



Optical Spectral Analysis of 4-Aminobenzenesulfonamide through Azo Coupling Reaction and its Commercial Chemical Applications

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Abstract

Determination of 4-aminobenzenesulfonamide (SFA) by azo-coupling with quercetin, producing a dark yellow color. The method is based on an azo-coupling reaction between SFA and quercetin. The SFA is first diazotized using hydrochloric acid and sodium nitrite at 0–5 °C, then coupled in an alkaline medium. The resulting dark yellow, water-soluble product exhibits a maximum absorbance at 413 nm. The experimental conditions were initially optimized using a univariate approach (OVAT) by examining the effects of individual variables. For further improvement, a central composite design was used to characterize system behavior and identify optimal conditions across multiple variables. The method demonstrated good linearity, with Beer's law obeyed over the concentration range of 1–10 µg.mL⁻¹. The detection limit was 1.12 µg.mL⁻¹. The molar absorptivity and Sandell's sensitivity were 2.53×10⁴ L.mol⁻¹.cm⁻¹ and 6.82×10⁻³ µg.cm⁻², respectively. The resulting color remained stable for more than one hour. The proposed method was successfully applied to sulfadiazine pharmaceutical formulations.

Keywords: 4-Aminobenzenesulfonamide, Central composite design, Diazotization, Quercetin, Response surface design.

1. Introduction

4-Aminobenzenesulfonamide (SFA) is a widely utilized synthetic pharmaceutical compound known for its pronounced antibacterial activity. It has a molecular formula of C₆H₈N₂O₂S and a molar mass of 172.205 g.mol⁻¹. Since its discovery in the 1930s by Gerhard Domagk, this compound has played a pivotal role in the development of antimicrobial chemotherapy. Under standard temperature and pressure, it is considerably stable; however, it is sensitive to strong oxidizing agents and light exposure^{2,3}. In terms of physicochemical properties, the compound displays limited solubility in water compared to organic solvents, with higher solubility in ethanol (~27 mg.cm×10⁻³) and acetone (~200 mg.cm×10⁻³)^{4,5}. It has a pKa value of 10.4, indicating weakly acidic behavior. This characteristic can be attributed to the strong electron-withdrawing effect of the sulfonyl (–SO₂) group and the resonance stabilization of the resulting anion⁶. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a naturally occurring flavonoid characterized by a structural framework consisting of two aromatic rings (A and B) linked through a heterocyclic pyrone ring (C)⁷. It appears yellow and is highly susceptible to oxidation, with a melting point of approximately 316 °C. Quercetin is practically insoluble in water but readily soluble in alkaline aqueous solutions and various organic solvents, including ethanol, acetone, pyridine, and acetic acid. It shows appreciable solubility in ether and methanol^{8,9}.

In addition to its structural features, quercetin is well recognized for its strong antioxidant activity, which arises from multiple hydroxyl (OH) groups that can donate electrons and stabilize free radicals. Beyond its biological significance^{10,11}, these properties also contribute to its chemical reactivity and relative stability, enhancing its suitability as a coupling agent. These

characteristics are particularly advantageous in spectrophotometric applications, where stable, intensely colored azo products are required. Diazonium salts are typically generated by reacting aromatic amines with nitrous acid in the presence of mineral acids under carefully controlled conditions. These reactions are generally conducted at low temperatures (commonly below 0–5°C) to prevent decomposition of the reactive intermediate. Aromatic diazonium salts are relatively stable because the positive charge is delocalized over the benzene ring. Many analytical methods, especially spectrophotometric techniques, depend on diazotization followed by azo coupling with suitable reagents to produce colored species that are measurable in the visible region^{12,13}. Azo coupling reactions have been studied in recent years as alternative techniques that may provide advantages such as enhanced sensitivity or simplified processes. However, diazotization-based methods remain widely used because they are reliable and have well-defined reaction mechanisms^{14,15}. Because both SFA (as an aromatic amine) and quercetin (as a phenolic coupling agent) have multiple functional groups, their interaction offers a promising route to stable azo chromophores with distinct spectroscopic properties¹⁶⁻¹⁹. Quercetin may serve as a coupling reagent¹⁷, yet its use in identifying this compound has not been thoroughly examined. This underscores the need to develop analytical methods that leverage its structural and electronic characteristics to improve sensitivity and selectivity. The current study seeks to establish a reliable spectrophotometric method for quantifying SFA, using a diazotization reaction followed by azo coupling with quercetin, yielding a stable colored compound. This study optimized the proposed method using univariate and multivariate approaches and used it to analyze pharmaceutical formulations, with the multivariate method showing superior accuracy and sensitivity.

2. Materials and Methods

2.1. Instrumentation

All absorbance measurements were conducted using a UV–Visible spectrophotometer (Shimadzu UV-1800) fitted with matched quartz cells. Weighing procedures were performed using an analytical balance (Sartorius BL 210S). A thermostatically controlled hot plate equipped with a magnetic stirrer (Germany) and a mechanical shaker operating at 500 rpm (China) were employed during the experimental work.

2.2. Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade and used as received without further purification. Quercetin (99.98% pure), sodium hydroxide (NaOH), sodium nitrite (NaNO₂), and hydrochloric acid (HCl) were obtained from BDH Chemicals (UK). Absolute ethanol (99.9%) from Sigma-Aldrich (Germany). Distilled water (DW) was used for all solution preparations and dilution steps. The reference standard, SFA, was supplied as a pure powder by the State Company for Pharmaceutical Industries and Medical Appliances (SDI) in Samarra, Iraq.

2.3. Preparation of standard and working solutions

2.3.1. Quercetin stock solution

A stock solution of quercetin was prepared at 1000 µg/mL by accurately weighing 0.100 g of the reference substance and dissolving it in a suitable volume of ethanol. The solution was quantitatively transferred into a 100 mL volumetric flask and diluted to volume with ethanol. Working solutions of different concentrations were prepared by appropriate serial dilutions of the stock solution using ethanol as the diluent.

2.3.2. Preparation of a synthetic sample of sulfanilamide drug

The synthetic sample was prepared by adding 0.00625 g of an interference mixture (equal weights of glucose, sucrose, lactose, and gum arabic) to 0.025 g of pure SFA.

Step 1: 0.025 g of pure SFA was dissolved in 10 mL of deionized water at approximately 70°C, with continuous heating until complete dissolution. The solution was then allowed to cool to room temperature.

Step 2: The mixture of the pure drug and the interfering substances was dissolved in 20 mL of deionized water under constant stirring until fully homogenized.

Step 3: The two solutions were mixed in a glass beaker with constant stirring. After confirming complete dissolution and mixing, the mixture was transferred to a 100 mL volumetric flask and made up to the mark with deionized water, following the same preparation procedure as the standard drug solution, to obtain a final concentration of $500 \mu\text{g.mL}^{-1}$. Then, 70 mL were withdrawn and diluted to 100 mL with DW in a volumetric flask to perform subsequent measurements, which provides a final concentration of $350 \mu\text{g.mL}^{-1}$.

