

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

Duaa Abdulzahraa Al-Rammahi

Department of Community Health Techniques, College of Health and Medical Techniques /Kufa, Al Furat AL Awsat Technical university, Najaf, 3100

Received: 2025, 15, Dec

Accepted: 2026, 21, Jan

Published: 2026, 27, Feb

Copyright © 2026 by author(s) and Scientific Research Publishing Inc. This work is licensed under the Creative Commons Attribution International License (CC BY 4.0).



Open Access

<http://creativecommons.org/licenses/by/4.0/>

Annotation: 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.

Keywords: Azo dye formation; Biomarker quantification; Colorimetric method; Human serum; Thymole.

1. 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 $RN_2^+X^-$, where R can be any chemical group, like an aryl or alkyl, and X can be an organic or inorganic anion, such as a halogen [9].

The process of creating diazonium compounds is known by the names diazotation, diazotization, 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 H_2SO_4 , HBF_4 , HCl , or $p-H_3CC_6H_4SO_3H$) 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.

2. 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

3. 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 stretching 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 imino 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.

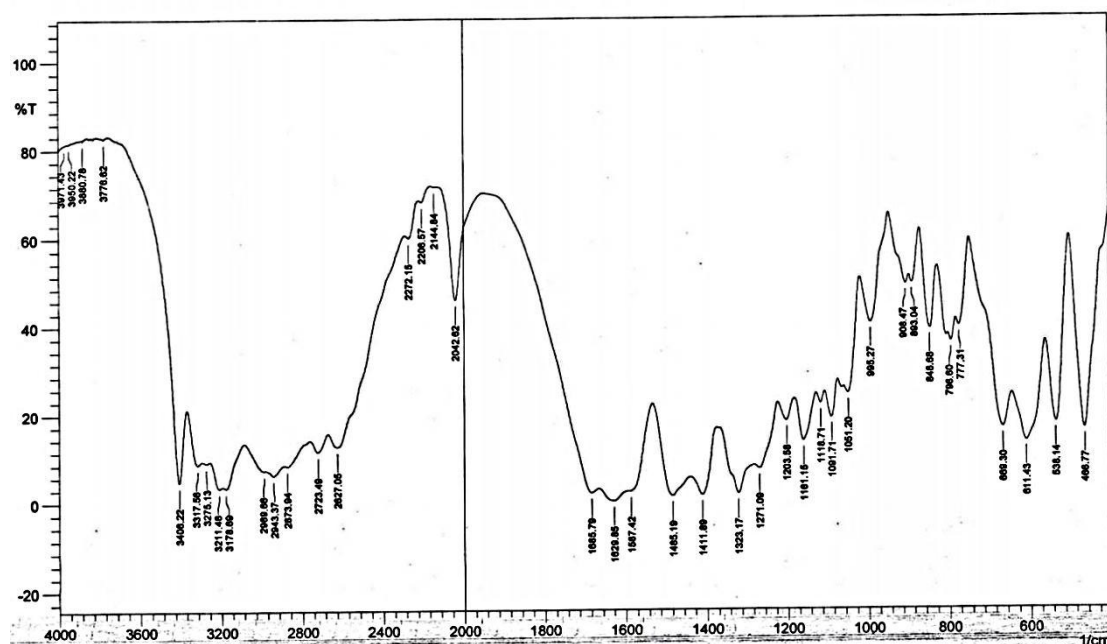


Fig. 1: FTIR spectrum of creatinine.

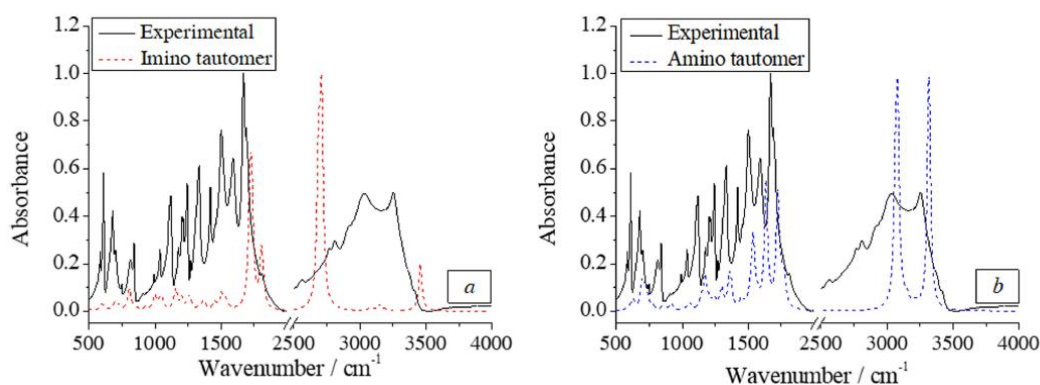


Figure 2: Comparison of the simulated counterpart derived for the imine (a) and amine (b) crystal structures inside the double-harmonic approximation using a periodic approach with the experimental infrared spectrum (between 500 and 4000 cm^{-1}) of the creatinine crystal in KBr pellet [17]

