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Determination Standardization and Validation of Optimized Spectrophotometric Assays for Total Antioxidant, Polyphenol, and Flavonoid Determination: A Kinetic and Methodological Framework

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Abstract

This study addresses persistent methodological inconsistencies in phytochemical assays by developing a harmonized, high-sensitivity framework for TAC, TPC, and TFC. Using the Ferric Bipyridyl Reducing Capacity (FBRC), Folin-Ciocalteu, and Aluminum Chloride methods, critical parameters—including maximum wavelength (λ_{max}), temperature, kinetics, pH, and stoichiometry—were systematically re-evaluated and optimized. . The novelty of this work lies in the transition from empirical "snapshot" measurements to kinetically optimized and AOAC-validated protocols that ensure inter-laboratory reproducibility. Optimized conditions for TAC were established at 520 nm, pH 4, and 30 minutes at 50°C. TPC was optimized at 750 nm (20 min at 50°C), while TFC achieved stability at 430 nm after 10 minutes at ambient temperature. Unlike conventional methods that often suffer from baseline drift, these refined protocols achieved superior sensitivity. Comprehensive validation in accordance with AOAC guidelines confirmed high precision, with repeatability (%RSD) ranges of 0.82–5.68% (TAC), 0.85–6.22% (TPC), and 0.29–5.09% (TFC). Linearity was established over concentration ranges of 0.2–5 ppm for TAC (ascorbic acid equivalent), 1–10 ppm for TPC (gallic acid equivalent), and 2–20 ppm for TFC (quercetin equivalent). All methods demonstrated excellent linearity (R^2 = 0.9978) and significantly lower limits of detection (LODs: 0.027, 0.164, and 0.065 ppm) than those reported in the literature. These protocols offer a rapid, cost-effective, standardized alternative to expensive chromatography. By resolving the kinetic and chemical variables that typically cause discrepancies in research, this study provides a validated "gold-standard" approach that ensures the global comparability of phytochemical data across diverse plant matrices.

Article Info

Keywords:
Spectrophotometry, Total antioxidant capacity, Phytochemicals, Validation, Kinetic optimization.

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1. INTRODUCTION

Antioxidants are stable molecules, ions, or radicals that delay or prevent oxidative damage to biomolecules, such as proteins, lipids, and DNA [1–3]. Oxidative stress, which arises from an imbalance between reactive oxygen species (ROS) and antioxidant defenses, can lead to chronic cellular damage [4–6]. Consequently, antioxidants are vital in the food, cosmetics, and pharmaceutical industries for neutralizing free radicals and interrupting damaging chain reactions [1–3].

The bipyridine (Bp) redox method is a key technique for determining Total Antioxidant Capacity (TAC); however, a comparative analysis of protocols from 2013 to 2020 [7–11] reveals profound methodological inconsistencies that undermine reproducibility. These include the use of varying iron precursors ($FeCl_3 \cdot 6H_2O$, $Fe_2(SO_4)_3$,

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$Fe(ClO_4)_3$) with different redox potentials, fluctuating pH ranges (3.74–4.6), incubation times (10–60 min), temperatures (ambient to 60 °C), detection wavelengths (λ_{max} 520–535 nm), and standards (folic acid, ascorbic acid, pyrogallic acid), which fragment quantitative comparability across studies.

Similarly, the Folin–Ciocalteu (F–C) assay, a cornerstone for total phenolic content (TPC), faces reliability challenges due to unharmonized protocols (2007–2020) [12–18], where variations in wavelengths (725–765 nm), incubation periods (15–120 min), temperatures (20–50 °C), reagent dilutions, and Na_2CO_3 concentrations (7–20%) risk incomplete chromophore formation and interference from non-phenolic reducing agents.

Furthermore, methodologies for total flavonoid content (TFC) determination vary, including the use of $AlCl_3$ (10% w/v) with $NaNO_2$/NaOH [19], $AlCl_3$ (10% w/v) in ethanol/NaOAc [20], and $AlCl_3$ (2% w/v) in ethanol: deionized water (DIW) (1:1) [21]. To select the approach yielding the most accurate, precise, stable, and sensitive findings, these three methods were compared in this study.

The lack of standardized, kinetically stable protocols creates a critical research gap that compromises data precision and inter-study comparability. Existing methods often overlook essential stoichiometric factors, such as iron-to-ligand ratios and reagent-alkali balances, which frequently result in unstable readings and baseline drifts. To address these challenges, this study aimed to replace inconsistent legacy protocols with a reliable, validated, high-throughput analytical framework.

2. MATERIALS AND METHODS

2.1. Instruments and Apparatus

A double-beam UV-Vis spectrophotometer (Specord 200, Analytik Jena, Germany) connected to an HP computer was used to measure the absorbance of TAC, TPC, and TFC. The scan mode was employed to generate the absorption spectra and determine λ_{max} for each assay. All pH measurements for the preparation of acetate and ammonium buffers were conducted using a Biotech Engineering HM Digital pH-80 digital pH meter (Biotech Engineering, USA).

2.2. Chemicals and solvents

All chemicals were of analytical grade. Ascorbic acid (99.7%, BDH), gallic acid (99.8%, ACS), quercetin (99.85%, P-Spark Scientific), 2,2'-bipyridyl (99.5%, P-Spark Scientific), ferric chloride hexahydrate (99.0%, Merck), sodium acetate (99.81%, BDH), glacial acetic acid (100.0%, BDH), sodium carbonate (99.5%, May & Baker), aluminum chloride hexahydrate (99.7%, Scharlau), methanol (99.5%, Scharlau), ammonium chloride (99.0%, BDH), and sodium nitrite (99.8%, BDH) were used. Deionized water (DIW) was obtained using a Direct-Q3 Millipore water purification system (Bedford, MA, USA).

2.3. Preparation of standard solutions and reagents

Stock solutions (1000 ppm) of ascorbic acid (TAC), gallic acid (TPC), and quercetin (TFC) were prepared in DIW and methanol. The working standards were obtained by appropriate dilution.

Acetate buffers (pH 4 and 6) were prepared by dissolving 3.0 g and 0.5 g of sodium acetate, respectively, in 1 L volumetric flasks, adding 12 mL of glacial acetic acid, and adjusting the volume with DIW. Ammonium buffers (pH 8 and 10) were prepared by dissolving 0.535 g and 0.6 g of ammonium chloride, respectively, in 1 L volumetric flasks and adjusting the pH with ammonia solution. The pH values were verified using an HM Digital pH-80 pH meter.

