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A Sensitive and Validated Hydroxytriazene-Based UV–Visible Spectrophotometric Method for Simultaneous Determination of Cu(II), Ni(II) and Co(II)

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Abstract : A simple, sensitive and validated UV–Visible spectrophotometric method was developed for the simultaneous determination of Cu(II), Ni(II) and Co(II) using a hydroxytriazene reagent as a chromogenic chelating agent. The analytical procedure was optimized by systematically investigating the effects of experimental variables, including pH, buffer composition, reagent concentration, reaction time and temperature. The metal–hydroxytriazene complexes exhibited intense absorption maxima at 545, 470 and 590 nm for Cu(II), Ni(II) and Co(II), respectively, while the free reagent showed an absorption maximum at 365 nm. The pronounced bathochromic shifts observed upon complex formation confirmed the formation of coloured metal–ligand complexes and provided well-separated analytical wavelengths suitable for simultaneous determination. An acetate–acetic acid buffer at pH 6.5 was selected as the optimum reaction medium, with 3.0 mL of 0.1% hydroxytriazene reagent and a reaction time of 15 min. The optimum temperature for colour development was found to be approximately 25–30 °C, and therefore the analytical measurements were performed at room temperature (25 ± 2 °C). The proposed method obeyed Beer's law in the concentration ranges of 0.2–1.6 µg mL^{-1} for Cu(II) and Ni(II), and 0.3–1.8 µg mL^{-1} for Co(II), with correlation coefficients of 0.9998, 0.9997 and 0.9995, respectively. The molar absorptivities were 1.86×10^4, 1.42×10^4 and 1.65×10^4 L mol^{-1} cm^{-1} for Cu(II), Ni(II) and Co(II), respectively. Sandell's sensitivities were found to be 0.0034, 0.0041 and 0.0036 µg cm^{-2} for Cu(II), Ni(II) and Co(II), respectively. The limits of detection were 0.018, 0.024 and 0.021 µg mL^{-1}, while the corresponding limits of quantification were 0.054, 0.072 and 0.063 µg mL^{-1}. Accuracy studies for Cu(II) gave recoveries of 99.6–100.5% with relative standard deviations below 1%, whereas intra-day and inter-day precision studies showed RSD values below 1.5%. The method also exhibited satisfactory robustness and ruggedness under small deliberate variations in experimental conditions. The proposed procedure was successfully applied to synthetic mixtures and real samples, including natural water, industrial effluent, pharmaceutical and alloy samples. The results demonstrate that the hydroxytriazene-based method provides a convenient, sensitive, precise and economical approach for the simultaneous spectrophotometric determination of Cu(II), Ni(II) and Co(II).

IndexTerms **- Hydroxytriazene; Cu(II); Ni(II); Co(II); UV–Visible spectrophotometry; Chromogenic reagent; Metal–ligand complex; Simultaneous determination; Trace metal analysis; Method validation.**

I. INTRODUCTION

The researcher studied the spectrophotometric behaviour of the hydroxytriazene compound, 3-hydroxy-3-(4-chlorophenyl)-1-(4-acetylsulfonyl)phenyltriazene, towards iron(III) ions. Importance of the study lies in the fact that the functional groups present in the hydroxytriazenes can interact strongly with metal ions and therefore can be used as sensitive analytical reagent. The authors were concentrated on the preparation of the iron (III) – hydroxytriazene complex and the spectrophotometric properties of it and showed that the synthesized ligand could be used for the determination of the metal-ions. Typically, such studies include looking at absorption maximum, reagent concentration, pH, complex formation, and analytical parameters related to the development of the colour and the absorbance. Chlorphenyl and acetylsulfonyl groups are incorporated to provide structural features that may

