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Spectrophotometric Determination of Creatinine Using a Modified Diazo Coupling Reaction: A Reliable Approach for Biomarker Analysis in Human Serum

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Abstract
This study introduces a novel and modified spectrophotometric method for the precise quantitative determination of creatinine in human serum samples, addressing the need for more specific and reliable renal function biomarkers. The proposed approach is centered on a Diazo coupling reaction, which is inherently characterized by high selectivity, sensitivity, and the formation of a stable, dark-colored product that is easily detectable. This method was developed to overcome the well-documented specificity limitations of the traditional Jaffe reaction, which is often susceptible to interference from other chromogens in serum. A key innovation of this work is the introduction of Thymole as a highly effective coupling agent, which, under optimized reaction conditions, significantly enhances the specificity towards creatinine. A systematic optimization of critical reaction parameters, including acid concentration, the choice of base, reaction time, and temperature, was performed to achieve maximum absorbance of the resultant yellow azo product at a wavelength of 403 nm. The developed method demonstrates excellent analytical performance, adhering to Beer–Lambert's law within a clinically relevant concentration range of 1.2–8.0 mg/dL, with a high coefficient of determination (R^2 = 0.9982). Furthermore, the low limit of detection (LOD = 0.39 mg/dL) underscores the method's superior sensitivity. Its efficacy was successfully validated through application to real human serum samples, showing a strong correlation with results obtained from the hospital-based Jaffe method. In conclusion, this simple, rapid, sensitive, and cost-effective technique offers a reliable and robust alternative for clinical and diagnostic applications, promising improved accuracy in creatinine measurement.
Key words: Azo dye formation; Biomarker quantification; Colorimetric method; Human serum; Thymole

Introduction

Chemicals found in living things that are essential for at least one biological activity, such as cellular division, morphogenesis, or growth, are referred to as biomolecules, often called biological molecules [1]. The large macromolecules (also known as polyanions) that comprise biomolecules include proteins, carbohydrates, metabolites, and natural constituents. The more general term for this class of materials is organisms. Biomolecules are essential components of living things [2], and when numerous and these It produces a biomolecule. internally Organisms frequently require external macromolecules, such as certain nutrients, to survive

[3]. Biomolecules exist in a variety of sizes and forms and carry out a wide range of tasks. Proteins, lipids, nucleic acids, and carbohydrates are the four primary categories of biomolecules [4]. The term "biomolecule" refers to any of the many chemicals produced by cells and living organisms. The human body is composed of only four elements: carbon, hydrogen, oxygen, and nitrogen. The bulk of biomolecules are organic substances. Nonetheless, trace amounts of a number of other elements are found, such as bio metals [5]. Because certain material types and metabolic pathways are consistent across a wide variety of organisms, the metabolic processes and biomolecules' functions are referred to as "biochemical principles" or "the concept of living organisms' substantial oneness," a unifying concept in biology that encompasses the development of cell theory [6].

One important biomarker that is frequently used to evaluate kidney function and identify metabolic diseases is creatinine. Serum creatinine measurement accuracy is crucial for determining glomerular filtration rate (GFR) and renal clearance [7]. Despite being straightforward, the Jaffe method has poor specificity because of interference from chemicals including ascorbic acid, bilirubin, and glucose. Numerous analytical methods, such as capillary electrophoresis, HPLC, enzymatic tests, and colorimetric procedures, have been reported for the quantification of creatinine. Chromatographic techniques, like LC-MS/MS, are very accurate, but they need costly equipment and skilled workers. Colorimetric sensors based on nanotechnology have also demonstrated promise, offering increased sensitivity through the catalysis of nanoparticles [8]. Despite these developments, spectrophotometric techniques are still used for everyday laboratory work because of their affordability and ease of use. By offering an alternate method for creating a colored complex that can be measured at a specific wavelength, the Diazo coupling reaction lessens the interferences that are frequently present in the Jaffe reaction. Diazonium salts, also called diazonium compounds, are functional groups of organic compounds with the formula RN2+X, where R can be any chemical X and group, like an aryl or alkyl, can be an organic or inorganic anion, such as a halogen [9].

