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Spectrophotometric determination of creatine in urine and blood human samples

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ABSTRACT **: *A new spectrophotometric method has been developed to accurately measure creatine levels in plasma, urine, and serum. This improved method is suitable for routine use in clinical laboratories and the pharmaceutical industry. The proposed method utilizes Alpha naphthol Sulpha Acetamide (ASA), a new azo dye, to react with creatine in order to produce an orange-colored complex that can be detected at a wavelength of 525 nm. The optimal conditions for this method have been thoroughly studied, including various factors such as pH, buffer, dye volume, sequence of addition, time, temperature, organic solvent, surfactant and interfering materials. It has been determined that the most optimal conditions for determining creatinine at room temperature are a temperature of 20°C after 5 minutes in the presence of citrate buffer pH(5) and Triton X-100 as a surfactant. The concentration range of (0.149-3.729) µg mL^{-1} with ASA dye obeys Beer's law, with a molar absorptivity of 11980 $L^{-1}.mol^{-1}.cm^{-1}$. This improved method is expected to contribute greatly to the clinical and pharmaceutical industries due to its accuracy and convenience.***

Keywords: **Creatine, A.S.A dye, Citrate buffer, a spectrophotometric method, time, Triton X-100.**

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I. INTRODUCTION

Creatine is a nitrogenous organic acid that plays a crucial role in supplying energy to cells, especially muscle cells [1]. It is an amino acid, which means it is a building block for proteins and contributes to muscle energy production. Creatine is mainly found in muscles and the brain, but the body's liver, pancreas, and kidneys can also produce around 1 gram of it daily. Creatine plays several key functions in the human body [2]. One of its primary roles is in energy production. During periods of high energy demand, creatine helps produce adenosine triphosphate (ATP), which is essential for cellular energy. Our ATP stores can only sustain about 10 seconds of work before needing replenishment, but by supplementing with creatine, ATP stores can be increased [3,4]. Another important function of creatine is its impact on muscle performance. It acts as a natural energy source that enables skeletal muscles to contract effectively, providing a steady supply of energy during exercise. Creatine supplements have been shown to enhance muscle performance in short-duration, high-intensity resistance exercises like weightlifting, sprinting, and cycling [5]. Creatine also plays a role in increasing anaerobic energy capacity. It has been found to have a beneficial effect on anaerobic energy production, allowing for improved performance during intense physical activity [6]. In addition to its effects on energy and muscle function, creatine has the added benefit of reducing protein breakdown in the body [7]. This can be particularly beneficial for individuals looking to build and maintain muscle mass. Furthermore, studies have shown that creatine exhibits neuroprotective effects and may potentially enhance cognitive function in older adults [8]. It has been suggested that creatine supplementation could have positive effects on brain health and function. While creatine is generally considered safe, it can have side effects such as stomach pain, nausea, and diarrhea. It is important to consult a healthcare provider, especially for individuals with a history of kidney disease or other medical conditions. Regarding the relationship between creatine and liver functions, the liver plays a crucial role in the generation of creatine [9]. Liver disorders may have a negative effect on creatine levels in the body. However, creatine supplementation has been shown to prevent the progression of nonalcoholic fatty liver and could have positive effects in reducing fat accumulation in the liver [10, 11]. Increased creatine intake may also be associated with lower ALP levels and an improved AST-to-ALT ratio, suggesting potential hepatoprotective effects [12]. It is worth noting that dietary exposure to creatine through a normal diet does not appear to be associated with an increased risk of liver disease in the general population.

Various methods are available for determining creatine levels in formulations. High-performance liquid chromatography (HPLC) and liquid chromatography (LC) with UV detection are commonly used methods to

simultaneously determine creatine and creatinine [13, 14]. Additionally, a creatine assay specifically designed for benchtop chemistry analyzers has been developed, utilizing various chemical assays and processes [15] and Enzymatic methods, utilizing different enzymes such as; creatininase, keratinase, urease, phosphoric acid enol pyruvic acid carboxylase and malate can also be employed for the determination of creatinine [16]. On-going research, a new spectrophotometric method is utilized for determining creatine.

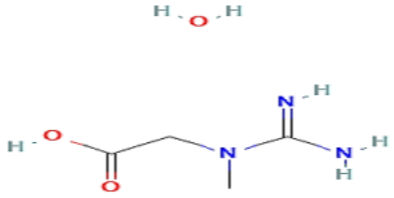


Figure 1. Chemical structure of Creatine monohydrate

II. MATERIALS AND METHODS

Reagents and chemicals

Analytical-grade reagents, which weren't subjected to any additional purification, were utilized as the chemicals and solvents in the research. All studies were performed using double-distilled water. Alpha chemika provided creatine monohydrate (with a purity of 99%, Mwt=149.15), BDH Company provided Sodium Hydroxide (Mwt=40), while Sigma-Aldrich provided Sulpha Acetamide (Mwt=214.24) and Alpha naphthol (Mwt=144.17). El-Nasr Company (Egypt) provided Methanol (Mwt=32.04), Ethanol 95% (Mwt=46.07), Di-methyl Formaldehyde (Mwt=73.09), 1-Butanol (Mwt=74.121), 2-Propanol (Mwt=60.09), Hydrochloric acid (Mwt=36.46), and Formaldehyde (Mwt=30.031).