2.3.3. 4-Aminobenzenesulfonamide (SFA) stock solution

A stock solution of SFA ($1000 \mu\text{g.mL}^{-1}$) was prepared by accurately weighing 0.100 g of the pure substance and dissolving it initially in 10 mL of deionized DW. To ensure complete dissolution, the solution was gently heated at 70°C for approximately 3 minutes under continuous stirring. The solution was then quantitatively transferred to a 100 mL volumetric flask and diluted to the mark with DW. Subsequent working solutions were prepared by serial dilution using DW as needed.

2.3.4. Preparation of auxiliary reagents

In 100 mL volumetric flasks, DW was used to prepare HCl ($\sim 2 \text{ M}$) and NaOH ($\sim 5 \text{ M}$) solutions, with additional dilutions as needed. 0.050 g of sodium nitrite was dissolved in DW and diluted to 100 mL in a volumetric flask to prepare a sodium nitrite solution ($500 \mu\text{g.mL}^{-1}$). As needed, additional dilutions were prepared. Every created solution was kept out of direct sunlight, in cold laboratory settings, and in firmly sealed amber glass containers. Every 48 hours, new solutions were made to guarantee analytical precision and chemical stability.

2.4. Preliminary investigation and determination of maximum absorption wavelength (λ_{max})

The maximum absorption wavelength (λ_{max}) of the colored azo coupling product was ascertained by preliminary investigations. To create the appropriate diazonium salt, SFA was first diazotized using sodium nitrite in an acidic solution at a low temperature ($0\text{--}5^{\circ}\text{C}$). Quercetin was then used as an effective coupling agent to couple this intermediate. Because quercetin has five active OH groups, stoichiometric principles were used to determine the molar ratios for different mixing scenarios. The reaction conditions were optimized by using these initial concentrations. After recording the product's absorption spectrum, λ_{max} was determined to be 413 nm.

2.5. Optimization of reaction conditions

2.5.1. Method 1: One variable at a time (OVAT) approach

To determine the ideal conditions, we examined the effects of experimental variables separately, including SFA concentration ($10 \mu\text{g.mL}^{-1}$), acid concentration, sodium nitrite concentration, coupling agent concentration, base concentration, reaction time, and reagent addition order. Ten mL volumetric flasks submerged in an ice bath kept between $0\text{--}5^{\circ}\text{C}$ were used for the experiments. The acid was added first, followed by SFA, then sodium nitrite for diazotization, then the coupling agent (quercetin), and finally NaOH to aid color development. After the color fully formed and stabilized, the absorbance of the colored product was measured at 413 nm after diluting to the appropriate level with deionized water.

2.5.2. Method 2: Central composite design (CCD)

Using specialized statistical software, a central composite design (CCD) was employed to assess the simultaneous effects and interactions of three important factors. In an ice bath ($0\text{--}5^{\circ}\text{C}$), 10 mL volumetric flasks were used for the tests. According to the volumes and concentrations specified in the experimental design matrix, the medication, NaOH, coupling agent, and sodium nitrite were added sequentially. The DW was then used to dilute the flasks to the appropriate level. Following absorbance measurements at 413 nm, analysis of variance (ANOVA) was performed in MATLAB to determine the optimal reaction conditions. The experimental design and statistical analysis were conducted in Minitab.

3. Results

3.1. Optimization of method (1) OVAT

The optimal experimental conditions for the color-producing reaction were studied using two approaches: univariate and multivariate. The influencing factors, including the concentrations and volumes of SFA, sodium nitrite, hydrochloric acid, coupling reagent, and NaOH, as well as the reaction temperature, heating time, and the type and amounts of acid and base, were carefully studied using a univariate approach for each factor. The results indicated that, among the various acids tested (HCl, HNO₃, H₂SO₄, and CH₃COOH), HCl was the most effective mediator for the diazotization of the studied substance, as shown in **Figure 1**. One mL of 0.2 M HCl solution with 1 mL of sodium nitrite solution (0.01% w/v) produced the best color response when the samples were placed in an ice bath at 0–5°C for 5–10 minutes; furthermore, the use of 1 mL of Querc. The solution (100 µg.mL⁻¹) was used as the coupling agent, as shown in **Figure 2**.