have an impact on ligand reactivity, selectivity and complex stability. Thus, organic chromogenic reagents for iron analysis have been developed and the role of structural modification of hydroxytriazenes for their better analytical applicability has been shown. It also serves as a basis for future studies on sulphonamide- and sulphone containing hydroxytriazenes and their transition-metal complexes [1]. The investigated the analytical utility of 1,1′-(sulfonyldibenzene-4,1- diyl)bis(3-hydroxy-3-phenyltriaz-1-en) (BHDPT) for spectrophotometric determination of Fe(III). The study illustrates the value of the synthesis of multifunctional hydroxytriazene reagents that have two or more reactive triazene groups. BHDPT has the two hydroxytriazene groups linked together by a sulphonyl incorporated aromatic system, with probably several possible co-ordination sites for metal and increased opportunities for the formation of a measurable coloured complex. The study examined the suitability of BHDPT for the analytical application to Fe(III) with particular focus on the structure of the ligands and the spectrophotometric responses. This type of analytical investigations is of great value since the determination of iron is needed in environmental, biological, industrial and pharmaceutical samples. The work shows that hydroxytriazene derivatives can be used as a practical alternative to the traditional complexing and chromogenic agents. The authors developed an analytical application for BHDPT and drew the attention to the possibility of using the structurally modified bis-hydroxytriazene compounds in the quantitative spectrophotometry of metal ions. [2]

The researcher reported synthesis and characterization of hydroxytriazene derivative, 3-hydroxy-3-(4-chlorophenyl)-1-(4-sulphonamido)phenyltriazene (HCNT) along with its Fe(III) complex. The study is important due to a combination of analytical chemistry and biological evaluation, which shows that hydroxytriazene derivatives may be metal-binding and antimicrobial. Fe(III) complex was synthesized by both conventional synthesis and mechanochemical method which represents one of the ‘solvent-minimized' approaches for the synthesis of complexes. From the spectrophotometric study a working wavelength of about 402 nm was determined and the composition of the Fe(III):HCNT was 1:2. The maximum absorbance for the complex was observed at mildly acidic pH which indicated that the pH plays important role in the interaction of metal–ligand and sensitivity of the analyses. Compounds were also tested against bacteria and fungi using microbiological assays. Results showed a higher possibility of being used as bactericidal rather than fungicidal agent. In summary, the study highlights the potential for the development of multifunctional hydroxytriazene derivatives with useful coordination, spectrophotometric and biological properties by incorporating a sulphonamide group into the hydroxytriazene architecture. [3]

Investigator studied Beyond analytical chemistry, research on the neglected drug classes of hydroxytriazenes is also important in medicinal chemistry. Before the biological evaluation, the hydroxytriazenes of sulphanilamide, sulphadiazine, sulphapyridine and other sulpha drugs were synthesized and characterized. The biological activities which are potentially useful were predicted by computational methods using the online tool PASS and then validated. α-amylase and α-glucosidase inhibitory assays were used to evaluate the antidiabetic activity while the DPPH and ABTS radical scavenging assays were used to assess the antioxidant properties. Experimental study of anti-inflammatory activity was also carried out. To consider the potential interactions related to antidiabetic activity as well as obtain a theoretical explanation for the observed biological effects, molecular docking studies were performed. Results indicated that hydroxytriazene functionalised sulpha drugs can be prepared with measurable biological activities. This work thus reinforces the idea of the hydroxytriazenes acting as multifunctional scaffolds and offers a link to the new pharmaceutical possibilities offered by these compounds and to the known coordination/spectrophotometric applications. [4]

Researcher presented detailed biochemical study of triacsin biosynthesis, and the formation of the unusual pharmacophore N-hydroxytriazene. On the basis of the previous identification of the triacsin biosynthetic gene cluster, the researchers identified the enzymes involved in the construction and incorporation of the N-hydroxytriazene functionality through mutagenesis and biochemical studies. They found that two enzymes that used ATP to provide energy also formed a pair of successive N–N bond-forming reactions. One, called Tri28, also uses glycine, and is found in the making of hydrazine, while the second enzyme, called Tri17, uses nitrite in an N-nitrosation reaction. These results offer a mechanistic understanding of how a structural group is formed that is very rare in natural products. The research is very relevant to the modern triazene chemistry as it shows that the biological system can form N–N bond in enzyme-mediated way. In addition to the biosynthetic relevance, there are possible applications to controlled synthesis of unusual nitrogen containing compounds and biocatalysis. This work thus marks an important leap forward from the identification of the genetic underpinning of triacsin production to the experimental delineation of the enzymatic machinery that gives rise to the unique N-hydroxytriazene pharmacophore. [5]