Instrumentation

The absorbance was measured using a T80 UV/Vis double beam spectrophotometer (PG Instruments Ltd Company, United Kingdom) with a spectral bandwidth of 2.0 nm and 1.0 cm matched quartz cells. Using an Adwa pH meter (Model AD 1030, Romania), the pH levels were adjusted.

Solutions

14.915g of creatine was dissolved in 100 ml of distilled water to create a stock solution at 1.00 M. Dilution from the stock solutions was used to create working solutions with lower concentrations.

By dissolving 0.1845 g of ASA dye in 500 ml of 95% ethanol, a stock solution with a concentration of 1.00×10^{-3} M was created.

Buffers

A group of buffer solutions including; Universal buffer (pH 2.0-12.0) was prepared by mixing the acids of (0.04 M boric, orthophosphoric, and acetic acids) with 0.2 M NaOH in appropriate proportions [17]. By mixing the appropriate ratios of 0.2 M boric acid and borax solutions with 0.2 M NaOH, borate buffer (pH 7.0-11.0) was created [18]. By mixing sodium acetate and 0.1 M acetic acid in appropriate amounts, acetate buffer (pH 3.0-6.0) was created. By adding sodium citrate and 0.1 M citric acid in appropriate amounts, citrate buffer (pH 3.0-6.0) was created. By mixing the proper ratios of 0.1 M potassium dihydrogen phosphate with 0.1 M HCl or NaOH, phosphate buffer (pH 7.0-11.0) was created [19]. Triton X-100, sodium lauryl sulfate (SLS), and cetyl trimethyl ammonium bromide (CTAB), among several different surfactant types, were synthesized at a concentration of (1.0×10^{-2} M) by dissolving a specific amount of each in 100 mL deionized water.

Procedures

Preparation of Azo dye (ASA):

Take 0.01 mol. of sodium nitrite in 5 ml of deionized water, add it drop by drop to 0.01 mol. of sulpha acetamide in 10 ml of hydrochloric acid (1:1), and then pour the mixture drop by drop on 0.01 mol. of alpha-naphthol in 10 ml of sodium hydroxide (2M). In an ice bath, the addition took place while being stirred. Using the Buchner funnel, the mixture was finally filtered. The precipitate was then collected, washed with distilled water, dried, and crystallized from ethanol to produce the compound N-((4-(2-(2-hydroxy naphthalene-1-yl)-1, 4, 2-diazynyl) phenyl) acetamide, which is reddish brown in color.

Synthesis and Determination of Creatine-ASA Complex:

The following ingredients were added to a 10 mL standard flask: 2.00 mL of ($1x10^{-3}$) M creatine, 3 ml of citrate buffer pH=5, 2.00 mL of ($1x10^{-3}$) M ASA dye solution, 10 mL of ethanol 95%, and all of it was thoroughly mixed. After five minutes at 30°C, measure the absorbance to the blank, which was made using the same composition but without creatine, by scanning the wavelength from 200 to 800 nm.

Factors affecting the procedure:

Numerous variables, including pH and buffer, time of reaction, volume of creatine provided, temperature, surfactants,

interference from external components, organic solvent, and order of addition, were investigated.

General procedure in the presence of surfactant:

2.00 mL of ($1x10^{-3}$) M creatine was added to a 10 mL standard flask accompanied by 3 mL of citrate buffer pH=5, 2.00 mL of ($1x10^{-3}$) M ASA dye solution, 2 mL of ($1x10^{-2}$) M Triton X-100, and ethanol 95% to the mark. The mixture was carefully mixed after all of the additions. When the five minutes are over, check the absorbance by comparing it to a blank made from the same composition but devoid of creatine.

Beer's law:

Using 2 ml of the reagent, 3 ml of a citric acid/sodium citrate buffer with a pH of 5, and 2 ml of TritonX-100 at a concentration of $1x10^{-2}$ M, a calibration graph for the quantification of creatine was created. 2 mL of various creatine concentrations were added to start the reaction, and then 95% ethanol was added to the mark and carefully mixed. Five minutes at 30°C were given for the reaction mixture to stand. The colored solution's absorbance was measured at 520 nm.

