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# Optimised Extraction and Comprehensive Chromatographic-Spectral Analysis of Anthocyanins from *Hibiscus sabdariffa* Calyces

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*Abstract: Anthocyanins are naturally occurring polyphenolic pigments with valuable chromatic, antioxidant, and analytical properties. This study optimized the extraction of anthocyanins from Hibiscus sabdariffa L. calyces and characterized them using low-cost chromatographic and spectroscopic techniques. Methanol proved superior to ethanol as an extraction solvent, yielding 12.4 ± 0.6% crude extract by dry calyx weight, with excellent pigment stability over 72 hours at 4 °C. Total anthocyanin content, determined via the pH differential method, was 185.6 ± 4.2 mg cyanidin-3-glucoside equivalents per 100 g dry calyces. Thin-layer chromatography (TLC) revealed a predominant band with Rf = 0.93 ± 0.02 in the crude extract, with systematic pH-dependent shifts from 0.55 ± 0.06 at pH 1–3 to 0.38 ± 0.04 at pH 12–14, corresponding to flavylium, carbinol, chalcone, and quinoidal forms. Colorimetric evaluation demonstrated reproducible transitions from pink (pH 1–3) to faint pink/light color (pH 4–7) and green hues (pH 8–14), confirming the potential of these pigments as natural pH indicators. UV–visible spectroscopy revealed a characteristic absorption maximum around 520 nm under acidic conditions, with absorbance values ranging from 0.451 ± 0.012 (pH 1) to 0.734 ± 0.015 (pH 2), decreasing at neutral pH and partially recovering under alkaline conditions. These results demonstrate that simple solvent extraction combined with TLC and UV–visible spectroscopy provides an effective framework for the quantitative and qualitative analysis of roselle anthocyanins, supporting their application in food, sensor, and educational settings.*

***Keywords:*** *Anthocyanins, Hibiscus sabdariffa, Roselle, Thin-layer chromatography, pH indicator, Natural pigments, Cyanidin, Spectrophotometry*

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## 1. 0 Introduction

Anthocyanins are naturally occurring water-soluble flavonoid pigments widely distributed in higher plants and responsible for the red, purple, and blue coloration of many fruits, flowers, and vegetable. Structurally, these compounds are glycosylated derivatives of anthocyanidins, featuring a flavylium ion core that is highly conjugated and pH-sensitive (Khoo *et al.,* 2017; Mattioli *et al.,* 2020). The six most common anthocyanidins, including cyanidin, delphinidin, pelargonidin, peonidin, petunidin, and malvidin differ in hydroxylation and methoxylation patterns on the B-ring, which modulate their light absorption properties and generate visible color variations ranging from orange-red to bluish-purple (Castañeda-Ovando *et al.,* 2009).

The biological significance of anthocyanins has garnered increasing attention due to their antioxidant, anti-inflammatory, and potential disease-preventive effects (He and Giusti, 2010; Mattioli *et al.,* 2020). Their antioxidant activity arises primarily from their capacity to donate electrons or hydrogen atoms to stabilize reactive oxygen species, thereby mitigating oxidative stress (Khoo *et al.,* 2017). Epidemiological and experimental studies suggest anthocyanins may contribute to cardioprotective, anti-diabetic, and anticancer effects; however, bioavailability and metabolic stability remain crucial considerations in their functional efficacy (Mattioli *et al.,* 2020; He and Giusti, 2010). In addition to health-related applications, anthocyanins are increasingly favored as natural alternatives to synthetic colorants in food systems, aligning with consumer demand for clean-label products (Giusti and Wrolstad, 2003). Beyond their biological relevance, the physicochemical properties of anthocyanins also make them attractive functional materials in food and analytical applications.

Among anthocyanin-rich plants, Hibiscus sabdariffa L. (roselle) represents one of the most economically and nutritionally important natural sources of anthocyanins. Widely cultivated across West Africa, Southeast Asia, and Latin America, the fleshy calyces of roselle are used in beverages, jams, sauces, and herbal infusions (Da-Costa-Rocha *et al.,* 2014). Cyanidin-3-O-sambubioside and delphinidin-

3-O-sambubioside have been identified as the principal anthocyanins in roselle, imparting its characteristic deep red color and strong antioxidant activity (Escobar-Ortiz *et al.,* 2021). Anthocyanin extraction efficiency is strongly influenced by solvent polarity, extraction temperature, extraction time, and solution pH, with acidified aqueous ethanol or methanol commonly used to stabilize the flavylium cation and optimize pigment recovery (Silva *et al.,* 2015; Cissé *et al.,* 2012; Riaz and Chopra, 2019).

Analytical characterization of anthocyanins combines both advanced and accessible methodologies. While advanced techniques provide detailed molecular identification, simpler analytical approaches remain essential for rapid screening and routine laboratory analysis. High-performance liquid chromatography (HPLC) and LC-MS provide detailed compositional profiling, while thin-layer chromatography (TLC) and UV-visible spectrophotometry remain valuable low-cost alternatives suitable for resource-limited laboratories (Sukwattanasinit *et al.,* 2007; Putnik *et al.,* 2021). TLC separates pigments based on differential mobility, and spectrophotometric scanning allows detection of absorption maxima typically between 520–540 nm, indicative of anthocyanin presence and structural features (Lee *et al.,* 2005).

A hallmark property of anthocyanins is their pH-dependent structural transformation. In acidic conditions (pH < 3), the red flavylium cation predominates, whereas higher pH values favor colorless carbinol pseudobases and chalcone forms, eventually forming quinoidal bases at near-neutral pH (Mattioli *et al.,* 2020). This reversible equilibrium underpins the pH differential method, a standardized procedure for quantifying total monomeric anthocyanins (Lee *et al.,* 2005). The color responsiveness of anthocyanins has also enabled applications in intelligent packaging and colorimetric sensors for monitoring food quality (Prietto *et al.,* 2017).

