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ARTICLE

Optimized Spectrophotometric Method for Creatine and Creatinine Quantification using Alpha Naphthol Sulpha Acetamide Dye

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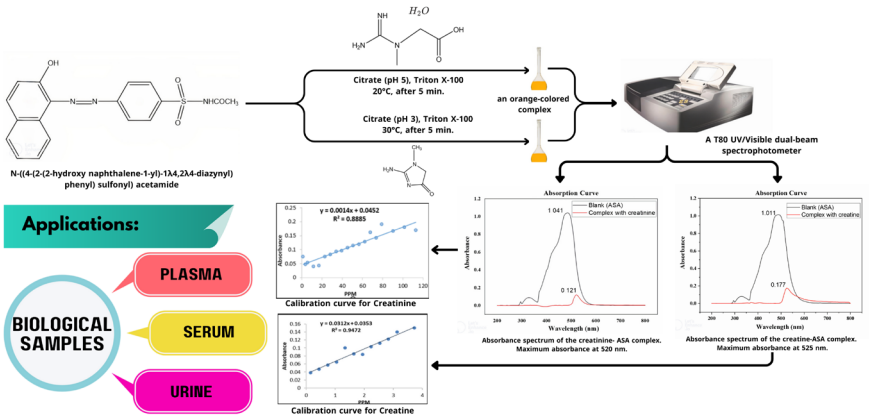

A new spectrophotometric method for the reliable determination of creatine and creatinine in plasma, urine and serum has been developed for routine clinical and pharmaceutical use. This technique makes use of the fact that creatine and creatinine can chemically interact with the azo dye Alpha naphthol Sulpha Acetamide (ASA), producing a compound that is an intense orange color and is measurable at 525 nm for creatine and 520 nm for creatinine, respectively. New features include Triton X-100 surfactant and ideal reaction conditions of 30 °C and pH 3 for creatinine and 20 °C and pH 5 for creatine, respectively. The method shows increased molar absorption within the specified concentration ranges and complies with Beer's law. This method is more friendly to the environment than traditional colorimetric, enzymatic, chromatographic, and immunoassay procedures because it utilizes less hazardous chemicals and biodegradable surfactants. Ethanol is a more environmentally friendly and less harmful solvent than traditional organic solvents utilized in many processes. The approach lowers energy consumption and waste by functioning at lower temperatures, consistent with the principles of green chemistry. This method guarantees accurate and dependable identification of creatine and creatinine while promoting environmentally sustainable laboratory practices.

Keywords: creatine, creatinine, alpha napthol sulpha acetamide dye, citrate buffer, surfactant, spectrophotometric method, serum

INTRODUCTION

Creatine and creatinine are key indicators with important clinical consequences. Creatine, which is required for muscular energy metabolism, has been demonstrated to increase muscle hypertrophy,

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strength, and performance, particularly in high-intensity sports.[1] It also exhibits potential neuroprotective effects, benefiting patients with neurodegenerative diseases.[2] Creatinine, on the other hand, is an important biomarker of renal function because it can be used to estimate glomerular filtration rate (GFR), which helps with the diagnosis and treatment of chronic kidney disease.[3] Elevated creatinine levels are also associated with cardiovascular risk, underscoring the significance of frequent monitoring.[4] These indicators are useful in clinical practice, providing information on muscle health, renal function, and cardiovascular risk.

Creatine, an organic compound containing nitrogen, is predominantly synthesized in the liver and other vital organs such as the kidneys and pancreas. The hepatic organ performs a central function in the formation of creatine through the utilization of amino acids such as glycine, arginine, and methionine. Following its synthesis, creatine is released into the bloodstream, facilitating its transportation to various tissues, including the muscular system.[5] On the contrary, the liver does not directly impact the levels of creatinine, which is a by-product of the breakdown of creatine in the muscles. Rather, creatinine is formed and enters the bloodstream, where it is eventually filtered by the kidneys for elimination through urine. However, it should be noted that the liver indirectly affects creatinine levels by playing a role in overall muscle health and metabolism.[6] Liver diseases or disorders can indirectly influence the metabolism of creatine and, subsequently, the production of creatinine. Conditions such as liver cirrhosis, hepatitis, or other forms of liver dysfunction can result in muscle wasting and reduced protein synthesis, both of which can have an impact on creatine levels. As a result, alterations in creatine metabolism can potentially affect the levels of creatinine in the bloodstream, thereby potentially influencing its diagnostic value in evaluating kidney function.[7] Understanding the interaction between creatine, creatinine, and liver function offers valuable insights into the intricate relationships within the body's systems.[8] Ongoing research and exploration by healthcare professionals aim to gain a deeper understanding of these connections, contributing to improved management strategies for liver diseases, muscle metabolism, and related diagnostic markers. Various techniques are available for determining creatine and creatinine levels in biological samples. Among the commonly used methods are enzymatic approaches which utilize specific enzymes to catalyze reactions involving creatine and creatinine, leading to the production of a measurable product.[9] For example, creatine can be converted to creatine phosphate in the presence of creatine kinase, and the resulting Adenosine diphosphate (ADP) can be quantified using spectrophotometric techniques. Similarly, creatinine can be enzymatically converted to creatine using creatininase, followed by creatine measurement. Enzymatic methods are often precise and sensitive. On the other hand, chromatographic techniques such as high-performance liquid chromatography (HPLC) and gas chromatography (GC) are frequently employed methodologies for the separation and quantification of creatine and creatinine. These techniques rely on the differential movement of compounds through a chromatographic column based on their physical and chemical properties. The separated compounds can then be detected and measured using appropriate detectors such as UV spectrophotometer or mass spectrometer.[10] Selective antibodies that target creatine or creatinine can be utilized in immunoassay techniques, like enzyme-linked immunosorbent assay or radioimmunoassay, to identify and measure the presence of these substances. These methods exhibit high sensitivity and specificity; however, they may necessitate the use of specialized reagents and equipment.[11] However, specific colorimetric or spectrophotometric reactions can be used to measure creatine and creatinine. For instance, Jaffe's reaction is a well-known method utilized for the determination of creatinine. Under alkaline conditions, creatinine undergoes a chemical reaction with picric acid, producing a red-colored complex. The amount of this complex can be quantitatively determined through spectrophotometric measurements, which involve analyzing the absorption of light by the colored solution.[12] These are a few examples of the available methods for determining the levels of creatine and creatinine. The selection of the method depends on factors such as sensitivity, specificity, cost, equipment availability, and the type of sample being analyzed. It is essential to select a suitable approach depending on the particular needs of the investigation and the intended degree of precision and accuracy. The primary goal of this research is to create a novel UV/Vis spectrophotometric method for precisely determining creatine and creatinine in biological samples using a newly synthesized azo dye, Alpha Naphthol Sulpha

Acetamide (ASA), which has improved sensitivity, accuracy, and simplicity for clinical and pharmaceutical applications.

