RESEARCH PAPER

A Novel Derivatization Uv Spectrophotometric Method Development And Validation For The Determination Of Caffeine By Condensation Method (Formation Of Schiff Base)

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ABSTRACT
The present study focused on the synthesis of Schiff base derivatives of caffeine and the development of UV-visible spectrophotometric methods for their quantitative estimation. The synthesized compounds were further evaluated through method validation in accordance with ICH Q2 (R1) guidelines to ensure reliability. Caffeine was reacted with phenyl hydrazine in ethanol containing acetic acid under reflux (1:1 molar ratio) to form Schiff base derivatives. The resulting product was isolated by cooling, filtered, and purified by recrystallization. A stock solution (100 mg/100 mL) was prepared, and working solutions were analyzed at 230 nm using UV spectrophotometry. The method was validated for parameters including accuracy, precision, and linearity, limit of detection (LOD), limit of quantification (LOQ), robustness, and ruggedness. The results demonstrated satisfactory accuracy, with percentage recoveries of 101.36%, 100.81%, and 98.51% at 80%, 100%, and 120% concentration levels, respectively. Precision studies showed excellent repeatability with %RSD values below 0.3%. Linearity was observed over the range of 1.5–7.5 µg/mL with a correlation coefficient (r^2) of 0.9998. LOD and LOQ were calculated as 3.17µg/mL and 9.6 µg/mL, respectively. Robustness and ruggedness studies (RSD < 0.3%) confirmed the consistency and reproducibility of the developed method.
Keywords: Caffeine, Schiff base, UV-visible spectrophotometry, ICH Q2(R1) validation, Analytical method development.
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INTRODUCTION

Caffeine, a naturally occurring xanthine alkaloid, is well known for its central nervous system–stimulating effects and is commonly incorporated into pharmaceutical and nutraceutical formulations. One promising strategy to enhance its pharmacological profile is the structural modification of caffeine, particularly through condensation reactions that generate Schiff bases. These compounds, formed via the reaction of a primary amine with a carbonyl group, are widely reported to exhibit diverse biological activities, such as antimicrobial, anti-inflammatory, and anticancer properties[2, 5, 8]. Physiologically, caffeine stimulates lipolysis and elevates adrenaline levels, effects that may contribute to sparing muscle glycogen during energy metabolism. It also enhances calcium release, a process essential for muscular contraction[9]. The principal mechanism underlying its central action, however, is antagonism of adenosine receptors in the brain, which leads to heightened alertness and neural stimulation[11]. The condensation of substituted anilines containing amino groups with carbonyl-substituted caffeine derivatives results in the formation of imine linkages (−C=N−), producing novel Schiff base derivatives[10]. These derivatives possess planar conjugated structures that significantly influence their electronic transitions, making them particularly suitable for characterization by UV-visible spectroscopy[3,13]. The simultaneous equation method in UV-visible spectrophotometry offers a robust quantitative approach for determining the concentration of multiple analytes in a mixture without requiring prior separation[15]. This technique is especially advantageous when the absorption spectra of the components show considerable overlap[15]. Spectrophotometric techniques, especially UV-visible spectroscopy, remain a preferred choice for pharmaceutical analysis owing to their simplicity, sensitivity, and cost-effectiveness[1,14]. However, proper validation of such methods is crucial and must align with International Council for Harmonization (ICH) guidelines Q2(R1), ensuring parameters such as precision, accuracy, linearity, specificity, and robustness are met[6,7]. The development and validation of UV spectroscopic methods

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for caffeine-derived Schiff bases enable both qualitative and quantitative evaluations while ensuring reproducibility and reliability in pharmacokinetic and quality control investigations [1,4]. Prior studies reporting the successful UV estimation of Schiff bases and xanthine derivatives provide a strong basis for continued research[12,14]. Accordingly, the present study emphasizes the synthesis of caffeine derivatives through Schiff base formation and the validation of UV spectrophotometric methods for their estimation[3,10]. This integrated approach offers a meaningful contribution to the growing domain of functional caffeine-based compounds with potential pharmaceutical applications [5,8].