Applications of Creatine:-

a- Determination of creatine in human serum:

Zagazig University Hospital supplied a sample of human blood serum for testing. Blood was taken in a certain volume (10 mL) from an adult individual. This sample was placed in the center of a centrifuge using a vacutainer tube that included a clot activator and a specific gel to separate serum from the cells. Iron and copper were masked by using 1ml sodium fluoride according to [20] finally; 2.00 mL of serum after diluted 10 times, 3.00 mL citrate buffer (pH 5), 2.00 mL from ASA (1.00×10^{-3} M), and 2ml of ($1.00x10^{-2}$ M) of Triton X-100 were added to a 10 ml standard flask, then completed with ethanol 95% to the mark. Absorbance was finally measured against the blank after 5 minutes at 20°C.

b- Determination of creatine in urine:

Three patients at Zagazig University Hospital—a man, a woman, and a little child—who have problems with their liver and kidney functions had urine samples taken. In a 10 mL standard flask, 2 mL of each urine sample was added along with 3.00 mL of citrate buffer (pH 5), 2.00 mL of ASA (1.00×10^{-3} M), 2 mL of TritonX-100 ($1x10^{-2}$ M), and ethanol (95% to the mark). After 5 minutes at 20°C, the absorbance was measured against the blank.

c- ***Determination of creatine in plasma:***

In the Zagazig University Hospital, a certain amount of blood (10 mL) was drawn from an adult human body. The samples was placed in a centrifuge using an EDTA-anticoagulant tube to help separate the plasma. The blood sample was added to the EDTA and put into a 10 mL standard flask. The amount of EDTA in the blood ranged from 1.2 to 2.0 mg/mL. To this, 3.00 mL of citrate buffer (pH 5), 2.00 mL of ASA (1.00×10^{-3} M), 2.00 mL of Triton X-100 ($1.00x10^{-2}$ M), and 95% ethanol were added. Absorbance was finally measured against the blank after 5 minutes at 20°C.

***** Guide I. Terms and definitions used in connection with reference materials, 30, 2nded, 1992.

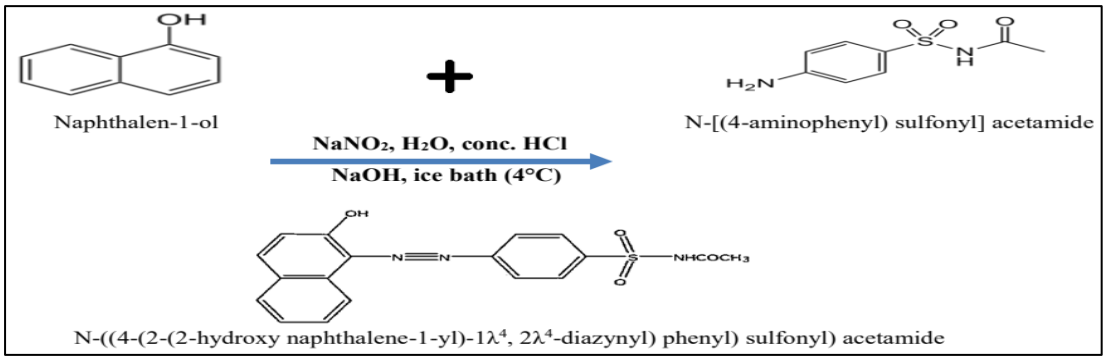
III. RESULTS AND DISCUSSION

3.1- Formation of the complex:

The structure of Azo dye (ASA) was supported by:

a) Correct analytical data, as shown in figures (2 and 3).
b) IR spectra of ASA, gave bands at 3554, 3436, 1697, 1539-1592, and 1177 cm^{-1} due to ט'-OH, ט'-NH, ט'-C=O, ט'-N=N, ט'-S=O, respectively as shown in figure (4) and table (II).

**** The proposed equation for the synthesis of a new azo dye of Alpha naphthol Sulpha Acetamide (ASA) was:-***

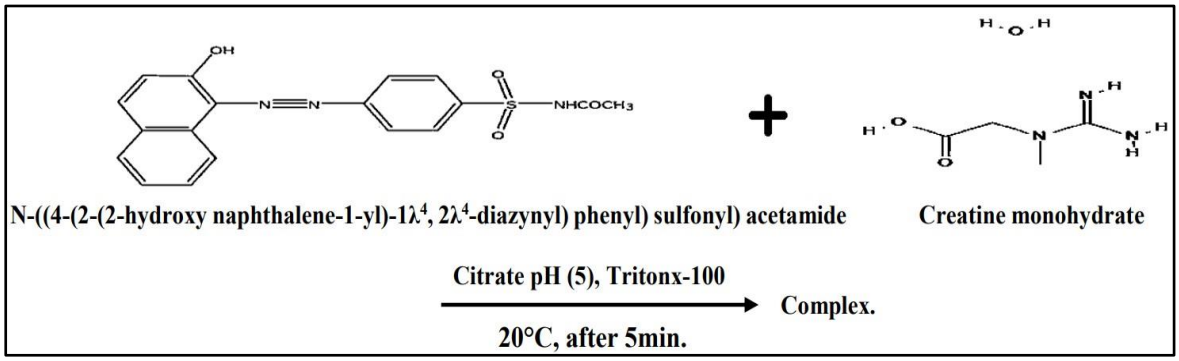


Scheme 1. Synthesis of A new Azodye.