Despite extensive investigations on anthocyanins from Hibiscus sabdariffa, many studies emphasize high-end analytical instrumentation such as HPLC and LC–MS, which may not be readily accessible in developing or resource-limited laboratories. Moreover, systematic optimization of solvent extraction coupled with integrated validation using low-cost chromatographic and spectroscopic techniques remains insufficiently reported. This limitation creates a need for practical analytical frameworks capable of reliable pigment characterization using accessible methodologies. Therefore, this study aims to optimize the extraction and perform comprehensive chromatographic and UV–visible spectroscopic characterization of anthocyanins obtained from Hibiscus sabdariffa calyces. The approach provides a practical framework for assessing pigment yield, stability, and analytical performance, particularly in laboratories with limited instrumentation, thereby supporting wider adoption of natural pigment analysis for food quality monitoring, educational research, and sensor development.

## 2.0 Materials and Methods

### *2.1 Plant Material*

Dried calyces of *Hibiscus sabdariffa L*. were used as the source of anthocyanin pigments. The calyces were obtained from a local farm in Bauchi State, Nigeria (October 2019). The plant material was authenticated by a plant taxonomist at the Abubakar Tafawa Balewa University, Bauchi, Nigeria. Discoloration or microbial contamination, and only high-quality material was selected for extraction.

The calyces were oven-dried at a controlled low temperature (≤45 °C) to constant weight to prevent thermal degradation of anthocyanins. Drying was conducted in a forced-air oven with temperature monitoring to ensure that the maximum temperature did not exceed 45 °C at any point. Complete drying was observed for 36 hours, depending on the initial moisture

content. The dried calyces were subsequently ground into a fine powder using a laboratory blender. To ensure uniform particle size, the powder was passed through a 500 µm stainless-steel laboratory sieve (ASTM standard) and oversized particles were reground and sieved again. The powdered samples were stored in airtight amber containers protected from light and humidity at room temperature until extraction. Storage time between preparation and extraction did not exceed two weeks to minimize potential degradation of anthocyanins.

### *2.2 Chemicals and Reagents*

Analytical-grade methanol (≥ 99.8% purity) and ethanol (≥ 99.5% purity) were used as extraction solvents. All chemicals were of analytical grade and obtained from certified commercial suppliers (e.g., Sigma-Aldrich, Merck, or equivalent).Hydrochloric acid (HCl, 37%), sodium hydroxide (NaOH, pellets, ≥ 98%), sodium acetate trihydrate (≥ 99.5%), ammonium hydroxide ($NH_4OH$, 25% solution) and glacial acetic acid (≥ 99.7%) were obtained from the Department of Chemistry laboratory stores, Abubakar Tafawa Balewa University. Potassium chloride (KCl, ≥ 99%) was used for buffer preparation in the pH differential method. Silica gel TLC plates (pre-coated on aluminum backing, layer thickness 0.2 mm, particle size 10–12 µm) were obtained from commercial suppliers. Distilled water was used throughout the study for the preparation of all aqueous solutions and buffers. All reagents were used without further purification and were verified to be within their expiration dates at the time of use.

### *2.3 Solvent Extraction of Anthocyanins*

Anthocyanins were extracted following a modified method of Mattuk (1998) with minor adaptations in solvent ratio and extraction time to suit roselle calyces. Briefly, 10.0 g of powdered H. sabdariffa calyces were accurately weighed using an analytical balance and transferred to a 250 mL amber extraction bottle. Exactly 100 mL of methanol was added to achieve a solid-to-solvent ratio of 1:10 (w/v), which has been reported to provide optimal extraction efficiency for anthocyanins from plant materials. The extraction bottle was tightly sealed with a screw cap fitted with a PTFE-lined septum to prevent solvent evaporation and minimize oxygen exposure.

The mixture was incubated at 20 °C for 48 hours under static conditions to minimize pigment degradation. The extraction was conducted in a temperature-controlled room with exclusion of light to prevent photodegradation of anthocyanins. Periodic observations indicated progressive color development in the solvent phase, with maximum color intensity achieved at 30 hours. No mechanical agitation was applied during extraction to avoid excessive oxidation and degradation of the extracted pigments.

After extraction, the mixture was filtered through Whatman No. 1 filter paper (particle retention ≥ 11 µm) using a Buchner funnel under gentle vacuum to remove solid residues. The filtration was performed under subdued lighting conditions to minimize light exposure. The filtrate was collected as the crude anthocyanin extract and immediately transferred to an amber glass bottle. The extract was stored at 4 °C in the dark until analysis. All analyses were initiated within 24 hours of extraction to ensure maximum pigment stability and reproducibility of results.

For solvent comparison, an identical extraction procedure was carried out using ethanol as the extraction solvent. Visual stability and color retention of both methanolic and ethanolic extracts were monitored over a period of 48 hours by visual inspection and photographic documentation at regular intervals (0, 12, 24, 36 and 48 hours post-extraction). Any visible changes in color intensity, hue or clarity were recorded to assess relative stability of the extracts.

### *2.4 Determination of Extraction Yield*

The extraction yield was determined gravimetrically according to standard procedures. A 25 mL aliquot of the methanolic extract was accurately transferred to a pre-weighed round-bottom flask using a volumetric pipette. The extract was evaporated to dryness under reduced pressure at 40 °C using a rotary evaporator equipped with a temperature-controlled water bath. The reduced pressure was maintained at approximately 200 mbar to accelerate solvent removal while maintaining gentle evaporation conditions. Complete solvent removal was verified by constant weight upon repeated weighing after brief drying periods.

