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# ANALYTICAL PERFORMANCE EVALUATION OF COLORIMETRIC METHODS FOR ESTIMATING INDOLE-3-ACETIC ACID, KINETIN, AND GIBBERELLIC ACID IN PLANTAIN (*MUSA × PARADISIACA* L.) TISSUES

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## ABSTRACT

This study evaluated the performance of three colorimetric procedures for estimating indole-3-acetic acid (IAA), gibberellic acid ($GA_3$), and kinetin in purified extracts of plantain corms (*Musa × paradisiaca* L.). The extracts were purified using C18 solid-phase extraction cartridges, and colorimetric responses were measured at 530, 540, and 570 nm, respectively. The retained calibration ranges were 0–40 µg $mL^{-1}$ for IAA, 0–60 µg $mL^{-1}$ for $GA_3$, and 0–80 µg $mL^{-1}$ for kinetin, with coefficients of determination ($R^2$) of 0.9976, 0.9886, and 0.9910, respectively. The estimated limits of detection (LOD) were 5.24, 8.26, and 11.71 µg $mL^{-1}$, while the limits of quantification (LOQ) were 15.88, 25.03, and 35.48 µg $mL^{-1}$, respectively. Repeatability and intermediate precision were assessed using coefficients of variation. Recovery tests showed variable results depending on the compound and spiking level. Some calculated concentrations, particularly for IAA and kinetin, exceeded the upper calibration limits and were therefore considered extrapolated estimates. The lack of demonstrated molecular selectivity limits the specific interpretation of the colorimetric responses obtained. These procedures may be used for relative or semi-quantitative comparisons, provided that the established calibration ranges are respected and results are expressed as equivalents of the corresponding standards. However, they cannot replace chromatographic methods when specific identification and quantification are required.

**Keywords:** Colorimetry; Phytohormones; Analytical performance; Semi-quantitative estimation; *Musa × paradisiaca L.*; C18-SPE.

## 1 INTRODUCTION

Phytohormones are a group of signaling molecules that, at low concentrations, regulate plant growth, development, and adaptive responses. Among them, auxins, cytokinins, and gibberellins play major roles in the regulation of key developmental processes, including cell division and elongation, meristematic activity, organogenesis, apical dominance, bud growth, and organ elongation. Quantitative analysis of these compounds and their spatiotemporal variations therefore represents an important approach for understanding the physiological mechanisms associated with plant development [1,2].

In banana and plantain (*Musa* spp.), vegetative propagation relies largely on the activation and development of meristems and buds associated with the corm. Understanding the physiological mechanisms underlying these processes requires, among other approaches, monitoring changes in growth regulators in the tissues involved. However, phytohormone analysis in plant matrices remains methodologically challenging because of their low abundance, the diversity of their physicochemical properties, and the presence of numerous endogenous compounds that may interfere with their extraction or detection [2]. Consequently, extraction, purification, and detection steps are critical for the reliability of the estimates obtained.

Advances in chromatography coupled with mass spectrometry have considerably improved the sensitivity and selectivity of phytohormone analysis. Methods based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) now allow the simultaneous quantification of several hormone classes from small amounts of plant tissue and are considered approaches of choice for hormone profiling [3,4]. Their separation capacity and ability to identify analytes make these techniques particularly suitable for complex plant matrices. However, their implementation requires access to specialized instrumentation, appropriate standards, and specific analytical expertise. In laboratories where such equipment is not routinely available, more accessible spectrophotometric methods may still be useful for certain applications, provided that their performance and limitations are adequately characterized.

Several historical colorimetric methods have been developed for the estimation of plant growth regulators. Gordon and Weber [5] proposed a colorimetric method for estimating indole-3-acetic acid (IAA) based on the development of a color reaction in the presence of a ferric reagent under strongly acidic conditions. Atkin and Wain [6] developed a colorimetric method for estimating kinetin, notably to overcome difficulties associated with ultraviolet-absorbing compounds present in plant extracts. Graham and Thomas [7], in turn, described a method for estimating gibberellic acid ($GA_3$) based on its reaction with 2,4-dinitrophenylhydrazine and the subsequent formation of a color measurable by spectrophotometry. These methods offer operational advantages owing to their simplicity and their reliance on UV-visible spectrophotometry. However, they lack the separation capability of chromatographic methods, and their responses may be affected by other matrix constituents. This limitation is particularly documented for the Salkowski reagent: Glickmann and Dessaux [8] showed that different formulations of this reagent responded not only to IAA but also to other indolic compounds, such as indole-3-pyruvic acid and indole-3-acetamide. Therefore, a colorimetric response cannot, without qualification, be equated with the specific molecular identification of the analyte.

Under these conditions, the usefulness of a colorimetric method for the analysis of plant matrices depends less on its simplicity alone than on the experimental characterization of its performance for the intended application. General principles of analytical validation recommend examining the relationship between concentration and analytical response, the measurement range, precision, accuracy, and detection and quantification capabilities, while also considering potential matrix interferences. In this regard, the ICH Q2(R2) guideline, although developed within a pharmaceutical regulatory framework, provides a useful conceptual framework based on the principle that an analytical procedure should be fit for its intended purpose ICH [9]. For colorimetric methods applied to phytohormones, such an assessment is particularly important to distinguish what the results can actually estimate from what would require identification or confirmation using a more selective separation-based method.