Ferric chloride (0.01 M) was prepared by dissolving 0.0689 g of $FeCl_3 \cdot 6H_2O$ in a 25 mL volumetric flask with DIW. 2,2'-Bipyridyl (0.1% w/v) was prepared by dissolving 0.0252g in a 25 mL volumetric flask with DIW. The Folin-Ciocalteu reagent (FCR) was prepared according to the literature. [22].

2.4. Optimization of antioxidant determination methods

2.4.1. Optimization of TAC assay parameters

The spectrophotometric method was systematically optimized across five key variables to ensure maximum sensitivity and stability. The optimal wavelength (λ_{max}) was first identified by scanning the colored complex across the visible spectrum (400–800 nm) against a reagent blank. To evaluate the pH effects, the absorbance was monitored using a range of acetate and ammonium buffers (pH 4.0–10.0). The kinetic stability of the reaction was then established by testing temperature and incubation time intervals from 25–60 °C and 10–60 min, respectively. Furthermore, the reagent volume of 2,2'-bipyridyl (0.1% w/v) was titrated between 0.5 and 3.0 mL to determine the ideal concentration. Finally, the volume of $FeCl_3$ (0.01 M) was fixed at 1.5 mL; this stoichiometric excess ensured that the reagent was never the limiting factor, thereby maintaining a linear response, where the resulting color development was strictly proportional to the antioxidant capacity of the sample.

2.4.2. Optimization of TPC assay parameters

Several published Folin-Ciocalteu methods have been evaluated [15–19]. The assay parameters were systematically optimized using 40 μL of a 1000 ppm gallic acid standard. All optimization tests were conducted in 10 mL volumetric flasks, and the absorbance of the result-

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ing molybdenum-tungsten blue complex was monitored. The optimal wavelength λ_{max}. The reaction mixture was scanned in the spectral range of 400–800 nm. The volume of the Folin-Ciocalteu reagent (FCR) was optimized by testing volumes ranging from 0.2 to 1.0 mL. Finally, to ensure maximum absorbance and complete reaction stability, the effects of temperature and incubation time were investigated over the ranges of 30–60 ℃ and 10–60 min, respectively.

2.4.3. Optimization of TFC assay parameters

Three $AlCl_3$ methods were compared: (I) sodium nitrite/sodium hydroxide [19], (II) sodium acetate/ethanol [22], and (III) $AlCl_3$ (2% w/v) in ethanol/DIW (1:1 v/v) [21].

The assay parameters were systematically optimized to ensure complete complexation of the quercetin standard. The optimal wavelength (λ_{max} The flavonoid-aluminum complex was scanned across a spectral range of 380–800 nm. The reagent volume was subsequently optimized by testing 1–3 mL of 2% w/v aluminum chloride ($AlCl_3$) to ensure sufficient reagent excess for the reaction. Finally, the effect of incubation time on the stability of the absorbance was monitored over a period of 5–60 min at ambient temperature.

2.5. Validation of optimized analytical methods for antioxidant determination

Linearity was assessed using standard curves (eight to nine concentrations) plotted as absorbance versus concentration. Precision was determined in triplicate and expressed as relative standard deviation (RSD, %). The limits of detection (LOD) and quantification (LOQ) were calculated as $3.0 \times (SD/S)$ and $10 \times (SD/S)$, respectively, where SD is the standard deviation of the blank and S is the calibration slope [23–25].

2.6. Statistical Analysis

Experimental data were processed in triplicate (n=3), and the results are presented as mean ± Standard Deviation (SD). The statistical significance of the calibration levels was confirmed using one-way ANOVA ($p < 0.05$), and the reliability of the mean absorbance values was established through 95% confidence intervals, demonstrating high instrumental precision across the validated linear range. All calculations and regression analyses were performed using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1. Optimization results for TAC determination

To enhance the method sensitivity and suitability, five parameters were optimized for the ferric 2,2'-bipyridyl reducing capacity (FBRC) method [7–11]. Five parameters were evaluated: wavelength, pH, incubation temperature, incubation time, and 2,2'-bipyridyl volume.

3.1.1. Wavelength selection

The optimal wavelength for the TAC assay was determined by scanning the reaction mixture across a range of 400–800 nm (Figure 1). The spectra illustrate the absorption of the TAC-FBRC complex and its corresponding reagent blanks. The analytical signal was determined by measuring the sample against a reagent blank to eliminate background interference from $FeCl_3$ and 2,2'-bipyridyl reagents. Maximum absorbance occurred at $\lambda_{max} = 520$ nm without interference, consistent with a previous study [13]. While the samples were scanned over the full range of 400-800 nm, the spectral figures are displayed over a focused range of 440-700 nm to clearly illustrate the characteristic peaks.

3.1.2. The Effect of pH

Buffers with pH values of 4, 6, 8, and 10 were tested (Figure 2). Maximum absorbance was achieved at pH 4, whereas pH 10 produced metal hydroxide precipitates, which is consistent with another study [9].

3.1.3. Effect of incubation temperature

Incubation at 50 ℃ provided the optimal thermal energy for reaction completion (Figure 3).

3.1.4. Effect of incubation time

Reaction times (10–60 min) were tested at 50 ℃ (Figure 4). The absorbance stabilized after 30 min, matching the value published by Othman *et al.* [7]. The complex exhibited sustained stability for up to 120 min post-incubation, verifying the reliability of the optimized kinetic window for routine analysis.

3.1.5. Effect of 2,2'-Bipyridyl Volume

A systematic evaluation of the reagent volumes was conducted to resolve the stoichiometric imbalances that are common in legacy protocols. For the TAC assay, the volume of 2,2'-bipyridyl (0.1% w/v) was varied from 0.5 to 3 mL (Figure 5). The results established that 1.5 mL was the optimal volume, as it yielded maximum absorbance and ensured complete complexation with the reduced iron species. This volume provides the necessary ligand-to-metal ratio to maintain both the sensitivity and kinetic stability of the resulting chromophore.