Table I. Chemical properties of new azodye.

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

- ***The proposed reaction to form a complex with creatine was:***



Scheme 2. Formation of complex with Creatine.

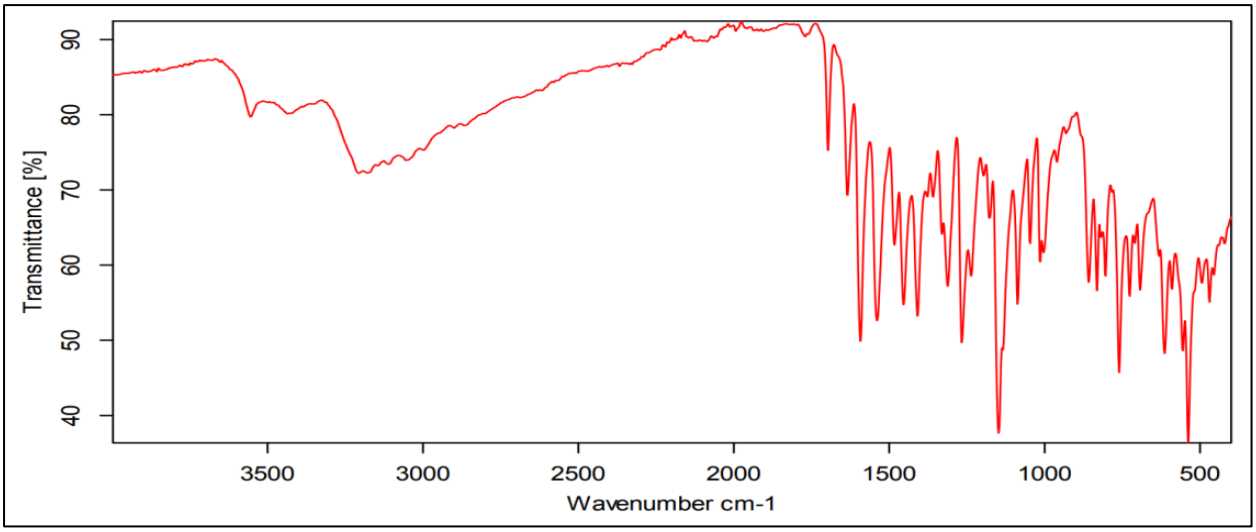


Figure 2. IR spectra of ASA dye.

Table II. Some selected Infrared frequencies and tentative assignments for 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		

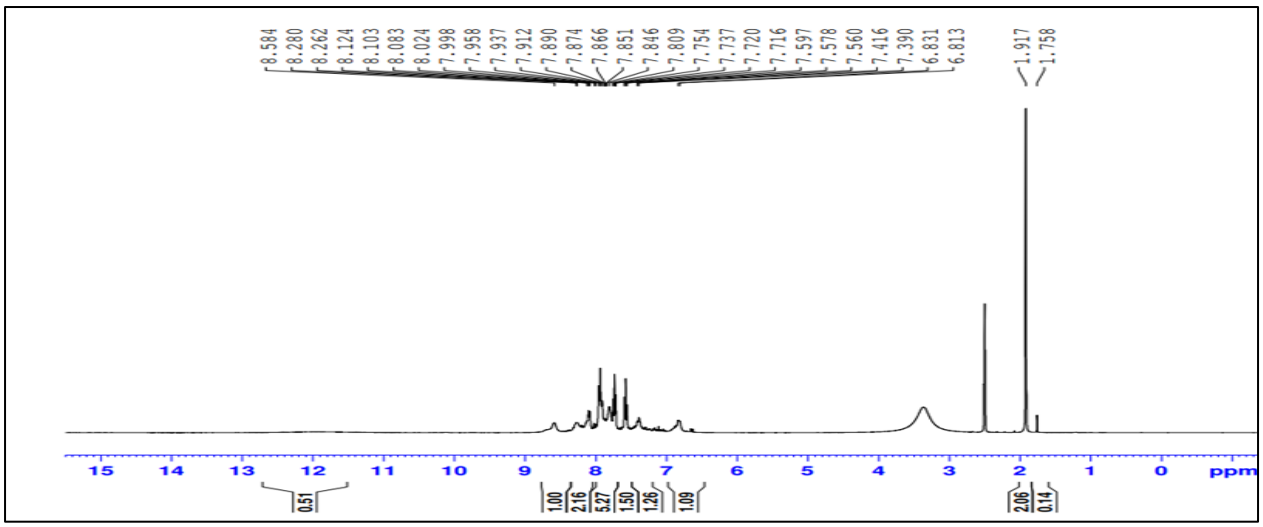


Figure 3. 1-H-HNMR of ASA dye.