The present study aimed to assess the performance and applicability range of three colorimetric procedures for estimating indole-3-acetic acid (IAA), kinetin, and gibberellic acid ($GA_3$) in purified extracts of plantain tissues, in a context where advanced chromatographic techniques are not routinely available. Specifically, the study aimed to: (i) characterize the concentration–response relationship, calibration range, estimated sensitivity, and precision of the three procedures; (ii) assess their recovery following spiking of plant extracts; and (iii) define the interpretative limitations of the equivalent concentrations obtained, particularly in the absence of structural identification of the analytes.

## 2 MATERIAL AND METHODS

### 2.1 Plant material

The plant material consisted of stem fragments (corms) obtained from sword suckers of the plantain cultivar Big Ebanga (*Musa* × *paradisiaca* L.). The suckers were collected from a stool plantation located in Azaguié, southern Côte d'Ivoire (5°39′10.73″ N and 4°05′43.67″ W). After preparation according to the principle of macropropagation through activation of latent buds from stem fragments, as described for plantain [10,11], the explants were maintained in a propagation bed and sampled during vegetative regeneration for the evaluation of the colorimetric procedures.

### 2.2 Reagents and preparation of standard solutions

The standards used were indole-3-acetic acid (IAA; CAS No. 87-51-4; purity 98%), kinetin (CAS No. 525-79-1; purity 99%), and gibberellic acid ($GA_3$; CAS No. 77-06-5; purity 90%). For each compound, a 1,000-µg $mL^{-1}$ stock solution was prepared in the appropriate solvent: ethanol for IAA and $GA_3$, and methanol for kinetin. Serial dilutions were subsequently prepared to obtain the solutions used to establish the calibration curves.

For IAA, six concentration levels were initially investigated: 0, 5, 10, 20, 40, and 60 µg $mL^{-1}$. Because a marked deviation from linearity was observed at 60 µg $mL^{-1}$, the 0–40 µg $mL^{-1}$ range was retained for fitting the linear model. For $GA_3$, the concentrations investigated were 0, 5, 10, 20, 40, and 60 µg $mL^{-1}$. For kinetin, the concentrations were 0, 10, 20, 40, 60, and 80 µg $mL^{-1}$. Each concentration level was analyzed in triplicate.

### 2.3 Extraction and purification of plant extracts

Five grams of fresh plant tissue were homogenized in 10 mL of extraction solvent. For IAA and kinetin, extraction was performed using 80% ethanol acidified with 1% acetic acid. $GA_3$ was extracted using 80% methanol. During extraction, 0.5 g of polyvinylpolypyrrolidone (PVPP) was added to reduce the potential influence of phenolic compounds in the plant matrix.

The homogenates were then filtered through Whatman No. 1 filter paper and centrifuged at 3,000 rpm for 10–15 min. The supernatants were purified by solid-phase extraction using C18 cartridges (SPE-C18). The cartridges were sequentially conditioned with 80% methanol and water acidified with 1% acetic acid (pH 3). After loading the extract, the cartridges were rinsed with acidified water to reduce the presence of hydrophilic matrix constituents. The retained compounds were then eluted with HPLC-grade methanol. The eluates were concentrated by evaporation in a water bath at 40 °C and subsequently reconstituted in a small volume of absolute ethanol before colorimetric analysis.

### 2.4 Colorimetric procedures

#### 2.4.1 Indole-3-Acetic Acid

IAA was estimated using a colorimetric reaction with Salkowski reagent, adapted from Gordon and Weber [5]. One milliliter of standard solution or purified extract was mixed with 2 mL of modified Salkowski reagent, consisting of 1 mL of 0.5 M $FeCl_3$ in 49 mL of 98% concentrated sulfuric acid. After incubation in the dark for 30 min, absorbance was measured at 530 nm against the reagent blank. Extract concentrations were expressed as IAA equivalents based on the calibration equation obtained using the pure standard.

#### 2.4.2 Gibberellic Acid

$GA_3$ was estimated using a colorimetric reaction with 2,4-dinitrophenylhydrazine (DNPH). The 0.5% DNPH reagent in an acidic medium was added to the standard solution or purified extract. After heating at a temperature between 80 and 100 °C for 5 min, a 10% alcoholic KOH solution was added to allow color development. Absorbance was measured at 540 nm against the reagent blank. The results obtained for the extracts were expressed as $GA_3$ equivalents based on the calibration curve established using the corresponding standard.

#### 2.4.3 Kinetin

Kinetin was estimated according to the colorimetric procedure actually used in the laboratory, based on a reaction with ninhydrin in the presence of glacial acetic acid. The resulting color was measured at 570 nm against the reagent blank. Standards and purified extracts were treated identically during each analytical series, and extract concentrations were expressed as kinetin equivalents based on the corresponding calibration curve.

However, the available experimental records do not allow the exact reagent volumes, ninhydrin concentration, or the duration and temperature of color development to be reconstructed with certainty. Therefore, none of these parameters was retrospectively reconstructed in the present manuscript.