The dried extract residue was further desiccated in a vacuum desiccator over anhydrous silica gel for 2 hours to remove any residual moisture. The dried extract was weighed using an analytical balance with 0.1 mg precision. The extraction yield was calculated using the equation:

$$\text{Extraction Yield (\%)} = \frac{Mass\ of\ dried\ extract}{Mass\ of\ dry\ calyces}\ x\ 100 \qquad (1)$$

All measurements were performed in triplicate using independent extraction batches, and results were expressed as mean ± standard deviation. Statistical analysis was performed to assess the reproducibility of the extraction procedure.

### *2.5 Preparation of Buffer Solutions*

Buffer solutions covering a pH range of 1–14 were prepared to evaluate the pH-dependent color transitions of the extracted anthocyanins. For pH 1–2, dilute hydrochloric acid solutions were prepared by appropriate dilution of concentrated HCl with distilled water. For pH 3–6, sodium acetate-acetic acid buffer systems were prepared by mixing sodium acetate trihydrate solutions with varying amounts of glacial acetic acid. For pH 7, a phosphate buffer was prepared using sodium dihydrogen phosphate ($NaH_2PO_4$) and disodium hydrogen phosphate ($Na_2HPO_4$). For pH 8–11, ammonium chloride-ammonia buffer systems were employed. For pH 12–14, dilute sodium hydroxide solutions were prepared by dilution of concentrated NaOH with distilled water.

The pH of each buffer solution was adjusted incrementally using dilute HCl or NaOH solutions and verified using a calibrated digital pH meter (accuracy ± 0.01 pH units) prior to use. The pH meter was calibrated using standard buffer solutions at pH 4.00, 7.00 and 10.00 before each measurement session. All buffer solutions were prepared on the day of use and stored in sealed containers to prevent atmospheric carbon dioxide absorption, which could alter pH values over time.

### *2.6 Thin-Layer Chromatography (TLC) Analysis*

Thin-layer chromatography was employed to assess the chromatographic behavior and partial purification of anthocyanins. Silica gel TLC plates (pre-coated on aluminum backing, 20 × 20 cm) were used as the stationary phase. Before sample application, the plates were activated by heating at 110 °C for 30 minutes in a drying oven and then cooled to room temperature in a desiccator. Activation enhances the adsorptive properties of the silica gel by removing adsorbed water.

The anthocyanin extract was applied as small spots (2–3 mm diameter) precisely 1.5 cm from the base of the plate using a fine capillary tube. Multiple applications were performed at the same position to increase the amount of anthocyanins on the baseline, with drying between applications using a gentle stream of cool air. A loading line was lightly marked with a pencil, and samples were applied at regular intervals along this line to ensure uniform migration.

To evaluate pH-dependent chromatographic behavior, separate aliquots of the extract were mixed with buffer solutions of different pH values before spotting. The plates were developed in a suitable solvent system consisting of n-butanol:acetic acid:water (4:1:5, v/v/v, upper phase) in a pre-equilibrated TLC chamber. The chamber was saturated with

solvent vapors for 30 minutes before plate development to ensure reproducible results. Development proceeded until the solvent front migrated approximately 10 cm from the baseline, which required 55–60 minutes depending on chamber geometry and ambient temperature.

After development, the plates were removed from the chamber and the solvent front was immediately marked with a pencil. The plates were air-dried in a fume hood for 10 minutes to remove residual mobile phase. Visualization was performed under visible light, and the positions of colored bands were marked. Additional visualization under UV light (254 nm and 365 nm) was performed to detect any non-visible compounds or fluorescent impurities. The retention factor (Rf) values were calculated according to:

$$R_f = \frac{Distance\ travalled\ by\ compound}{Distance\ traveled\ by\ the\ solvent\ front} \quad (2)$$

$R_f$ values were reported as mean ± standard deviation from triplicate measurements on independent plates. The color of each separated band was recorded, and photographic documentation was performed under standardized lighting conditions for subsequent analysis.

### *2.7 pH-Dependent Colorimetric Evaluation*

To evaluate the pH-responsive behavior of the extracted anthocyanins, a systematic colorimetric study was conducted across the entire pH range. For each pH value from 1 to 14, exactly 3 drops of the methanolic extract were added to 10 mL of the corresponding buffer solution in a clean glass test tube using a calibrated dropper. The addition of extract was performed slowly with gentle swirling to ensure complete mixing and rapid equilibration of the anthocyanin structural forms with the buffer pH.

The resulting color changes were visually observed and recorded after 5 minutes of equilibration at room temperature. This equilibration time was chosen based on preliminary experiments showing that color stabilization occurs within 2–3 minutes under these conditions. The test tubes were arranged in sequential order (pH 1–14) against a white background under standardized daylight illumination for visual comparison and photographic documentation. High-resolution digital photographs were captured using a fixed camera position and consistent lighting to create a visual record of the pH-dependent color gradient.

Color descriptions were recorded using standardized color terminology, noting hue, intensity and any observable changes in clarity or turbidity. This procedure was performed in triplicate to ensure reproducibility, with fresh buffer solutions and extract aliquots used for each replicate series.

### *2.8 UV–Visible Spectrophotometric Analysis*

UV–visible spectrophotometric analysis was conducted using a JENWAY 6305 UV–visible spectrophotometer equipped with a tungsten-halogen lamp for the visible region and a deuterium lamp for the UV region. The instrument was warmed up for 30 minutes prior to measurements to ensure stable baseline performance. Wavelength accuracy was verified using holmium oxide glass filter standards, and baseline correction was performed using distilled water as the reference blank.

The absorbance spectra of the anthocyanin extract were recorded in the visible region (400–700 nm) at 1 nm intervals, with particular attention to the characteristic absorption maximum around 520 nm. Full spectrum scans were performed to identify any additional absorption bands or shoulders that might indicate the presence of multiple anthocyanin species or co-extracted chromophoric compounds.