The process of creating diazonium compounds is known by the names diazotation, dizoniation, and diazotization. [10]. After first describing the reaction in 1858, Peter Griess discovered several reactions of this novel family of chemicals in order to create diazonium salts, aromatic amines are frequently treated with nitrous acid and additional acid [11–13]. To create nitrous acid, salt nitrate and an excess of metallic acid (commonly aqueous H2SO4, HBF4, HCl, or p-H3CC6H4SO3H) are normally mixed in situ (in the same flask) [14]. One common kind of chemical reaction that employs strong acids as a catalyst is the Diazo coupling reaction [15]. The diazotization of volatile primary amines and the coupling reaction of diazonium salts with phenols or aromatic amines are the two main procedures used to make azo colors [16]. In order to determine creatinine in serum quickly and selectively, the current work intends to develop an effective spectrophotometric approach that employs a modified Diazo coupling process with Thymole as the coupling reagent.

Materials and Methods

Creatinine Preliminary Tests Using Selected Compounds

In order to investigate the proposed reaction by forming an azo coupling product, a large number of materials were chosen based on their structures. β-naphthol, naphthalene, quinolone, phenol, thymol, resorcinol, p-benzoquinone, nicotinic acid, m-toluic acid, benzoic acid, and picric acid were among the compounds that were examined. Only Thymole exhibits the capacity to participate in the intended reaction among these substances.

Optimizing the optimal creatinine assessment conditions

The following criteria must be met in order to determine creatinine:

Creatinine, Thymole, $NaNO_2$, and HCl concentrations, Volumes of Thymole, creatinine, $NaNO_2$, and HCl Ideal pH Diazotization and final product reaction times

Results and Discussion

Creatinine characteristics

Fourier transform infrared spectroscopy (FT-IR) spectroscopy

The solid creatinine FTIR scan is shown in Figure (1). As is well known, the secondary amine exhibits a single band, but the main aromatic amine displays two starching bands in the 3300–3400 cm-1 range. Figure (1) gives the impression of having a single band; however, this is not the case. Figure 2 illustrates the infrared spectrum of creatinine in both amino and amino forms, as well as the theoretical spectrum of the two forms, in accordance with the study of Iker Leon et al. [17]. The FTIR spectrum of creatinine confirmed characteristic bands corresponding to the amine and carbonyl groups, indicating structural purity, . It is observed that the theoretical spectrum is the only area where the two forums differ, with the practical spectrum being fairly comparable. In reference to the creatinine utilized in this investigation, it is present as amino.