### 2.5 Evaluation of analytical performance

The performance of the three procedures was evaluated based on the general principles for the characterization of analytical procedures described in the ICH Q2(R2) guideline. This reference was used as a methodological framework and not as a claim of regulatory validation of the procedures. The characteristics examined were: (i) the concentration–response relationship and calibration range; (ii) estimated limits of detection and quantification; (iii) within-day repeatability; (iv) intermediate precision across days; and (v) recovery following spiking of plant extracts.

#### 2.5.1 Concentration–response relationship and calibration range

For each compound, the absorbance obtained at each concentration level was determined in triplicate. The results were expressed as the mean of the three measurements, and the relationship between concentration and absorbance was fitted by linear regression according to the equation:

$$y = ax + b \quad (1)$$

where y represents absorbance, $x$ represents the standard concentration (µg $mL^{-1}$), a represents the slope, and b represents the intercept.

The descriptive quality of the fit was characterized using the coefficient of determination ($R^2$), without considering it as a sufficient criterion on its own. The calibration ranges used for quantitative estimation were 0–40 µg $mL^{-1}$ for IAA, 0–60 µg $mL^{-1}$ for $GA_3$, and 0–80 µg $mL^{-1}$ for kinetin. For IAA, the 60-µg $mL^{-1}$ level, which was initially investigated, was retained as an experimental observation but excluded from the regression used for calibration because of a marked deviation from the linear behavior observed at lower concentrations.

#### 2.5.2 Estimation of the limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were estimated from the residual dispersion around the regression model and the slope of the calibration curve. The parameter $S_Y/_x$ corresponded to the residual standard deviation of the individual observations around the regression line. The estimates were calculated using the following equations:

$$LOD = \frac{3.3S_{y/x}}{a} \quad (2)$$

$$LOQ = \frac{10S_{y/x}}{a} \quad (3)$$

where $S_{y/x}$ is the residual standard deviation of the regression model and a is the slope of the calibration curve. The resulting values are referred to as estimated LOD and LOQ, as no independent experimental confirmation was performed at the calculated concentrations.

#### 2.5.3 Within-day repeatability

Repeatability was assessed using independent measurements performed on the same day under the same analytical conditions. For IAA, the unspiked extract and extracts spiked at 10, 20, and 40 µg $mL^{-1}$ were considered. For $GA_3$, the unspiked extract and extracts spiked at 10 and 20 µg $mL^{-1}$, for which corresponding within-day measurements were available, were used. For kinetin, the unspiked extract and extracts spiked at 10, 20, and 30 µg $mL^{-1}$ were evaluated. The variability among replicate determinations was expressed as the coefficient of variation (CV). When the calculated equivalent concentration exceeded the calibration range, the CV was retained as an indicator of the repeatability of the analytical response, whereas the corresponding concentration was considered an extrapolated value.

$$CV\ (\%) = \frac{s}{\bar{x}} \times 100 \quad (4)$$

where $s$ represents the standard deviation and $\bar{x}$ represents the mean of the determinations.

#### 2.5.4 Intermediate precision across days

Intermediate precision was assessed using measurements performed over three consecutive days (D1, D2, and D3). Extraction solutions and reagents were prepared on each day of analysis. For each concentration level studied, the mean obtained for each day was calculated, and variability across the three days was expressed as the coefficient of variation of the daily means. Values falling outside the calibration range were interpreted only as indicators of the stability of the analytical response and not as reliable quantitative measurements.

### 2.5.5 Recovery after Spiking

Recovery was assessed using the spike-recovery method on plant extracts that had undergone the extraction and C18-SPE purification procedure. For IAA and $GA_3$, three spiking levels were investigated: 10, 20, and 40 µg $mL^{-1}$. For kinetin, the spiking levels were 10, 20, and 30 µg $mL^{-1}$. For each spiking level, recovery was calculated using the following equation:

$$R\ (\%) = \frac{C_{spiked} - C_{initial}}{C_{added}} \times 100 \qquad (5)$$

where $C_{initial}$ corresponds to the equivalent concentration measured in the unspiked extract, $C_{spiked}$ to the concentration measured after addition of the standard, and $C_{added}$ to the theoretical concentration added.

Recovery was evaluated separately at each spiking level. Quantitative interpretation was limited to levels for which the calculated equivalent concentration after spiking remained within the calibration range of the procedure. When the calculated concentration exceeded the upper calibration limit, the corresponding concentration was considered an extrapolated value, and the resulting recovery was reported for exploratory purposes only.

### 2.5.6 Treatment of values outside the calibration range

Equivalent concentrations were calculated from the regression equations established using the corresponding pure standards. Values falling within the respective calibration range were considered suitable for quantitative interpretation. When a calculated equivalent concentration exceeded the upper limit of the calibration range, it was identified as an extrapolated estimate. Such extrapolated values were not considered reliable for quantitative interpretation and were therefore not used as evidence of the quantitative accuracy of the corresponding procedure.

