



VALIDATED UV-VISIBLE SPECTROPHOTOMETRIC DETERMINATION OF COPPER(II), NICKEL(II) AND COBALT(II) USING A HYDROXYTRIAZENE REAGENT

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ABSTRACT

A sensitive, accurate and validated UV-Visible spectrophotometric method was developed for the determination of Cu(II), Ni(II) and Co(II) using a hydroxytriazene chromogenic reagent. The analytical procedure was optimized by a classical univariate approach, in which the effects of individual experimental variables were systematically investigated. The metal ions reacted with the hydroxytriazene reagent under controlled conditions in acetate-acetic acid buffer at pH 6.5, producing coloured metal-ligand complexes suitable for spectrophotometric measurement. The optimum reaction conditions included 3.0 mL of 0.1% hydroxytriazene reagent and a standing time of 15 min. The proposed method exhibited good linearity over the concentration ranges of 0.2–1.6 $\mu\text{g mL}^{-1}$ for Cu(II) and Ni(II), and 0.3–1.8 $\mu\text{g mL}^{-1}$ for Co(II), with correlation coefficients of 0.9998, 0.9997 and 0.9995, respectively. The molar absorptivity values were found to be 1.86×10^4 , 1.42×10^4 and 1.65×10^4 $\text{L mol}^{-1} \text{cm}^{-1}$ for Cu(II), Ni(II) and Co(II), respectively. The limits of detection were 0.018, 0.024 and 0.021 $\mu\text{g mL}^{-1}$, while the corresponding limits of quantification were 0.054, 0.072 and 0.063 $\mu\text{g mL}^{-1}$. Accuracy studies by the standard-addition method gave recoveries of 99.6–100.5% with relative standard deviations below 1%. Intra-day and inter-day precision studies showed RSD values below 1.5%. The method also demonstrated satisfactory robustness against small deliberate variations in pH, reagent volume, wavelength and standing time, while ruggedness studies showed good agreement between different analysts and instruments. The method was successfully applied to the determination of Cu(II), Ni(II) and Co(II) in water, industrial effluent, pharmaceutical and alloy samples. The proposed hydroxytriazene-based spectrophotometric procedure provides a simple, sensitive, precise and reliable analytical approach for the determination of these metal ions using conventional UV-Visible spectrophotometric instrumentation.

KEYWORDS: Hydroxytriazene; Cu(II); Ni(II); Co(II); UV-Visible spectrophotometry; Metal-ligand complex; Simultaneous determination; Method validation; Trace metal analysis.

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. Chlorophenyl 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 triacin biosynthesis, and the formation of the unusual pharmacophore N-hydroxytriazene. On the basis of the previous identification of the triacin 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 triacin production to the experimental delineation of the enzymatic machinery that gives rise to the unique N-hydroxytriazene pharmacophore.^[5]

The researcher 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 diaryltriazenes skeleton as a way to generate biologically active substances. On the general field of hydroxytriazenes 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 hydroxytriazenes derivatives. This research is an important component of the analytical development of hydroxytriazenes 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–hydroxytriazenes 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 hydroxytriazenes 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]

2. 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 Hydroxytriazenes Reagent

The hydroxytriazenes 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 hydroxytriazenes 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 hydroxytriazenes 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 (λ_{max}) 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 λ_{max} , 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

Method Validation

The proposed method was validated in accordance with

the principles laid down in the ICH guidelines, evaluating linearity, accuracy, precision, detection and quantification limits, robustness and ruggedness.

Linearity and Working Range

The analytical performance characteristics derived from the calibration data are compiled in

Analytical and regression characteristics of the proposed method.

Parameter	Cu(II)	Ni(II)	Co(II)
Beer's law range ($\mu\text{g mL}^{-1}$)	0.2–1.6	0.2–1.6	0.3–1.8
Molar absorptivity ($\text{L mol}^{-1} \text{cm}^{-1}$)	1.86×10^4	1.42×10^4	1.65×10^4
Slope	0.543	0.417	0.489
Intercept	0.006	0.004	0.008
Correlation coefficient (r)	0.9998	0.9997	0.9995
Sandell's sensitivity ($\mu\text{g cm}^{-2}$)	0.0034	0.0041	0.0036

Accuracy (Recovery Studies)

Accuracy was assessed by the standard-addition technique, in which known amounts of metal were

added to previously analysed samples and the total recovered amount determined. The results are shown in Table 4.13.

Table Recovery study for the determination of Cu(II) by the standard-addition method.

Amount present ($\mu\text{g mL}^{-1}$)	Amount Added	Total found	Recovery (%)	RSD (%)
0.40	0.20	0.598	99.7	0.8
0.40	0.40	0.803	100.4	0.6
0.40	0.60	0.996	99.6	0.9
0.60	0.40	1.005	100.5	0.7
0.80	0.40	1.198	99.8	0.5

Recoveries in the range 99.6–100.5% with relative standard deviations below 1% confirmed the excellent accuracy of the method and the absence of any systematic error. The standard-addition technique is particularly appropriate for assessing accuracy in the presence of a sample matrix, because it evaluates the recovery of analyte added to the actual sample rather than to a pure standard, and thereby detects proportional matrix effects that a simple external calibration might miss. The fact that the recovery of the added analyte was essentially quantitative at every level, and independent of the amount already present, shows that the matrix did

not suppress or enhance the colour reaction and that the calibration slope obtained from pure standards is applicable to the real samples. This is strong evidence for the freedom of the method from multiplicative interference and underpins the reliability of the real-sample results presented in Section 4.8.