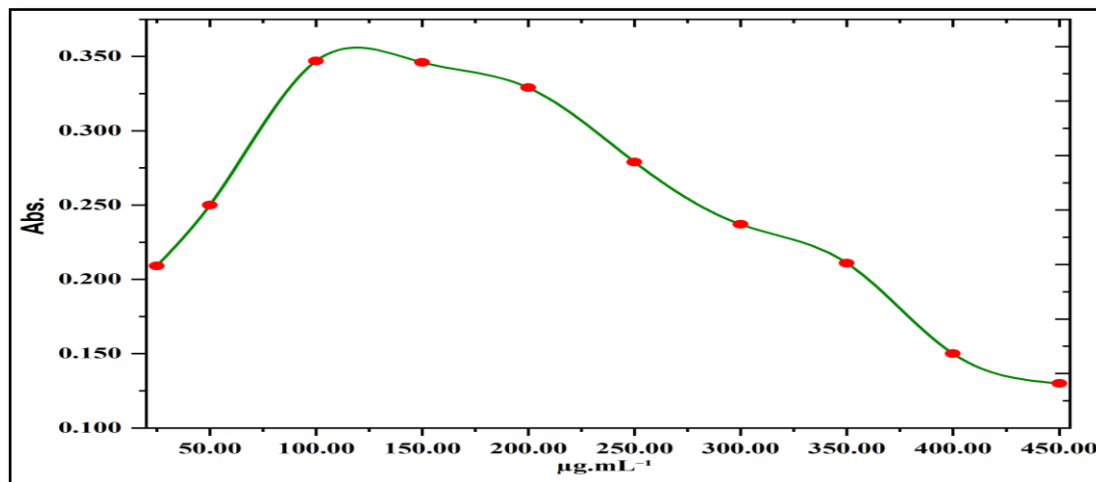


Figure 1. Effect of SFA concentration on the absorption of the colored product

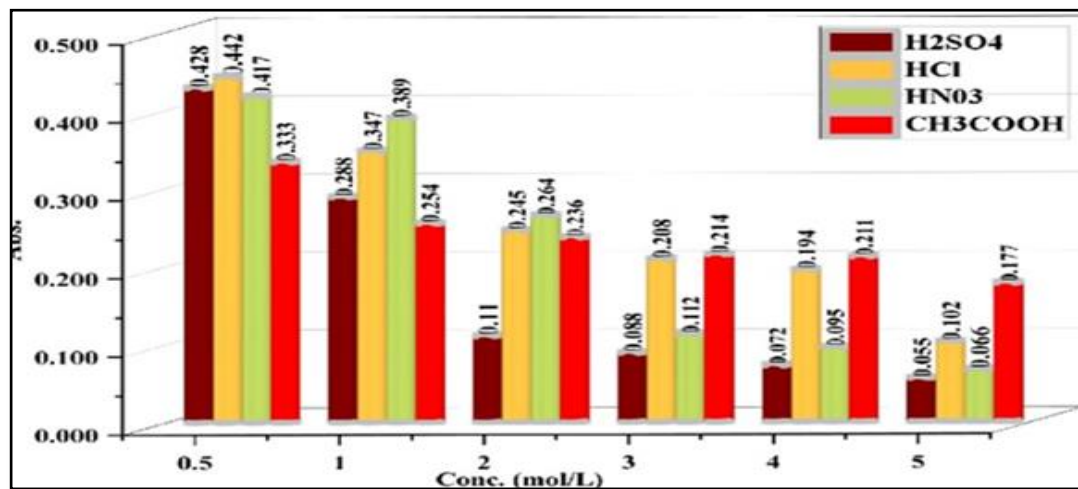


Figure 2. The effect of different acid types on the absorbance of the colored product under experimental conditions. In general, to efficiently couple the diazo-conjugated substance with the coupling reagent, it was necessary to optimize the pH and temperature of the coupling medium, given the reaction's sensitivity (**Figure 3**). Accordingly, different volumes of alkaline solutions (NaOH, KOH, Na₂CO₃, and NH₄OH) were tested (**Figure 4**). Among these, 1 mL of NaOH solution (3 M) was chosen because it provided the highest absorbance, as shown in **Figure 5**.

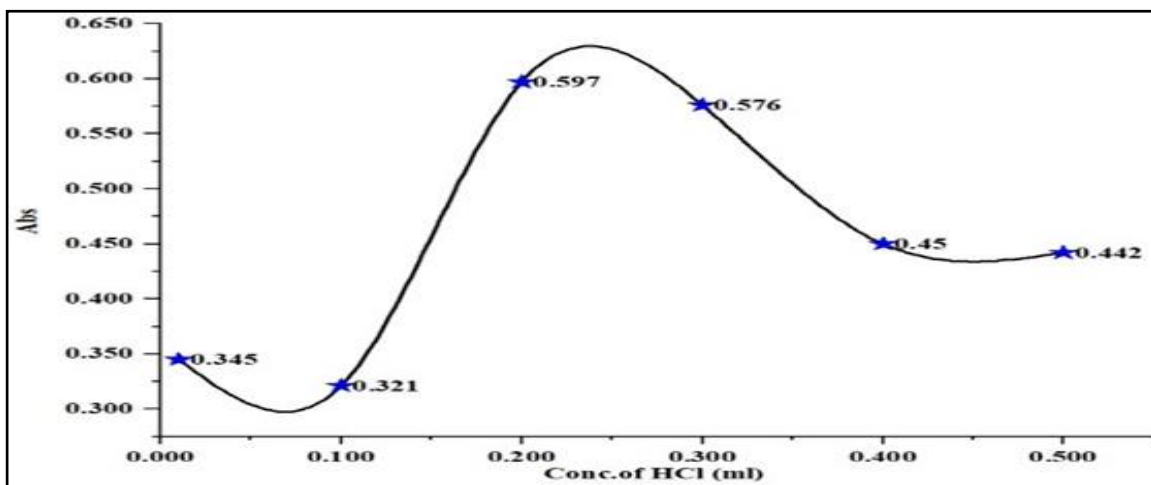


Figure 3. The effect of 1 mL of HCl concentration on the absorption of the colored product under experimental conditions, showing the highest absorbance of HCl, which is 0.597 at λ_{max} = 413 nm.

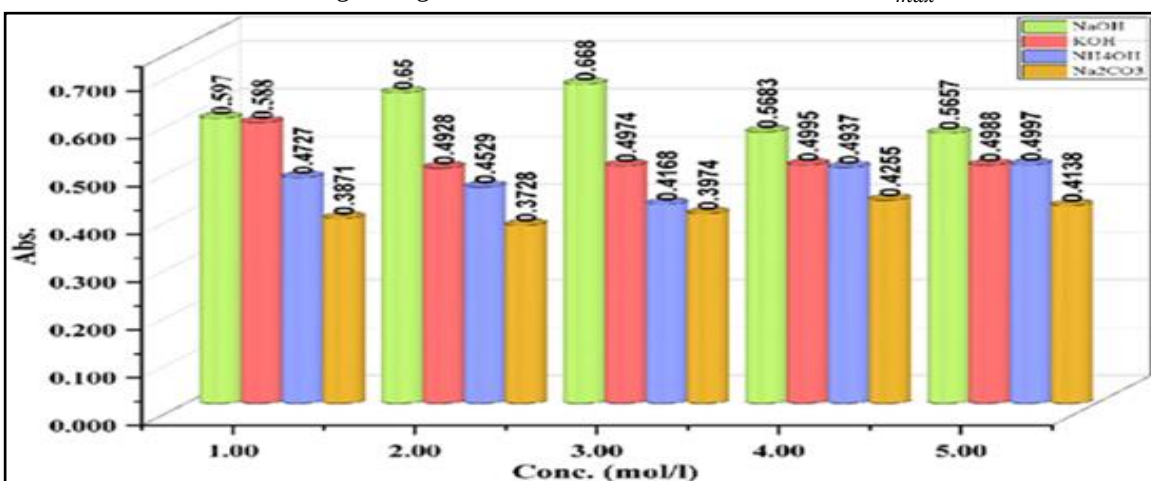


Figure 4. The effect of different base concentrations (1-5) M on the absorption of the colored product

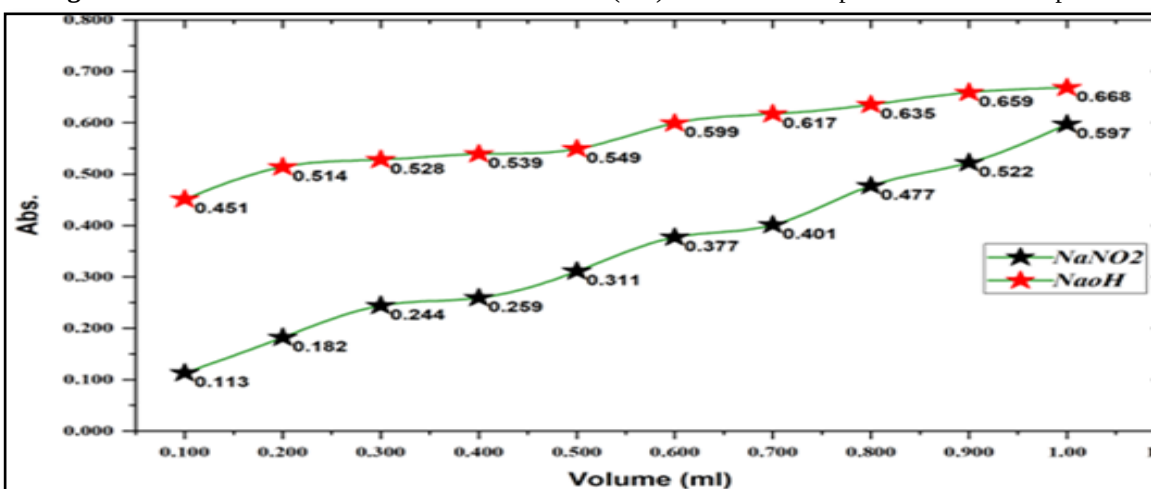


Figure 5. The optimal volume of NaOH is 3 M (1 mL) and NaNO₂ solution with a concentration of (0.011%) on the absorption of the colored product under experimental conditions.