### 2.6 Colorimetric response of purified plant extracts

Unspiked purified plant extracts were analyzed under the same analytical conditions as the corresponding standards. For each compound, the absorbance of the extract was measured in triplicate and compared with the reagent blank and the response range established using the pure standard. The results were expressed as equivalent concentrations of the corresponding standard. Concentrations falling within the calibration range were considered suitable for quantitative interpretation, whereas values at or beyond the upper limit of the calibration range were interpreted cautiously and classified as extrapolated estimates in accordance with Section 2.5.6.

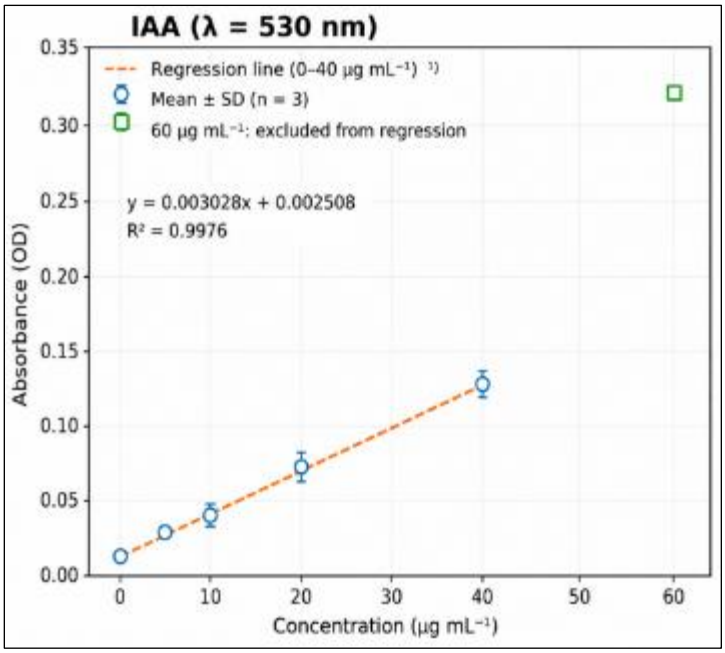
### 2.7 Data analysis

Calibration curve parameters were determined by linear regression using the least-squares method. For each calibration level, triplicate measurements were used to calculate the mean and standard deviation. Within-day repeatability and intermediate precision across days were expressed as coefficients of variation (CV). Recovery was calculated separately for each spiking level. Values calculated beyond the calibration range were identified as extrapolated estimates and excluded from conclusions regarding the quantitative accuracy of the corresponding procedure. Experimental data were recorded and organized using Microsoft Excel 2019, while numerical analyses were performed using Python version 3.10.

## 3 RESULTS

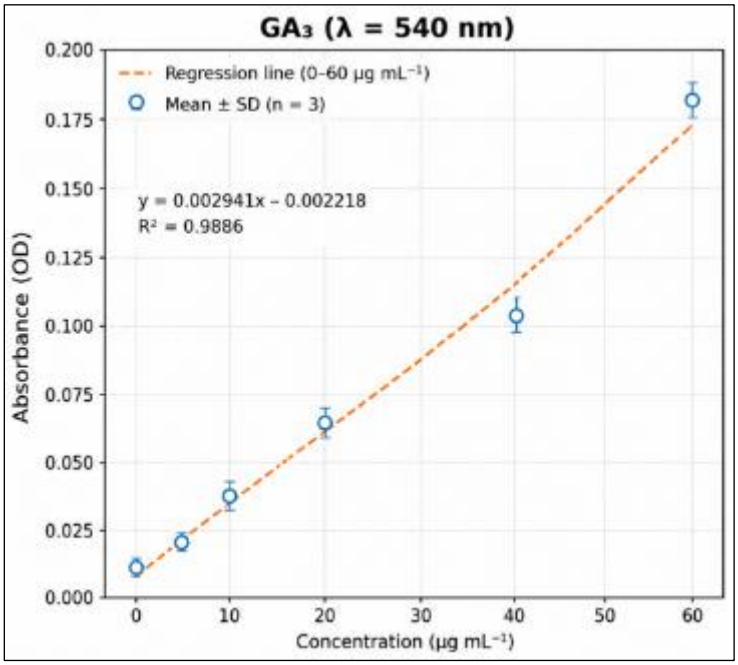
### 3.1 Concentration–response relationship and calibration ranges

An increase in absorbance with increasing standard concentration was observed for the three colorimetric procedures (Figures 1–3). For IAA, a linear relationship was observed over the 0–40 µg $mL^{-1}$ range, with the regression equation ($y = 0.003028x + 0.002508$) and a coefficient of determination of ($R^2 = 0.9976$) (Figure 1). The 60-µg $mL^{-1}$ concentration, which was initially investigated, was excluded from the linear regression because of its marked deviation from the linear relationship observed at lower concentrations. For $GA_3$, the response over the 0–60 µg $mL^{-1}$ range was described by the regression equation ($y = 0.002941x - 0.002218$), with ($R^2 = 0.9886$) (Figure 2). For kinetin, the relationship over the 0–80 µg $mL^{-1}$ range was described by the regression equation ($y = 0.001088x + 0.004905$), with ($R^2 = 0.9910$) (Figure 3). The calibration slopes were 0.003028 for IAA, 0.002941 for $GA_3$, and 0.001088 for kinetin. The corresponding coefficients of determination were 0.9976, 0.9886, and 0.9910, respectively.