MATERIALS AND METHODS

Reagents and chemicals

All substances and solutions used were analytical grade reagents that were not subjected to additional purification. Distilled water was utilized in all trials. Creatine monohydrate (purity 99%) (Mwt = 149.15 g/mol) was obtained from Alpha Chemika (Mumbai, Maharashtra, India). Creatinine anhydrous (purity 99.90%) (Mwt = 113.12 g/mol) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide as a basic compound (purity 97%) (Mwt = 40 g/mol) was obtained from VWR Chemicals, BDH Company (in North America), and sulpha acetamide (purity 98%) (Mwt = 214.24 g/mol) and alpha naphthol (purity 99%) (Mwt = 144.17 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol (purity 99.85%) (Mwt = 32.04 g/mol), ethanol 95% (Mwt = 46.07 g/mol), di-methyl formamide (purity 98%) (Mwt = 73.09 g/mol), 1-butanol (purity 99.8%) (Mwt = 74.121 g/mol), 2-propanol (purity 99.8%) (Mwt = 60.09 g/mol), hydrogen chloride as acidic compound (purity 33%) (Mwt = 36.46 g/mol), and formaldehyde (purity 37-41%) (Mwt = 30.031 g/mol) were obtained from El-Nasr Company (Egypt). Octoxynol-9 (Triton X-100) (purity 100%) (Mwt = 625 g/mol and density 1.07 g/cm^3), sodium lauryl sulfate (SLS) (purity 99%) (Mwt = 288.37 g/mol), and cetyl trimethyl ammonium bromide (CTAB) (purity 99%) (Mwt = 364.45 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mucilage was obtained from canola (rapeseed) at the Analytical Chemistry Laboratory, Faculty of Science, Zagazig University, Egypt.

Samples

All biological samples were collected in accordance with applicable laws and institutional guidelines (profession and ethics legislation, Ministry of health and population Resolution No. 23812003). The protocol was approved by the Institutional Review Board in the Faculty of Medicine (Zagazig University, Egypt).

Instrumentation

A T80 UV/Visible dual-beam spectrophotometer (PG Instruments Ltd Company, United Kingdom) with a spectral bandwidth of 2.0 nm and 1.0 cm matched quartz cells was employed for absorbance determinations. The acidity levels were modified utilizing an Adwa pH gauge (Model AD 1030, Romania).

Solutions

A solution containing 1.00 mol L^{-1} of creatinine was prepared by dissolving 11.312 g in a 100 mL volumetric flask of distilled water. Dilutions of lower concentrations were prepared by diluting from the original solutions.

A solution containing 1.00 mol L^{-1} of creatine was prepared by dissolving 13.113 g in a 100 mL volumetric flask of distilled water. Dilutions of lower concentrations were prepared by diluting from the original solutions.

A solution with a concentration of 1.00×10^{-3} mol L^{-1} of ASA dye was prepared by dissolving 0.1845 g in 500 mL of ethanol.

Buffers

Assorted buffer solutions, such as the Universal Buffer (pH 2.0-12.0), have been prepared by mixing 0.04 mol L^{-1} of boric, orthophosphoric, and acetic acids with 0.2 mol L^{-1} NaOH and completed to 100 mL with distilled water. The borate buffer (pH 7.0-11.0) formulation required mixing precise amounts of 0.2 mol L^{-1} boric acid and borax solutions with 0.2 mol L^{-1} NaOH, as described in other referenced documents.[13,14] Likewise, the citrate buffer (pH 3.0-6.0) preparation involved combining suitable ratios of 0.1 mol L^{-1} citric acid with sodium citrate. The phosphate buffer (pH 7.0-11.0) preparation included mixing precise measurements of 0.1 mol L^{-1} potassium dihydrogen phosphate with either 0.1 mol L^{-1} HCl or NaOH, as specified in a particular publication.[15] Preparation of the phosphate buffer (pH 7.0-11.0) was performed by

mixing exact measurements of 0.1 mol L^{-1} potassium dihydrogen phosphate with either 0.1 mol L^{-1} HCl or NaOH, as indicated in a particular publication.[15]

Surfactants

Different types of surfactants, such as a non-ionic surfactant Triton X-100, an anionic surfactant sodium lauryl sulfate (SLS), and a cationic surfactant cetyl trimethyl ammonium bromide (CTAB) and mucilage of rapeseed were prepared. To prepare these surfactant solutions, a precise amount of each surfactant was dissolved in 100 mL of deionized water. The concentration of each surfactant solution was 0.01 mol L^{-1}.

Synthesis of the azo dye ASA

Take (0.01 mol) (0.688 g) of sodium nitrite in 5 mL of deionized water, and add it drop by drop on (0.01 mol) (2.142 g) of sulpha acetamide in 10 mL of 6 mol L^{-1} concentration of hydrochloric acid (1:1). The solution was added slowly to (0.01 mol) (1.4417 g) of alpha-naphthol in a 10 mL solution of 2 mol L^{-1} concentration of sodium hydroxide while stirring in an ice bath. Subsequent to this, the concoction underwent filtration utilizing a Buchner funnel, succeeded by the gathering of the precipitate via filtration, rinsing with ethanol 95% at 25 ºC several times, and left to dry in the air to produce a reddish-brown solid. In this procedure, the yielded product was 90.48%.

Synthesis and determination of the creatinine-ASA complex

In a 10 mL volumetric flask, 2.00 mL of creatinine having a concentration of $1x10^{-3}$ mol L^{-1} was added, followed by 3 mL of citrate buffer at pH=3. Next, 2.00 mL of ASA dye solution with a concentration of $1x10^{-3}$ mol L^{-1} was added, and the flask was topped up to 10 mL with 95% ethanol before being thoroughly mixed. After incubating for 5 minutes at 30 ºC, the absorbance was measured by using UV/Vis spectrophotometric technique by scanning wavelengths from 200 to 800 nm against a blank prepared using the same composition without creatinine.

Synthesis and determination of the creatine-ASA complex

In a standard flask of 10 mL capacity, 2.00 mL of creatine at a concentration of $1x10^{-3}$ mol L^{-1} was introduced, followed by 3 mL of citrate buffer at a pH of 5. Subsequently, 2.00 mL of ASA dye solution at a concentration of $1x10^{-3}$ mol L^{-1} was added, and the volume was made up to 10 mL using 95% ethanol, ensuring thorough mixing. Subsequent to a 5-minute incubation period at 30 °C, the absorbance levels were quantified by scanning the wavelength spectrum ranging from 200 to 800 nm against a blank that was formulated with the same constituents minus creatine.

Factors affecting the determination of creatine and creatinine

Several factors were investigated, such as pH and buffer, reaction time, volume of creatinine or creatine added, temperature, surfactants, interference from foreign materials, organic solvent and order of addition.

Determination of creatine and creatinine in the presence of surfactant

In a 10 mL volumetric flask, specific volumes of various solutions were combined sequentially. First, 2 mL of a 1 mM creatinine solution was added. Then, 3 mL of a citrate buffer (mixture of 0.1mol L^{-1} of citric acid and 0.1ml L^{-1} of sodium citrate) at pH 3 was introduced. After that, 2 mL of a 1 mM ASA dye solution was poured in. Next, 2 mL of a 10 mM Triton X-100 solution was added. Finally, the flask was filled to the 10 mL mark with 95% ethanol and the contents thoroughly mixed. Following a 5-minute wait period, the absorbance spectrum of this final solution was measured by scanning wavelengths from 200 to 800 nm, using an identically prepared solution without creatinine as the blank reference. Alternatively, in a separate 10 mL volumetric flask, introduce 2 mL of a 1 mM creatine solution, followed by 3 mL of a citrate buffer at pH 5. Next, add 2 mL of a 1 mM ASA dye solution, and then 2 mL of a 10 mM Triton X-100 solution. Fill the flask to the 10 mL mark with 95% ethanol, ensuring thorough mixing of the contents. After allowing 5

minutes for the reaction to occur, measure the absorbance spectrum from 200 to 800 nm, using a blank solution prepared identically but excluding the creatine component as the reference.