A volume of 1.5 mL ensured that the 1:3 iron-to-ligand stoichiometric requirement was exceeded, providing a broad and reliable analytical range that is sufficiently

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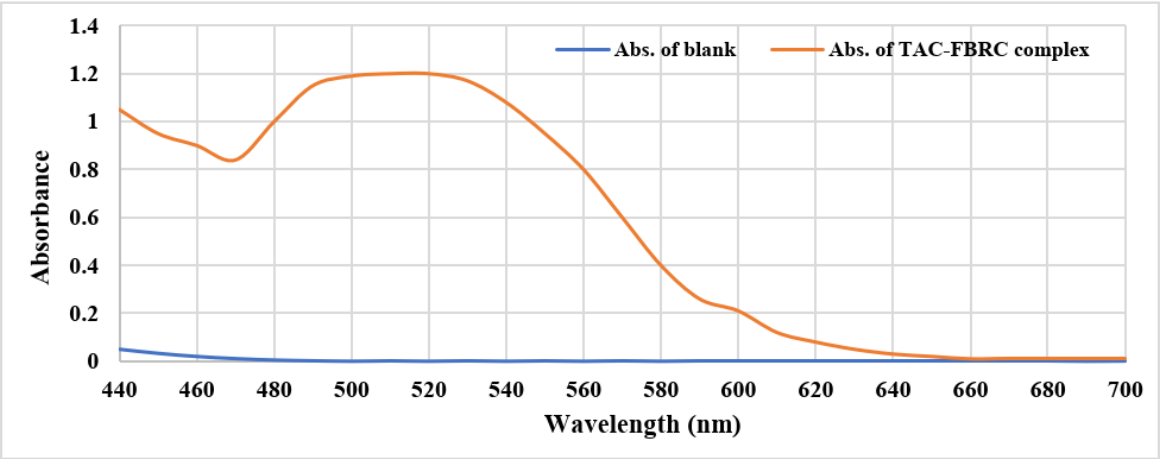


Figure 1. UV-Vis absorption spectra of the TAC-FBRC complex and the reagent blank.

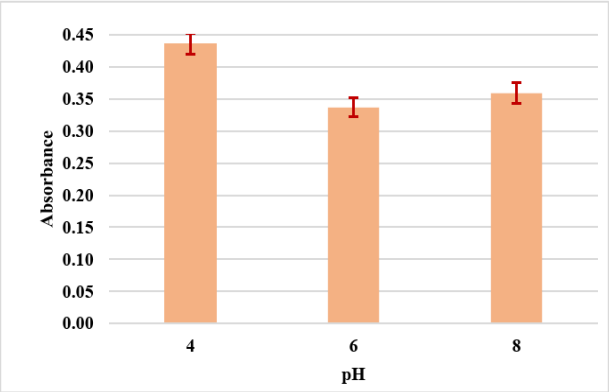


Figure 2. Effect of pH on the absorbance of the $[Fe(bpy)_3]^{2+}$ complex (using 2.0 ppm ascorbic acid standard)

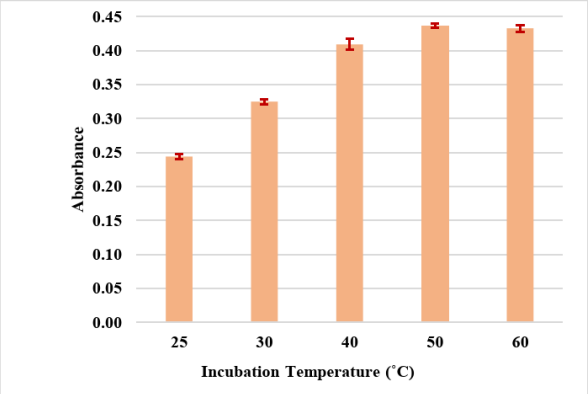


Figure 3. Effect of incubation temperature on the absorbance of the $[Fe(bpy)_3]^{2+}$ complex (using 2.0 ppm ascorbic acid standard)

stable for real sample applications, regardless of the naturally high antioxidant levels.

The Ferric Reducing Power method using bipyridyl quantifies the total antioxidant reducing power through $Fe^{3+} \rightarrow Fe^{2+}$ reduction. 2,2'-Bipyridyl serves as the chromogenic ligand, forming a stable, intense red $[Fe(bpy)_3]^{2+}$ complex (1:3 stoichiometry) at the optimized pH of 4.0. This acidity ensures quantitative complexation, yielding absorbance increases at 520 nm that are directly proportional to the reducing capacity of the sample [7, 10].

Optimized TAC conditions: 520 nm, pH 4, 50 ℃, 30 min, 1.5 mL each of $FeCl_3$ (0.01 M) and 2,2'-bipyridyl (0.1% w/v).

3.2. Optimization Results for TPC Determination

Four parameters were optimized for the Folin-Ciocalteu method.

3.2.1. Selection of wavelength

The results are shown in Figure 6. The spectrum illustrates the absorption of the blue complex and its corresponding reagent blank. The optimal wavelength (λ_{max}) was identified as 750 nm, where the analytical signal was maximized and background interference from the reagents was negligible. The wavelength for TPC quantification was selected by scanning the reaction product of gallic acid and the Folin-Ciocalteu reagent against a blank. As shown in Figure 6, all subsequent measurements were performed against a blank to ensure that only the molybdenum blue complex formed by the phenolics was quantified, thereby enhancing the accuracy of the phytochemical profiling.

3.2.2. Effect of incubation temperature

The results are shown in Figure 7. The maximum absorbance was obtained when the reaction mixture was heated in a water bath at 50 ℃. This result is consistent with that of a previous study [18]. Further heating the absorbance at 60 ℃ was almost stable.

3.2.3. Effect of Incubation Time

The results are shown in Figure 8. The results showed that the optimal incubation time was 20 min.

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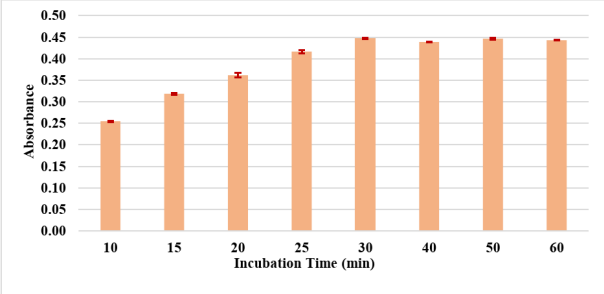


Figure 4. Kinetic study showing the effect of reaction time on the absorbance of the $[Fe(bpy)_3]^{2+}$ complex (using 2.0 ppm ascorbic acid standard)

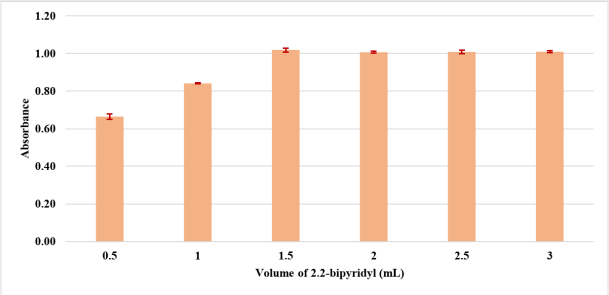


Figure 5. Effect of 2,2'-bipyridyl volume on the absorbance of the $[Fe(bpy)_3]^{2+}$ complex (using 2.0 ppm ascorbic acid)