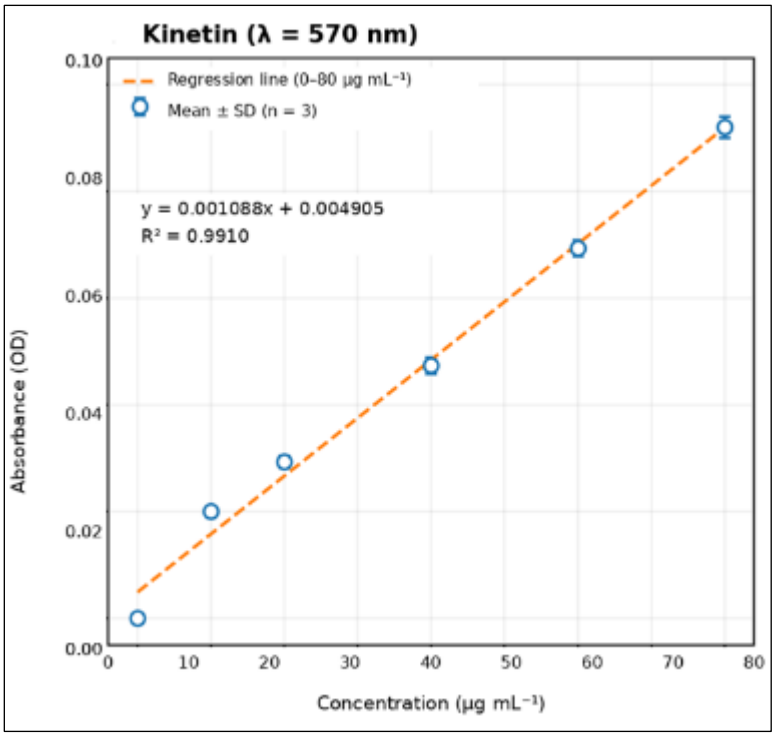

Points represent the mean values ± SD of three measurements (n = 3). The regression line was fitted over the 0–40 μg $mL^{-1}$ range. The 60-μg $mL^{-1}$ concentration was excluded from the regression.

**Figure 1: Relationship between IAA standard concentration and absorbance**




Points represent the mean values ± SD of three measurements (n = 3). The regression line was fitted over the 0–60 μg $mL^{-1}$ range.

**Figure 2: Relationship between $GA_3$ standard concentration and absorbance.**




Points represent the mean values ± SD of three measurements (n = 3). The regression line was fitted over the 0–80 µg $mL^{-1}$ range

**Figure 3: Relationship between kinetin standard concentration and absorbance.**

### 3.2 Estimated analytical sensitivity

The estimated limits of detection and quantification differed among the three procedures (Table 1). For IAA, the estimated LOD and LOQ were 5.24 and 15.88 µg $mL^{-1}$, respectively. For $GA_3$, the corresponding values were 8.26 and 25.03 µg $mL^{-1}$, respectively. For kinetin, the estimated LOD and LOQ were 11.71 and 35.48 µg $mL^{-1}$, respectively. The slopes of the concentration–response relationships were 0.003028 for IAA, 0.002941 for $GA_3$, and 0.001088 for kinetin. The main analytical characteristics of the three colorimetric procedures are presented in Table 1.

**Table 1: Main analytical characteristics of the three colorimetric procedures**

| Parameter | IAA | $GA_3$ | Kinetin |
|---|---|---|---|
| Wavelength (nm) | 530 | 540 | 570 |
| Explored range (µg $mL^{-1}$) | 0–60 | 0–60 | 0–80 |
| Retained calibration range (µg $mL^{-1}$) | 0–40 | 0–60 | 0–80 |
| Slope | 0.003028 | 0.002941 | 0.001088 |
| Intercept | 0.002508 | −0.002218 | 0.004905 |
| $R^2$, based on triplicate means | 0.9976 | 0.9886 | 0.9910 |
| $R^2$, individual observations | 0.9892 | 0.9877 | 0.9861 |
| $S_{y/x}$ | 0.00481 | 0.00736 | 0.00386 |
| Estimated LOD (µg $mL^{-1}$) | 5.24 | 8.26 | 11.71 |
| Estimated LOQ (µg $mL^{-1}$) | 15.88 | 25.03 | Kinetin |

$R^2$, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification; IAA, indole-3-acetic acid; $GA_3$, gibberellic acid; $S_{Y/x}$, residual standard deviation of the individual observations around the regression line.