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

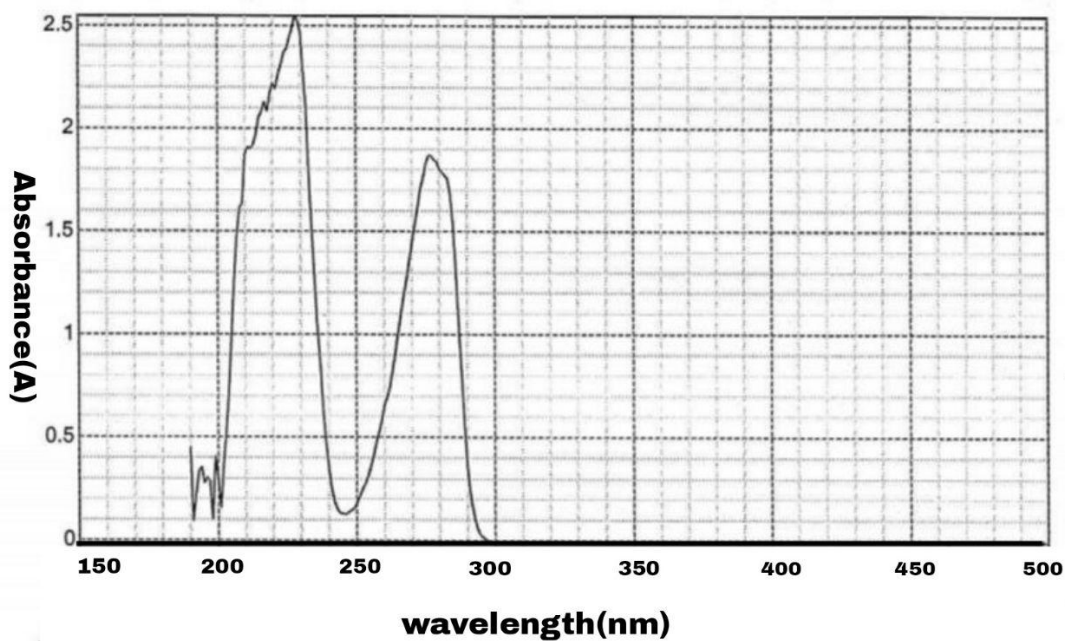


Figure 3: Thymole UV-Vis spectrum in an alkaline solution.