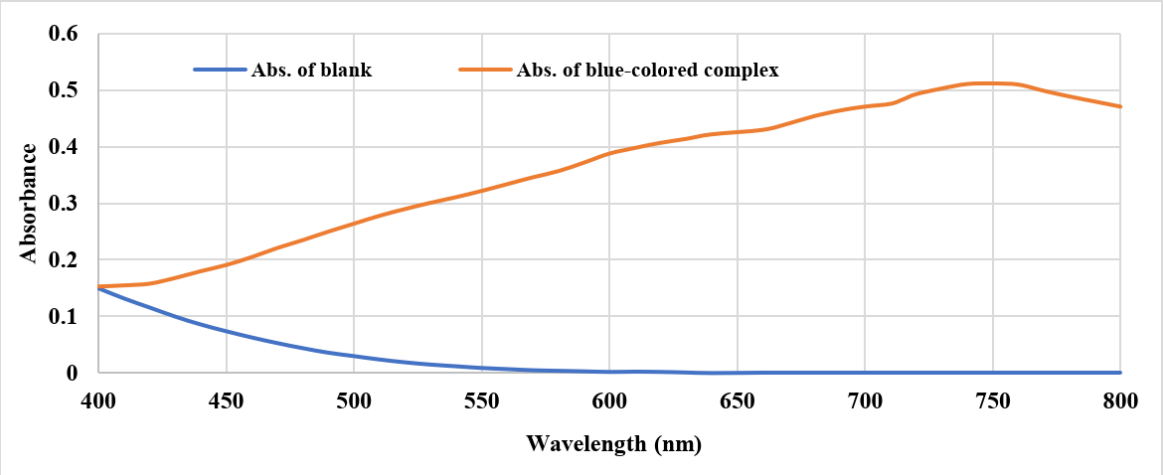


Figure 6. UV-Vis absorption spectra of the TPC-molybdenum blue complex and the reagent blank.

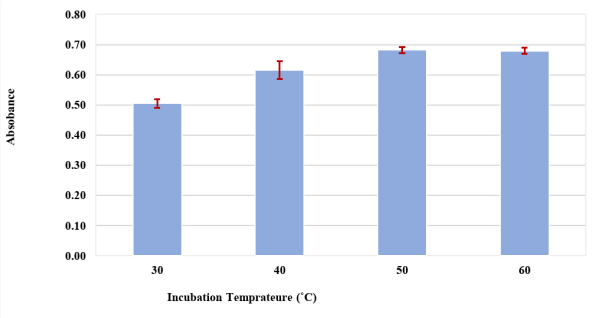


Figure 7. Effect of incubation temperature on the absorbance of the TPC-Folin complex (using 5.0 ppm gallic acid)

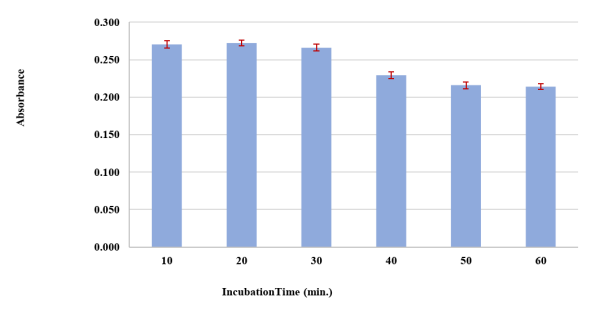


Figure 8. Kinetic study showing the effect of reaction time on the absorbance of the TPC-Folin complex (using 5.0 ppm gallic acid)

3.2.4. Effect of Folin-Ciocalteu reagent volume

The volume of the Folin-Ciocalteu reagent (FCR) was optimized by testing a range between 0.2 and 1.0 mL within a 10 mL reaction system. As illustrated in Figure 9, the highest absorbance was consistently achieved using 0.4 mL FCR.

TPC determination employs the FCR via single electron transfer (SET), where phosphotungstate/phosphomolybdate accepts electrons from polyphenols under optimized alkaline conditions. This reduces yellow Mo (VI)/W(VI) to blue Mo(V)/W(V) oxides, forming a molybdenum blue complex with λ_{max} [11, 12].

The Optimized TPC conditions: 750 nm, 50℃, 20 min, and 0.4 mL FCR.

3.3. Optimization results for TFC determination

3.3.1. Comparison of different determination methods for TFC

Based on a comparative analysis of three established aluminum chloride protocols, Method III (utilizing 2% w/v

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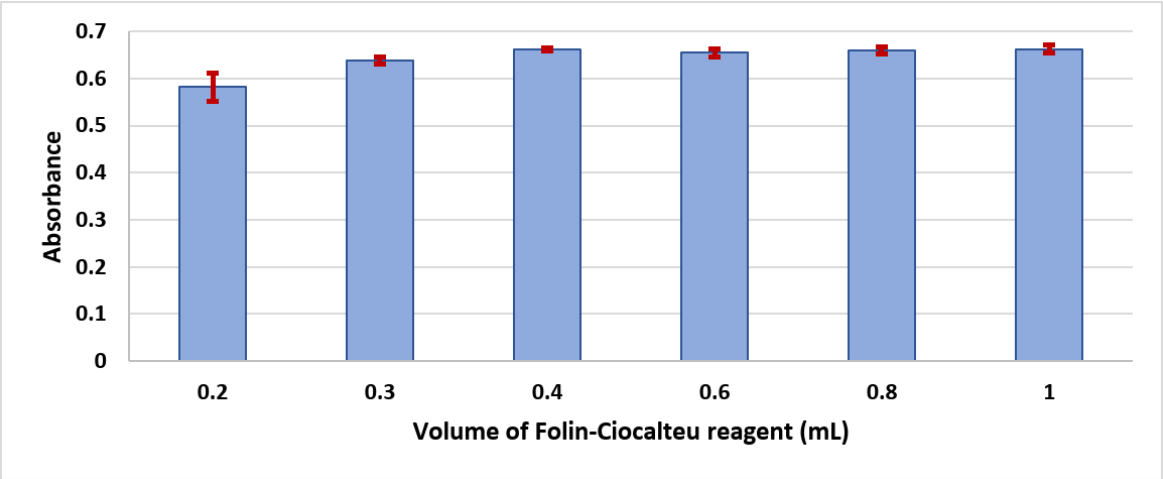


Figure 9. Effect of Folin-Ciocalteu reagent volume on the absorbance of the TPC-Folin complex (using 5.0 ppm gallic acid)