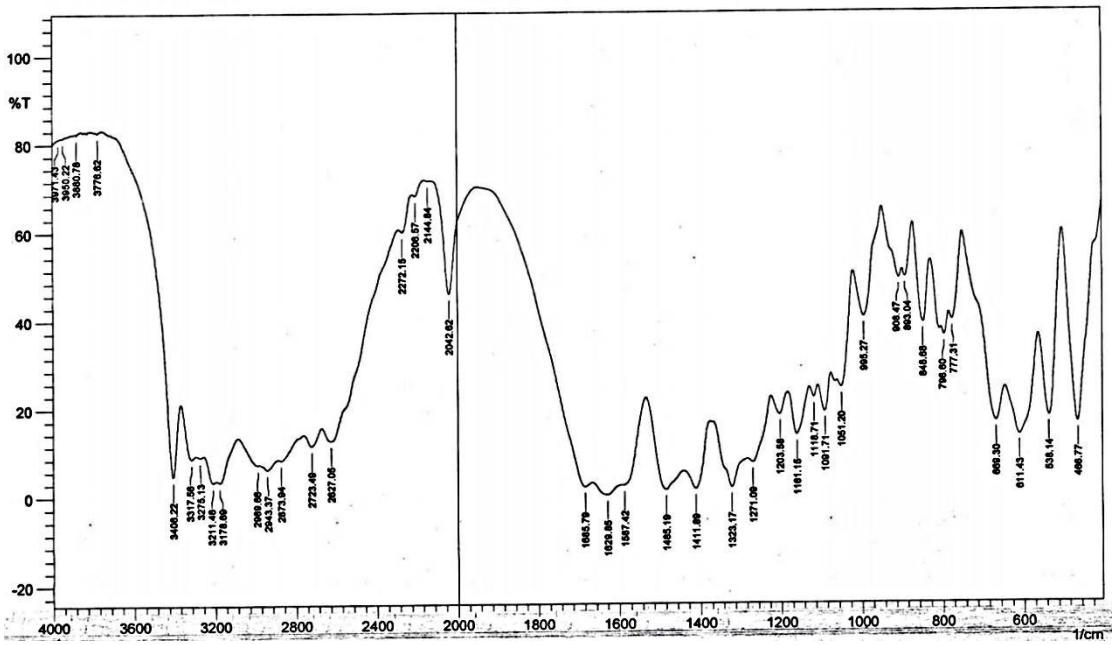

Fig. 1: FTIR spectrum of creatinine.

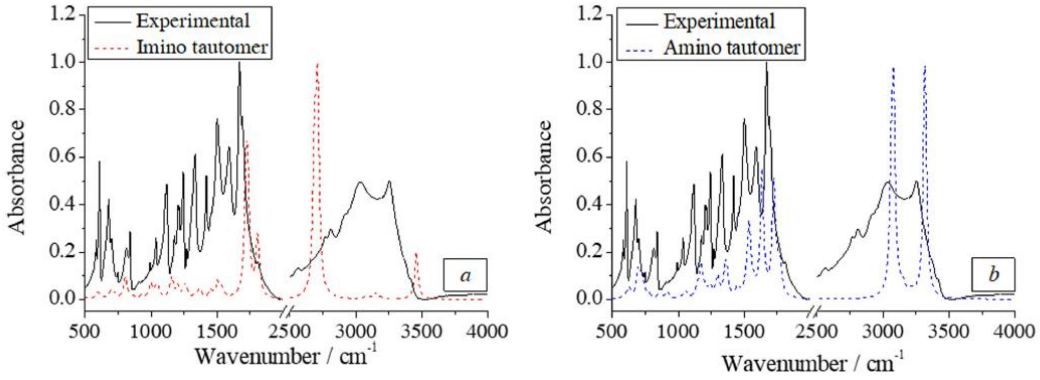

Figure 2: Comparison of the simulated counterpart derived for the imine (a) and amine (b) crystal structures inside the double-harmonic approximation using a periodic approach with the experimental infrared spectrum (between 500 and 4000 cm-1) of the creatinine crystal in KBr pellet [17]

UV Vis Spectrophotometer

Electronic UV-Visible was studied in further detail. To fix the formation of the desired product, figures (3) and (4) were measured in more than likelihood. UV–Visible spectra of creatinine, Thymole, and the resulting azo product verified product formation. Thymole demonstrated superior coupling behavior compared to other aromatic compounds such as phenol, quinolone, and β-naphthol