MATERIALS

All chemicals and reagents employed in this study were procured from standard commercial suppliers. Caffeine (1,3,7-trimethylxanthine) was obtained from Oxford Lab Fine Chem Ltd., while acetic acid was supplied by Loba Chem Pvt. Ltd. The primary amine used for Schiff base synthesis, phenyl hydrazine, was purchased from Nice Chemical Pvt. Ltd. Additional reagents included sodium hydroxide (Oxford Lab Fine Chem LIP) and ethanol (Cian Agro), the latter serving both as a reaction medium and solvent for spectroscopic analysis. The synthesized compounds and intermediate products were characterized and quantified using a UV-Visible spectrophotometer (Shimadzu UV-1900i). Standard laboratory apparatus utilized during synthesis and analysis included a reflux condenser for controlled heating, an analytical balance (Weigh Pro – Prime series) for accurate mass determination, as well as calibrated burettes, pipettes, volumetric flasks, beakers, and measuring cylinders for solution preparation and titrimetric procedures.

METHODOLOGY

Preparation of Reaction Mixture

A precisely measured amount of caffeine (1 g) was weighed, and phenyl hydrazine solution was prepared in ethanol containing a few drops of acetic acid. The synthesis was performed using an equimolar (1:1) ratio of caffeine to phenyl hydrazine. The reactants were then combined in a conical flask and mixed thoroughly to facilitate Schiff base formation.

Heating the Reaction Mixture

In such reactions, refluxing is generally performed in the presence of ethanol and acetic acid to achieve complete dissolution of the reactants and to enhance the reaction rate. Phenyl hydrazine reacts primarily with the carbonyl group of caffeine, and occasionally with nitrogen atoms, resulting in the formation of aniline or hydrazone derivatives. The advancement of the reaction is often indicated by a distinct color change and the formation of a solid product, confirming the successful synthesis of the desired compound.

Cooling and isolation

The reaction mixture was heated for a suitable period, usually 1–2 hours, and then allowed to cool to room temperature. When a solid product formed, it was collected by vacuum filtration. If no solid was observed, the reaction was either subjected to further refluxing or the solvent was evaporated to promote the formation of the product.

Purification

If the reaction yield a solid product purify it by recrystallization using solvent ethanol