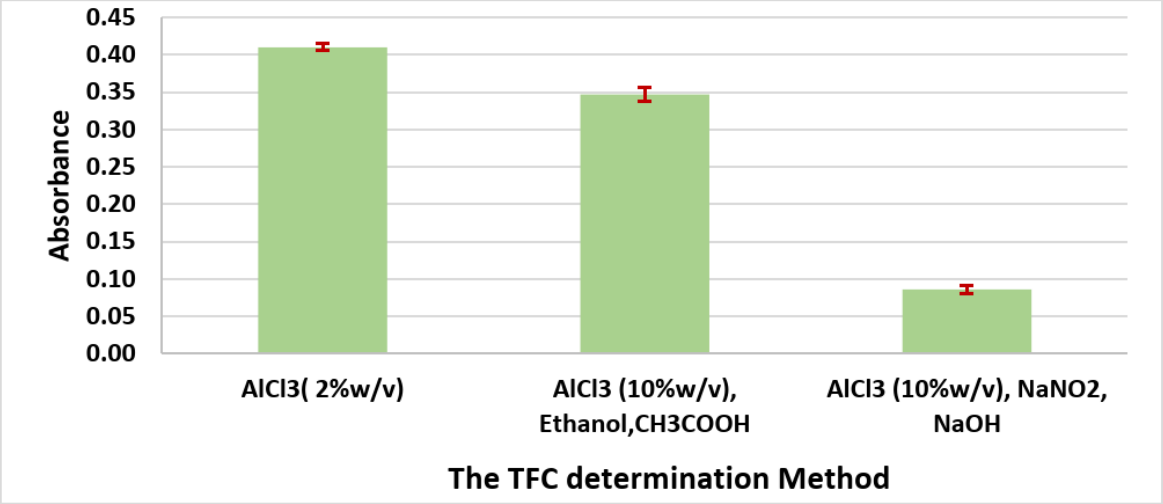


Figure 10. Comparison of TFC determination methods

$AlCl_3$ in an ethanol: DIW 1:1 mixture) was selected as the optimal approach. A systematic comparison revealed that Method III consistently yielded superior absorbance at $\lambda = 430$ nm compared to the sodium nitrite/sodium hydroxide (Method I) and sodium acetate/ethanol (Method II) systems. As illustrated in Figure 10, a modified version of the 2% w/v aluminum chloride method [21] provided enhanced sensitivity and signal stability over previously reported methodologies [19, 20]. The selection of Method III ensured high reproducibility and minimized potential interference from matrix-specific alkaline conditions found in alternative protocols.

3.3.2. TFC assay optimization

3.3.2.1. Wavelength selection

To determine the optimal wavelength (λ_{max}), the flavonoid-aluminum complex was scanned against a reagent blank over a range of 400–800 nm. The spectra illustrate the absorption of the flavonoid-aluminum complex and its corresponding reagent blank. Although the full spectral scan covered this broad spectrum, the figures highlight the 380–600 nm range to better emphasize the characteristic peaks. As illustrated in Figure 11, the complex exhibited a primary absorption peak at 430 nm. Measuring against the reagent blank, which contains all components except the sample, is critical in this assay to negate any inherent absorbance from aluminum chloride at lower wavelengths, thereby ensuring the specificity of flavonoid quantification.

3.3.2.2. Effect of incubation time

From Figure 12, results indicate that the reaction between quercetin and 2% aluminum chloride reached stability within 10 min when maintained at ambient temperature (25°C).

3.3.2.3. Effect of aluminium chloride volume

The optimum amount of aluminum chloride reagent, which served as a stable complexing/coloring agent, was 2 mL, exhibiting stable complexation and a fixed color (Figure 13).

The TFC assay exploits selective Al^{3+} chelation with flavonoids, primarily at the C-4 keto and adjacent C-3/C-5 hydroxyls of flavones/flavonols (stable, acid-resistant

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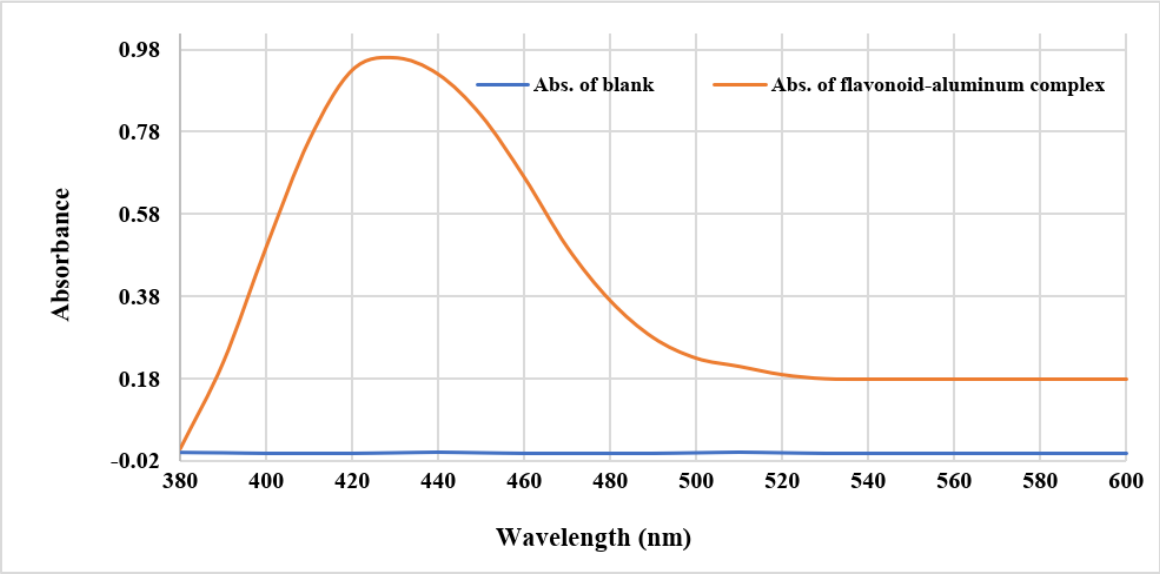


Figure 11. UV-Vis absorption spectra of the flavonoid-aluminum complex and the reagent blank.