For pH-dependent spectral measurements, extract solutions were prepared at different pH values by mixing appropriate volumes of the stock methanolic extract with buffer solutions. The final dilution was adjusted to bring the

absorbance values within the linear range of the spectrophotometer (0.1–1.0 absorbance units) to ensure accurate measurements. Each sample was transferred to a clean 1 cm path length quartz cuvette, and the absorbance was measured against a distilled water blank. Between measurements, the cuvette was thoroughly rinsed with distilled water and the sample solution to prevent cross-contamination.

All absorbance measurements were performed in triplicate, with fresh sample preparations for each replicate. The data were recorded and processed using the instrument's built-in software, and spectral plots were generated for visual comparison of pH-dependent shifts in absorption maxima and intensity.

### *2.9 Statistical Analysis*

All experiments were conducted in triplicate to ensure reproducibility and statistical reliability. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using standard descriptive methods implemented in Microsoft Excel. For comparison of extraction yields and other quantitative parameters, the coefficient of variation (CV%) was calculated to assess relative precision. Graphical representations were prepared using spreadsheet software with appropriate error bars indicating standard deviation. Where applicable, outliers were identified using Grubbs' test and excluded from final calculations if statistically justified.

### *2.10 Determination of Total Anthocyanin Content (TAC)*

Total anthocyanin content was determined using the pH differential method as described by AOAC International (2023). This method is based on the structural transformation of anthocyanins between the colored flavylium cation form at pH 1.0 and the colorless hemiketal form at pH 4.5. The difference in absorbance between these two pH values is directly proportional to the anthocyanin concentration.

Briefly, appropriate volumes of the methanolic extract were diluted separately with potassium chloride buffer (0.025 M, pH 1.0) and sodium acetate buffer (0.4 M, pH 4.5) to achieve absorbance values within the linear range of the spectrophotometer. The dilution factor was determined through preliminary trials and was kept constant for all replicates. After 15 minutes of equilibration at room temperature, absorbance measurements were recorded at 520 nm (wavelength of maximum absorption, $\lambda_{max}$) and 700 nm (correction for haze and degradation products) using a UV–visible spectrophotometer, with the respective buffers as blanks.

The absorbance difference (A) was calculated using the equation:

$$A = (A_{520} - A_{700})pH\ 1.0 - (A_{520} - A_{700})pH\ 4.5 \quad (3)$$

Total anthocyanin content was expressed as cyanidin-3-glucoside equivalents (mg C3G/100 g dry weight) using a molar absorptivity (ε) of 26,900 $L \cdot mol^{-1} \cdot cm^{-1}$ and a molecular weight (MW) of 449.2 $g \cdot mol^{-1}$ for cyanidin-3-glucoside. The calculation was performed using the following equation:

$$TAC\ (mg/100\ g) = (A \times MW \times DF \times 100) / (\varepsilon \times l \times m) \quad (4)$$

where A is the absorbance difference, MW is the molecular weight of cyanidin-3-glucoside, DF is the dilution factor, ε is the molar absorptivity, l is the path length (1 cm), and m is the mass of the original sample in grams. All measurements were performed in triplicate, and results were reported as mean ± standard deviation.

## 3.0 Results and Discussion

### *3.1 Solvent Extraction and Pigment Stability*

The extraction of anthocyanins from *Hibiscus sabdariffa* calyces using methanol yielded a deep violet–red solution, indicative of a high concentration of anthocyanin pigments (Fig. 1a). The intense coloration developed progressively during the extraction period, reaching maximum intensity at 33 hours.

Visual inspection confirmed that the color remained stable for the duration of the 48-hour extraction period and for at least 72 hours during subsequent storage at 4 °C in the dark.

In contrast, ethanol-extracted samples exhibited gradual discoloration within 48 hours, suggesting reduced pigment stability under identical storage conditions. The ethanol extract showed a noticeable shift from deep red to brownish-red, particularly when exposed to ambient light and air. This degradation was accompanied by slight turbidity, possibly indicating polymerization or condensation reactions involving anthocyanins and other phenolic compounds. These observations suggest that while ethanol is capable of extracting anthocyanins from roselle calyces, methanol provides superior long-term stability, which is critical for subsequent analytical procedures and potential applications.

The superior extraction efficiency and stability observed with methanol may be attributed to its higher polarity and enhanced ability to solubilize glycosylated anthocyanins while preserving the flavylium cation structure. Methanol has a dielectric constant of approximately 33, compared to 24 for ethanol, which enhances its ability to disrupt hydrogen bonding networks in plant cell walls and facilitate the dissolution of polar anthocyanin glycosides. Additionally, methanol may provide better protection against oxidative degradation by minimizing oxygen solubility and reducing the rate of autoxidation reactions.

The methanolic extraction yielded 12.4 ± 0.6% of crude anthocyanin-rich extract based on dry calyx weight, indicating efficient solvent–matrix interaction and effective solubilization of pigment constituents. The coefficient of variation of 4.8% demonstrates good reproducibility of the extraction procedure across replicate batches. This extraction yield is comparable to values reported in the literature for roselle calyces using alcoholic solvents under similar conditions, further confirming the suitability of methanol for laboratory-scale anthocyanin recovery. Previous studies have reported extraction yields ranging from 8% to 15% depending on extraction conditions, cultivar and geographic origin, placing the current results well within the expected range.