Preparation of the calibration curves

A calibration curve was constructed to estimate creatinine levels, using 2 mL of reagent, 3 mL of $1x10^{-3}$ mol L^{-1} citric acid/sodium citrate buffer (pH 3), and 2 mL of $1x10^{-2}$ mol L^{-1} TritonX-100. The reaction began with the addition of 2 mL of varying creatinine concentrations, and ended with the addition of 95% ethanol up to the mark, ensuring thorough mixing. The reaction mixture stood at 30 °C for 5 minutes. The absorbance of the coloured solution was measured at 520 nm.

For creatine, a calibration curve was constructed using 2 mL of reagent, 3 mL of a citric acid/sodium citrate buffer ($1x10^{-3}$ mol L^{-1}), and 2 mL of TritonX-100 ($1x10^{-2}$ mol L^{-1}). The process began with the addition of 2 mL of different creatine concentrations, followed by the completion of adding 95% ethanol up to 10 mL calibrated flask and thoroughly mixing. Then, the resulting reaction mixture was allowed to sit and incubate for 5 minutes at a temperature of 30 ºC. The absorption of the tinted solution was measured at 520 nm.

Determination of creatinine and creatine in human serum

Different samples of human blood serum were collected from the medical diagnostic laboratory named Main lab in Zagazig University Hospital. The specimen was placed at the centrifuge within a vacutainer tube equipped with a clot activator and a unique gel for the purpose of segregating the serum from the cells. Iron and copper were masked by using 1 mL sodium fluoride.[16] Then, 2.00 mL of serum after diluted 10 times, 3.00 mL citrate buffer (pH 3), 2.00 mL from ASA (1.00×10^{-3} mol L^{-1}), and 2 mL of ($1.00x10^{-2}$ mol L^{-1}) of Triton X-100 were added then completed with ethanol 95% to a 10 mL calibrated flask. Subsequent to a 5-minute period of incubation at a temperature of 20 °C, the measurement of absorbance was carried out utilizing the blank as a point of reference. However, for the purpose of examining creatine, a substitution was made by replacing 3.00 mL of pH 3 citrate buffer with 3.00 mL of pH 5 citrate buffer.

Determination of creatinine and creatine in urine

Urine human specimens were collected from the main lab at Zagazig University Hospital who suffered from disturbances in liver and kidney functions. 1 mL of each urine sample was diluted by adding 10 mL of deionized water. In a 10 mL standard flask, 1.00 mL from the diluted solution, 3.00 mL citrate buffer (pH 3), 2.00 mL from ASA (1.00×10^{-3} mol L^{-1}), and 2 mL of ($1x10^{-2}$ mol L^{-1}) of TritonX-100 were added and completed with ethanol 95% to 10 mL, following 5 minutes at 30 °C, the absorbance was recorded compared to the blank. Conversely, when working with creatine, we used 3.00 mL of citrate buffer (pH 5) instead of 3.00 mL of citrate buffer (pH 3).

Determination of creatinine and creatine in plasma

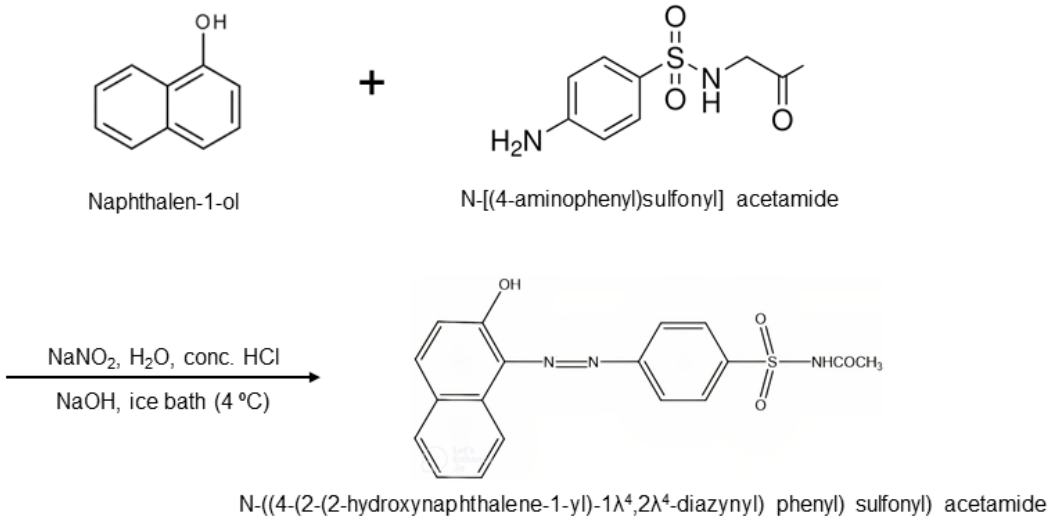
A specific volume of blood (10 mL) was acquired from mature human individuals at Zagazig University Hospital. The particular specimens, which were obtained from the main lab of Zagazig University Hospital, were placed in a centrifuge with a tube containing EDTA as an anticoagulant to help separate the plasma. EDTA was integrated into the blood sample and positioned in a 10 mL conventional flask. The EDTA concentration fluctuated between 1.2 and 2.0 mg mL^{-1} of blood, accompanied by 2.00 mL of plasma, 3.00 mL of citrate buffer (pH 3), 2.00 mL from ASA (1.00×10^{-3} mol L^{-1}), and 2 mL of ($1.00x10^{-2}$ mol L^{-1}) of Triton X-100, followed by the introduction of ethanol 95% to a 10 mL volumetric flask. Subsequently, after a duration of 5 minutes at 30 °C, the absorbance was gauged against the blank. Conversely, concerning creatine, the substitution of 3.00 mL citrate buffer (pH 3) with 3.00 mL citrate buffer (pH 5) was performed.

RESULTS AND DISCUSSION

Synthesis of the azo dye ASA

Scheme 1 shows the proposed reaction for synthesising the new azo dye. Alphanapthol reacts with

sulpha acetamide under the conditions detailed in the 'Materials and Methods' section, subsection 'Synthesis of the azo dye ASA' of this article, to give the azo dye compound called N-((4-(2-(2-hydroxy naphthalene-1-yl)-1**λ**^{4},2**λ**^{4}-diazynyl) phenyl) sulfonyl) acetamide (ASA). The chemical properties of the azo dye ASA are given in Table I.



Scheme 1. Proposed reaction for the synthesis of the new ASA azo dye.

Table I. Chemical properties of the new ASA azo dye

IUPAC Name	N-((4-(2-(2-hydroxy naphthalene-1-yl)-1λ^4,2λ^4-diazynyl) phenyl) sulfonyl) acetamide
Chemical Formula	$C_{18}H_{15}N_3O_4S$
Extract Mass	369.08
Molecular Weight	369.40
m/z	369.08(100.0%), 370.08(19.5%), 371.07(4.5%), 371.09(1.8%), 370.08(1.1%)
Elemental Analysis	C,58.53; H,4.09; N,11.38; O,17.32; S,8.68

Valuable insights into the functional groups and molecular structure of the azo dye ASA are provided by the IR spectrum in Figure 1 and the assignment Table II. The presence of a strong absorption band at 3554 cm^{-1} indicates the existence of a hydroxyl (-OH) group in the molecule. This could be associated with phenol. The absorption band at 3436 cm^{-1} suggests the presence of an amine (-NH-) group, such as a primary or secondary amine. The strong absorption band at 1697 cm^{-1} is characteristic of a carbonyl (-C=O) group, which could be part of a ketone. The band at 1539 cm^{-1} is indicative of an azo (-N=N-) group, which is commonly found in dyes and pigments. The absorption band at 1592 cm^{-1} is associated with the C=C stretching vibrations of an aromatic ring, suggesting the presence of an aromatic or conjugated system in the molecule. The band at 1454 cm^{-1} is characteristic of the C-H bending vibrations of a methyl (-CH3) group. The absorption band at 1177 cm^{-1} is indicative of the S=O stretching vibration, which of a sulfoxide or sulfone functional group.