Reaction

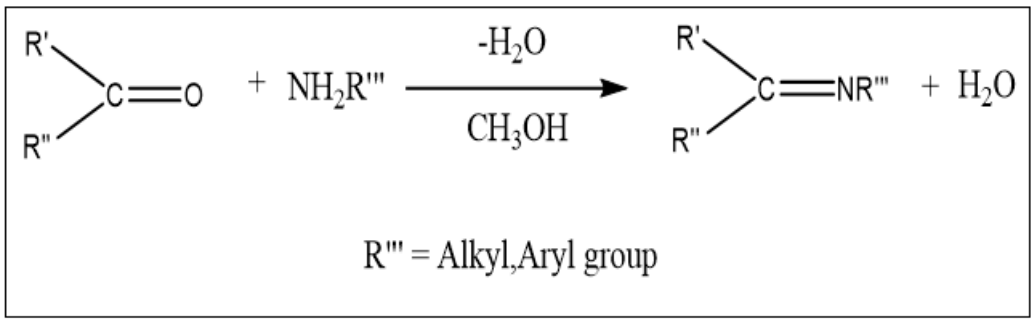


Figure-1: General Reaction

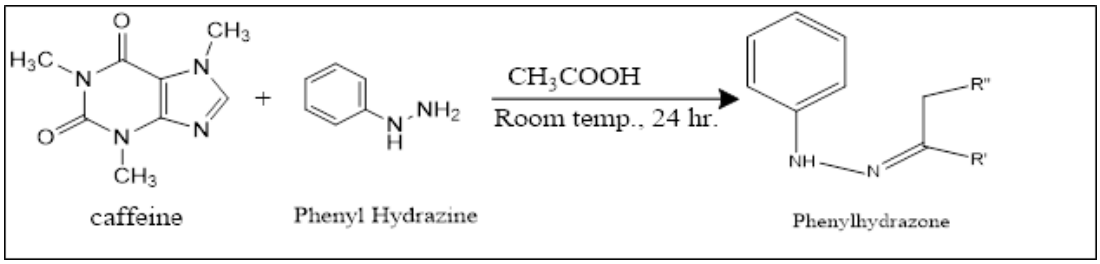


Figure -2: Caffeine & Phenyl Hydrazine Reaction

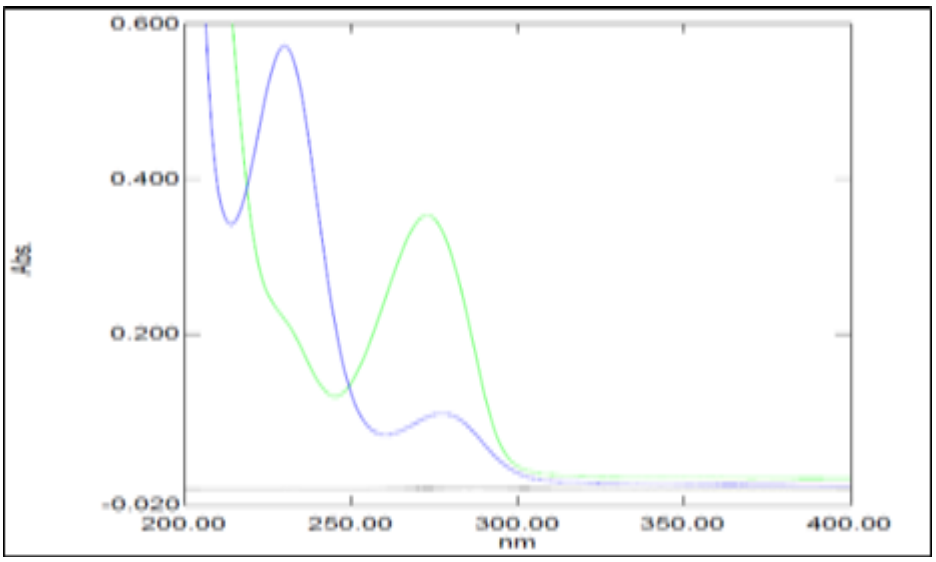


Figure -3: Synthesis of Caffeine Derivatives

Method Development

A precisely weighed 100 mg of the synthesized compound was dissolved in 100 mL of distilled water in a 100 mL volumetric flask to prepare the stock solution. From this stock, 1 mL was transferred into another 100 mL volumetric flask and diluted to the mark to obtain a 10 ppm solution. The absorbance of the 10 ppm solution was measured using UV spectroscopy, but it was found to be outside the desired range. Consequently, 4.5 mL of the 10 ppm solution was taken and diluted to 10 mL in a volumetric flask to prepare a 4.5 ppm solution. The absorbance of this solution fell within the desired range, and therefore, the 4.5 ppm solution was selected for use in subsequent experiments.

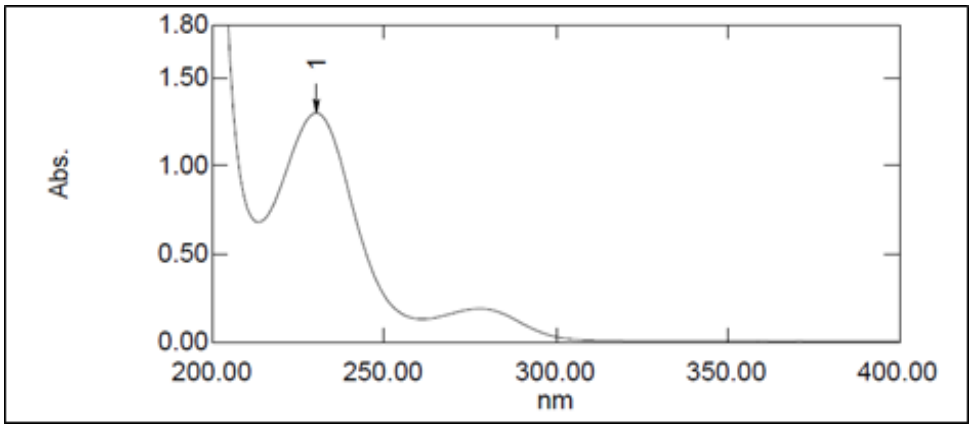


Figure -4: UV Spectra of 10PPM of Solution Mixture

Method validation

Validated documentation ensures a high degree of confidence that a particular process will consistently produce a product that meets its predefined specifications and quality attributes. Due to the diverse range of procedures, processes, and activities that require validation, the field is divided into several categories, including process validation, analytical method validation, equipment validation, and facility validation. In the present study, analytical method validation was conducted, evaluating the method across multiple parameters to confirm its accuracy, precision, and overall reliability.