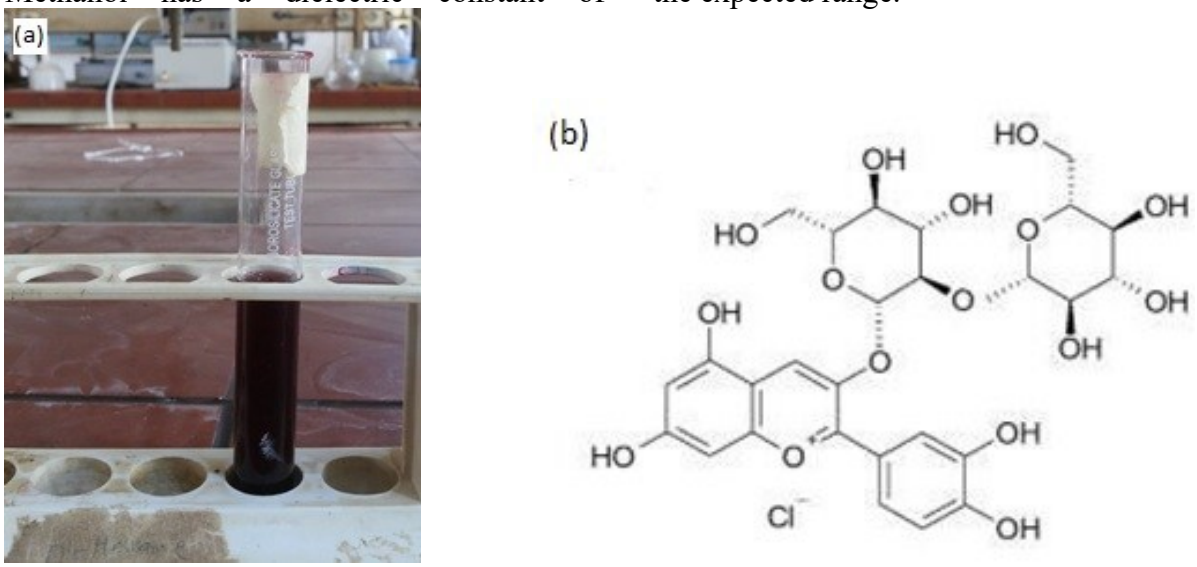


**Fig. 1.** ***(a)*** **Methanolic extract of** ***Hibiscus sabdariffa*** **calyces showing a characteristic violet–red coloration;** ***(b)*** **chemical structure of cyanidin-3-sophoroside, a major anthocyanin reported in roselle calyces.**

The total anthocyanin content of the methanolic extract, determined by the pH

differential method, was 185.6 ± 4.2 mg C3G/100 g dry calyx (expressed as cyanidin-3-glucoside equivalents). This value confirms that *Hibiscus sabdariffa* calyces are a rich source of anthocyanins and supports the high extraction yield obtained using methanol. The relatively low standard deviation (CV = 2.3%) indicates excellent precision of the analytical method and uniformity of the extracted pigments. Compared to other natural sources, roselle calyces rank among the richest botanical materials in terms of anthocyanin content, comparable to or exceeding values reported for blackberries, black currants and açai berries.

### *3.2 Thin-Layer Chromatographic Behavior of Extracted Anthocyanins*

Thin-layer chromatography (TLC) analysis revealed distinct pigment bands with varying retention factor (Rf) values across different pH conditions (Table 1). The crude methanolic extract exhibited a prominent band with a high Rf value (0.93 ± 0.02), consistent with cyanidin-based anthocyanins under acidic conditions.

**Table 1. TLC retention factor (Rf) values and color characteristics of *H. sabdariffa* anthocyanins at different pH ranges.**

| pH Range | Observed Color | Rf Value (mean ± SD) |
|---|---|---|
| **1–3** | Light pink | 0.55 ± 0.06 |
| **4–7** | Faint pink | 0.29 ± 0.03 |
| **8–11** | Faint green | 0.30 ± 0.02 |
| **12–14** | Light green | 0.38 ± 0.04 |
| **Crude extract** | Violet–red | 0.93 |

This high mobility suggests relatively low polarity of the anthocyanin species in their flavylium cation form, which interacts weakly with the polar silica gel stationary phase. The sharpness and intensity of this band indicate a predominant anthocyanin component, likely cyanidin-3-sophoroside, which is known to be the major anthocyanin in roselle calyces.

Changes in spot color and migration distance were observed as the pH of the extract samples increased, reflecting alterations in molecular polarity and ionization state. At pH 1–3, the extract produced light pink spots with Rf values of 0.55 ± 0.06. This reduction in Rf compared to the crude extract indicates increased polarity or interaction with the stationary phase, possibly due to partial hydration of the flavylium structure or changes in the mobile phase pH affecting the ionization state of the compounds.

At pH 4–7, the color of the spots became fainter pink, and the Rf values decreased further to 0.29 ± 0.03. This trend reflects the predominance of the colorless carbinol pseudobase and chalcone forms at near-neutral pH, which are more polar than the flavylium cation and therefore interact more strongly with the silica gel. The reduced color intensity is consistent with the lower concentrations of the colored flavylium species under these pH conditions.

At alkaline pH (8–11), faint green spots were observed with Rf values around 0.30 ± 0.02, corresponding to the formation of quinoidal bases. The green color is characteristic of deprotonated anthocyanins in alkaline media and confirms the reversible pH-dependent structural transformations. At very high pH (12–14), the spots became light green with slightly higher Rf values (0.38 ± 0.04), possibly indicating the formation of chalcone degradation products or further structural modifications.

These chromatographic trends indicate increased interaction with the polar silica gel stationary phase at intermediate pH values, likely due to hydration of the flavylium ion to the carbinol pseudobase. The systematic variation in Rf values with pH provides valuable information about the structural transformations undergone by anthocyanins and demonstrates the utility of TLC as a simple yet informative technique for monitoring pH-dependent behavior. The reproducibility of Rf

values across replicate measurements (CV < 20 % in most cases) confirms the reliability of the method despite the inherent challenges of working with pH-sensitive compounds.