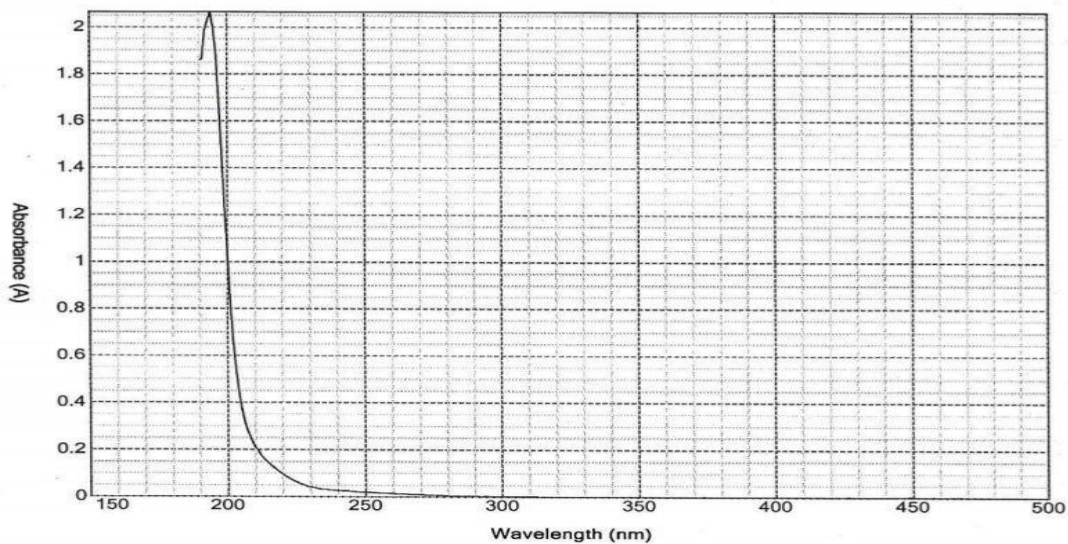


Fig. 3-4: UV-Vis. spectrum of creatinine

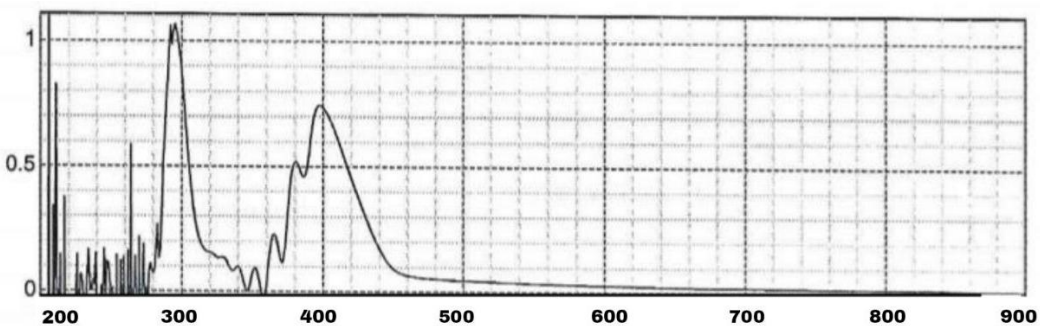


Fig. 3-5: UV-Vis. Spectrum of the yellow product

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 NaNO₂, 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 ₃ COOH	H ₂ SO 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 ₄ OH
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 NaNO₂ 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 deproteinized 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 NaNO₂ 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 In 25 ml D. WT	0.56g In 25 ml
	In 25 ml and diazotized for 10 min at iced bath water				

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 water 0.5, 1, 1.5, 2, 2. and 3mL were pipetted 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 water 0.5, 1, 1.5, 2, 2, 3. and 3.5mL were pipetted 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 water 6, 8, 10 and 12mL were pipetted and completed to 20mL.				

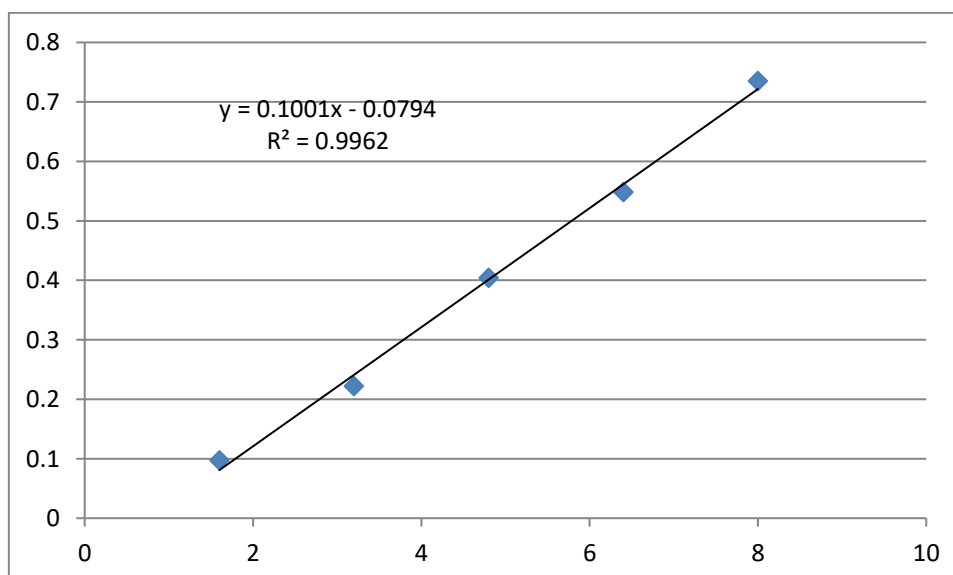


Figure 3-6: Calibration curve for creatinine within range 1.6-8mg/dL .