The reserchers studied the synthesis, structural characterisation and evaluation of the antimicrobial and antifungal activity of diaryltriazene-derived triazene compounds. Triazenes, the compounds rich in N, have unique chemical and physical properties and have been known to have significant biological implications; hence, they are intriguing compounds to be explored as drugs and pharmaceuticals. This study examined 1,3- diaryltriazene derivatives with methoxy and nitro substituents, and provided an assessment of the effect of structural variation on biological behaviour. Molecular and crystalline structures were determined, and relationships between chemical structure and the activity observed were determined. Then the biological assays were conducted for antimicrobial and antifungal activity. This study has significance in showing that considerable changes in biological activity can be achieved by varying the aromatic substituents attached to the triazene: It also adds to general knowledge about the correlation between molecular architectures, physicochemical behaviours and biological responses. The results encourage further studies on the diaryltriazene skeleton as a way to generate biologically active substances. On the general field

of hydroxytriazene research, this is a complementary piece of work showing that triazene-based molecules can have significant biological activity as well as their many synthetic and analytical applications. [6]

The investigator studied the spectrophotometric reagents for determination of metal ions based on hydroxytriazene derivatives. This research is an important component of the analytical development of hydroxytriazene chemistry since the N–N–N functional system and hydroxyl group can provide appropriate donor sites for the coordination with transition-metal ions. The formation of a metal–hydroxytriazene complex may sometimes form a distinguishable colour or absorbance maximum for the quantitative determination of the complex using UV–visible spectrophotometry. The study is relevant to the development of simple analytical methods because spectrophotometric techniques are relatively easily available and the techniques are fast and economical when compared to many advanced instrumental techniques. The study of variables like reagent concentration, pH, wavelength, stoichiometric composition and the stability of the complex is a fundamental need for the establishment of the analytical performance. The potential selectivity and sensitivity of hydroxylamine reagents containing the hydroxytriazene group can be altered by replacing the aromatic groups and functional groups. The work, therefore, adds to the general trend of development of selective organic reagents for the determination of metal-ions. It is also an excellent starting point for future use of hydroxytriazenes for environmental, pharmaceutical, biological and industrial samples. [7]

I. RESEARCH METHODOLOGY

The analytical procedure was developed using a classical univariate optimisation approach. In this strategy, individual experimental parameters were systematically varied while keeping the remaining conditions constant. The optimum values obtained for the individual variables were subsequently combined to establish the final analytical procedure. Analytical-reagent-grade chemicals were used throughout the investigation, and double-distilled water, free from trace-metal contamination, was employed for the preparation of all solutions [8].

2.1 Apparatus

Absorbance measurements were carried out using a double-beam UV–Visible spectrophotometer equipped with matched 1 cm quartz cells. Spectral measurements were recorded in the wavelength region of 350–700 nm. Before measurements, the instrument was allowed to warm up for approximately 30 min, and its wavelength accuracy was periodically verified. The pH of the solutions was determined using a digital pH meter fitted with a combined glass electrode. The pH meter was calibrated with appropriate standard buffer solutions before use.

An analytical balance with a precision of 0.0001 g and Class A volumetric glassware were employed for quantitative measurements. A thermostatically controlled water bath was used whenever temperature-dependent experiments were performed. To minimise trace-metal contamination, laboratory glassware was immersed overnight in dilute nitric acid and subsequently washed thoroughly with double-distilled water.

2.2 Synthesis of the Hydroxytriazene Reagent

The hydroxytriazene reagent was prepared through a conventional diazotisation–coupling reaction. In the first step, the selected aromatic amine was diazotised using sodium nitrite in dilute hydrochloric acid under cooled conditions, with the temperature maintained below 5 °C. The freshly prepared diazonium salt was then coupled with a second aromatic amine under continuous stirring. During the coupling reaction, the pH was controlled by the gradual addition of sodium acetate.

Following completion of the coupling reaction, the product was separated by neutralisation and filtration. The crude material was purified by recrystallisation using aqueous ethanol and dried under vacuum until a constant melting point was obtained. The purified reagent was stored in a dark container to minimise degradation. Its identity and purity were assessed from the melting point, elemental composition, infrared spectral characteristics of the triazene and hydroxyl functionalities, and the characteristic UV absorption of the free ligand near 365 nm [9].

2.3 Preparation of Standard and Reagent Solutions

The hydroxytriazene reagent solution was prepared by accurately weighing the required amount of reagent, dissolving it in a small volume of ethanol and subsequently diluting it with double-distilled water. The prepared solution was stored in an amber-coloured bottle to protect it from light. The use of hydroxytriazene solutions as chromogenic reagents in spectrophotometric metal-ion determination, including their preparation and use under controlled reaction conditions, has been reported in previous studies.