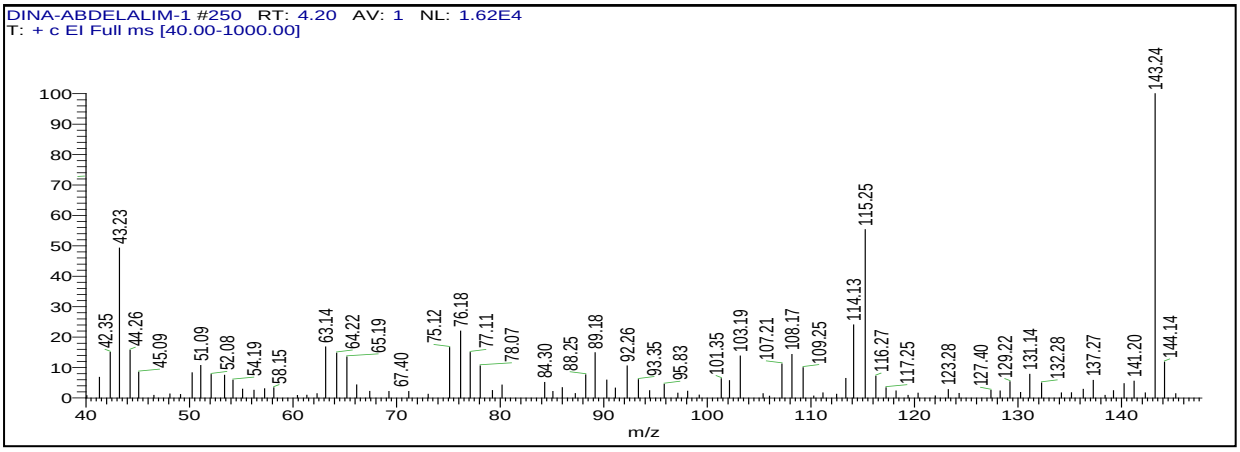


Figure 4. Mass Spectroscopy of ASA dye.

3.2- Absorption curve:

Creatine was measured spectrophotometrically using the ASA reagent, which produced an orange-colored stable combination of creatine at 525 nm, as shown in Fig. (5); it occurred bathochromic shift due to the formation of complex between creatine and ASA dye.

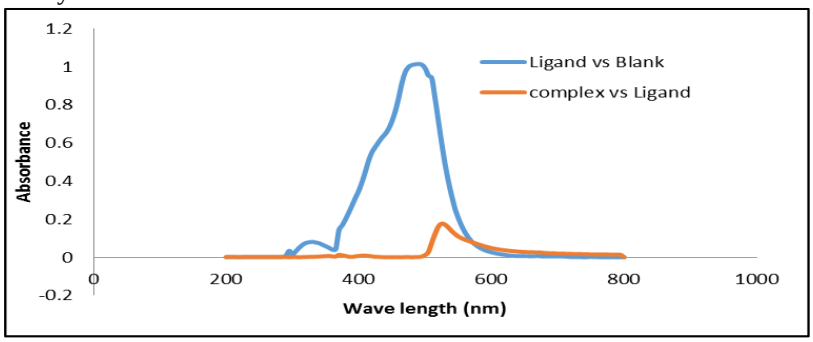


Figure 5. Absorption curve of Alpha Naphthol Sulpha Acetamide with Creatine.

3.3- Effect of pH and buffer:

The complex is best suited for a pH range of 1 to 7, with a maximum value at pH = 4 as shown in Fig. (6), according to the investigation of the impact of pH on the color intensity of the complex. As shown in Fig. (7), studies investigating the effects of various buffer types over the pH range of (3-7) revealed that citrate buffer at a pH of 5 produces the highest absorbance value.

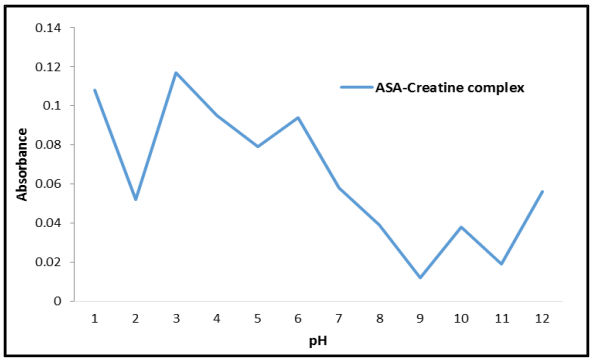


Figure 6. Effect of pH on the formation of the creatine-ASA complex.