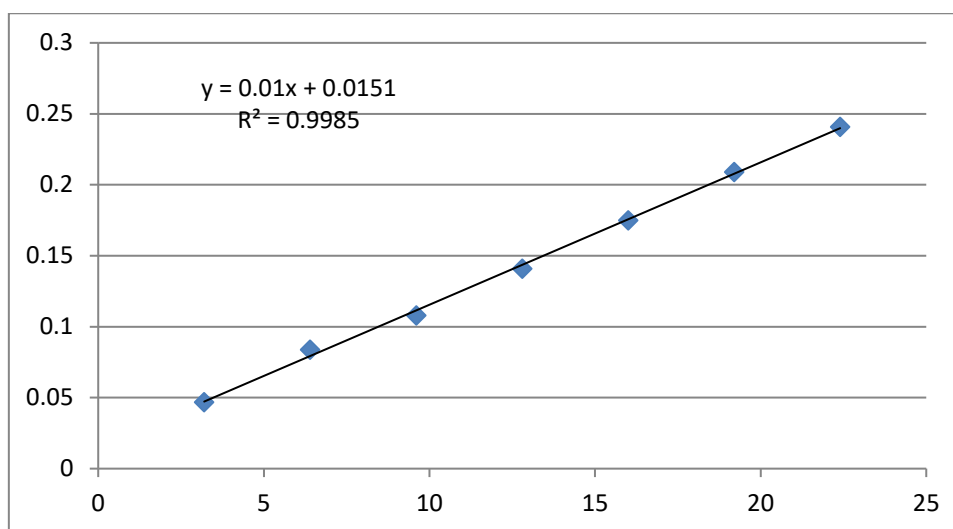


Figure 3-7: Calibration curve for creatinine within range 3.2-22.4mg/dL .

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 $(3 \times \text{SD}/\text{slope})$ and $(10 \times \text{SD}/\text{slope})$, 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
	$\Sigma X = 3.399$		$\Sigma(X - \bar{X})^2 = 0.0016029$
	$\bar{X} = 0.3399$		

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

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

$$S.D = 0.013$$

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

$$\text{RSD}\% = (0.013/0.3399)100\%$$

$$\text{RSD}\% = 3.93$$

5. 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 °C—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.

6. References

1. Gayon J, Sununta LS, Rattanarat P, Chailapakul O, Praphairaksit N. Microfluidic paper-based analytical devices for determination of creatinine in urine samples. *Anal Sci.* 2018;34(1):109–13.
2. Bunge M. *Treatise on Basic Philosophy. Volume 4, Ontology II: A World of Systems.* Dordrecht: D. Reidel Publishing Company; 1979. p. 61–2.
3. Voon CH, Sam ST. 2.1 Biosensors. In: *Nanobiosensors for Biomolecular Targeting.* Elsevier; 2019.
4. Farlex, Inc. Endogeny [Internet]. *Segen’s Medical Dictionary. The Free Dictionary by Farlex;* 2011 [cited 2019 Jun 27]. Available from: <https://medical-dictionary.thefreedictionary.com/endogeny>
5. Rogers K. Biomolecule [Internet]. *Encyclopedia Britannica.* 2020 Mar 18. Available from: <https://www.britannica.com/science/biomolecule>
6. Green DE, Goldberger R. *Molecular Insights into the Living Process.* New York, NY: Academic Press; 1967.
7. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev.* 2000;80(3):1107–213.
8. Bishop ML, Fody EP, Schoeff LE. *Clinical Chemistry: Principles, Techniques, and Correlations.* Philadelphia, PA: Lippincott Williams & Wilkins; 2013.
9. Boer DP, de Rijke YB, Hop WC, Cransberg K, Dorresteijn EM. Reference values for serum creatinine in children younger than 1 year of age. *Pediatr Nephrol.* 2010;25(10):2107–13.
10. Arias JJ, Perez JP, Pont MLP, Montelongo FG. [Article Title]. *Microchem J.* 1894;29:119–25.
11. Suzuki A. Palladium-Catalyzed Cross-Coupling Reactions of Organoboron Compounds. In: Ramachandran PV, Brown HC, editors. *Organoboranes for Syntheses.* Washington, DC: American Chemical Society; 2001. p. 80–93.
12. Miyaura N. Palladium-Catalyzed Cross-Coupling Reactions of Organoboron Compounds. In: Liebeskind LS, editor. *Advances in metal-organic chemistry. Vol 6.* London: Jai Press; 1998. p. 187–243.

13. Benkhaya S, M'rabet S, El Harfi A. Classifications, properties, recent synthesis and applications of azo dyes. *Heliyon*. 2020;6(1):e03272.
14. Rahimizadeh M, Eshghi H, Shiri A, Ghadamyari Z, Matin MM, Oroojalian F, et al. $\text{Fe}(\text{HSO}_4)_3$ as an efficient catalyst for diazotization and diazo coupling reactions. *J Korean Chem Soc*. 2012;56(3):287–92.
15. King AO, Yasuda N. Palladium-catalyzed cross-coupling reactions in the synthesis of pharmaceuticals. In: Ikariya T, Murata S, editors. *Organometallics in Process Chemistry*. Heidelberg: Springer; 2008. p. 205–245.
16. Filarowski J, Pavlovic G, Racane L, Cicak H. Resonance. *Resonance*. 2010;15(9):850–5.
17. Leon I, Tasinato N, Spada L, Alonso ER, Mata S, Balbi A, et al. Looking for the elusive imine tautomer of creatinine: Different states of aggregation studied by quantum chemistry and molecular spectroscopy. *Chempluschem*. 2021;86(8):1374–86.