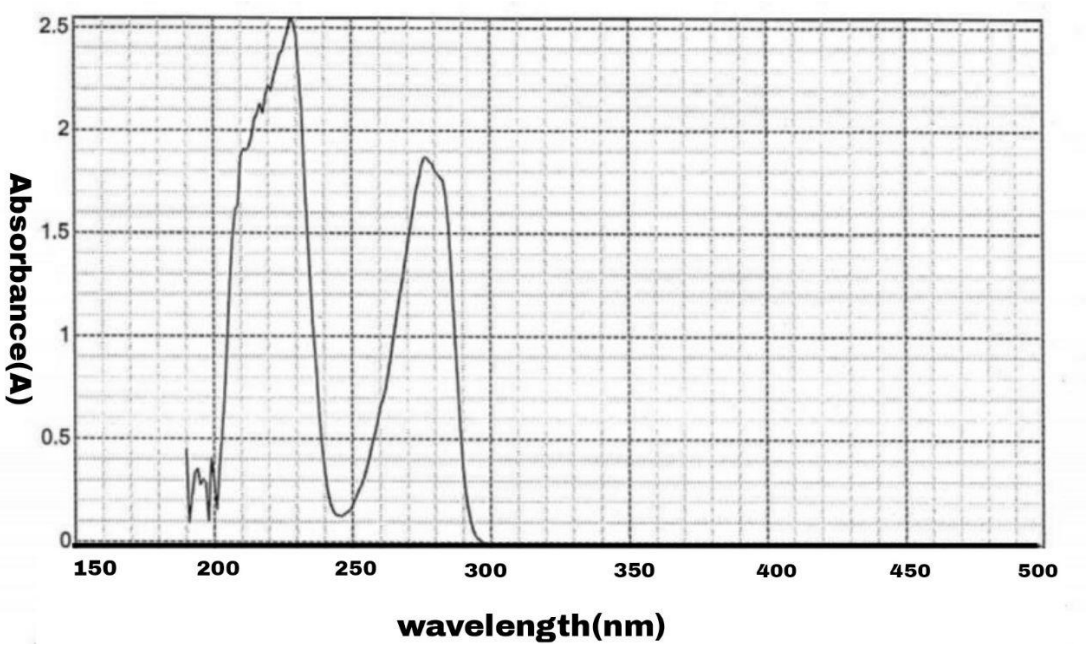

Figure 3: Thymole UV-Vis spectrum in an alkaline solution.