### 3.3 Within-day repeatability

Within-day variability was generally low for the three procedures (Table 2). For IAA, CVs ranged from 1.59 to 3.48%. The unspiked extract (22.24 µg $mL^{-1}$) and the +10-µg $mL^{-1}$ spiking level (30.86 µg $mL^{-1}$) were within the calibration range. The concentrations calculated at the +20 and +40 µg $mL^{-1}$ spiking levels (40.32 and 56.11 µg $mL^{-1}$, respectively) slightly or markedly exceeded its upper limit and were therefore considered extrapolated for quantitative

interpretation. For $GA_3$, CVs were 3.94, 3.50, and 3.25% for the unspiked extract and the +10 and +20 µg $mL^{-1}$ spiking levels, respectively; the corresponding equivalent concentrations (33.31, 45.91, and 56.98 µg $mL^{-1}$) remained within the 0–60 µg $mL^{-1}$ range. For kinetin, the unspiked extract showed a concentration of 80.13 µg $mL^{-1}$ (CV = 5.26%), corresponding to the upper limit of the calibration range. The concentrations calculated after spiking (90.72 to 112.84 µg $mL^{-1}$) exceeded this range; their CVs, which ranged from 1.73 to 4.16%, therefore provide information on the repeatability of the analytical response but not on the accuracy of the extrapolated concentrations.

**Table 2: Within-day repeatability of the three colorimetric procedures**

| Compound | Spiking level (µg $mL^{-1}$) | Mean equivalent concentration (µg $mL^{-1}$) | SD | CV (%) |
|---|---|---|---|---|
| IAA | 0 | 22.24 | 0.77 | 3.48 |
| | 10 | 30.86 | 0.77 | 2.51 |
| | 20 | 40.32* | 0.64 | 1.59 |
| | 40 | 56.11* | 0.92 | 1.63 |
| $GA_3$ | 0 | 33.31 | 1.31 | 3.94 |
| | 10 | 45.91 | 1.60 | 3.50 |
| | 20 | 56.98 | 1.85 | 3.25 |
| Kinetin | 0 | 80.13† | 4.21 | 5.26 |
| | 10 | 90.72* | 3.37 | 3.71 |
| | 20 | 101.31* | 4.21 | 4.16 |
| | 30 | 112.84* | 1.95 | 1.73 |

* Calculated concentration outside the calibration range; the CV provides information on the repeatability of the analytical response, whereas the concentration is extrapolated. † Value at the upper limit of the calibration range.; IAA, indole-3-acetic acid; $GA_3$, gibberellic acid; SD, standard deviation; CV, coefficient of variation.

### 3.4 Intermediate precision across days

Intermediate precision over three consecutive days showed variability depending on the compound and the level tested (Table 3). For IAA, the between-day CVs were 0.63, 0.27, and 0.90% for the unspiked extract and the +10 and +20 µg $mL^{-1}$ spiking levels, respectively; the +40-µg $mL^{-1}$ spiking level showed a CV of 11.04%, with calculated concentrations outside the calibration range.

**Table 3: Intermediate precision of the procedures over three consecutive days**

| Compound | Spiking level (µg $mL^{-1}$) | Day 1 | Day 2 | Day 3 | Between-day CV (%) |
|---|---|---|---|---|---|
| IAA | 0 | 21.68 | 21.41 | 21.52 | 0.63 |
| | 10 | 30.20 | 30.09 | 30.25 | 0.27 |
| | 20 | 39.25 | 39.32 | 38.68 | 0.90 |
| | 40 | 56.36* | 55.83* | 46.02* | 11.04 |
| $GA_3$ | 0 | 20.17 | 21.94 | 20.06 | 5.09 |
| | 10 | 30.54 | 32.31 | 28.42 | 6.40 |
| | 20 | 38.91 | 39.68 | 37.80 | 2.44 |
| | 40 | 55.38 | 56.15 | 54.26 | 1.72 |
| Kinetin | 0 | 74.91† | 79.00† | 75.45† | 2.91 |
| | 10 | 88.73* | 87.91* | 85.32* | 2.04 |
| | 20 | 98.73* | 97.91* | 103.32* | 2.92 |
| | 30 | 108.42* | 105.91* | 110.17* | 1.98 |

*Calculated values outside the calibration range; the CVs are reported as indicators of between-day stability of the analytical response. †Values within the calibration range but close to its upper limit. IAA, indole-3-acetic acid; $GA_3$, gibberellic acid; CV, coefficient of variation.

For $GA_3$, CVs ranged from 1.72 to 6.40%, and the calculated concentrations remained within the calibration range. For kinetin, between-day CVs ranged from 1.98 to 2.92%. The unspiked extract was close to the upper calibration limit, whereas the spiked levels exceeded this limit; these CVs were therefore considered indicators of between-day stability of the analytical response rather than validation of the extrapolated concentrations.

### 3.5 Recovery after Spiking

Recovery values varied according to the compound and spiking level (Table 4). For IAA, the recoveries calculated at the 10 and 20 µg $mL^{-1}$ spiking levels were 70.70 and 74.40%, respectively, with spiked equivalent concentrations of 28.62 and 39.62 µg $mL^{-1}$, which were within the calibration range. At the + 40 µg $mL^{-1}$ spiking level, the calculated concentration reached 57.18 µg $mL^{-1}$; the corresponding recovery (84.93%) was therefore based on an extrapolated concentration and was retained for exploratory purposes only.