Standard stock solutions of Cu(II), Ni(II) and Co(II) were prepared from analytical-grade sulphate or chloride salts. The salts were dissolved in a small quantity of dilute acid to minimise hydrolysis and then diluted to the required volume with double-distilled water. Where required, the concentrations of the standard solutions were confirmed by independent standardisation. Fresh working solutions were obtained daily by appropriate serial dilution of the stock standards.

An acetate–acetic acid buffer was employed to maintain the reaction medium at pH 6.5. This reaction condition is consistent with the reported hydroxytriazene spectrophotometric determination of Cu(II), Ni(II) and Co(II), for which the colour reaction was optimized in acetate buffer at pH 6.5. **[10]** Appropriate masking solutions were also prepared for controlling the

interference of foreign metal ions. Fluoride or citrate was used for Fe, thiourea for Zn, tartrate for Mn and iodide for Cd. These masking reagents were prepared as concentrated aqueous solutions and diluted as required during the analysis. The need for masking agents in hydroxytriazene-based metal determinations has been documented because several foreign metal ions can interfere with the analytical response. For example, interference from Co(II), Zn(II), Ni(II), Mn(II) and other metal ions has been reported in hydroxytriazene spectrophotometric/complexometric procedures. **[4]**

2.4 General Procedure for Colour Development

For colour development, a suitable aliquot of the metal-ion solution within the selected working concentration range was transferred into a 25 mL volumetric flask. The required quantity of acetate–acetic acid buffer was first added to establish the reaction medium at pH 6.5. Subsequently, an excess amount of the hydroxytriazene reagent was introduced; typically, 3.0 mL of 0.1% reagent solution was used.

The contents were thoroughly mixed and allowed to stand for 15 min to ensure complete development of the coloured metal–ligand complex. The solution was then diluted to the calibration mark with double-distilled water and mixed thoroughly. Absorbance was measured against a reagent blank at the corresponding analytical wavelength (λmax) of each metal.

For samples containing more than one metal ion, absorbance measurements were recorded at the respective analytical wavelengths. The resulting values were subsequently used in simultaneous-equation calculations for the determination of the individual metal concentrations. All measurements were performed in triplicate, and the average absorbance was used for quantitative calculations.

Preliminary experiments established the order of reagent addition. Addition of the buffer before the hydroxytriazene reagent produced slightly higher and more reproducible absorbance values than the reverse sequence; therefore, this order was maintained throughout the analytical procedure. **[4]**

2.5 Stoichiometric Studies, Calibration and Method Validation

The stoichiometry of the metal–hydroxytriazene complexes was investigated using Job's method of continuous variation and the mole-ratio method. In Job's method, the combined molar concentration of the metal ion and reagent was maintained constant while their respective mole fractions were systematically varied. In the mole-ratio method, the concentration of the metal ion was kept constant while progressively increasing amounts of the reagent were added. **[11]**

Conditional stability constants of the resulting complexes were determined from continuous-variation data under controlled ionic-strength conditions. Calibration curves were prepared individually for each metal at its corresponding λmax, and the analytical data were evaluated by linear regression.

The applicability of the simultaneous-equation approach was initially examined using synthetic binary and ternary mixtures containing known concentrations of the investigated metal ions. After satisfactory recovery was obtained, the method was extended to real samples.

Analytical performance was evaluated in terms of linearity, accuracy, precision, detection limit (LOD), quantification limit (LOQ), robustness and ruggedness. Accuracy was examined by the standard-addition method, while intra-day and inter-day precision were assessed from replicate measurements. LOD and LOQ were calculated using the standard deviation of the blank and the slope of the corresponding calibration curve. Robustness was investigated by introducing small deliberate changes in the experimental conditions, whereas ruggedness was assessed by comparing results obtained using different analysts and instruments [10].

2.6 Analysis of Real Samples

The developed and validated analytical method was further applied to different real samples, including natural water, industrial effluent, a commercial multi-mineral pharmaceutical formulation and a standard alloy sample.

Water and effluent samples were initially passed through a 0.45 µm membrane filter and subsequently acidified and diluted as necessary before analysis. The pharmaceutical formulation was subjected to acid digestion to decompose the organic matrix and release the metal ions into solution. The alloy sample was dissolved using a suitable acid mixture prior to analysis. Appropriate masking agents were incorporated whenever potential interfering ions were present in the sample matrix.