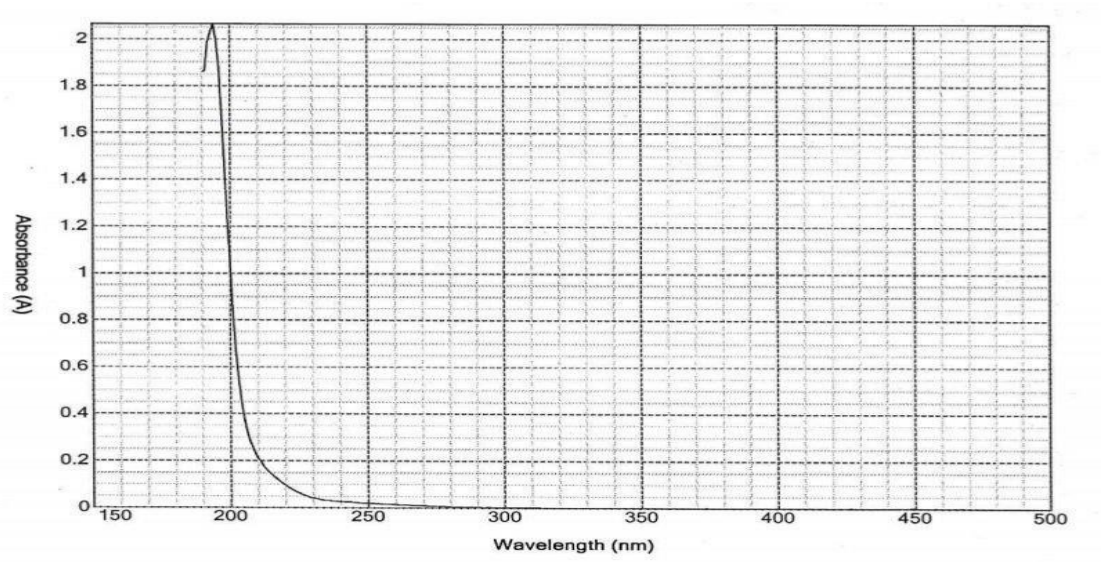

Fig. 3-4: UV-Vis. spectrum of creatinine

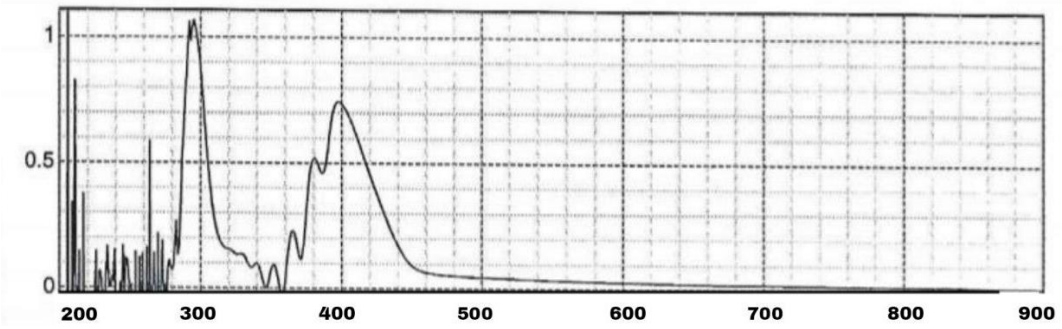

Fig. 3-5: UV-Vis. Spectrum of the yellow product

Optimal circumstances for measuring creatinine

Choosing the perfect acid for the diazotization procedure

Various acids were applied in a final volume of 25 milliliters of diazotized solution for this experiment. The following acids were used: hydrochloric acid, nitric acid, sulfuric acid, and acetic acid. Additionally, 0.01g creatinine, 0.085g $NaNO_2$, 0.075g Thymole, and 0.56g base were used in this operation. The diazotized solution and Thymole had respective volumes of 25 mL. The results of the optimal acid for creatinine evaluation are displayed in Table 1.

Table 1: Acids used for selecting the best acid for creatinine assessment.

Acid	**CH_3COOH**	**H_2S0_4**	**HNO_3**	**HCl**
ABS.	0.200	0.900	0.700	1.700

The data displayed in table (1) make it clear that hydrochloric acid is more appropriate and was selected for additional testing.

Best Volume of hydrochloric acid for diazotization process

Different volumes of 6M hydrochloric acid were tested in order to determine the ideal volume of this acid, which was chosen as the optimum acid for the diazotization procedure.

We used volumes 1, 1.5, 2, 2.5, and 3 mL. The impact of the HCl volumes used in this investigation is displayed in Table 3-5.

Table 2: Effect of volumes of HCl(6M) on diazotization process.

Volume, mL	1	1.5	2.0	2.5	3.0
ABS.	0.500	0.400	0.500	0.800	0.600

Choosing the perfect base for product formation

Several bases were used in this inquiry. The bases that were used were sodium hydroxide, potassium hydroxide, and ammonium hydroxide solution. A final volume of 25 mL of diazotized solution was prepared using the following conditions: 2 mL of 6M hydrochloric acid, 0.01 g creatinine, 0.085 g NaNO2, 0.075 g Thymole, and 0.56 g of a base. Table 3 lists the bases that were used to determine which was best for measuring creatinine.

Table 3: Bases used for selecting the suitable base for creatinine assessment.

Base	NaOH	KOH	NH_4OH
ABS.	0.600	1.300	N.R

Best Volume of potassium hydroxide for diazotization process

different volumes used this test from appropriate concentration of potassium hydroxide for detect the perfect volume of strong base. the volumes that used are (1.5,2,2.5 and 3 mL). the effect volumes of strong base are shown in the table (4).