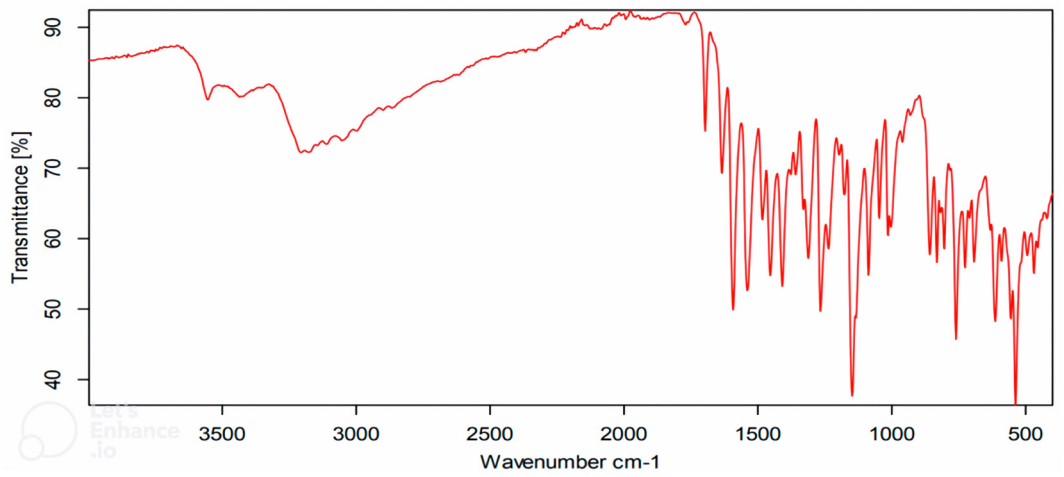


Figure 1. IR spectrum of the new ASA azo dye.

Table II. Some specific infrared wavelengths and provisional designations for the ASA dye

Assignments	Frequency (cm^{-1})	Assignments	Frequency (cm^{-1})
υ(-OH)	3554	υ(-S=O)	1177
υ(-NH-)	3436	υ(-C=C-) aryl subst.	1592
υ(-C=O)	1697	υ($-CH_3$)	1454
υ(-N=N-)	1539		

The combination of these functional group assignments provides valuable information about the structural features of the compound, which could be useful in elucidating its identity or understanding its chemical properties and potential applications. Further analysis and integration with other analytical techniques such as ^{1}H-NMR and mass spectrometry were performed to fully characterize the compound as shown in Figure 2 and Figure 3, respectively.

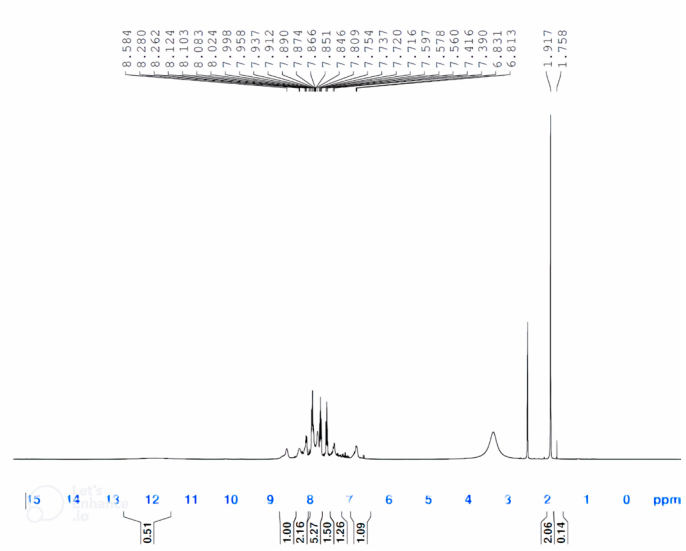


Figure 2. ^{1}H-NMR spectrum of the ASA azo dye.

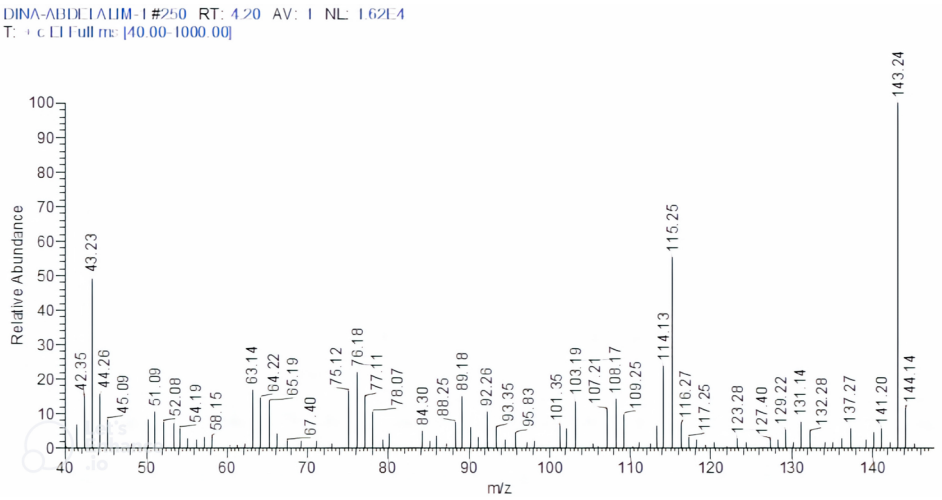


Figure 3. Mass spectrum of the ASA dye.

Synthesis of the ASA complexes of creatine and creatinine

The optimum reaction conditions for the formation of a complex between the ASA reagent and creatine are pH 5 citrate buffer, Triton X-100, 5-minute incubation period at 20 ºC.

The optimum reaction conditions for the formation of a complex between the ASA reagent and creatinine are pH 3 citrate buffer, Triton X-100, 5-minute incubation period at 30 ºC.

Absorption curve

The determination of creatinine and creatine was performed by colorimetric analysis of the orange stable complexes of creatinine and creatine at 520 nm and 525 nm, respectively, as shown in Figures 4 and 5.

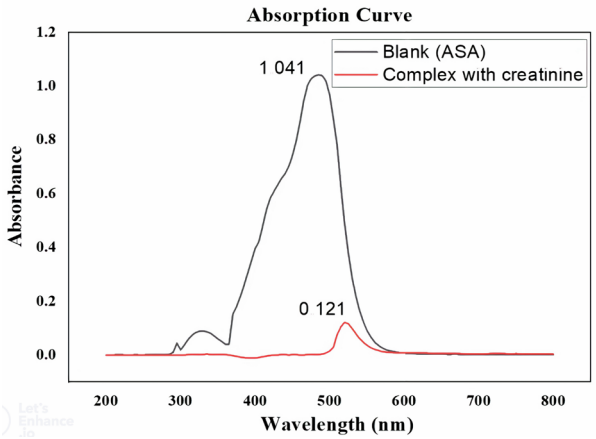


Figure 4. Absorbance spectrum of the creatinine-ASA complex. Maximum absorbance at 520 nm.