For $GA_3$, the three spiked equivalent concentrations (28.50, 39.37, and 53.62 µg $mL^{-1}$) remained within the 0–60 µg $mL^{-1}$ calibration range. The corresponding recovery values were 64.40, 86.55, and 78.90%, respectively.

For kinetin, the concentration of the unspiked extract was already close to the upper calibration limit (79.45 µg $mL^{-1}$), and all calculated concentrations after spiking (86.17–101.35 µg $mL^{-1}$) exceeded the 0–80 µg $mL^{-1}$ calibration range. The corresponding recovery values (67.20–78.00%) were therefore considered exploratory.

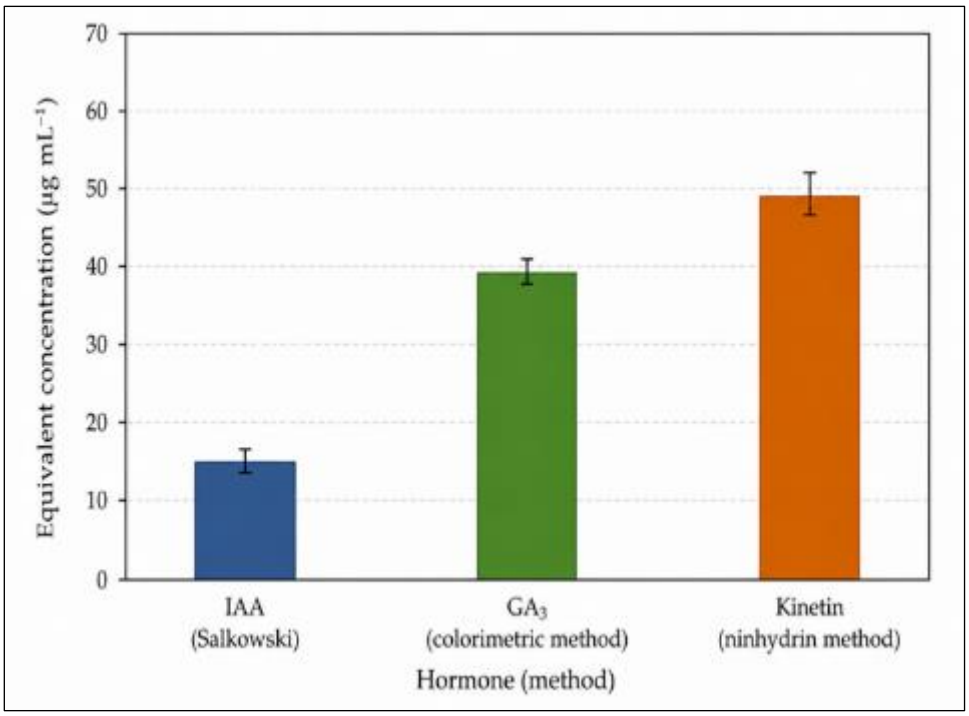
**Table 4: Recovery of standards added to purified plant extracts**

| Compound | Standard added (µg $mL^{-1}$) | $C_{initial}$ | $C_{spiked}$ | Recovery (%) |
|---|---|---|---|---|
| IAA | 10 | 21.55 | 28.62 | 70.70 |
| IAA | 20 | 24.74 | 39.62 | 74.40 |
| IAA | 40 | 23.21 | 57.18* | 84.93* |
| $GA_3$ | 10 | 22.06 | 28.50 | 64.40 |
| $GA_3$ | 20 | 22.06 | 39.37 | 86.55 |
| $GA_3$ | 40 | 22.06 | 53.62 | 78.90 |
| Kinetin | 10 | 79.45† | 86.17* | 67.20* |
| Kinetin | 20 | 79.45† | 95.05* | 78.00* |
| Kinetin | 30 | 79.45† | 101.35* | 73.00* |

*Calculated spiked concentration outside the calibration range; the corresponding recovery is reported for exploratory purposes only. †Initial concentration within the calibration range but close to its upper limit. IAA, indole-3-acetic acid; $GA_3$, gibberellic acid; Cspiked, equivalent concentration measured in the unspiked extract; Cspiked, equivalent concentration measured after standard addition.

### 3.6 Response of Purified Plant Extracts

Unspiked purified plant extracts produced a measurable colorimetric response with each of the three procedures (Figure 4). Under within-day repeatability conditions, the mean equivalent concentrations of IAA (22.24 µg $mL^{-1}$) and $GA_3$ (33.31 µg $mL^{-1}$) were within their respective calibration ranges. The equivalent concentration of kinetin (80.13 µg $mL^{-1}$) was at the upper limit of the investigated range (0–80 µg $mL^{-1}$) and should therefore be interpreted with particular caution.




