm, band 6 mm, space 6 mm, starting position 10 mm and development over a path of 8 cm. Solvents of different solvent strength (S_T) and selectivity (P_S) values were combined in various proportions and tested under the same experimental conditions with 10 mL of the mobile phase. The solvents tested include toluene, ethyl acetate, *n*-hexane, acetone, cyclohexane and methanol. HPTLC precoated 10 cm × 10 cm glass silica gel 60 F_{254} plates were used to evaluation the effects of developing chamber on the separation. The optimum mobile phase of toluene-ethyl acetate 6:4 v/v was used. The procedure included a plate width of 100 mm, band 8 mm, space 5 mm, starting position 15 mm, 6 sec/µL and development over a path of 8 cm. 10 mL of the solvent was used for the twin-trough chamber and 2.5 mL for the horizontal chambers.

HPLC analysis: The samples were analyzed with an HPLC Waters Tm 717 autosampler, equipped with a photodiode array detector set at 240 nm (Waters Corp., Milford, MA, USA). The instrument comprises a binary pump, vacuum degasser and a dwell volume of 6.16 mL. The wavelength for the UV spectra was 255 nm. The simulation with DryLab software was done with DryLab 2010 version 3.1 (LC Resources Inc., Alamo, CA, USA; in Europe: Molnar, Berlin, Germany). 5 mg/mL concentration of the extract was injected into the column, with a total run time of 25 minutes. The solvents and instrumental conditions are shown in Table 6.

Table 6: HPLC conditions of test runs for *Piper guineense* extracts.

Mobile phase	Acetonitrile: water
Gradient range	5 – 100% of organic solvent
Flow rate	1 mL/min
Column	C18, 5 µm, 250 mm x 4.6 mm
Wavelength	255 nm
Gradient time	30 and 60 min
Temperature	Ambient, approx. 20–25°C
Injection volume	20 µL

Supplementary data: Figures 3a, 3b and 4 showing the separation of *Piper guineense* seed extract, using a 30 min, 60 min and 19 min gradient at 255 nm are included in the Supporting information.

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