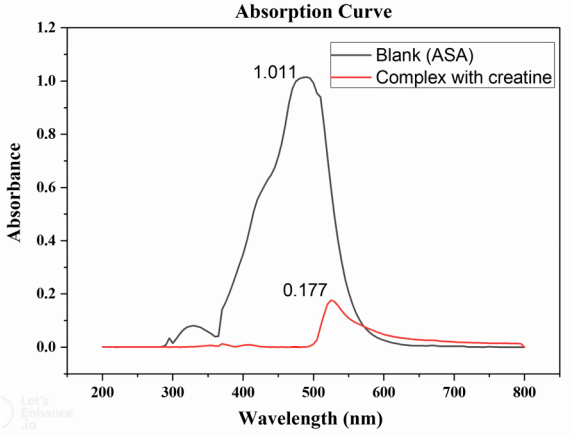


Figure 5. Absorbance spectrum of the creatine-ASA complex. Maximum absorbance at 525 nm.

Effect of pH and type of buffer on the color intensity of the ASA complexes

The investigation of the effect of acidity on the color intensity of the ASA-creatinine complex showed that the suitable pH range for this complex is from 1 to 7 with an optimum value at pH = 4 as shown in Figure 6. Examination of the influence of various buffer categories within the interval of pH 3 – 7 demonstrated that the highest absorbance value is achieved utilizing citrate buffer at pH = 3 as depicted in Figure 7.

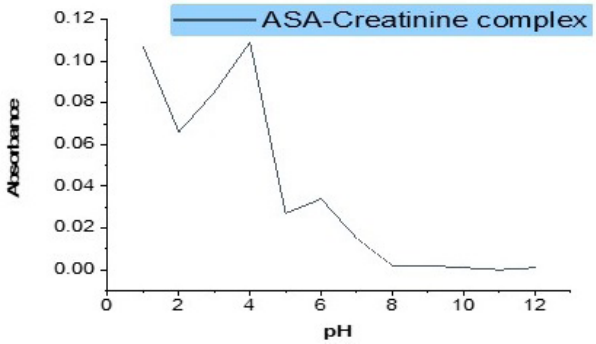


Figure 6. Effect of pH on the color intensity of the creatinine-ASA complex.

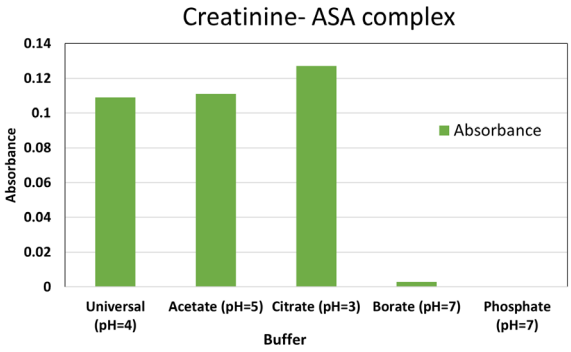


Figure 7. Effect of different buffer categories on the development of the creatinine-ASA complex.

In the scenario involving creatine, the analysis of the influence of acidity on the intensity of color of the ASA-creatine complex revealed that the appropriate pH range for the complex is from 1 to 7 with an optimum value at pH = 4 as presented in Figure 8. Investigating the impact of various buffer categories within the range of pH 3 – 7 revealed that the maximum absorbance value is acquired utilizing citrate buffer at pH = 5 as illustrated in Figure 9.

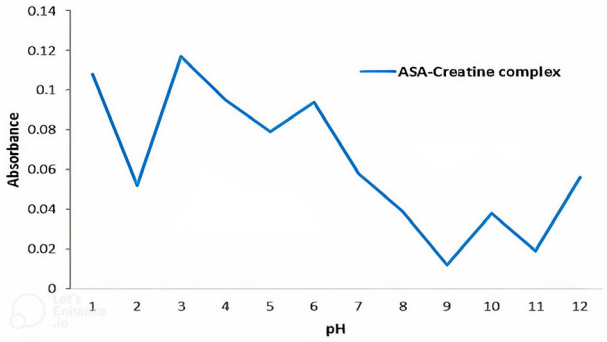


Figure 8. Effect of pH on the formation of the creatine-ASA complex.

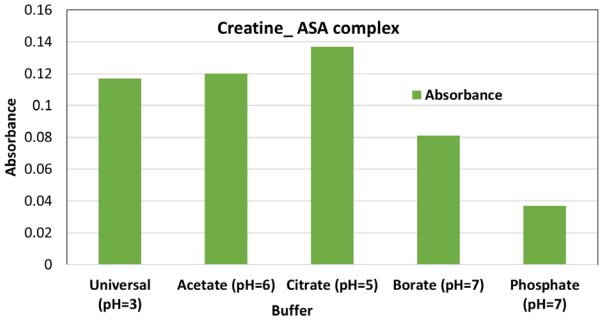


Figure 9. Effect of different buffer categories on the development of the creatine-ASA complex.

Effect of addition sequence

The effect of the order of addition of the reagents on the formation of the creatine-ASA composite showed that the creatine-buffer-reagent order gave the maximum absorbance measurement due to the complete formation of a composite. For creatinine, the effect of the order of addition on the formation of the creatinine-ASA composite showed that the creatinine-buffer-reagent order gave the highest absorbance, as shown in Table III. This result is expected because buffered creatinine or buffered creatine solutions are suitable media to form complexes with better color intensity. The reaction consisted of addition solutions of 2 mL creatine or creatinine at a concentration of 0.001 mol L^{-1}, 3 mL of buffer, 2 mL of ASA reagent at a concentration of 0.001 mol L^{-1} and ethanol 95% to 10 mL.

Table III. Effect of addition sequence on the determination of creatine and creatinine

Creatine		Creatinine	
Sequence	**Absorbance**	**Sequence**	**Absorbance**
Reagent + Creatine + Buffer	0.081	Reagent + Creatinine + Buffer	0.100
Reagent + Buffer + Creatine	0.079	Reagent + Buffer + Creatinine	0.107
Creatine + Buffer + Reagent	0.126	Creatinine + Buffer + Reagent	0.112
Creatine + Reagent + Buffer	0.084	Creatinine + Reagent + Buffer	0.096

Effect of time and temperature

Investigation of the effect of reaction time and temperature on the stability and color intensity of the creatine-ASA complex showed that this complex is formed rapidly and that the light absorbance peaked after 5 minutes at 20 ºC, as shown in Figures 10 and 11 respectively.

In the case of creatinine, the complex formed between creatinine and ASA showed that it resembled creatine in time but at 30 ºC, as shown in Figures 12 and 13 respectively. As shown in Figures 11 and 13, the color intensity of the complex decreased with increasing temperature due to the formation of crystals resulting in a decrease in absorbance.

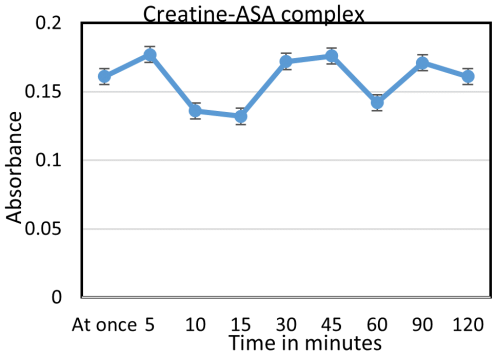


Figure 10. Effect of time on the absorbance of the creatine-ASA complex.

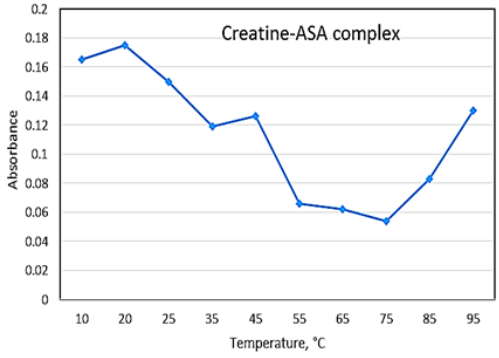


Figure 11. Effect of temperature on the absorbance of the creatine-ASA complex.