Volume, mL	1.5	2.0	2.5	3.0
ABS.	0.537	0.498	0.736	0.665

Assessment of serum creatinine in actual participant samples

Serum samples were examined to determine the creatinine content in order to examine the validity of the existing procedure. Some tests were carried out with faulty deproteinization or without the use of a precipitating agent for the protein present in serum. The outcomes of such techniques produced data that was undesirable. Using 0.084g of $NaNO_2$ and 5mL of 7M HCl, 1mL of serum was collected and diazotized straight without deproteinization. After allowing the contents to sit in cooled bath water for ten minutes, a volumetric flask containing ten milliliters was created. One milliliter of this solution was then combined with one milliliter of alkaline Thymole solution, which was made by dissolving 0.065 grams of Thymole in 30 milliliters of KOH solution. When the absorbance was measured at 400 nm after two minutes of mixing, it was discovered to be extremely high and incompatible with the actual value. The results of these tests are displayed in Table 5.

Table 5: Creatinine estimation without deproteinization (no precipitating agents used).

Serum no.	Conc. mg/dL	
	Current method	referenced method
1	2.400	1.2
2	0.600	0.6
3	2.478	1.0
4	2.999	0.8

2 mL of the identical serum samples listed in table 6 were obtained, and 3 mL of strong hydrochloric acid was added to achieve deproteinization. Following that, the diazotization procedure was carried out using the previously mentioned diazotization conditions, and a volume of 8 mL was created. One milliliter of alkaline Thymole solution was then added to one milliliter of the resultant solution. When absorbance was tested two minutes later, the result was lower than in the first scenario, but it was still high compared to the real one table 7.

Table 6: Creatinine estimation using conc. HCl for deproteinization

Serum no.	Conc. mg/dL	
	Current method	referenced method
1	1.600	2.0
2	1.474	1.4
3	-0.168	1.3
4	0.174	0.6

Then, deproteinization process was achieved using organic solvents method. Therefore, ethanol was used for this purpose. This solvent was adapted for subsequent procedures since the result of the current method was compared by the reference method (all measurements which conducted by reference method were obtained using

Biomarker	Current method	Reference method	%Erel
Creatinine,	1.800, mg/dL	1.809, mg/dL	4.59

Although the percent relative error within allowed value (less than 5%), but may refer to be as high value. This may be to effect of proteins in creatinine estimation using reference(Jaffe) method[178] . However, from this result concluded that the method of deproteinization is acceptable and can be applied easily with low costs. Volume of 1mL of serum was taken and deproteinizated using 1.5 or 2mL of ethanol then the mixture was centrifuged for 10min at 4000rpm. The supernatant was poured into 10mL volumetric flask. Diazotization process then conducted using 0.085g of $NaNO_2$ and 2mL of 6M HCl. The contents were left for 10min at iced bath water and the total volume was made. After that 1.5mL of this solution was mixed with 1.5mL alkaline Thymol solution (prepared through dissolving 0.075g of Thymole in 25mL of 0.56g KOH solution). After 2min of mixing the absorbance was measured at 403nm. Table 3-9 shows results of these tests.

Preparation of standard creatinine solutions for calibration curve construction.
This table summarizes the quantities and experimental conditions used for preparing standard creatinine solutions. Each standard solution was subjected to the optimized Diazo coupling procedure using Thymole as the coupling agent and potassium hydroxide as the base. The mixtures were diazotized for 10 minutes in an ice bath before measuring absorbance at 403 nm.

Exp.	Creat.	$NaNO_2$	HCl	thymol	Base
1	0.008g	0.085	2 ml (6M)	0.075g In 25 ml	0.56g In 25 ml
	In 50 ml and diazotized for 10 min at iced bath water				
Exp.	Creat.	$NaNO_2$	HCl	thymol	Base
2	0.01g	0.085	2 ml (6M)	0.075g	0.56g

	In 25 ml and diazotized for 10 min at iced bath water	In 25 ml D. WT	In 25 ml

Calibration curve for creatinine

To construct the calibration curve, a series of standard creatinine solutions with different concentrations were prepared under the optimized reaction conditions. Each experiment involved varying the volume of the standard creatinine solution while keeping the quantities of other reagents constant. The Diazo coupling reaction was carried out using Thymole as the coupling agent and potassium hydroxide as the base. The reaction mixture was diazotized for 10 minutes in an ice bath before measuring the absorbance of the resulting yellow azo product at 403 nm.