Accuracy

Accuracy is defined as the closeness of a measured value to the accepted true value and is commonly related to bias error. In this study, 100 mg of the synthesized compound was precisely weighed and dissolved in 100 mL of distilled water in a 100 mL volumetric flask to prepare the stock solution. From this stock, volumes of 3.6 mL, 4.5 mL, and 5.6 mL were transferred into separate 10 mL volumetric flasks and diluted to the mark to obtain solutions with concentrations of 3.6 µg/mL, 4.5 µg/mL, and 5.6 µg/mL, each prepared in triplicate. The absorbance of these solutions was measured, and the percentage recovery was calculated, expected to be within 98–102%. The relative standard deviation (RSD) was also determined, with values below 2% considered acceptable, to verify the precision of the method.

Precision

Precision denotes the degree of agreement among multiple measurements of the same homogeneous sample under defined conditions, reflecting the reproducibility or repeatability of a method. In the present study, only repeatability was evaluated. For this purpose, 100 mg of the synthesized compound was accurately weighed and dissolved in a 100 mL volumetric flask. Subsequently, 4.5 mL of this solution was transferred into a 10 mL volumetric flask and diluted to the mark. The absorbance of this solution was measured six times, and the mean along with the relative standard deviation (RSD) was calculated, with an RSD below 2% considered acceptable to demonstrate the precision of the method.

Linearity

Linearity describes a method's capability to yield results that are directly proportional to the analyte concentration within a specified range. In this study, 100 mg of the synthesized compound was precisely weighed and dissolved in a 100 mL volumetric flask to prepare the stock solution. From this stock, five concentrations—1.5, 3, 4.5, 6, and 7.5 µg/mL—were prepared by transferring 1.5 mL, 3 mL, 4.5 mL, 6 mL, and 7.5 mL, respectively, into separate 10 mL volumetric flasks and diluting each to the mark. The absorbance of these solutions was recorded at 230 nm, and the resulting data were used to generate a calibration curve. From this curve, the slope, intercept, correlation coefficient, standard error, and standard deviation of the intercept were calculated to assess the method's linearity.

Limit of detection (LOD)

The limit of detection (LOD) refers to the minimum concentration of an analyte that an analytical method can reliably identify. It is usually determined using statistical approaches, either by assessing the signal-to-noise ratio or by employing formulas derived from blank sample measurements and their corresponding standard deviations.

LOD = (3.3 X Std. Deviation of Intercept) / Slope

Limit of Quantification (LOQ)

The limit of quantification (LOQ) refers to the minimum concentration of an analyte that can be determined with acceptable levels of precision and accuracy. It defines the point at which the method can consistently and reliably measure the analyte's concentration. This differs from the limit of detection (LOD), which merely identifies the presence of the analyte without allowing for accurate quantification.

LOQ = (10 X Std. Deviation of Intercept) / Slope

Robustness

Robustness is defined as the ability of an analytical method to remain unaffected by small, intentional variations in procedural parameters. In this study, 100 mg of the synthesized compound was precisely weighed and dissolved in a 100 mL volumetric flask to prepare the

stock solution. From this stock, 4.5 mL was aliquot into three separate 10 mL volumetric flasks and diluted to the mark. The absorbance of these solutions was measured at three different wavelengths (229 nm, 230 nm, and 231 nm), and the mean absorbance along with the percentage relative standard deviation (RSD) was calculated to evaluate the method's robustness.

Ruggedness

Ruggedness is defined as the ability of a method to remain reliable, durable, and consistent under varying conditions. In the context of analytical procedures, it assesses the reproducibility of results under normal operational variations, including differences between analysts and laboratories. In this study, two analysts measured the same concentration (4.5µg/mL) independently, recording the absorbance six times each. The mean values and percentage relative standard deviation (%RSD) were calculated separately for both analysts, with a %RSD of less than 2% considered acceptable, confirming the method's ruggedness.