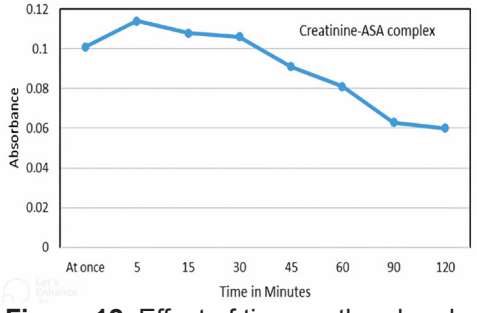


Figure 12. Effect of time on the absorbance of the creatinine-ASA complex.

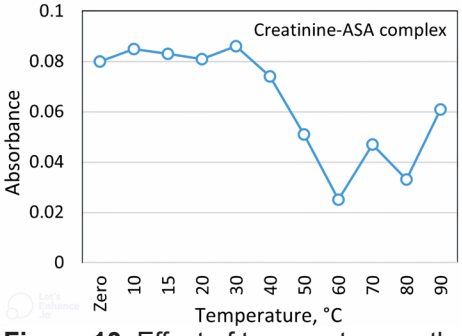


Figure 13. Effect of temperature on the absorbance of the creatinine-ASA complex.

Effect of organic solvent

Different organic solvents were tested in order to select the ideal organic solvent in the absorption spectra. Ethanol, methanol, 2-propanol, 1-butanol, dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were tested and the results showed that ethanol increased the absorbance of the creatine and creatinine complexes.

Ethanol's polarity and hydrogen bonding capacity effectively dissolve the complexes and result in fewer intermolecular interactions, increasing the number of molecules available for absorption, as shown in Figures 14 and 15, respectively.

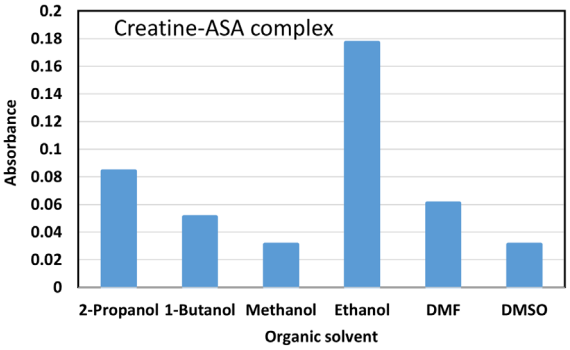


Figure 14. Effect of organic solvent on the absorbance of the creatine-ASA complex.

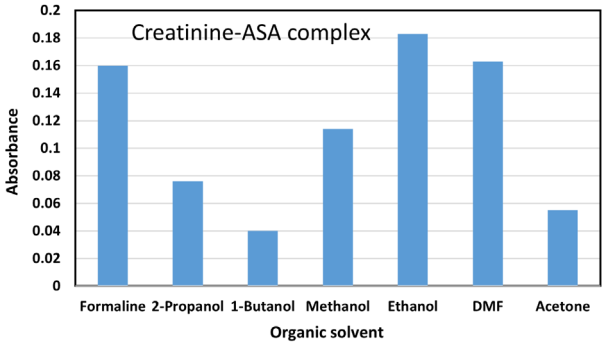


Figure 15. Effect of organic solvent on the absorbance of the creatinine-ASA complex.

Effect of surfactant

In the case of both creatine and creatinine, the outcomes indicated that the existence of 2.00 mL of Triton X-100 as a surface-active agent moved the absorption peak towards hyper and bathochromic shift. This might be attributed to the creation of micelles or a creation of a double or triple compound with the surface-active agent itself, as shown in Figures 16 and 17, respectively.

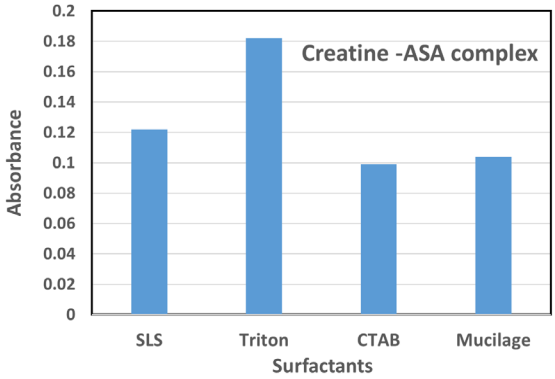


Figure 16. Effect of surfactant on the absorbance of the creatine-ASA complex.

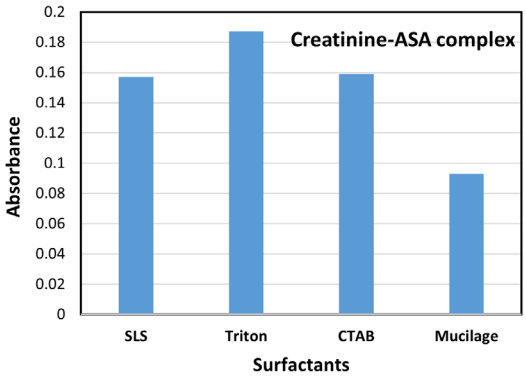


Figure 17. Effect of surfactant on the absorbance of the creatinine-ASA complex.

Effect of interferents

The utilization of interfering substances such as aluminium, calcium, carbonate, urea, magnesium, glucose, potassium, sodium, chloride, phosphate, and EDTA of 0.01 mol L^{-1} with 0.001 mol L^{-1} of either creatine or creatinine was investigated. In the case of creatine, the results showed that there was interference, except for aluminium and urea where there was no interference. In the case of creatinine, the results showed that there was no interference with aluminium, calcium, and urea with creatinine in the presence of ASA dye, while carbonate, magnesium, glucose, potassium, sodium, chloride, phosphate, and EDTA interfered, as shown in Table IV and Table V, respectively.

Table IV. Effect of interfering materials on the determination of creatine

Interfering Ion	Absorbance	Selectivity coefficient ($K_{(A, Xa)}$)*	Interfering Ion	Absorbance	Selectivity coefficient ($K_{(A, Xa)}$)*
Without interfering	0.177	Zero	Glucose	0.156	0.088
Aluminium	0.147	0.083	Potassium	0.147	0.083
Calcium	0.145	0.082	Sodium	0.113	0.064
Carbonate	0.147	0.083	Chloride	0.113	0.064
Urea	0.165	0.093	Phosphate	0.137	0.077
Magnesium	0.157	0.089	EDTA	0.137	0.077

*A: concentration of creatine; Xa: concentration of interfering material; $K_{(A, Xa)}$: selectivity coefficient of A over Xa – the smaller the selectivity coefficient, the less interference by foreign material Xa.

Table V. Effect of interfering materials on the determination of creatinine

Interfering Ion	Absorbance	Selectivity coefficient ($K_{(B, Xb)}$)*	Interfering Ion	Absorbance	Selectivity coefficient ($K_{(B, Xb)}$)*
Without interfering	0.187	Zero	Glucose	0.068	0.036
Aluminium	0.187	0.1	Potassium	0.049	0.026
Calcium	0.201	0.11	Sodium	0.08	0.043
Carbonate	0.156	0.083	Chloride	0.08	0.043
Urea	0.178	0.095	Phosphate	0.058	0.031
Magnesium	0.046	0.025	EDTA	0.02	0.011

*B: concentration of creatinine; Xb: concentration of interfering material; $K_{(B, Xb)}$: selectivity coefficient of B over Xb – the smaller the selectivity coefficient, the less interference by foreign material Xb.