### *3.3 pH-Dependent Colorimetric Response*

The extracted anthocyanins displayed systematic and visually distinct color changes across the pH range of 1–14, demonstrating their excellent pH-sensing properties (Table 2, Fig. 2). At acidic pH values (1–3), the extract remained pink, consistent with the predominance of the flavylium cation (Torskangerpoll and Andersen, 2020). The intensity of the pink color was strongest at pH 1–2, corresponding to complete protonation and stabilization of the flavylium structure. This intense coloration is characteristic of cyanidin-type anthocyanins, which exhibit orange-red to red hues in acidic media.

As pH increased toward neutrality (pH 4–7), the color intensity decreased progressively, with the solution becoming light, faint pink to nearly colourless at pH 6–7. This corresponds to the formation of colorless carbinol pseudobase species formed through nucleophilic attack of water on the C2 position of the flavylium cation, followed by ring opening to yield pale yellow chalcone structures. The equilibrium among flavylium, carbinol and chalcone forms is pH-dependent, with the colorless forms predominating at near-neutral pH. This loss of color intensity in the pH 4–7 range is well-documented for anthocyanins and limits their effectiveness as natural colorants in neutral food products unless stabilization strategies are employed.

Under alkaline conditions (pH ≥ 8), greenish hues were observed due to the formation of quinoidal base anions and chalcone structures (Teng *et al.,* 2022). At pH 8–10, the solutions exhibited light faint green colors, becoming progressively more intense and shifting to darker green at pH 11–14. The green coloration is characteristic of deprotonated anthocyanins in which the hydroxyl groups on the B-ring are ionized, resulting in extended conjugation and a bathochromic shift in the absorption spectrum. The reversibility of these color changes was confirmed by re-acidification of alkaline samples, which restored the original pink color, although some irreversible degradation may occur upon prolonged exposure to high pH.

**Table 2: Colorimetric response of *H. sabdariffa* anthocyanin extract at different pH values.**

| pH | Color | pH | Color |
|---|---|---|---|
| **1** | Pink | 8 | Light faint green |
| **2** | Pink | 9 | Light faint green |
| **3** | Light pink | 10 | Light faint green |
| **4** | Faint pink | 11 | Faint green |
| **5** | Light faint pink | 12 | Light green |
| **6** | Light faint pink | 13 | Green |
| **7** | Light pink | 14 | Green |

This high mobility suggests relatively low polarity of the anthocyanin species in their flavylium cation form, which interacts weakly with the polar silica gel stationary phase. The sharpness and intensity of this band indicate a predominant anthocyanin component, likely cyanidin-3-sophoroside, which is known to be the major anthocyanin in roselle calyces.

The clarity and reproducibility of these color transitions make roselle anthocyanins excellent candidates for pH indicator applications, particularly in the pH range 2–12 where distinct and visually differentiable colors are produced. The smooth color gradient from pink through colorless to green encompasses the physiological pH range and common environmental pH values, making these extracts potentially useful as educational pH indicators, food freshness indicators or environmental monitoring sensors.

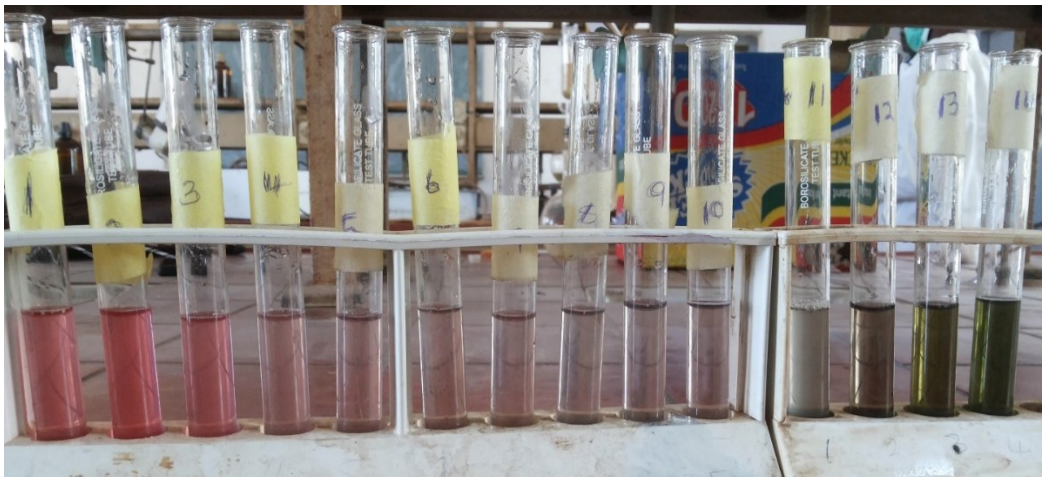

**Fig. 2. Visual appearance of *Hibiscus sabdariffa* anthocyanin extract across pH values 1–14, illustrating progressive chromophoric transitions.**

### *3.4 UV–Visible Spectral Characterization*

The natural origin and non-toxicity of these pigments are additional advantages for such applications, particularly in contexts where synthetic dyes may be undesirable or prohibited.

UV–visible spectrophotometric analysis revealed a characteristic absorption maximum around 520 nm under acidic conditions (pH < 4), confirming the presence of cyanidin-type anthocyanins (Ifie *et al.,* 2020; Cissé *et al.,* 2021). The wavelength of maximum absorption (λmax) is diagnostic of the anthocyanidin aglycone structure, with cyanidin derivatives typically absorbing in the range 510–535 nm, delphinidin derivatives at 520–545 nm and pelargonidin derivatives at 490–520 nm. The observed λmax of approximately 520 nm in acidic solution is therefore consistent with cyanidin-3-sophoroside and related cyanidin glycosides as the predominant pigments.

At pH 2, the extract exhibited the highest absorbance value (0.734 ± 0.015), reflecting maximum concentration of the colored flavylium cation form. The high absorbance intensity at this pH is consistent with the visual observations of intense pink coloration. At pH 1, the absorbance was slightly lower (0.451 ± 0.012), possibly due to partial protonation-induced aggregation or salting-out effects at very high ionic strength. This slight reduction in absorbance at extremely low pH has been reported previously for some anthocyanin systems and may reflect non-ideal behavior under highly acidic conditions.