RESULT AND DISCUSSION

Accuracy

Table No-1:- Accuracy data of three concentration (80%, 100 %, 120%)

% Concentration	Concentration	Absorbance	% Recovery	Range (98-102)
80%	3.6 µg/ml	0.463	101.1%	101.36
		0.466	101.6%	
		0.469	101.38%	
100%	4.5µg/ml	0.570	100.44%	100.81
		0.572	100.88%	
		0.572	101.11%	
120%	5.6µg/ml	0.688	98.21%	98.51
		0.690	98.57%	
		0.691	98.75%	

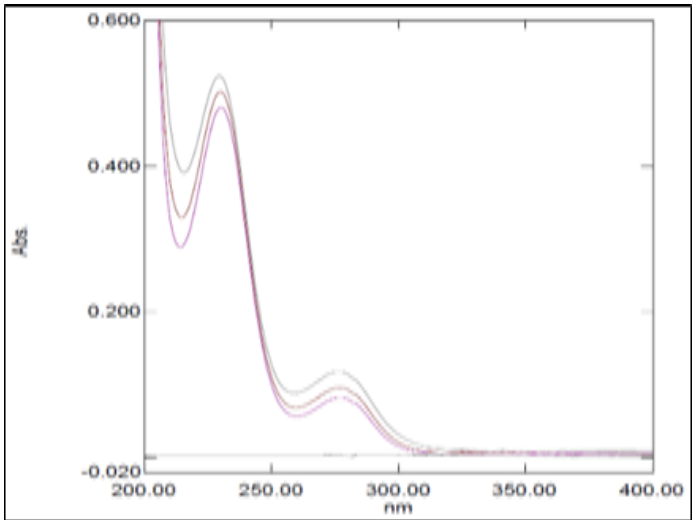


Figure -5: UV Spectra of 4.5PPM of Solution Mixture

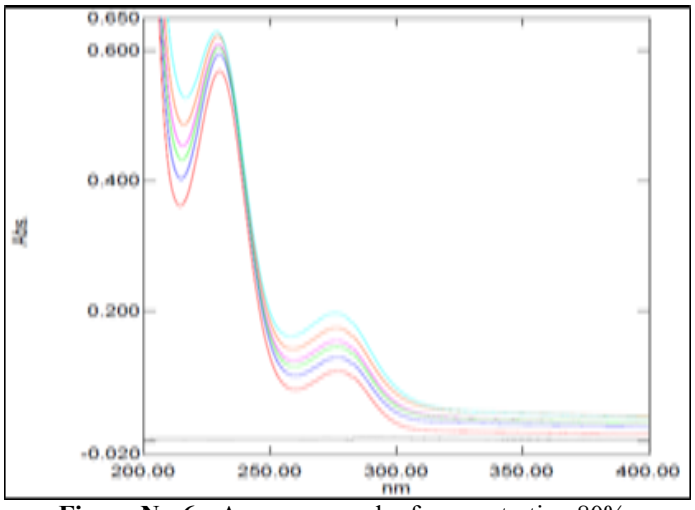


Figure No-6:- Accuracy graph of concentration 80%.

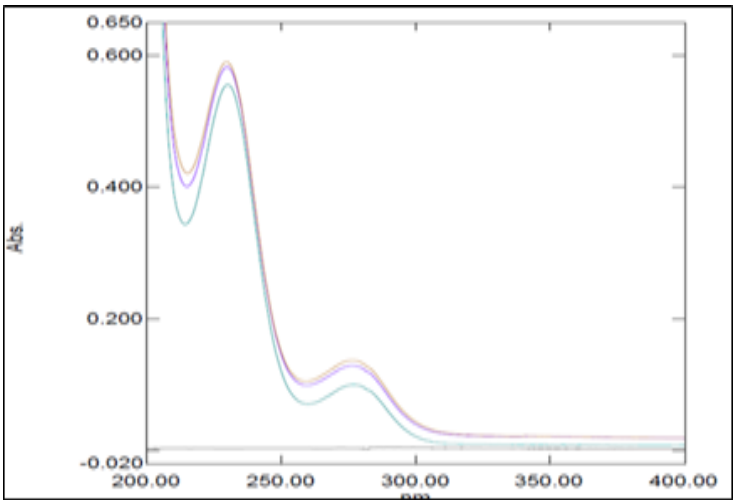


Figure No-7:- Accuracy graph of concentration 100%.