Determination of stoichiometric ratio

Molar ratio and continuous variation method

A sequence of remedies was formulated in which the creatine level was maintained constant at 1 mL (1×10^{-3} mol L^{-1}), while that of the ligand was systematically altered from 0 to 5 mL of 1×10^{-3} mol L^{-1}. The optical density of these remedies was measured at 525 nm with all optimal settings. In Figure 18, the absorbance-molar ratio graph showed that creatine formed a 1:4 complex with the ASA dye.[17] Whereas in the continuous modification situation, a sequence of remedies was formulated by mixing equimolar remedies of creatine and ligand in different proportions from 1.2 mL to 3.6 mL (1×10^{-3} mol L^{-1}) of the same concentration while preserving the total molar concentration constant. A plot of absorbance measured at the suggested wavelength versus mole fraction was used to determine the creatine-reagent ratio. Figure 19 shows that the maximum optical density was obtained at a molar ratio of 1:5 for the complex.[18] In the case of creatinine, a sequence of remedies was formulated in which the creatinine level was maintained constant at 1 mL (1×10^{-3} mol L^{-1}), while that of the ligand was systematically altered from 0 to 3.5 mL of 1×10^{-3} mol L^{-1}. The optical density of these remedies was measured at 520 nm with all optimal settings. In Figure 20, the absorbance-molar ratio graph showed that creatinine formed a 1:4 complex with the ASA

dye.[17] In the continuous modification situation, a sequence of remedies was formulated by mixing equimolar remedies of creatinine and ligand in various proportions from 1.2 mL to 4 mL (1×10^{-3} mol L^{-1}) of the same concentration, while preserving the total molar concentration constant. A plot of absorbance measured at the suggested wavelength versus mole fraction was used to determine the creatinine-reagent ratio. Figure 21 shows that the maximum optical density was acquired at a molar ratio of 1:4 for the complex.[18]

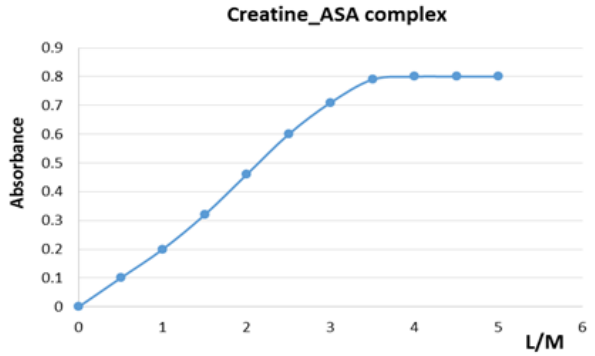


Figure 18. Molar ratio method of creatine-ASA complex.

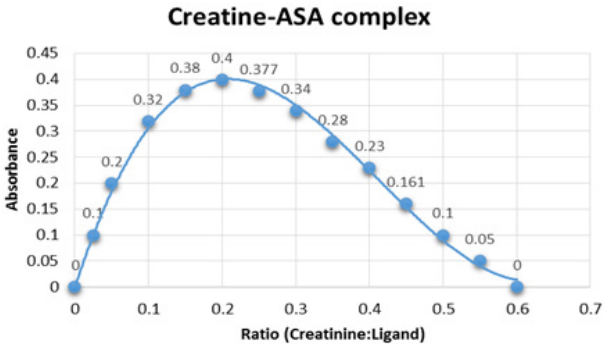


Figure 19. Job's method of continuous variation of creatine-ASA complex.

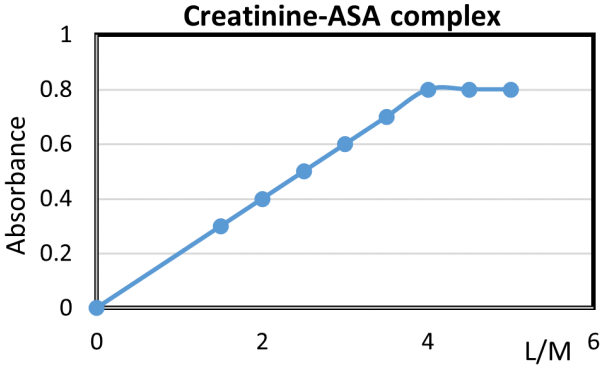


Figure 20. Molar ratio method of creatinine-ASA complex.

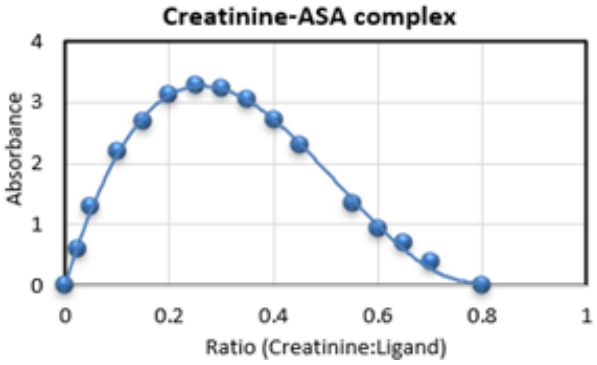


Figure 21. Job's method of continuous variation of creatinine-ASA complex.

Calibration curve (Beer-Lambert law)

The calibration curves were created using spectrophotometric data taken after optimizing all of the generated complex's optimal parameters as detailed above. Different concentrations of creatine and creatinine with ASA dye were used to study Beer's law. The results showed that the levels of Beer's law were (0.149-3.729) µg mL^{-1} and (3.4-101.808) µg mL^{-1}, respectively, as shown in Figures 22 and 23.

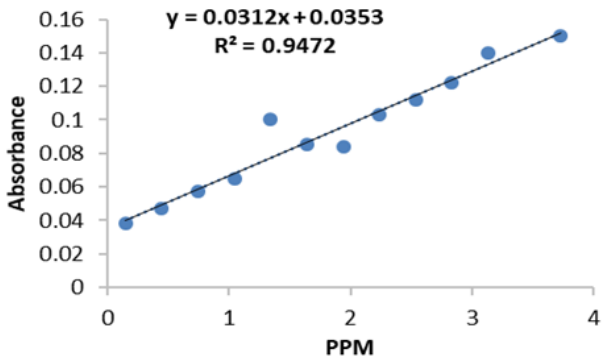


Figure 22. Standard curve of creatine determination.

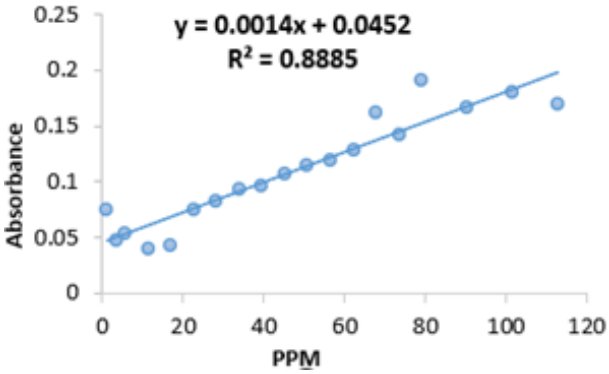


Figure 23. Standard curve of creatinine determination.