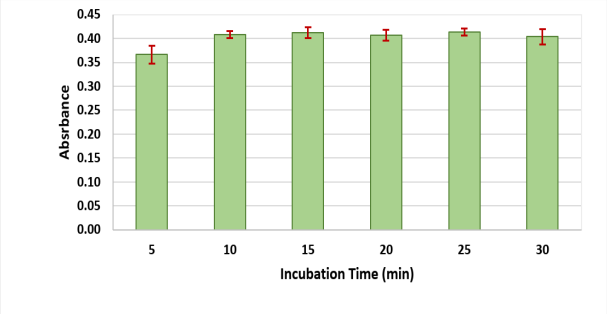


Figure 12. Kinetic study showing the effect of reaction time on the absorbance of the TFC-$AlCl_3$ complex (using 10.0 ppm quercetin)

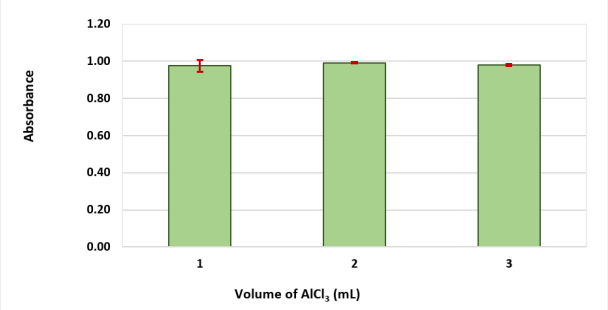


Figure 13. Effect of aluminium chloride volume on the absorbance of the TFC-$AlCl_3$ complex (using 10.0 ppm quercetin)

complexes), and acid-labile ortho-dihydroxyl sites in A/B-rings. This induces a bathochromic shift, yielding a yellow aluminum-flavonoid complex at λ_{max} 0 nm [20, 21]. Unlike redox assays, complexation completes efficiently at 25℃ within 10 min, offering kinetic advantages for high-throughput phytochemical analysis [20].

Optimized TFC conditions: 430 nm, 25℃, 10 min, and 2 mL $AlCl_3$ (2% w/v).

3.4. Validation of Optimized Methods

To ensure the reliability and accuracy of the results, the analytical methods used to determine the TAC, TPC, and TFC were validated. Parameters, including linearity, sensitivity (limit of detection, LOD; limit of quantitation, LOQ), and precision, were evaluated according to the Association of Official Agricultural Chemists (AOAC) guidelines.

3.4.1. TAC Method Validation

The linearity of the TAC analysis method was established by plotting the instrument response against the ascorbic acid concentrations (0.2–5 ppm), as shown in Figure 14. The resulting linear regression equation $(y = 0.1718x + 0.0062)$. The results showed good linearity over the studied concentration range, with a correlation coefficient of determination (R^2) of 0.9978, which is satisfactory for the quantitative analysis of the analyte, as shown in Table 1.

The values of (%RSD) ranged between 0.82 and 5.68%. The low %RSD values were due to the high repeatability of the measurements. The optimized TAC method showed high precision, with 95% confidence intervals ranging from ± 0.004 to ± 0.063 absorbance units across the linear range. The LOD and LOQ values were 0.027 and 0.089 ppm, respectively, indicating a higher sensitivity for the determination.

3.4.2. TPC Method Validation

The linearity of the instrument was determined by analyzing a series of eight concentrations, and the instrument response was plotted versus the concentrations of the analysis in the range of 1-10 ppm. As illustrated in Figure 15, the TPC method demonstrated excellent linearity over the studied range of gallic acid, and the regression coeffi-

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Table 1. TAC validation method results

Ascorbic Acid Conc.	Absorbance, n=3				SD	% RSD	95% CI (±)
	Reading1	Reading2	Reading3	Average			
0.2(ppm)	0.036	0.034	0.037	0.03567	0.001528	4.28	0.004
0.4	0.072	0.069	0.071	0.07067	0.001528	2.16	0.004
0.6	0.101	0.109	0.113	0.10767	0.006110	5.68	0.015
0.8	0.151	0.149	0.153	0.15100	0.002000	1.32	0.005
1.0	0.201	0.197	0.211	0.20300	0.007211	3.55	0.018
2.0	0.315	0.326	0.331	0.32400	0.008185	2.53	0.020
4.0	0.695	0.684	0.692	0.69033	0.005686	0.82	0.014
5.0	0.892	0.882	0.844	0.872667	0.025325	2.90	0.063
(Ascorbic acid, 0.2-5 ppm); Linear equation: y = 0.1718x + 0.0062; R^2: 0.9978; LOD: 0.027 ppm; LOQ: 0.089 ppm. CI: 95% Confidence Intervals, 95% CI=Average ±(t×SD/$\sqrt{n}$),where t = 4.303 for n=3.							

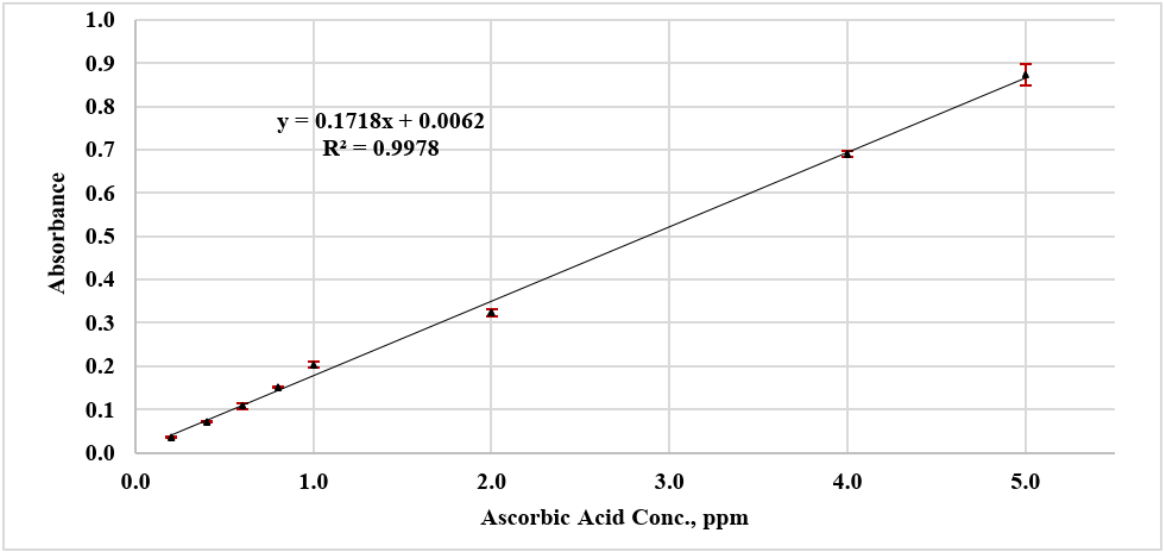


Figure 14. Calibration Curve for TAC Assay

cient was calculated from the linear regression equation y = 0.0659x–0.0055. The obtained results showed good linearity over the range of concentrations studied, with a correlation coefficient of determination R^2 equal to 0.9988, which is satisfactory for the quantitative analysis of the analyte. The repeatability of the analysis method was determined by analyzing the gallic acid standard solution at different concentrations in triplicates. The obtained values of %RSD were between 0.85 and 6.22 %. The low values of %RSD are attributed to the high repeatability of measurements. With 95% confidence intervals ranging from ± 0.009 to ± 0.029 absorbance units across the linear range, the obtained data are illustrated in Table 2.