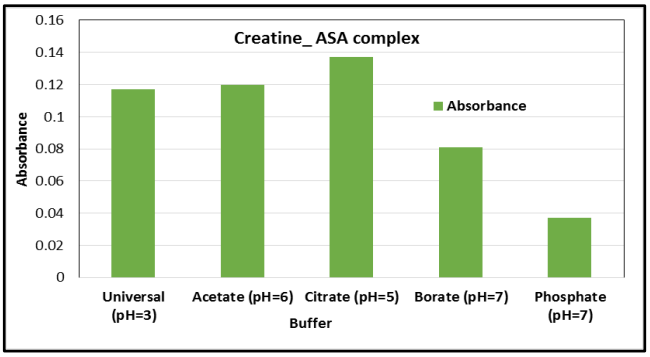


Figure 7. Effect of different buffer types on the formation of creatine-ASA complex.

From the results shown Figure 6 and 7; it was found that the absorbance reached its maximum value in Acidic medium than in alkaline medium, and by studying different buffer types over the pH range, it was found that the complex was formed completely in presence of citrate buffer at pH (5).

3.4- Effect of a sequence of addition:

The order (creatine-buffer-reagent) produced the maximum absorbance value, due to it contributed in complete formation of complex, according to the study of the effect of the addition sequence on the formation of the creatine-ASA complex, as shown in Table III.

Table III. Effect of a sequence of addition on the determination of creatine

Sequence	Absorbance
Reagent + Creatine + Buffer	0.081
Reagent + Buffer + Creatine	0.079
Creatine + Buffer + Reagent	0.126
Creatine + Reagent + Buffer	0.084

3.5- Effect of time and temperature:

Studying how temperature and time affect the stability and color intensity of the complex formed by creatine and ASA showed that the complex formed instantly and that the absorbance reached its highest value after 5 minutes at a temperature of 20 ^{o}C. According to Figures 8 and 9, respectively.

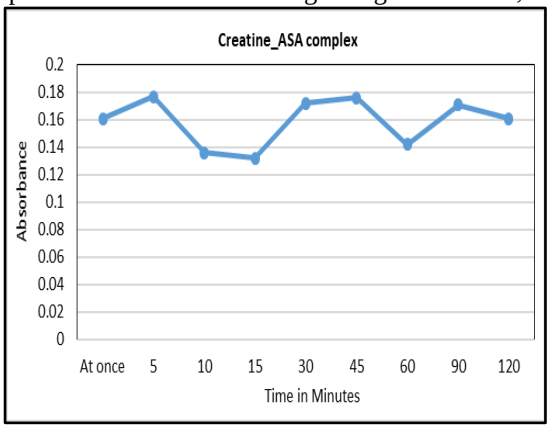


Figure 8. Effect of time on the determination of creatine.

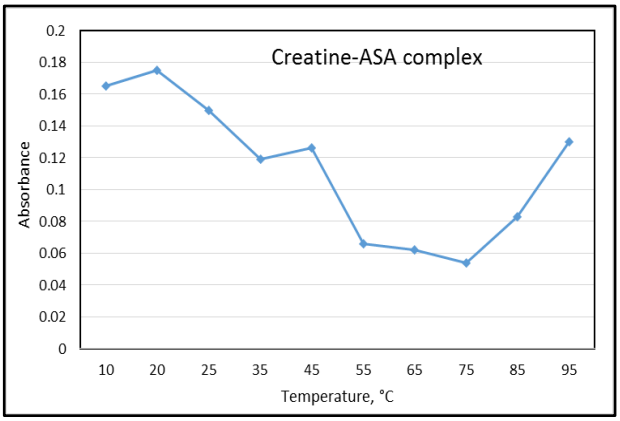


Figure 9. Effect of temperature on the determination of creatine.

3.6- Effect of organic solvent and Surfactant:

Different organic solvents, including ethanol, methanol, 2-propanol, 1-butanol, DMF, and DMSO, were utilized to find the best organic solvent based on the absorption spectra. The results revealed that ethanol organic solvent enhanced the absorbance of creatine according to Figure 10. While, in case of surfactant; the results shown that the absorption peak was shifted to a hyper and bathochromic shift in the presence of 2.00 ml of Triton X-100 as a surfactant. As seen in Figure 11, this might be the result of the creation of micelles or a double- or triple-complex with the surfactant itself.

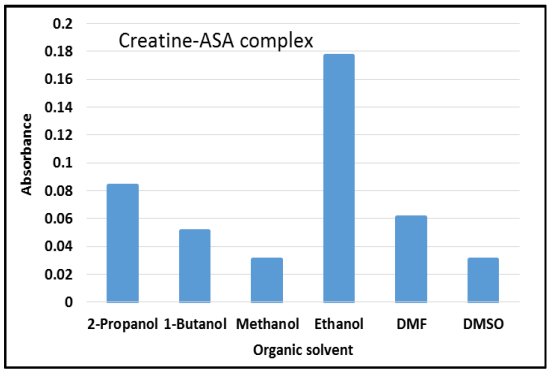


Figure 10. Effect of organic solvent on the determination of creatine.