When the reaction mixture was kept in an ice bath for 30 s before absorbance measurement, as shown in **Figure 6**, this also revealed the sequence of additions and their effects on absorbance (**Figure 7**)—the optimum conditions for Method I were established based on the maximum absorbance observed.

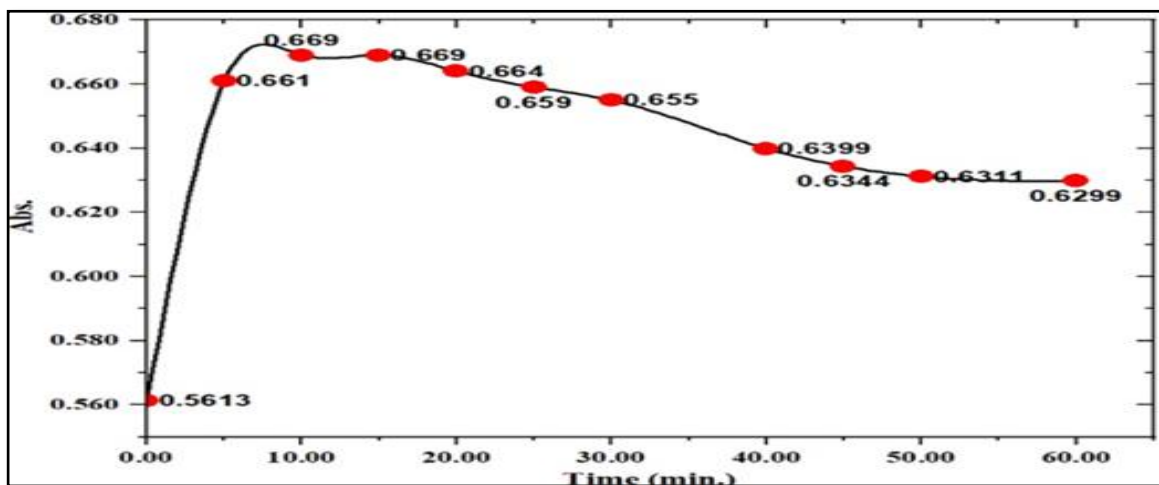


Figure 6. The optimal time range for achieving maximum absorbance is at a wavelength of 413 nm

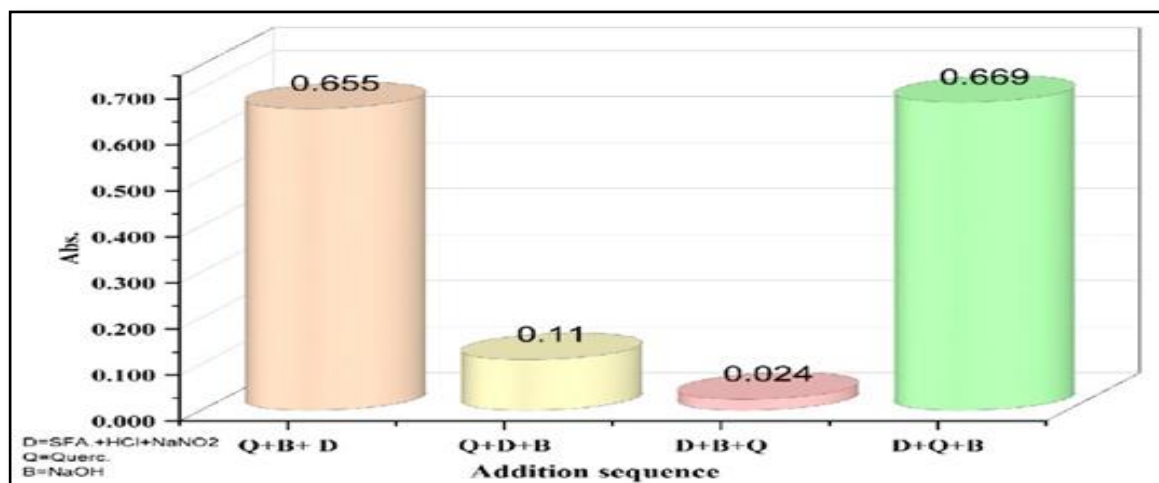


Figure 7. The sequence of addition and its effect on absorbance at a wavelength of 413 nm

3.1. Final absorption spectrum

Figure 8 compares the absorption spectra of the compound from the initial study under optimal single-variable conditions and under multivariable conditions based on the CCD results, against the blank solution at the same wavelength.

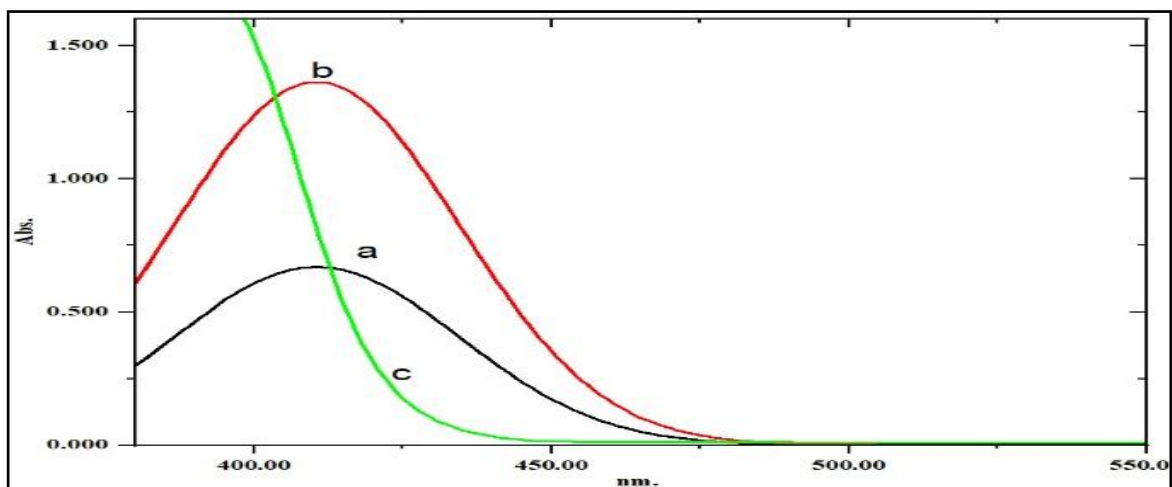


Figure 8. Absorption spectra of the chromogenic product ($\lambda_{\text{max}} = 413 \text{ nm}$) under optimal conditions using (a) method 1, (b) method 2, and (c) reagent blank

3.2. Calibration curve under conditions obtained via OVAT and CCD of method

Figure 9 illustrates the final spectra of the color product obtained under the OVAT and CCD optimization methods, respectively, demonstrating the compliance (obedience) to Beer's law in terms of linearity, accuracy, and precision.