The LOD and LOQ values were 0.164 and 0.547 ppm, respectively. The results are presented in Table 2. Lower LOD and LOQ values indicate a higher sensitivity of the determination.

3.4.3. TFC Method Validation

The linearity of the analysis method was determined by analyzing a series of nine concentrations in the range of 2–20 ppm of quercetin (Figure 16). The linear range for quercetin solution and the regression coefficient were 0.9992, and the linear regression equation was $y = 0.0461x - 0.0124$. The obtained results showed that the curve had good linearity over the studied range of concentrations, with a correlation coefficient of determination R^2 equal to 0.9992, which is satisfactory for the quantitative analysis of the analyte. The obtained values of %RSD ranged between 0.29 and 5.09 %. The low values of % RSD are attributed to the high repeatability, with 95% confidence intervals ranging from ± 0.002 to ± 0.038 absorbance units across the linear range. The obtained data are presented in Table 3.

The obtained LOD and LOQ were (0.065 and 0.217 ppm, respectively), indicating the high sensitivity of this method. The results are presented in Table 3. Lower LOD and LOQ values indicate a higher sensitivity of the determination.

All methods demonstrated excellent linearity (R^2 > 0.9978), precision (%RSD < 7%), and sensitivity (LOD < 0.2 ppm), confirming their suitability for routine phytochemical analyses.

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Table 2. TPC method validation results

Conc. of Gallic Acid, ppm	Absorbance				SD	%RSD	95% CI (±)
	Reading 1	Reading 2	Reading 3	Average			
1.0	0.054	0.061	0.059	0.05800	0.003606	6.22	0.009
2.0	0.114	0.123	0.125	0.12067	0.005859	4.86	0.015
3.0	0.198	0.182	0.195	0.19167	0.008505	4.44	0.021
4.0	0.263	0.276	0.271	0.27000	0.006557	2.43	0.016
5.0	0.339	0.317	0.322	0.32600	0.011533	3.54	0.029
6.0	0.394	0.386	0.398	0.39267	0.006110	1.56	0.015
8.0	0.513	0.512	0.505	0.51000	0.004359	0.85	0.011
10.0	0.649	0.658	0.663	0.65667	0.007095	1.08	0.018
(Gallic acid, 1.0-10.0 ppm); regression equation y = 0.0659x - 0.0055; R^2: 0.9988; LOD: 0.164 ppm; LOQ: 0.547 ppm. CI: 95% Confidence Intervals, 95% CI=Average ±(t×SD/$\sqrt{n}$),where t = 4.303 for n=3							

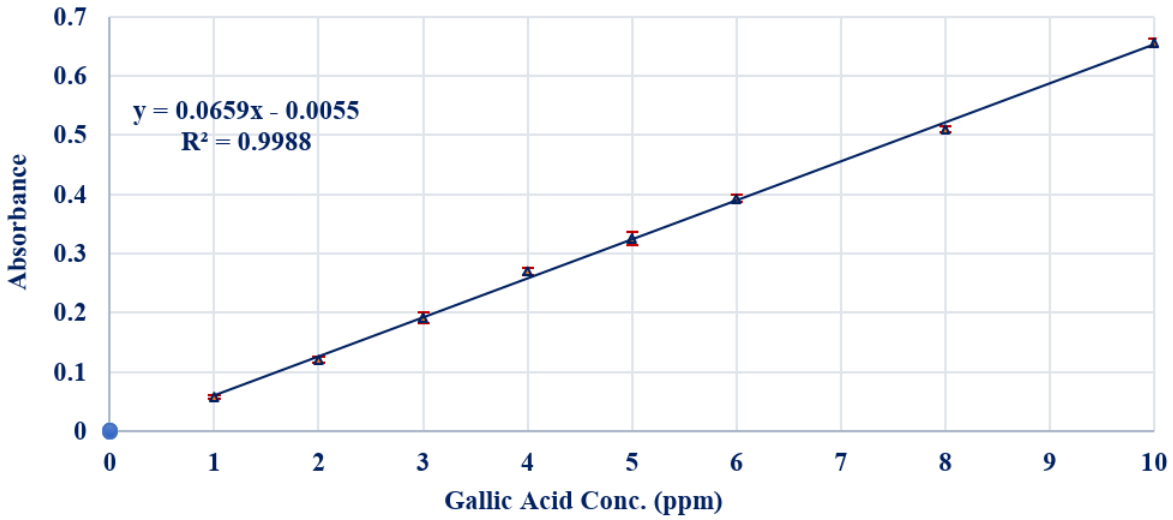


Figure 15. Calibration Curve for Polyphenols Determination

Table 3. TFC method validation results

Conc. of Quercetin, ppm	Absorbance, n=3				SD	%RSD	95% CI (±)
	Reading 1	Reading 2	Reading 3	Average			
2.0	0.083	0.082	0.084	0.08300	0.001000	1.20	0.002
2.5	0.088	0.091	0.095	0.09133	0.003512	3.85	0.009
4.0	0.175	0.180	0.164	0.17300	0.008185	4.73	0.020
5.0	0.225	0.207	0.217	0.21633	0.009018	4.17	0.022
6.0	0.275	0.255	0.250	0.26000	0.013229	5.09	0.033
8.0	0.350	0.380	0.360	0.36333	0.015275	4.20	0.038
10.0	0.472	0.454	0.466	0.46400	0.009165	1.98	0.023
15.0	0.683	0.679	0.681	0.68100	0.002000	0.29	0.005
20.0	0.907	0.903	0.897	0.90233	0.005033	0.56	0.012
(Quercetin, 2.0-20.0 ppm); linear regression equation y = 0.0461x - 0.0124; R^2: 0.9992; LOD: 0.065 ppm; LOQ: 0.217 ppm. CI: 95% Confidence Intervals,95% CI=Average ±(t×SD/$\sqrt{n}$),where t = 4.303 for n=3.							

3.5. Comparative Performance of Optimized TAC/TPC/TFC Assays against Literature Benchmarks

To evaluate the performance of the proposed method, its validation parameters were compared with those of previously established spectrophotometric methods (Table 4). Legacy spectrophotometric methods for phytochemical quantification suffer from unharmonized parameters, such as variable pH, temperature, and incubation times, leading to baseline drift and poor sensitivity. This study overcomes these limitations by establishing strictly optimized, kinetically locked frameworks.