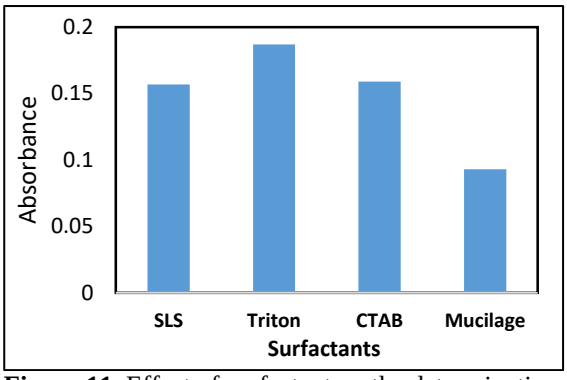


Figure 11. Effect of surfactant on the determination of creatine.

3.7- Effect of Interference:

The interaction of 1×10^{-3}M concentrations of creatine with 1×10^{-2}M concentrations of interfering substances such as aluminum, calcium, carbonate, urea, magnesium, glucose, potassium, sodium, chloride, phosphate, and EDTA was studied. The results demonstrated that there was interference, with the exception of aluminum and urea, according to Table IV.

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

Interfering Materials	Absorbance	% ± Interference	Interfering Materials	Absorbance	% ± Interference
Without interfering materials	0.177	Zero	**Glucose**	0.156	11.9%
Aluminum	0.174	1.7%	**Potassium**	0.147	17%
Calcium	0.145	18.1%	**Sodium**	0.113	36.2%
Carbonate	0.147	17%	**Chloride**	0.113	36.2%
Urea	0.165	6.8%	**Phosphate**	0.137	22.6%
Magnesium	0.157	11.3%	**EDTA**	0.137	22.6%

3.8- Determination of stoichiometric ratio:

a. Molar ratio and Continuous variation method:

The concentration of the ligand was regularly changed from 0 to 5 ml of 1×10^{-3} M, while the Creatine concentration was maintained at 1 ml (1×10^{-3} M) in a series of solutions. These solutions' absorbance is measured at 525 nm under ideal circumstances. The absorbance-molar ratio curve in Fig. (12) demonstrated that creatine and ASA dye formed a 1:4 complex [21]. For continuous variation, a series of solutions were made by mixing equimolar solutions of creatine and ligand in varying ratios from 1.2 ml to 3.6 ml (1×10^{-3} M) of the same concentration while maintaining the total molar concentration constant. The creatine-reagent ratio was calculated by plotting absorbance at the appropriate wavelength vs mole fraction. A molar ratio of 1:5 for the complex [22] resulted in the highest absorbance, as shown in Fig. (13).

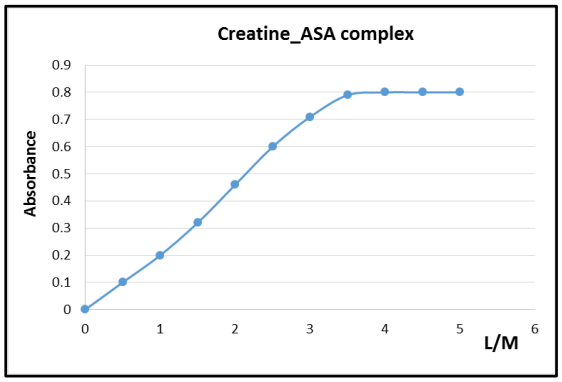


Figure 12. Molar ratio method of creatine-ASA Complex.

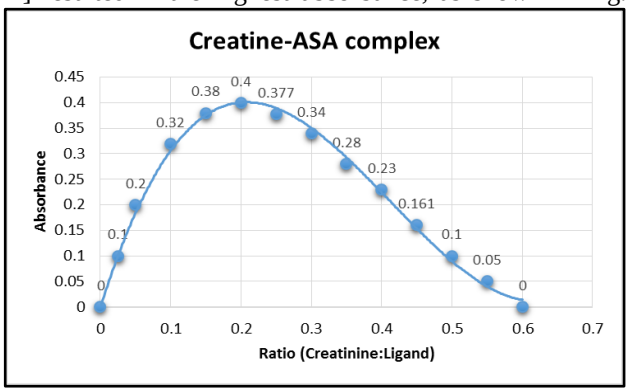


Figure 13. Job's method of Continuous variation of creatine-ASA Complex.

b- Calibration curve (Beer's law):

After optimizing all of the above-mentioned produced complex's ideal parameters, the calibration curve was made using spectrophotometric data. Beer's law was investigated using various creatine concentrations and ASA dye. The findings revealed that the range for Beer's law was between 0.149 and 3.729 µg mL^{-1} so the method was suitable for determination of creatine in low concentrations. Figure (14) illustrates this.