The analytical results obtained by the proposed method were compared with certified values, labelled concentrations or values obtained using established reference methods. Statistical evaluation of the results was performed using Student's *t*-test to assess possible systematic bias and the variance-ratio *F*-test to compare the precision of the results [12].

RESULT DISCUSSION:

Table Effect of temperature on the absorbance of the Cu(II)–hydroxytriazene chelate.

Temperature (°C)	Absorbance	Observation
10	0.700	Slow kinetics
20	0.800	Good
25	0.860	Optimum
30	0.862	Optimum
40	0.855	Slight decrease
50	0.830	Decrease
60	0.780	Partial decomposition
70	0.700	Marked decomposition

Selection of Suitable Buffer System

Several buffer systems capable of maintaining the solution near pH 6.5 were compared with respect to the intensity and reproducibility of the colour, as summarised in Table.

Table Comparison of buffer systems at pH 6.5 for the Cu(II)–hydroxytriazene chelate.

Buffer system	Absorbance	RSD (%)	Suitability
Acetate–acetic acid	0.862	0.6	Excellent
Phosphate	0.848	1.1	Good
Ammonia–ammonium chloride	0.790	2.4	Fair (masking)
Hexamine	0.855	0.9	Good
Borate	0.760	3.0	Poor

The acetate buffer gave the highest and most reproducible absorbance with the lowest relative standard deviation, and did not itself compete with the reagent for the metal ion. It was therefore selected as the buffering medium for all subsequent experiments. Ammoniacal buffers, although capable of maintaining the desired pH, tended to lower the absorbance through partial formation of metal–ammine species, which compete with the chelation equilibrium.

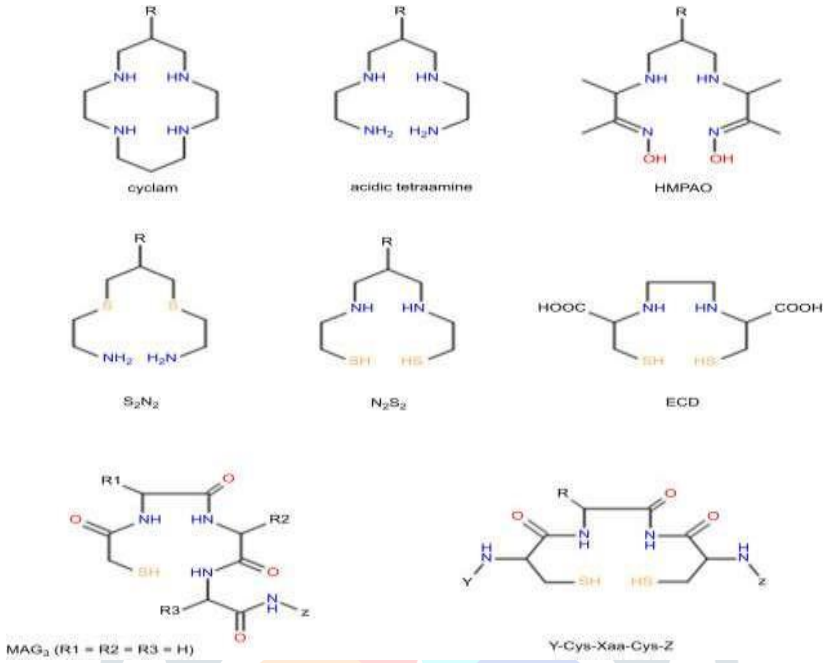
The choice of buffer is not a trivial matter in chelometric spectrophotometry, because the buffer anion may itself act as a ligand and enter into competing equilibria with the metal ion. The acetate ion is a weak, monodentate ligand that forms only feeble complexes with the metals studied, so that it does not appreciably divert the metal from the chelation reaction; at the same time it provides excellent buffering capacity at the working pH of 6.5, close to the pKa of acetic acid. The phosphate and borate buffers, by contrast, form appreciably stronger complexes with the transition-metal ions and consequently depressed the absorbance, while the ammoniacal buffer suffered from the additional drawback of ammine formation noted above. Hexamine performed almost as well as acetate but offered no compensating advantage. On the combined criteria of maximum absorbance, minimum relative standard deviation and freedom from competing complexation, the acetate–acetic acid system was the clear choice.