Precision (Intra-day and Inter-day)

Precision was evaluated by analysing replicate samples within a single day (repeatability) and on five successive days (intermediate precision), as summarised in Table 4.14.

Table Intra-day and inter-day precision of the proposed method (n = 6).

Metal	Concentration ($\mu\text{g mL}^{-1}$)	Intra-day RSD (%)	Inter-day RSD (%)
Cu(II)	0.80	0.62	1.05
Ni(II)	0.80	0.71	1.18
Co(II)	0.80	0.84	1.32

Relative standard deviations below 1.5% in every case demonstrated the good repeatability and intermediate precision of the method. The distinction between intra-day and inter-day precision is important in method validation. The intra-day, or repeatability, figure reflects the scatter arising from the manipulative steps of a single analyst working with a single instrument and set of reagents within a short period, and is generally the smaller of the two. The inter-day, or intermediate-precision, figure additionally incorporates the day-to-day variability of reagent solutions, ambient conditions and instrument calibration, and is therefore expected to be somewhat larger, as indeed observed. That both figures remained below 1.5% indicates that the

method is not unduly sensitive to these sources of variation and that results obtained on different days can be meaningfully compared. The good precision is a direct consequence of the stability of the colour, the sharpness of the plateaux in the optimisation curves, and the freedom of the method from critical timing or temperature control.

Limit of Detection (LOD)

The limit of detection was calculated from the standard deviation of the blank (σ) and the slope of the calibration line (m).

Metal	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)
Cu(II)	0.018	0.054
Ni(II)	0.024	0.072
Co(II)	0.021	0.063

Low LOD and LOQ values indicate that the method is able to determine the metals at trace level that is well below the lower range of Beer's law range. The limit of detection (LOD) is the lowest concentration that can be reliably distinguished from the blank with a signal of 3.3 standard deviations of the blank, and the limit of quantification (LOQ) is the lowest concentration that can be measured with acceptable precision with a signal of 10 standard deviations of the blank. the traditional ratio of false-positive and false-negative detection. Both limits

are inversely related to the slope of the calibration line, which also is directly related to the molar absorptivity, and the high molar absorptivities of the hydroxytriazene chelates are all directly related to low limits of detection and limits of quantification. Actually, the method is able to detect the metals at concentrations one order of magnitude lower than the lower limit of the calibrated range and more than sufficient for the trace-analysis applications, which were considered.

Robustness

The robustness of the method was tested by deliberately introducing small variations in pH, reagent volume and wavelength, and observing their effect on the recovery, as shown in Table.

Table Robustness of the method under small deliberate variations of conditions.

Parameter varied	Nominal	Altered	Recovery (%)	RSD (%)
pH	6.5	6.3 / 6.7	99.6 / 100.2	0.7
Reagent volume (mL)	3.0	2.8 / 3.2	99.8 / 100.1	0.6
Wavelength (nm)	545	543 / 547	100.0 / 99.7	0.5
Standing time (min)	15	12 / 18	99.9 / 100.0	0.4

The recovery remained within $\pm 1\%$ of the nominal value under all the small variations examined, establishing that the method is robust and tolerant of minor changes in operating conditions. Robustness is a measure of the capacity of an analytical procedure to remain unaffected by small, deliberate variations in method parameters, and provides an indication of its reliability during normal use. The insensitivity of the result to small changes in pH, reagent volume, wavelength and standing time is a direct consequence of the design decisions taken during optimisation, namely the choice of a working pH on the plateau of the pH-absorbance curve, the use of an excess of reagent beyond the plateau of the reagent-concentration curve, the measurement at the flat maximum of the absorption band, and the exploitation of the wide window of colour stability. Each of these choices was made specifically so that the operating point would lie in a region where the absorbance is stationary with respect to the parameter concerned, and the robustness data confirm that this objective was achieved.

Ruggedness

Ruggedness was investigated by performing the determination with two analysts on two instruments on different days. Means results were in good agreement ($\text{RSD} < 1.4\%$) showing that the method is rugged, and transferable between operator and instrument. While robustness refers to the impact of deliberate method parameter changes, ruggedness refers to the ability of the method to give the same result when various operators, instruments, reagent batches and days run the method in routine operating conditions. A robust and rugged method can be communicated with confidence from one laboratory to another and can be expected to provide comparable results—a necessity if the method is to be used routinely. The lack of any obvious operator or

instrument bias in the present data shows that this method does not require unusual skill on the part of the analyst, or particularly sensitive instruments, or exacting demands for the accuracy of the instrument, and is suitable for use with conventional laboratory equipment.

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