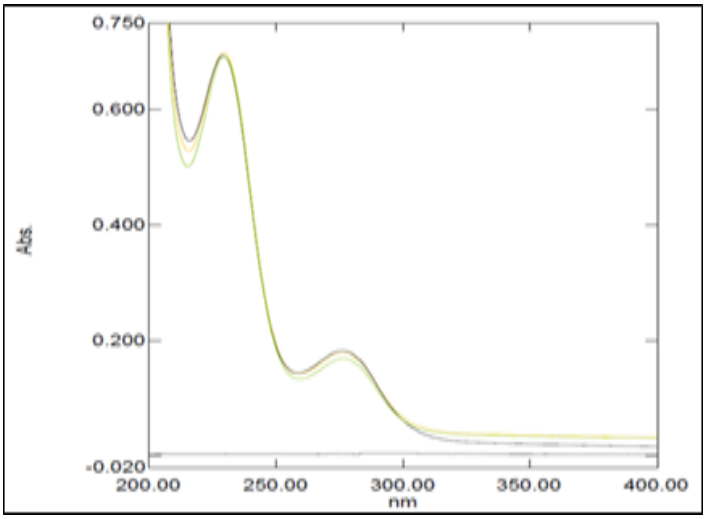


Figure No-8:- Accuracy graph of concentration 120%.

DISCUSSION

The percentage of recovery obtained from 80%, 100%, 120% concentrations are 101.36% ,100.81%, 98.51%.

Precision / Repeatability

Table No-2: Repeatability data of concentration 100 %

Solution (4.5µg/ml each)	Absorbance	Mean	%RSD
Solution 1	0.573	0.573	0.27%
Solution 2	0.572		
Solution 3	0.574		
Solution 4	0.573		
Solution 5	0.575		
Solution 6	0.576		

Discussion

The % of RSD obtained is 0.27%

Linearity

Table No-3: Linearity data

Concentration (ppm)	Absorbance
1.5	0.201
3	0.386
4.5	0.572
6	0.748

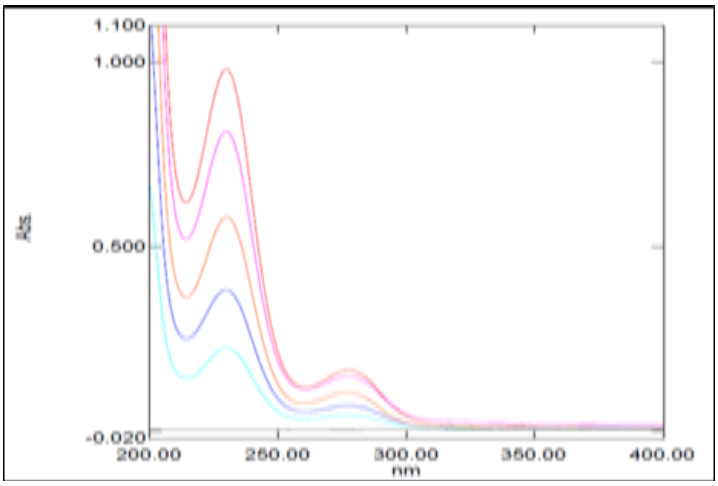


Figure No-9: Repeatability graph of concentration 100%

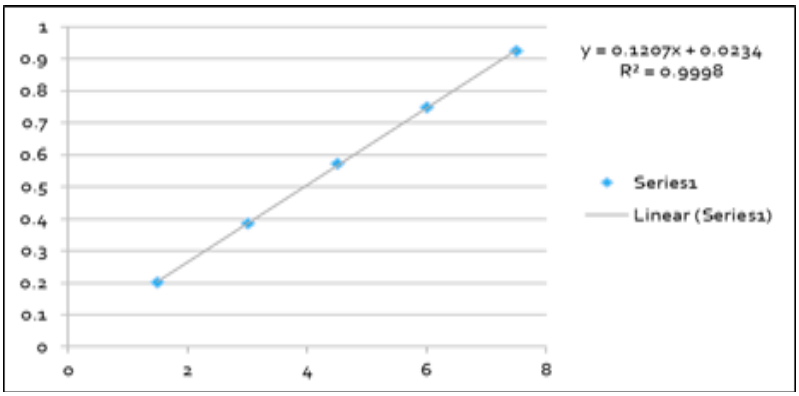


Figure No-10: Linearity graph

Figure No-11: Linearity graph of different concentration

Calculation

Y= 0.01207X + 0.0234, Slope = 0.01207, Intercept = 0.0234, r^2 =0.9998, Standard error of intercept = 0.052022204, Standard deviation of intercept