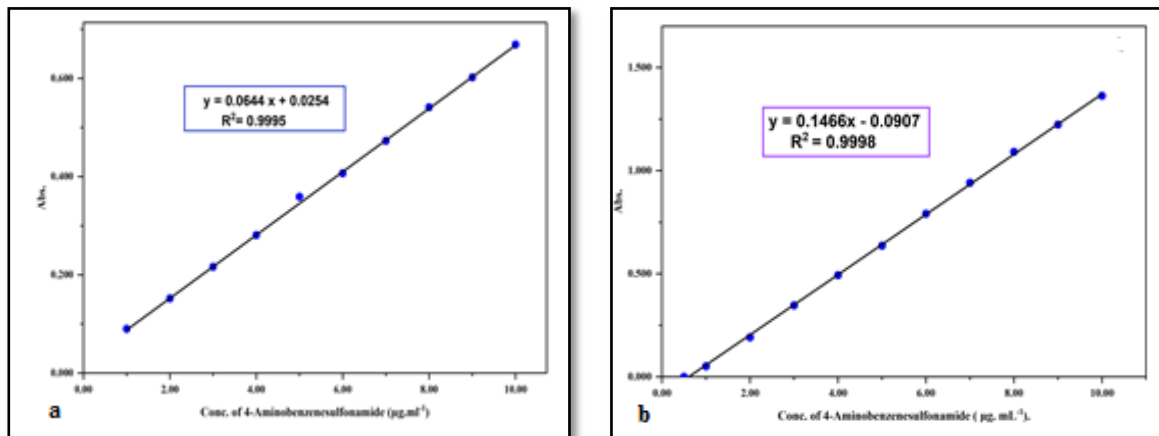


Figure 9. Final absorption spectrum of a. Method 1, b. Method 2

3.3. Analytical parameters of the OVAT and CCD methods

For the spectrophotometric determination of color product using OVAT and CCD approaches under the optimization conditions, the calibration range was obtained, and **Table 1** summarizes the optical and statistical characteristics of both optimization methods.

Table 1. Analytical Parameters of the OVAT and CCD methods

Parameter	Value of OVAT method	Value of CCD method
Equation of a straight line	$Y=0.0643X+0.0254$	$y = 0.1466x - 0.0907$
Color	Dark yellow	
Molar absorptivity, ϵ ($L \cdot mol^{-1} \cdot cm^{-1}$)	1.11×10^5	2.53×10^4
Sandell's sensitivity, S ($\mu g \cdot cm^{-2}$)	0.001	6.82×10^{-3}
Slope ($mL \cdot \mu g^{-2}$)	0.0643	0.14603
Correlation coefficient, r	0.9994	0.9997
Coefficient of determination, R^2	0.9995	0.9998
Beer's Law Limit (Linearity range, $\mu g \cdot mL^{-1}$)	1-10	1-10
Limit of Detection (LOD) ($\mu g \cdot mL^{-1}$)	0.0036	0.0769
Limit of Quantitation (LOQ) ($\mu g \cdot mL^{-1}$)	0.01089	0.2328
Standard error (σ)	0.00007	0.0034

3.4. Accuracy and precision of the OVAT and CCD methods

The precision and accuracy of the OVAT and CCD Approaches were evaluated by performing three replicates. Analysis of pure drug solutions was performed at three different concentration levels within the Beer's law limits. The percent error (RE%) and relative standard deviation (RSD) values are presented in **Table 2**.

Table 2. Accuracy and precision (n=3) of Method1 and Method 2(CCD) under optimal conditions

	Taken Conc.	Found Conc.	Recovery	Relative Error (%)	RSD% precision
	$\mu g \cdot mL^{-1}$				
OVAT Method	2	1.9378	96.890	-3.11	3.76
	5	5.1726	103.452	3.45	2.11
	8	8.0233	100.291	0.29	0.47
CCD Method	2	2.0438	102.19	2.1905	3.76
	5	5.0208	100.420	0.4163	0.91
	8	8.0766	100.96	0.96	0.90

3.5. Optimization of the multivariate/central composite design (CCD) method

A multivariate approach using CCD was employed to create a predictive model describing the changes in the responses within the experimental domain, to investigate the simultaneous variations of the independent selected factors X_1 : SFA concentration, X_2 : sodium nitrite concentration, and X_3 : quercetin concentration on the dependent response Y (absorbance). The response surface was defined, and the response for any circumstance in the experimental space was predicted using a second-order polynomial that explains the mathematical relationship between the factors and the response. Each factor had three levels-upper, central point, and lower (-1, 0, +1) and the ranges of values used in the design yielded a total of 20 different values. **Figure 8** shows a 3D drawing of the response surface results. Therefore, the highest response value was observed under the optimal experimental conditions: an SFA concentration of 350 $\mu\text{g/mL}$, an NaOH concentration of 3 M, and a sodium nitrite volume of 1 mL at a concentration of 100 $\mu\text{g/mL}$.

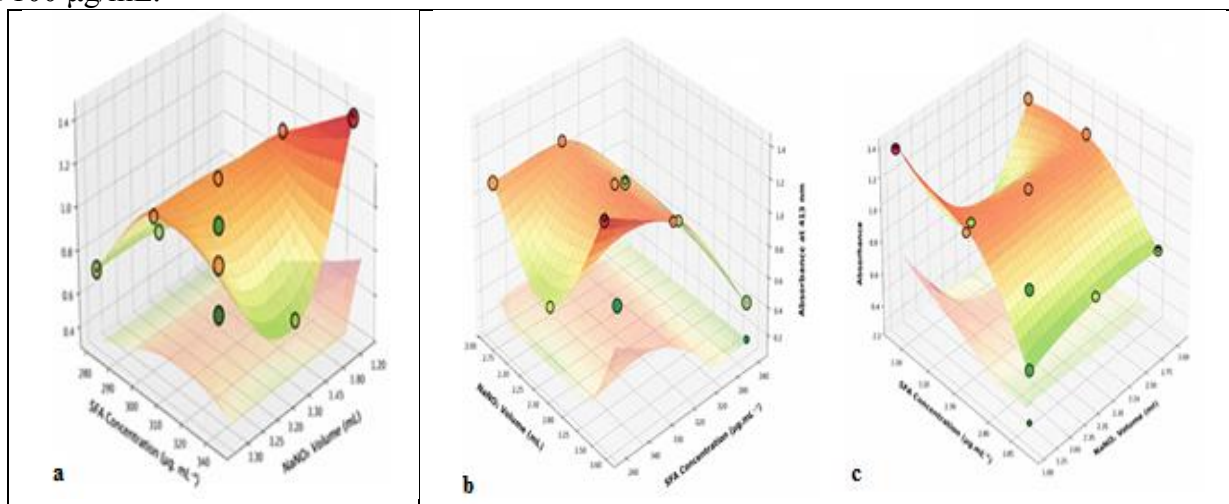


Figure 10. Response surface plots for absorbance as a function of any two of the studied variables while the third variable was held constant. A. Front view; b. side view; c. back view. The variables are defined as follows: X_1 : SFA concentration ($\mu\text{g.mL}^{-1}$), X_2 : sodium nitrite volume (mL), X_3 : NaOH concentration ($\mu\text{g.mL}^{-1}$).