Exp.	**Creat.**	**$NaNO_2$**	**HCl**	**thymol**	**Base**
10	0.008g	0.085	2 ml (6M)	0.075g In 25 mL	0.56g In 25 mL
	In 50 mL and diazotized for 10 min at iced bath water0.5, 1, 1.5, 2, 2.and 3mL were piptted and completed to 5mL.				
Exp.	Creat.	$NaNO_2$	HCl	thymol	Base
11	0.01g	0.085	2 ml (6M)	0.075g In 25 ml D. WT	0.56g In 25 ml
	In 25 ml and diazotized for 10 min at iced bath water0.5, 1, 1.5, 2, 2, 3.and 3.5mL were piptted and completed to 20mL.				
Exp.	Creat.	$NaNO_2$	HCl	thymol	Base
12	0.02g	0.017	4 ml (6M)	0.075g In 25 ml D. WT	0.56g In 25 ml
	In 50 ml and diazotized for 10 min at iced bath water6, 8, 10 and 12mL were piptted and completed to 20mL.				

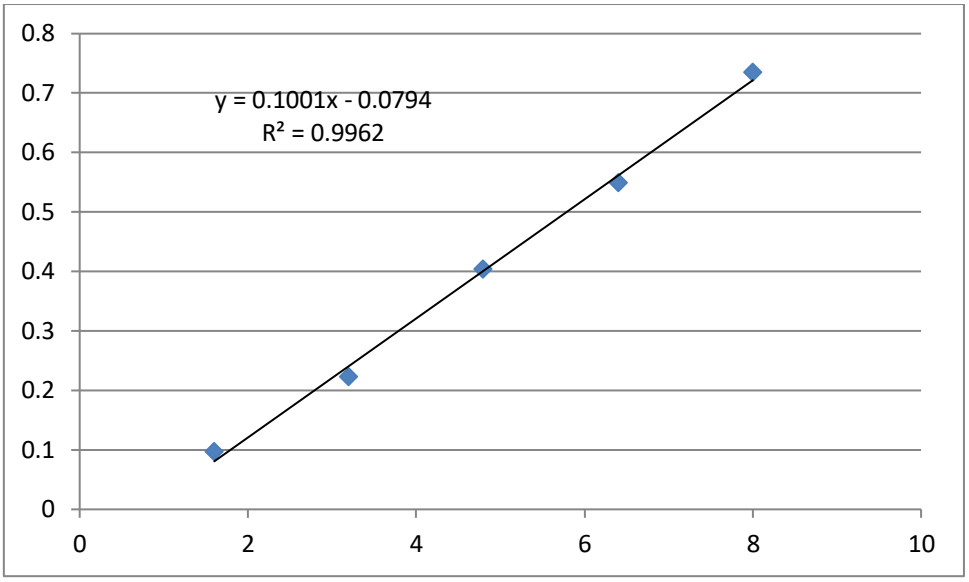

Figure 3-6: Calibration curve for creatinine within range 1.6-8mg/dL .