=0.052022204 × √5

=0.052022204 ×2.23606

=0.1163

Limit of Detection (LOD)

LOD = (3.3X Std. Deviation of Intercept) / Slope

= (3.3×0.1163)/0.1207

= 3.17 µg/ml

Limit of Quantification

LOQ = (10X Std. Deviation of Intercept) / Slope

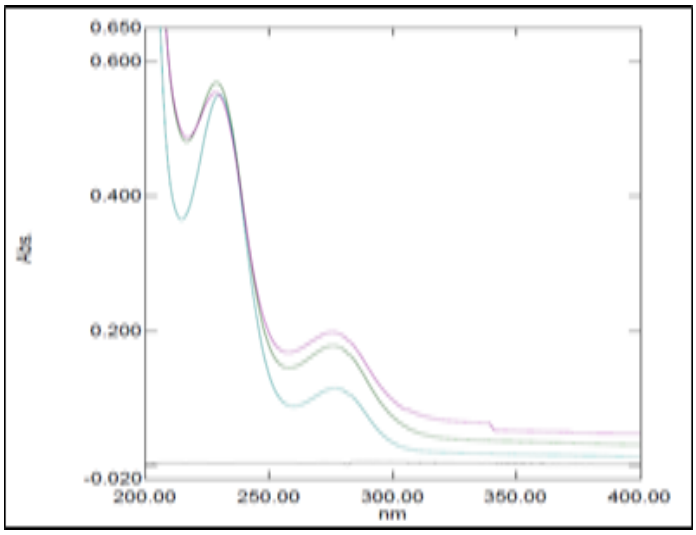
= (10× 0.1163)/0.1207

= 9.6 µg/ml

Robustness

Table No-4: Robustness table data at different wavelength

Wavelength	Concentration	Absorbance	Mean	% of RSD
229nm	4.5µg/ml	0.562	0.56	0.22%
		0.560		
		0.563		
230nm	4.5µg/ml	0.567	0.567	0.20%
		0.569		
		0.567		
231nm	4.5µg/ml	0.560	0.561	0.22%
		0.561		
		0.563		



At 229 nm

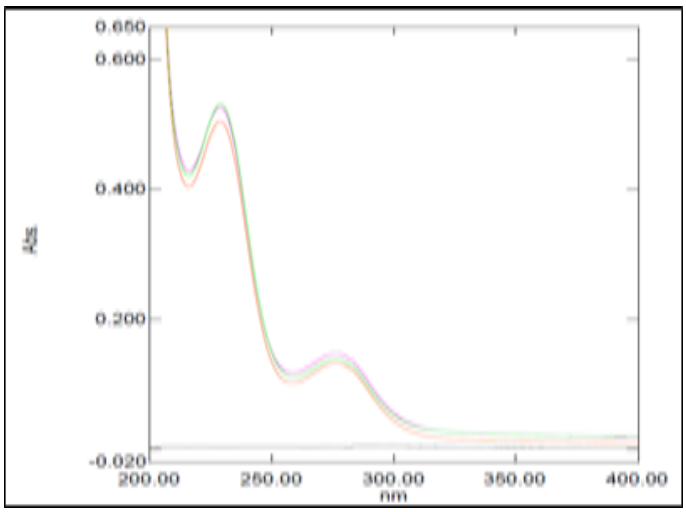


Figure No-12: Absorbance at 229 nm.

At 230 nm

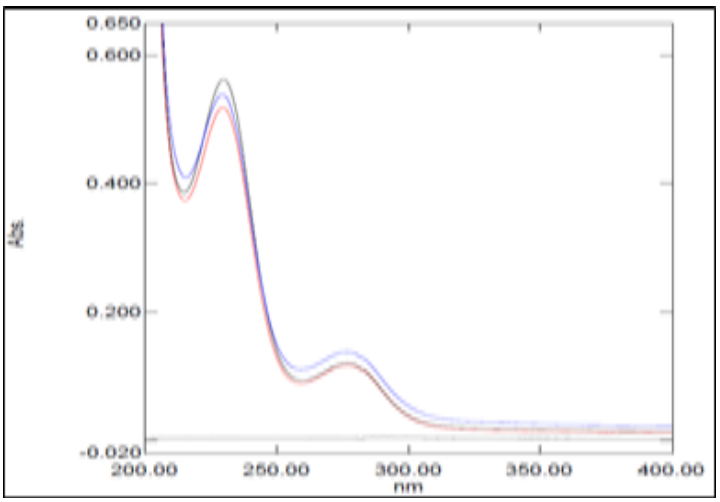


Figure No-12: Absorbance at 230 nm.