3.6. Polynomial curve (Central Composite Design) (MATLAB software)

The calibration data used to construct the polynomial model are presented in **Table 3**. A fourth-degree polynomial equation was obtained based on eight calibration points.

Table 3. Determination of the linear equation using the CCD approach based on only eight data points

Point No.	Absorbance	Concentration $\mu\text{g.mL}^{-1}$
2	0.412	250
4	0.600	250
9	0.591	250
11	1.055	350
14	1.385	350
17	0.602	250
19	0.178	250

The obtained polynomial equation is shown below:

Regression coefficients:

$$\beta_0 \text{ (Constant)} = 193.4580, \beta_1 \text{ (A)} = 586.6542, \beta_2 \text{ (A}^2\text{)} = -1893.7344, \\ \beta_3 \text{ (A}^3\text{)} = 2300.0184, \beta_4 \text{ (A}^4\text{)} = -851.7043$$

Regression equation:

$$C = 193.46 + 586.65A - 1893.73A^2 + 2300.02A^3 - 851.70A^4$$

while Mean Absolute Error (MAE) = 0.17 $\mu\text{g. mL}^{-1}$

3.7. ANOVA results of response surface analysis

The statistical results of the fourth-degree equation for surface response have been summarized in **Table 4**. ANOVA confirmed agreement between spiked and found concentrations, as shown in **Figures 11** and **12**.

Table 4. Analysis of variance (ANOVA)

Source	SS	df	MS	F	p-value
Regression	18749.61	4	4687.40	36207.0549	0.0001
Residual	0.39	3	0.13		
Total	18750.00	7			

Significance: $p < 0.001$.

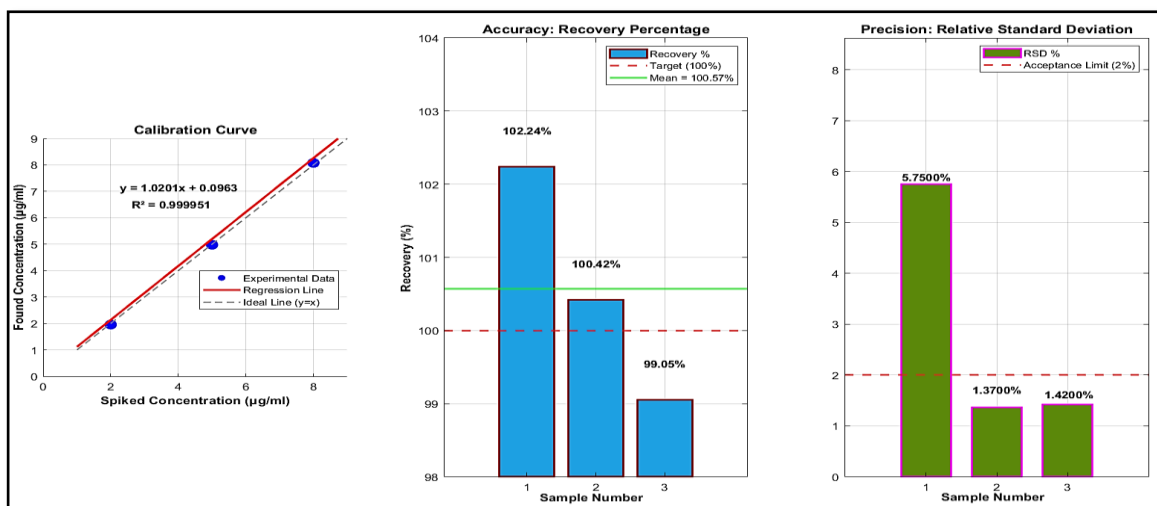


Figure 11. Results of analytical method validation

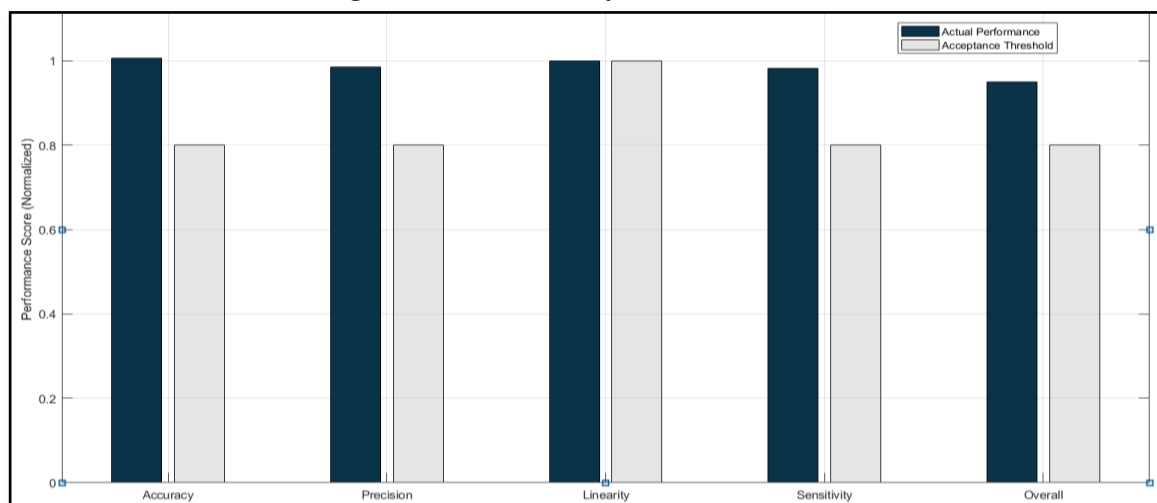


Figure 12. Performance comparison against acceptance criteria

3.8. Study of the formula of the formed azo dye

The optimal reactant ratio for the formation of the azo-coupling product was investigated using the continuous variation and molar ratio methods. Absorbance measurements from mixing different volumes of SFA and Querc. solutions at equal molar concentrations indicated that each compound had a molar concentration of 3.3×10^{-3} M. In contrast, the total moles of the two compounds remained constant, as shown in **Figure 13** and **Table 5**.