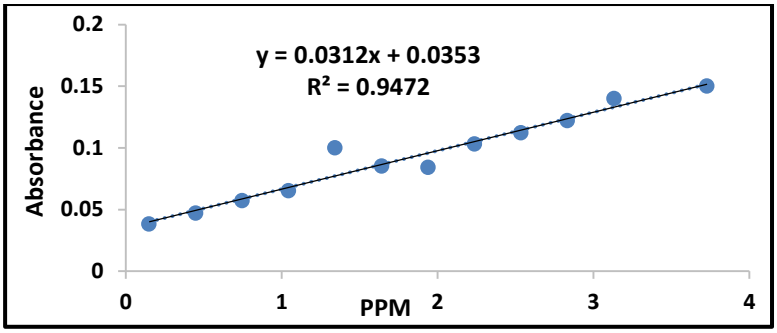


Figure 14. Effect of creatine concentration with ASA.

3.9- Statistical treatment for determination of Creatine:

Under ideal circumstances, the procedure had good repeatability for a set of nine measurements of 2.24 µg.mL^{-1} of creatine with ASA. Table IV provides a summary of many statistical variables including standard deviation, molar absorptivity, Sandell's sensitivity, student's t-test, limit of detection (LOD), and limit of quantification (LOQ). The results demonstrated that the correlation coefficient is 0.9472, indicating the linearity between the two variables. The precision of the present method was evaluated by determining different concentration of creatine (each analyzed at least nine times). The relative standard deviation (n=9) was 0.122% for (1.49 and 37.29) µg of creatine in 10-ml indicating that this method is highly precise and reproducible.

Table V. Statistical treatment for spectrophotometric determination of creatine

Parameters	Creatine	Parameters	Creatine
Molar absorptivity L.mol^{-1}. cm^{-1}	11980	**Standard error of the mean (SEM)**	0.000913
Sandell's sensitivity µg cm^{-2}	0.0125	**Student t-test**	1.095
Mean value ($\bar{x}$)	2.241	**Slope**	0.0312
Standard deviation (SD)	0.00274	**Limit of detection (µg / ml)**	0.2898
Relative Standard Deviation (RSD %)	0.122	**Limit of quantification (µg / ml)**	0.8782

3.10- Applications of Creatine in Urine, Plasma, and Serum by using ASA:

To measure creatinine in human urine, plasma, and serum, the suggested method used ASA dye. The results showed that the suggested technique could be applied because they were in good agreement with the suggested method, due to good recoveries in the range from (99.43 to 99.96) % obtained for creatine in these samples are given in Table (V).

Table VI. Applications of Creatine by using ASA dye (the recommended method according to**

Sample	ASA dye				
	Taken (µg / ml)	Found (µg / ml)	Recommended method	%Recovery	%RSD
Man urine	2.238	2.237	2.235	99.96	0.045
Woman urine	1.047	1.044	1.046	99.71	0.096
Child urine	0.77	0.746	0.745	96.88	0.134
Plasma	32.9	32.82	32.80	99.76	0.0031
Serum	21	20.88	20.86	99.43	0.0048

** **Fabiny DL, Ertinghausen G.** Automated reaction-rate method for determination of serum creatinine with the CentrifiChem. ClinChem1971; 17:696-700.

Bartels H, Böhmer M. Micro-determination of creatinine. Clin Chim Acta 1971; 32:81-85.

Table VII. Comparison of the suggested methods with other reported detectability spectrophotometric methods for the determination of creatine.

Reagent	λ_{max} (nm)	pH	Surfactant	Time	Temperature	References
Alpha napthol sulpha acetamide (ASA)	525	Acidic medium	Triton X-100	5 min.	20°C	Proposed method
Alpha naphthol-Di acetyl	530	Alkaline medium (Maleate buffer)	Triton X-100	30 min.	30°C	[23]
Alpha naphthol-biacetyl	520	Alkaline medium	___	9 min.	40°C	[24]

IV. Conclusion

The data obtained demonstrated the method's accuracy and sensitivity. It didn't need pricey chemicals. The technique determined a low concentration of creatine and did not require a high temperature. We suggested this

technique as a quick and easy way to estimate creatine levels. Long after the reaction, the color remained constant. Data supported the method's ability to determine micro concentration. The created system's ease of use and the method's inexpensive measurement costs were its key benefits. Also, it was found from the results in table (VII) the novelty of the suggested method for determination of creatine. Finally, this technique made it simple to find creatine in human serum, plasma, and urine.

V. Acknowledgements

I appreciate the help from the Chemistry Department of Zagazig University's Faculty of Science. Additionally, I would like to thank Dr. Adel Mansour El-Gendy, an organic chemistry professor at Zagazig University's Chemistry Department and Faculty of Science, for his invaluable assistance and perceptive criticism that considerably improved the quality of this work.

VI. Declarations

Compliance with ethical standards: The study involved human-derived samples from the clinical pathology department at the Zagazig University hospitals.

Conflict of interest: The authors declare no competing interests.

VII. References

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