Statistical treatment for determination of creatine and creatinine

The technique demonstrated excellent replicability for a series of nine measurements of 2.24 µg mL^{-1} of creatine with ASA under ideal circumstances. Various statistical characteristics like variance, molar absorptivity, Sandell's responsiveness, student's *t*-test, the detection threshold (LOD), and the quantification threshold (LOQ) are outlined in Table VI. The information shows the suggested approach's superb straightness, elevated responsiveness, satisfactory precision, and exactitude. Concerning creatinine, the approach also exhibited fine consistency for a series of seven readings of 62.22 µg mL^{-1} of creatinine with ASA under ideal circumstances. Various distinct statistical parameters like variance, molar absorptivity, Sandell's selectivity, student's *t*-test, the detection limit (LOD), and the quantification limit (LOQ)[19] were consolidated in Table VI. The data demonstrated the outstanding linearity, elevated selectivity, satisfactory accuracy, and precision of the suggested approach.

Table VI. Statistical treatment for spectrophotometric determination of creatine and creatinine

Parameters	Creatine	Creatinine	Parameters	Creatine	Creatinine
Molar absorptivity L mol^{-1} cm^{-1}	11980	425.388	Standard error of the mean (SEM)	0.0009	0.0008
Sandell's sensitivity(µg cm^{-2})	0.0125	0.266	Student *t*-test	1.095	1.225
Mean value	2.241	62.221	Slope	0.0312	0.0014
Standard deviation (SD)	0.00274	0.0022	Limit of detection (LOD) (µg mL^{-1})	0.2898	5.092
(RSD %)	0.122	0.0035	Limit of quantification (LOQ) (µg mL^{-1})	0.8782	15.4304

Determination of creatine and creatinine in urine, plasma, and serum by using ASA

The suggested method used ASA dye to quantify the levels of creatine and creatinine in human urine, plasma, and serum. The results in Table VII were in strong concordance with the suggested approach, indicating that the suggested approach could be used.

Table VII. Applications of creatine and creatinine by using ASA dye

	Creatine samples											
	Urine				Plasma				Serum			
	Man	Woman	Child	Pregnant Woman	Man	Woman	Child	Pregnant Woman	Man	Woman	Child	Pregnant Woman
Taken (μg mL^{-1})	3.29	1.047	0.77	3.72	2.24	0.445	0.147	3.43	2.87	1.65	1.34	3.21
Found (μg mL^{-1})	3.28	1.044	0.746	3.7	2.237	0.449	0.148	3.428	2.84	1.63	1.343	3.24
Recommended method[19,20]	3.27	1.046	0.745	3.7	2.235	0.444	0.148	3.41	2.86	1.6	1.341	3.15
%Recovery	99.7	99.71	96.88	99.46	99.87	100.9	100.7	99.94	98.95	98.79	100.2	100.93

	Creatinine samples											
	Urine				Plasma				Serum			
	Man	Woman	Child	Pregnant Woman	Man	Woman	Child	Pregnant Woman	Man	Woman	Child	Pregnant Woman
Taken (μg mL^{-1})	90.45	56.54	22.6	101.79	45.25	39.593	5.66	50.89	73.52	62.2	50.87	65.8
Found (μg mL^{-1})	90.49	56.57	22.61	101.81	45.24	39.59	5.68	50.91	73.53	62.22	50.9	66
Recommended method[20]	90.47	56.58	22.6	101.8	45.25	39.592	5.67	50.9	73.52	62.24	50.88	65.9
%Recovery	100	100.1	100	100	99.98	100	100.4	100.04	100.5	100.03	100.1	100.3

As shown in Table VIII, a comparison between the proposed methods and other spectroscopic methods for the quantification of creatine and creatinine shows the advantages and novelty of our proposed methods.

Table VIII. Evaluation of the proposed techniques with alternative documented detectability spectroscopic approaches for the quantification of creatine and creatinine

Determination of creatine							
Reagent	**λ_{max} (nm)**	**pH**	**Surfactant**	**Time (min)**	**Temp. (ºC)**	**Concentration range**	**Reference**
Alpha naphthol sulpha acetamide	525	Acidic medium (pH 5)	Triton X-100	5	20	0.149-3.729 μg mL^{-1}	Proposed method
Alpha naphthol-Di acetyl	530	Alkaline medium (pH 7.4)	Triton X-100	30	30	5 nmol	21
Alpha naphthol-biacetyl	520	Alkaline medium (pH 7.4)	___	9	40	30 mg L^{-1}	22
Determination of creatinine							
Reagent	**λ_{max} (nm)**	**pH**	**Surfactant**	**Time (min)**	**Temp. (ºC)**	**Concentration range**	**Reference**
Alpha naphthol sulpha acetamide	520	Acidic medium (pH 3)	Triton X-100	5	30	3.4-101.81 μg mL^{-1}	Proposed method
ρ-methyl aminophenol sulfate	530	Acidic medium (pH 5.4)	___	30	27	4.4 to 620 μM	23
Picric acid of Folin &Wu	520	Alkaline medium (pH 7.4)	___	20	20	400 μg/100 mL	24

A comparison between the proposed method and traditional alternatives, based on environmental criteria, is presented in Table IX.

Table IX. Comparison between the proposed method and traditional alternatives based on environmental criteria

Aspect	Proposed method	Traditional alternatives
Reagent	Alpha-naphthol sulpha acetamide (for creatine and creatinine)	Alternatives such as picric acid (used in Folin & Wu method)[24] have environmental concerns due to hazardous waste production.
Solvent/Surfactant	Triton X-100 (less hazardous surfactant used in acidic medium)	Traditional methods often do not use surfactants or rely on solvents that might be less environmentally friendly.
Reaction time	5 minutes (faster reaction time)	Traditional methods often require longer reaction times, such as 20-30 minutes (e.g., picric acid-based methods), increasing energy consumption.
Temperature	20–30 °C (mild conditions)	Some traditional methods require higher temperatures (e.g., 40 °C in the alpha naphthol-bi-acetyl method[22]), which increases energy demand.
Waste generation	Minimal due to lower reagent use and surfactant involvement	Traditional methods may produce more waste, particularly hazardous byproducts like picric acid residues.
Sensitivity	μg mL^{-1} detection range (sensitive)	Some traditional methods (e.g., Folin & Wu method) are less sensitive and require larger sample sizes, resulting in greater chemical consumption.
Sustainability	Designed with green metrics in mind	Older methods often lack sustainability considerations, with higher energy and resource consumption.

As shown in Table IX, the proposed method has significant advantages in terms of environmental sustainability, offering an eco-friendlier alternative to traditional approaches such as:

- Eco-Friendly Reagents: The proposed method uses alpha-naphthol sulpha acetamide, less harmful than toxic alternatives like picric acid.
- Energy Efficiency: Faster reaction times and mild temperatures reduce energy consumption, making the method more sustainable.
- Waste Reduction: Lower chemical and solvent usage align with green analytical chemistry principles.

CONCLUSION

The enhanced spectrophotometric method reported in this study is accurate, sensitive, and efficient for detecting creatinine and creatine in biological samples. It provides a quick and cheap alternative to conventional treatments, consistent reaction products, and great sensitivity across a large concentration range. Notably, a new advancement in spectrophotometric analysis uses alpha-naphthol sulpha acetamide as a reagent. Potential clinical uses for this method include tracking muscular and kidney health and enabling more precise evaluations of metabolic disorders. The straightforward process and widely accessible chemicals make it perfect for routine laboratory usage, even with limited resources. Furthermore, identifying analytes at low and high concentrations with minimal interference from common biological and chemical pollutants demonstrates the instrument's durability and reliability.

Future research could look into merging this process with automated analytical systems to increase throughput and uniformity. This would increase its use in pharmaceutical quality control and clinical diagnostics, contributing to analytical and biomedical science advances.

Conflicts of interest

The authors do not report any conflicting concerns.

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