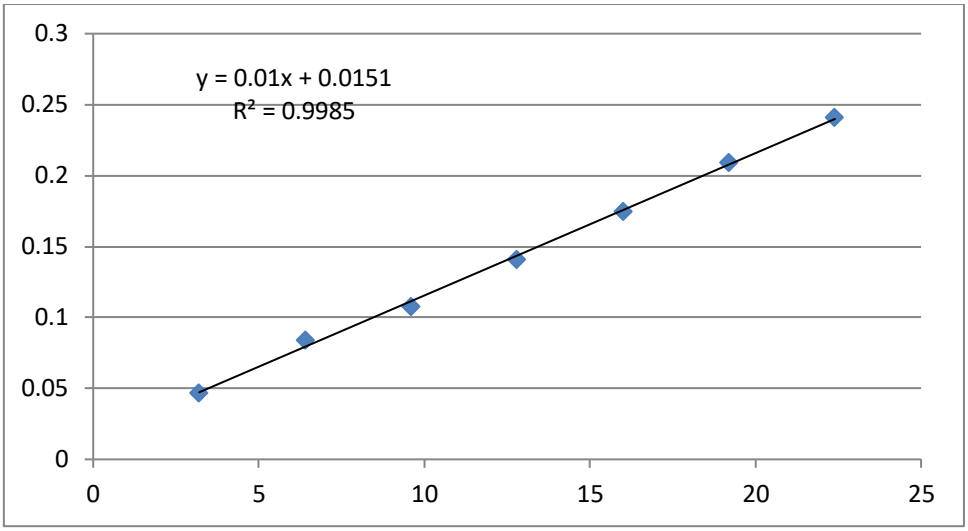

Figure 3-7: Calibration curve for creatinine within range 3.2-22.4mg/dL.

Analytical performance studies

For ten repetitions with the same creatinine concentration, the precision of the procedure was represented as standard deviation and percent relative standard deviation. A value of 3.93 for the percentage RSD is acceptable and falls within the permitted analytical technique error range. Limits of Quantitation, LOD, and Detection. LOQ, the method's LOD and LOQ were calculated to be (3×SD/slope) and (10×SD/slope), respectively., additional analytical parameters were displayed.

Table 8: Calculating percent relative standard deviation of the current method.

Exp.	X	$X - \bar{X}$	$(X - \bar{X})^2$
1	0.332	-0.0079	0.00006241
2	0.334	0.0041	0.00001681
3	0.369	0.0291	0.00084681
4	0.336	-0.0039	0.00001521
5	0.319	-0.0209	0.00043681
6	0.352	0.0121	0.00014641
7	0.341	0.0011	0.00000121
8	0.345	0.0051	0.00002601
9	0.333	-0.0069	0.00004761
10	0.338	-0.0019	0.00000361
	ΣX=3.399		$\Sigma(X - \bar{X})^2$= 0.0016029
	$\bar{X}$ = 0.3399		

$$S.D = \frac{\sqrt{\Sigma(x-x)^2}}{n-1}$$

$$S.D = \frac{\sqrt{0.0016029}}{9}$$

S.D = 0.013

$$RSD\% = \frac{S.D}{mean} \times 100\%$$

RSD% = (0.013/0.3399)100%

RSD% = 3.93

Conclusions

The developed spectrophotometric method based on a modified Diazo coupling reaction using Thymole as the coupling agent offers a precise, sensitive, and selective approach for the quantitative estimation of creatinine in human serum. Optimization of key reaction parameters—specifically the use of 6 M hydrochloric acid for diazotization, potassium hydroxide as the alkaline medium, and a reaction temperature of 10 ℃—resulted in the formation of a stable azo complex with maximum absorbance at 403 nm. The analytical performance of the method showed excellent linearity ($R^2 = 0.9982$), compliance with Beer–Lambert's law, and a low detection limit (0.39 mg/dL). When applied to actual serum samples, the results closely matched those obtained using the conventional Jaffe method, confirming the reliability of this technique. Furthermore, the modified Diazo coupling approach effectively reduced interferences from biomolecules such as glucose and amino acids, which commonly hinder the accuracy of traditional colorimetric assays. In summary, this method represents a valuable, low-cost, and efficient alternative for clinical laboratories, particularly in resource-limited settings, and provides a foundation for future spectrophotometric developments targeting additional diagnostic biomarkers.

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