Determination of Maximum Absorption Wavelength (λmax)

The wavelengths of maximum absorption for the reagent and its chelates are listed in Table In every case a large bathochromic shift accompanied chelation, confirming coordination of the metal to the reagent Table 4.6 Absorption maxima of the hydroxytriazene reagent and its metal chelates.

Species	λmax (nm)	Colour	Bathochromic shift (nm)
Free reagent (HT)	365	Pale yellow	–
Cu(II)–HT	545	Green	180
Ni(II)–HT	470	Yellow-brown	105
Co(II)–HT	590	Reddish	225

This means that the complexation induces a significant red-shift of 100-225nm, which can be attributed to the extension of the conjugated system on the chelate ring closure, and also to the participation of the d-orbitals of the metal in charge transfer transitions. These different peaks correspond to the analytical wavelengths that will be used to separate mixtures. The size of the bathochromic shift is also a diagnostic indicator of the strength of the metal–ligand bond and in this way, the larger the shift, the stronger the bond and more covalent it is. On this basis the cobalt chelate, which undergoes the greatest shift, would be expected to be the most covalently bonded and the nickel chelate the least – a trend that is generally reflected in the stability constants. From the analyst's point of view it is fortunate that the three maxima are so well separated across the visible spectrum, since it is this separation that allows the separation of the three and the simultaneous determination to be possible. If the maxima were in phase, however, no mathematical manipulation could have been used to separate out the individual contributions and the reagent would have been of no value for analysis of multicomponent systems even though it was highly sensitive.



Scheme 4.2 Chelation of a divalent metal ion by two hydroxytriazene ligands giving a neutral 1:2 (O,N)-bonded chelate.

Sandell's Sensitivity

Sandell's sensitivity, S, defined as the mass of analyte (in µg) that gives an absorbance of 0.001 over a 1 cm path, was computed from the molar absorptivity using

$$S = M / \varepsilon$$

where M is the relative atomic mass of the metal. The results are presented in Table 4.8; the smaller the value of S, the more sensitive the method.

Table 4.8 Sandell's sensitivity of the metal–hydroxytriazene chelates.

Chelate	Atomic mass (g mol^{-1})	ε (L mol^{-1} cm^{-1})	Sandell's sensitivity (µg cm^{-2})
Cu(II)–HT	63.55	1.86×10^4	0.0034
Ni(II)–HT	58.69	1.42×10^4	0.0041
Co(II)–HT	58.93	1.65×10^4	0.0036

The low Sandell sensitivity values, all below 0.005 µg cm^{-2}, confirm that trace levels of the metals can be estimated with the proposed reagent. Sandell's sensitivity provides a convenient, instrument-independent measure of the intrinsic sensitivity of a colour reaction, expressed in terms of the mass of analyte per unit area of the light path rather than in terms of concentration. Because it is inversely proportional to the molar absorptivity, it decreases as the colour reaction becomes more intense; a small value therefore denotes a highly sensitive reaction. The values obtained here, all in the range 0.003–0.005 µg cm^{-2}, are comparable with or better than those of many reagents in routine analytical use and confirm that the hydroxytriazene method is well suited to the determination of the metals at the microgram-per-millilitre level and below. Together with the molar absorptivity and the detection limit, Sandell's sensitivity completes the characterisation of the fundamental analytical performance of the reagent. The maximum and constant absorbance obtained in the temperature range 25-35 °C, below which the reaction rate of chelation was

slow, and above 40 °C the coloured product formed decreased progressively, possibly due to the decomposition of the chelate complex and partial oxidation of the reagent. Therefore, measurements have been done at room temperature (25 ± 2 °C).

This temperature dependence is characteristic of the well-known competition between kinetics and thermodynamics. At low temperatures the chelation rate is slow, and during the set standing time the reaction is not completed, and the absorbance measured is less than the equilibrium absorbance. As the temperature is raised the reaction rate increases and the equilibrium is reached within the standing period, giving the observed plateau. At the higher temperatures, however, beyond approximately 40 °C, the stability constant of the chelate decreases (as is the general rule in exothermic complexation) and the increased thermal motion of the reacting species favours dissociation of the chelate and possible side reactions such as the oxidation of reagent by dissolved O2; the result is a decrease in absorbance. Thus the optimum operating condition is room temperature as it is high enough to allow the chelation to take place fairly rapidly but low enough to keep the coloured species intact. A further practical advantage of the method is the work that can be done at ambient temperature without the need for thermostatts).

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