Table 3. **Absorbance values of *H. sabdariffa* anthocyanin extract measured at 520 nm under different pH conditions.**

| pH | Absorbance | pH | Absorbance |
|---|---|---|---|
| **1** | 0.451 | 8 | 0.571 |
| **2** | 0.734 | 9 | 0.547 |
| **3** | 0.513 | 10 | 0.510 |
| **4** | 0.534 | 11 | 0.768 |
| **5** | 0.349 | 12 | 0.463 |
| **6** | 0.317 | 13 | 0.333 |
| **7** | 0.388 | 14 | 0.462 |

As pH increased beyond 4, significant variations in absorbance intensity were observed, reflecting structural transformations of the anthocyanin chromophore system. At pH 5–7, the absorbance decreased markedly (0.349, 0.317 and 0.388 respectively), corresponding to the formation of colorless carbinol and chalcone species. The minimum absorbance was observed at pH 6 (0.317 ± 0.008), representing the pH at which the equilibrium is most strongly shifted toward the colorless forms. This pH corresponds to the isoelectric point region where anthocyanins are

least stable and most susceptible to degradation.

At alkaline pH (8–14), the absorbance values increased again, although the wavelength of maximum absorption shifted to longer wavelengths (bathochromic shift), reflecting the formation of quinoidal bases. At pH 11, a pronounced increase in absorbance was observed (0.768 ± 0.019), corresponding to the formation of intensely colored quinoidal anions. This high absorbance is consistent with the visual observation of intense green coloration. At pH 12–14, the absorbance decreased somewhat (0.463, 0.333 and 0.462 respectively), possibly reflecting degradation or further structural modifications under extreme alkaline conditions.

These spectral variations correspond to transitions between flavylium, quinoidal and chalcone forms, demonstrating the strong pH sensitivity of the extracted anthocyanins. The systematic changes in absorption intensity and wavelength maxima as a function of pH provide quantitative support for the visual color observations and confirm the reversible structural interconversions characteristic of anthocyanins. The spectrophotometric data also provide a basis for developing calibration curves for pH determination using roselle anthocyanins as natural indicators.

### *3.5 Comparison with Literature Values*

The results obtained in this study are in good agreement with values reported in the literature for *Hibiscus sabdariffa* anthocyanins. The total anthocyanin content of 185.6 mg C3G/100 g dry weight falls within the range of 150–250 mg/100 g reported by various researchers for roselle calyces from different geographic origins (Cissé *et al.,* 2021; Ifie *et al.,* 2020). Variations in anthocyanin content among studies can be attributed to differences in cultivar, growing conditions, harvest time, post-harvest handling and analytical methods. The extraction yield of 12.4 % is also consistent with literature reports for methanolic extracts of roselle, confirming the effectiveness of the extraction protocol employed.

The observed $\lambda_{max}$ of approximately 520 nm (Fig. 3) matches the values reported for cyanidin-3-sophoroside, which is known to be the predominant anthocyanin in roselle calyces.

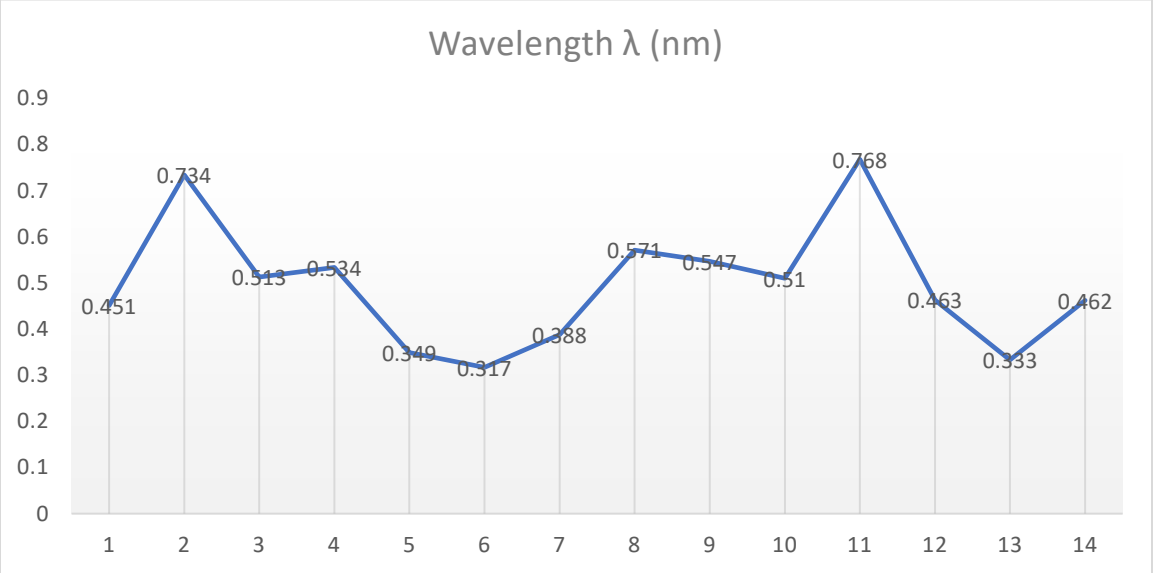


**Fig. 3. UV–visible absorbance spectra of *H. sabdariffa* anthocyanin extract at different pH values, showing pH-dependent spectral shifts and intensity changes.**

Previous HPLC-MS studies have identified cyanidin-3-sophoroside as comprising 70–85 % of total anthocyanins in *H. sabdariffa*, with cyanidin-3-sambubioside and delphinidin-3-sambubioside as minor components. While the simple analytical techniques employed in this study do not permit resolution and quantification of individual anthocyanins, the spectral and chromatographic behavior observed is fully consistent with a cyanidin-dominant anthocyanin profile.