Bars represent means ± standard deviation (n = 3). Concentrations are expressed as equivalents of the corresponding standards and do not constitute specific molecular quantification of the phytohormones. IAA: calibration range, 0–40 µg $mL^{-1}$; $GA_3$: calibration range, 0–60 µg $mL^{-1}$; kinetin: calibration range, 0–80 µg

**Figure 2: Equivalent concentrations of IAA, $GA_3$, and kinetin in purified plantain corm extracts.**

## 4 DISCUSSIONS

The three colorimetric procedures showed concentration–response relationships that could be described by linear regression within the experimentally retained calibration ranges. IAA exhibited the highest coefficient of determination ($R^2$ = 0.9976) over the 0–40 µg $mL^{-1}$ range, followed by kinetin ($R^2$ = 0.9910) and $GA_3$ ($R^2$ = 0.9886). However, high $R^2$ values alone are not sufficient to establish analytical validity. The deviation observed for IAA at 60 µg $mL^{-1}$ illustrates the need to define the calibration range experimentally under the specific conditions of each procedure. Glickmann and Dessaux [8] showed that the response of the Salkowski reagent depends on its formulation and is not specific to IAA. A recent comparative study also reported substantial discrepancies between Salkowski-based estimates and measurements obtained by chromatography coupled with mass spectrometry [12].

Estimated analytical sensitivity also differed among the procedures. IAA showed the lowest estimated LOD and LOQ (5.24 and 15.88 µg $mL^{-1}$, respectively), whereas kinetin showed the highest values (11.71 and 35.48 µg $mL^{-1}$). $GA_3$ showed intermediate values (8.26 and 25.03 µg $mL^{-1}$). These differences were accompanied by calibration slopes of 0.003028 for IAA, 0.002941 for $GA_3$, and 0.001088 for kinetin. Because LOD and LOQ were estimated from the residual standard deviation and calibration slope, both parameters contributed to the calculated values. These limits should nevertheless be regarded as estimates because no independent experimental confirmation was performed at the calculated concentrations.

Precision results showed that variability depended on the compound and concentration level. Within-day CVs ranged from 1.59 to 3.48% for IAA, 3.25 to 3.94% for $GA_3$, and 1.73 to 5.26% for kinetin. Between-day CVs ranged from 0.27 to 11.04% for IAA, 1.72 to 6.40% for $GA_3$, and 1.98 to 2.92% for kinetin. However, values calculated outside the calibration ranges require cautious interpretation. The corresponding CVs describe the variability of the analytical response under the tested conditions but cannot support the accuracy of extrapolated concentrations. Thus, precision should not be equated with accuracy.

Recovery also varied according to compound and spiking level. For IAA, recoveries within the calibration range were 70.70 and 74.40% at the +10 and +20 µg $mL^{-1}$ levels, respectively, whereas the 84.93% value at +40 µg $mL^{-1}$ was based on an extrapolated concentration. For $GA_3$, recoveries ranged from 64.40 to 86.55% and all calculated concentrations remained within the calibration range. For kinetin, recoveries ranged from 67.20 to 78.00%, but all spiked concentrations exceeded the upper calibration limit. The experimental design does not allow incomplete recoveries to be attributed to a specific step; they may reflect extraction, C18-SPE purification, matrix effects, or the colorimetric reaction itself.

The principal limitation of the three procedures is analytical selectivity. A linear response obtained with a pure standard does not demonstrate that the signal from a plant extract originates exclusively from the target phytohormone. This limitation is well documented for the Salkowski reagent, which can respond to several indolic compounds [8,12]. The same consideration applies to the ninhydrin-based kinetin procedure and, in the absence of chromatographic confirmation, to the $GA_3$ procedure. Therefore, concentrations obtained from purified extracts should be regarded as equivalent concentrations relative to the corresponding standards rather than unequivocal measurements of endogenous phytohormones.

An additional limitation concerns the kinetin procedure. Because the available experimental records did not contain all operational parameters of the ninhydrin reaction, its external reproducibility is less well documented than that of the IAA and $GA_3$ procedures.

Overall, the three procedures produced reproducible concentration-dependent colorimetric responses under controlled conditions, but their analytical scope is limited by the established calibration ranges, estimated LOD and LOQ values, extrapolated concentrations, and lack of molecular selectivity. They should therefore not be regarded as methods for absolute and specific quantification of endogenous IAA, $GA_3$, or kinetin in plantain tissues. Their use may nevertheless be relevant for relative or semi-quantitative comparisons when samples have the same matrix and are processed under strictly standardized conditions. For specific identification and quantification of endogenous phytohormones, chromatographic methods coupled with mass spectrometry remain more appropriate because of their higher selectivity and sensitivity [3,4].

## 5 CONCLUSION

This study showed that the three colorimetric procedures exhibited usable concentration–response relationships and generally satisfactory repeatability within the investigated ranges. However, incomplete recoveries, extrapolated values outside the established calibration ranges, and, most importantly, the lack of demonstrated molecular selectivity limit their use for the absolute and specific quantification of IAA, $GA_3$, and kinetin in plantain tissues. The procedures may nevertheless be useful for comparative or semi-quantitative estimation when samples of the same matrix are processed under strictly standardized conditions, the established calibration ranges are respected, and results are expressed as equivalents of the corresponding standards. When specific identification and quantification of endogenous phytohormones are required, more selective chromatographic approaches, particularly LC-MS/MS, are more appropriate. Further comparison with a reference instrumental method would help define more precisely the applicability and limitations of these colorimetric procedures.

## STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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The authors declare that they have no competing interests that could have influenced the design, conduct, analysis, or interpretation of the study.

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