For the TAC assay, standardizing conditions to pH 4.0, 50℃, and 30 min eliminated baseline drift and achieved an LOD of 0.027 ppm (AAE), substantially surpassing widely unreported or higher LODs in legacy protocols. In the TPC assay, enforcing a 20-minute kinetic window at 50℃ ensured complete chromophore formation, reduced baseline drift, and improved the LOD to 0.164 ppm (GAE) with > 96% complex stability, replacing the inconsistent 15–120 minute incubations common in prior studies.

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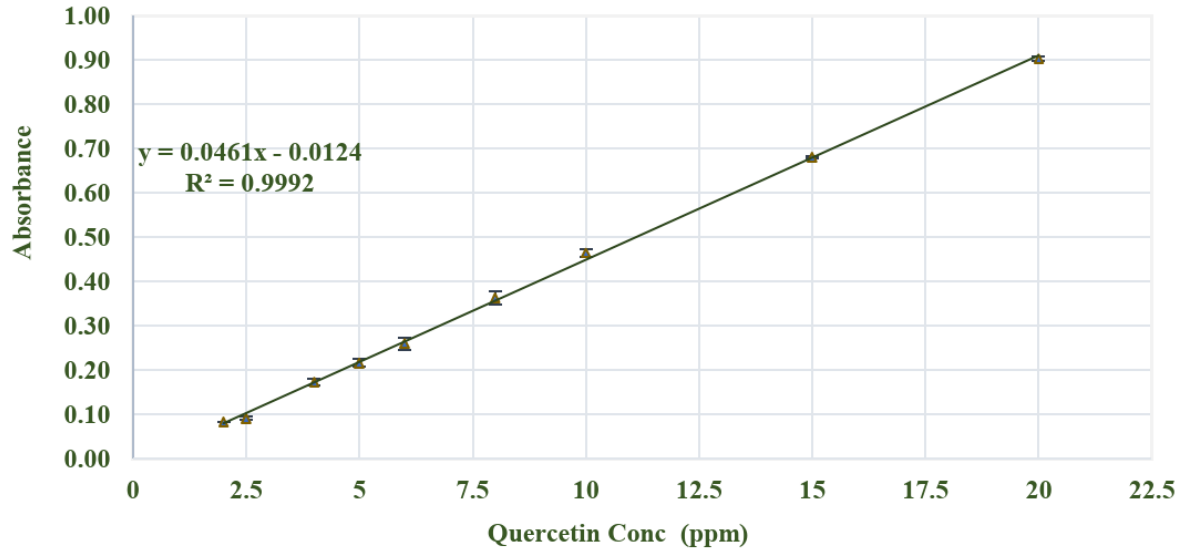


Figure 16. Calibration Curve for Flavonoids Determination.

Table 4. Comparative Analysis of Optimized Parameters and Statistical Analysis against Literature Benchmarks

Assay	Category	Current Study (Optimized)	Previous Studies (Legacy Protocols)	Literature References
TAC	Optimized Parameters	λ_{max}: 520 nm pH: 4.0 Temp: 50°C Time: 30 min	λ_{max}: 520–535 nm pH: 3.7–4.6 Temp: Ambient–60°C Time: 10–60 min	[7–11]
TPC	Statistical Analysis Optimized Parameters	LOD: 0.027 ppm (AAE) R^2: 0.9978 %RSD: 0.82–5.68% Recovery: 98–102% λ_{max}: 750 nm Temp: 50°C Time: 20 min Reagent: 0.4 mL FCR	LOD: Generally higher or unreported Precision: Unharmonized; poor inter-study comparability λ_{max}: 725–765 nm Temp: 20–50°C Time: 15–120 min Reagent: Highly variable dilution	[12–18]
TFC	Statistical Analysis Optimized Parameters	LOD: 0.164 ppm (GAE) LOQ: 0.547 ppm R^2: 0.9988 Stability: > 96% over 24h Method: 2% $AlCl_3$in EtOH:DIW λ_{max}: 430 nm Temp: 25°C Time: 10 min	LOD: Inconsistent reporting Precision: Baseline drift due to incomplete chromophore formation Method: $AlCl_3$+ $NaNO_2$/NaOH or NaOAc λ_{max}: 415–520 nm Temp/Time: Variable	[19–21]
	Statistical Analysis	LOD: 0.065 ppm (QE) LOQ: 0.217 ppm R^2:0.9992 %RSD: 0.29–5.09%	LOD: 0.1–1.5 ppm Precision: Matrix interference from multifaceted reagents	

AAE = Ascorbic Acid Equivalents; GAE = Gallic Acid Equivalents; QE = Quercetin Equivalents.
DIW = Deionized Water; R^2= Regression Coefficient; % RSD = Relative Standard Deviation;
FCR = Folin-Ciocalteu Reagent; LOD = Limit of Detection; LOQ = Limit of Quantification.

For the TFC assay, the study replaced complex, interference-prone reagent systems (e.g., $NaNO_2$/NaOH or NaOAc) with a streamlined 2% aluminum chloride matrix in ethanol–water at ambient temperature. This simplification eliminates competing side reactions, shortens the analysis time to 10 min, and lowers the LOD to 0.065 ppm (QE).

Collectively, these optimized protocols offer a 2- to 10-fold improvement in analytical sensitivity and a 6-fold reduction in operational throughput time for the TPC assay. By replacing empirical fragmented methodologies with these validated protocols, this study provides a rigor-

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ous and standardized benchmark for generating globally comparable phytochemical data across complex plant matrices.

3.6. Application to Real Samples and Method Comparison

To ensure their suitability for real-world applications, the optimized methods were practically applied and tested on diverse botanical sources, specifically Yemeni almond [24], grape [25], and mulberry [26], prior to the publication of this core methodological framework. The successful analysis of these complex matrices, as detailed in our previously published work, confirms the accuracy and high precision of the standardized assays established in this study.

4. CONCLUSION:

In this study, we established a standardized and optimized framework for the spectrophotometric determination of Total Antioxidant Capacity (TAC), total polyphenol content (TPC), and total flavonoid content (TFC). By systematically refining critical parameters such as incubation temperature, reaction time, and pH, and rigorously validating the protocols according to AOAC guidelines, this study provides a highly stable, reliable, and cost-effective alternative to expensive chromatographic techniques.

As the first comprehensive optimization of these widely used assays under unified, standardized conditions, this study highlights the primary merit of this investigation in the field of phytochemical analysis. These validated methods ensure high-throughput efficiency and enhance the international comparability of the research data. Given the high precision and sensitivity demonstrated, the application of these protocols to diverse plant matrices represents a logical and necessary next step for future studies aimed at exploring the bioactive potential of various natural products.

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