### *3.6 Integrated Interpretation and Analytical Significance*

The agreement between chromatographic behavior, visible color transitions and UV–visible spectral responses provides strong evidence for the successful extraction and characterization of anthocyanins from *Hibiscus sabdariffa* calyces. The consistency of the data across multiple analytical techniques highlights the reliability of the extraction method and confirms the suitability of roselle calyces as an accessible source of anthocyanin pigments for analytical applications.

The high total anthocyanin content obtained is consistent with the pronounced pH-dependent colorimetric and spectral responses observed, confirming that the extracted pigments are anthocyanin-dominant and chemically intact. The reproducibility of extraction yields, Rf values and spectrophotometric measurements across replicate experiments demonstrates the robustness of the analytical procedures and supports their applicability in resource-limited laboratory settings.

From a practical standpoint, this work demonstrates that meaningful chemical characterization of natural pigments can be achieved without reliance on expensive instrumentation such as HPLC-MS or NMR spectroscopy. The combination of simple extraction, TLC separation and UV–visible spectrophotometry provides sufficient information to confirm the identity and purity of anthocyanin extracts, assess their pH-responsive properties and evaluate their potential for various applications. This approach is particularly valuable for educational laboratories, where students can gain hands-on experience with natural product chemistry, chromatographic separations and spectroscopic analysis using accessible and affordable techniques.

### *3.7 Potential Applications and Future Directions*

The anthocyanin extracts characterized in this study exhibit properties that make them suitable for various practical applications. The intense, pH-dependent coloration and reversible structural transitions suggest potential use as natural pH indicators in educational, analytical and industrial contexts. Unlike synthetic indicators, which may pose environmental or health concerns, roselle anthocyanins are derived from a food-grade plant source and are generally recognized as safe. Their use as pH indicators in school chemistry laboratories, food quality monitoring or environmental testing could provide sustainable and non-toxic alternatives to conventional synthetic dyes.

In the food industry, roselle anthocyanins could serve as natural colorants for acidic beverages, confectionery, dairy products and other applications where intense red coloration is desired. Their antioxidant properties may provide additional functional benefits beyond color, potentially extending shelf life and enhancing nutritional value. However, their poor stability at neutral and alkaline pH limits their use in certain product formulations and strategies for color stabilization through complexation, co-pigmentation or encapsulation would be needed for broader applicability.

Recent interest in smart packaging has highlighted the potential of anthocyanins as freshness indicators for perishable foods. Changes in pH associated with microbial spoilage, protein degradation or fat oxidation could be detected through visible color changes in anthocyanin-containing indicator films or

labels. Such systems would provide consumers with direct visual evidence of food quality without requiring specialized equipment or training.

Future research directions building on this work could include optimization of extraction conditions to maximize anthocyanin yield and stability, development of purification methods to isolate individual anthocyanin species, investigation of complexation or co-pigmentation strategies for color stabilization, encapsulation studies to protect anthocyanins from degradation, and fabrication of anthocyanin-based indicator films or sensors for food packaging applications. Additionally, comparative studies examining anthocyanin profiles and properties of roselle varieties from different geographic origins could provide insights into cultivar selection for specific applications.

## 4.0 Conclusion

This study successfully demonstrated the extraction and analytical characterization of anthocyanins from *Hibiscus sabdariffa* calyces using simple and accessible laboratory techniques. Methanol was identified as a superior extraction solvent, yielding a stable violet–red extract with enhanced pigment preservation compared to ethanol. The methanolic extraction produced a crude anthocyanin-rich extract at 12.4 ± 0.6 % yield based on dry calyx weight, with a total anthocyanin content of 185.6 ± 4.2 mg C3G/100 g, confirming roselle as an excellent source of natural anthocyanin pigments.

Thin-layer chromatographic analysis revealed distinct anthocyanin bands with pH-dependent retention behavior, providing valuable information about the polarity and structural transformations of the extracted pigments. The Rf values obtained were consistent with cyanidin-based anthocyanins, supporting the known anthocyanin profile of roselle calyces. UV–visible spectrophotometric measurements confirmed the presence of cyanidin-type anthocyanins through characteristic absorption around 520 nm in acidic media.

The pronounced and reversible color transitions observed across a wide pH range highlight the intrinsic pH sensitivity of the extracted anthocyanins and reflect well-established structural interconversions between flavylium, quinoidal and chalcone forms. The systematic changes in color from pink (acidic) through colorless (neutral) to green (alkaline) demonstrate the excellent pH-sensing properties of roselle anthocyanins and support their potential use as natural indicators in educational, analytical and commercial applications.

The strong agreement between chromatographic, colorimetric and spectroscopic data underscores the reliability of the experimental approach and validates the effectiveness of low-cost analytical methods for anthocyanin characterization. This work demonstrates that meaningful chemical insights can be obtained without reliance on advanced instrumentation, making these techniques particularly suitable for resource-limited laboratory settings in developing countries and for educational purposes where students can gain practical experience with natural product chemistry.

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**Declaration**

**Consent for publication**

Not Applicable

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The publisher has the right to make the data public

**Conflict of Interest**

The authors declared no conflict of interest

**Ethical Considerations**

Not applicable

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**Authors' Contributions**

J. D. and O. O. A. conceptualized and designed the study. A. H. I., H. A., and W. M. I. conducted solvent extraction and sample preparation. M. M., Y. S. T., and J. I. S. performed TLC and pH-dependent colorimetric analyses. B. L., A. E. O., D. M., and H. A. G. contributed to UV–visible spectral measurements, data analysis, and manuscript drafting. All authors reviewed and approved the final manuscript.