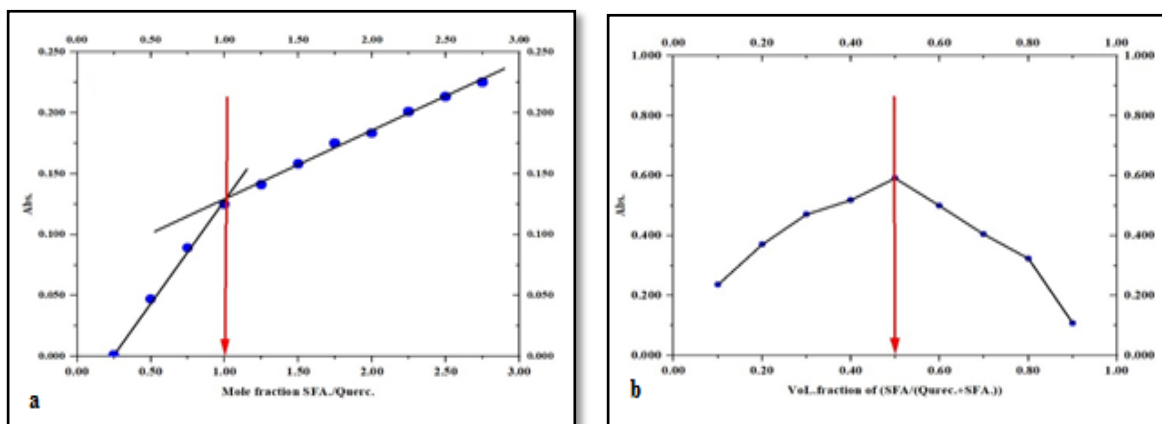


Figure 13. The complexation ratio between a reagent with compound, A/ Mole ratio for Azo complex, B/ Job's method for azo complex

Table 5. The equivalence of the proposed method

Molar ratio method				Job's method (Continuous various method) The same molarity concentration			
Using each method, the molar concentration of Querc. = 3.3×10^{-3} M, SFA.= 3.3×10^{-3} M							
$Vol_{(mL)}$ of Querc.	Vol of SFA (mL)	$\frac{V_{SFA.(mL)}}{V_{Querc.(mL)}}$	Abs.	$Vol_{(mL)}$ of SFA	Vol. of Ml	$\frac{V_{SFA..(mL)}}{V_{t(V_{SFA.}+V_{Querc.})}}$	Abs.
1	0.25	0.25	0.001	0.1	0.9	0.1	0.2362
1	0.5	0.5	0.047	0.2	0.8	0.2	0.3711
1	0.75	0.75	0.089	0.3	0.7	0.3	0.4701
1	1	1	0.125	0.4	0.6	0.4	0.5188
1	1.25	1.25	0.141	0.5	0.5	0.5	0.5907
1	1.5	1.5	0.158	0.6	0.4	0.6	0.5001
1	1.75	1.75	0.175	0.7	0.3	0.7	0.4037
1	2	2	0.1832	0.8	0.2	0.8	0.322
1	2.25	2.25	0.2007	0.9	0.1	0.9	0.107

3.9. Stability constant of the reaction product

To calculate the stability constant of the formed reaction product, it is assumed that the reaction between the substance (D) and the reagent (R) proceeds according to the following equilibrium:

$D + R \rightleftharpoons DR$, Accordingly, the stability constant K is given by

$$K = \frac{[DR]}{[D][R]} \quad (1)$$

where K represents the stability constant. If the degree of dissociation is assumed to be α and C represents the initial molar concentration (M) of sulfanilamide, the above equation can be rewritten as:

$$K = \frac{(1-\alpha)C}{(\alpha C)(\alpha C)} \Rightarrow k = \frac{(1-\alpha)}{\alpha^2 C} \quad (2)$$

The degree of dissociation α is calculated using the following relation:

$$\alpha = \frac{A_m - A_s}{A_m} \quad (3)$$

Where A_m and A_s are the absorbance values of the solution containing an excess amount of reagent and the stoichiometric amount of reagent, respectively. Based on the above equations, the calculations were carried out as follows: While $\alpha = 0.20$, $A_m = 1.363$, $A_s = 1.0911$.

The concentration of SFA was 2.0424×10^{-4} M. Therefore, the stability constant of the colored reaction product was found to be: The stability constant of the colored reaction product was calculated to be:

$K = 9.84 \times 10^4$ mol/L To evaluate the spontaneity of the reaction, the thermodynamic

$$\Delta G = -RT \ln K_{eq}$$

$$\Delta G = -RT \ln K_{eq} \Rightarrow \Delta G = -28883.60 \text{ (J.mol}^{-1}\text{)} \quad (4)$$

3.10. Study of the application of the method to a pharmaceutical preparation

The proposed method was applied to determine SFA ($10 \mu\text{g.mL}^{-1}$) in the presence of various excipients ($100 \mu\text{g.mL}^{-1}$) as shown in **Table 6**, with recovery percentages ranging from 99.26% to 104.03%. The precision of the method for determining SFA ($350 \mu\text{g.mL}^{-1}$) in a pharmaceutical preparation is summarized in **Table 7**. Furthermore, the standard addition method (**Figure 14** and **Table 8**) confirmed the suitability of the proposed method for determining SFA in a synthetic sample, with an original concentration of $34.5 \mu\text{g.mL}^{-1}$ and a recovery of 100.48%. Recoveries with sucrose and acacia were close to 100%, while glucose and lactose showed slightly higher recoveries. Proposed mechanism for the nitrification reaction of SFA substance and Querc reagent is showed in **Scheme 1**.

Table 6. Effect of interferences study

Excipient ($100 \mu\text{g mL}^{-1}$)	Conc. of SFA, $\mu\text{g mL}^{-1}$		Rec. %
	Present	Found	
Sucrose	10	9.9259	99.26
Glucose	10	10.4034	104.03
Lactose	10	10.3279	103.28
Acacia	10	9.9415	99.42

Table 7. Precision of the proposed method for SFA determination

Pharmaceutical Preparation	present Conc.	Found Conc.	SD	E (%)	RSD%	Rec (%)	RSD (%)
	$\mu\text{g. mL}^{-1}$						
350 ($\mu\text{g mL}^{-1}$)	3	3.0175	0.0000	0.5844	0.0498	100.58	0.0000
	7	7.0762	0.0001	1.0885	0.0369	101.09	0.0014
	9	9.0005	0.0002	0.0053	0.0422	100.01	0.0022

*Average of 6 determination, SD = Standard Deviation, RSD = Relative Standard Deviation

Table 8. Estimation of SFA in commercial pharmaceutical formulations using the standard addition method

Name	Regression Equation	Correlation coefficient	Found concentration after addition ($\mu\text{g.mL}^{-1}$)	Original Conc. ($\mu\text{g.mL}^{-1}$)	Recovery %
Synthesis	$y = 0.11557x + 0.11219$	0.9997	34.6653	34.5	100.48