At 231 nm **Figure No-13: Absorbance at 231 nm.**

Discussion

The percentage of RSD obtained at 3 different Wavelength are 0.22%, 0.20%, 0.22%.

Ruggedness

Concentration	Absorbance	Mean	% of RSD
4.5µg/ml	0.573	0.573	0.27%
	0.572		
	0.574		
	0.573		
	0.575		
	0.576		

Table No-6: Analyst -2 data for Ruggedness

Concentration	Absorbance	Mean	% of RSD
4.5µg/ml	0.567	0.569	0.27%
	0.569		
	0.572		
	0.568		
	0.570		
	0.569		

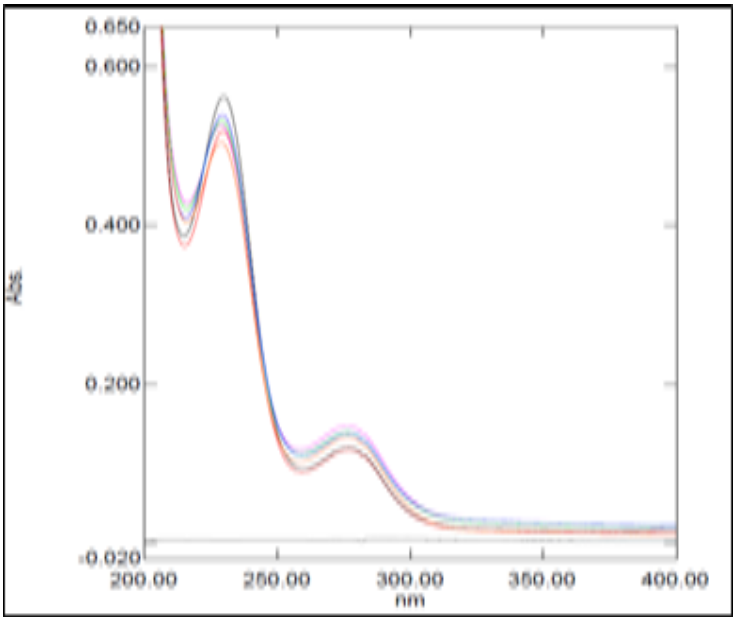


Figure No-14: Analyst -1 graph for Ruggedness

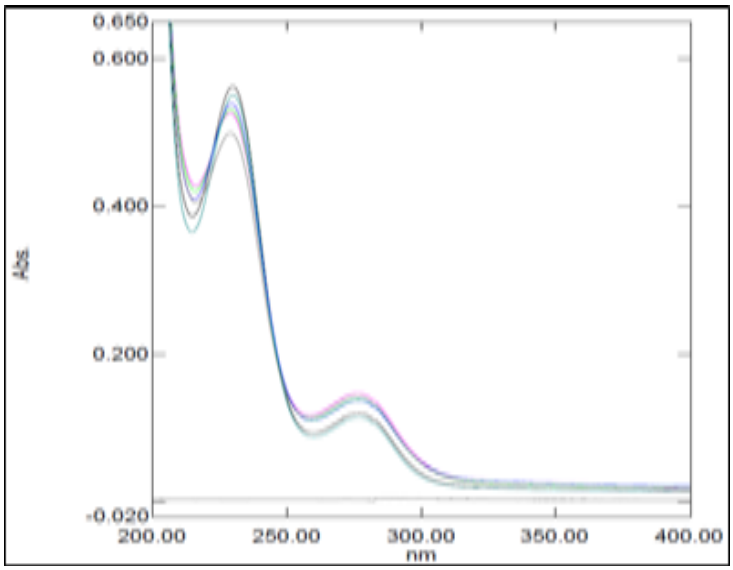


Figure No-15: Analyst -1 graph for Ruggedness

CONCLUSION

The caffeine derivatives is synthesized by condensation method and various analytical method of estimation of caffeine derivative is develop and validated by various parameters such as accuracy, precision, linearity, limit of detection, limit of quantification, Ruggedness and Robustness.

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CONFLICT OF INTEREST

There is no conflict of interest

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