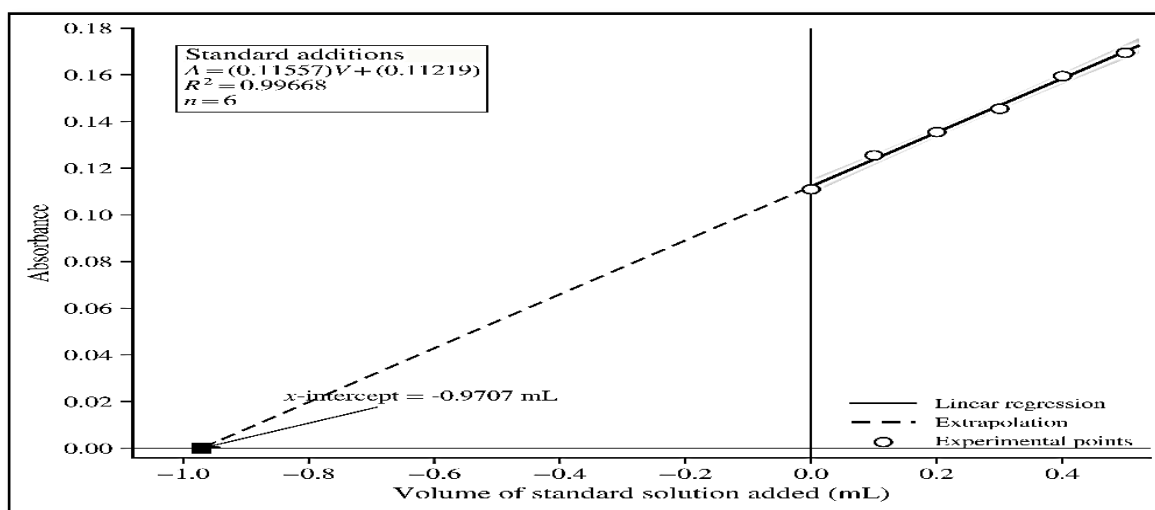
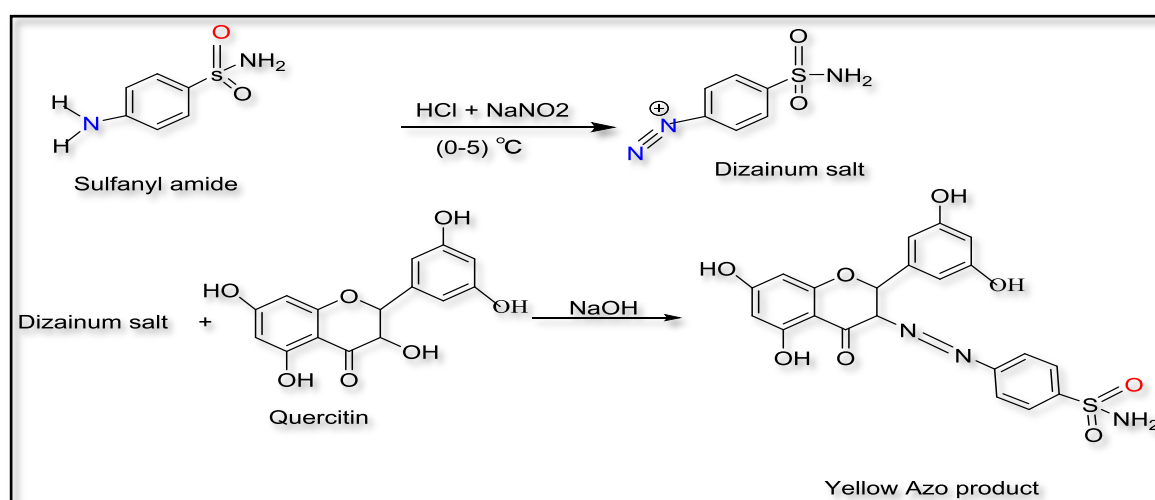


Figure 14. Determination of SFA in synthetic sample by standard additions method

Table 9. Results of previous studies for estimating SFA

Analytical method	Comment	LOD	Linear range ($\mu\text{g.mL}^{-1}$)	RSD%
Derivative method	Determination of sulfanilamide and Furosemide	0.1	1-30	3.46%
Spectrophotometry	Configuration of a charge-transfer complex using perylmelic dihydride (PMDA) as a π acceptor	0.0136	0.2 – 35	>0.6
HPLC–UV	Liquid-liquid extraction by salting (SALLE) followed by HPLC-UV analysis at a wavelength of 260 nm	0.3 ng.mL^{-1} ($0.0003 \mu\text{g. mL}^{-1}$)	0.001 – 10	1.55%
Spectrophotometry	Diazotization and coupling reaction with diphenylamine in an acidic medium	0.016	0.5 – 7.0	-
Voltammetry	Electrochemical probe composed of cobalt-phthalocyanine-chitosan on a glassy carbon tip	~ 0.046	$\sim 0.17\text{-}9.1$	-

**Scheme 1.** Proposed mechanism for the nitrification reaction of SFA substance and Querc reagent

4. Discussion

The results demonstrated the reliability of the single-spectral method for drug estimation, as an optimal absorption response was achieved under the selected conditions. This is attributed to the formation of a stable-colored product when SFA reacts with quercetin. Absorbance intensity is directly influenced by experimental variables, including pH (via acid and base adjustments)^{18,19}, the concentrations of SFA, quercetin, and sodium nitrate, and reaction time, consistent with diazotization-coupling reactions. The multivariate method was more efficient for optimizing conditions. The response surface design (RSM)²⁰ allowed for the study of interactions between variables, resulting in more accurate optimal conditions compared to the single-spectral method. This, in turn, improved sensitivity and response. The experimental design was based on CCD, which enables efficient estimation of quadratic models and interaction effects²¹. The calibration relationship was linear within a specific concentration range, with deviations observed at higher concentrations due to deviations from Beer's law. Consequently, the polynomial model more accurately represented the experimental data, improving quantitative estimation. Accuracy and control assessments indicated good reliability for both methods compared with previous studies, with the multivariate method demonstrating superior performance, attributed to its comprehensive control of influencing factors and reduced experimental variance. The molar ratio study (using Job's method and the continuous variation method) confirmed that the reaction proceeds according to a specific stoichiometric ratio,

indicating the formation of a stable complex. This finding is fundamental to interpreting the reaction. Based on the above, a plausible reaction mechanism is proposed via azo coupling between the drug's active functional group and the reagent, yielding a colored product that absorbs at the maximum wavelength. The molar ratio and spectral results support this mechanism and are consistent with previous studies on similar reactions. The method was applied to a pharmaceutical formulation, where it successfully determined the analyte without significant interference from excipients, indicating its selectivity and practical applicability.

5. Conclusion

This study aimed to develop a sensitive and reliable spectrophotometric method based on diazotization followed by azo coupling with quercetin to determine SFA in pure form using a UV-Visible spectrophotometer. The proposed method was developed using two approaches: the first relied on univariate optimization, while the second incorporated multivariate optimization. The findings showed that the multivariate method was superior in sensitivity, accuracy, and absorbance, while also requiring less time and effort. The method was successfully applied to determine SFA in two commercial pharmaceutical products: sulfadiazine tablets and sulfadiazine sodium syrup. The results confirmed the method's suitability for routine quality control analysis. Overall, the proposed method proved to be simple, rapid, and highly reliable for precise quantitative analysis.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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