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RESEARCH ARTICLE

STUDIES ON THE STRUCTURAL CHARACTERISTICS OF VARIOUS COMPLEXES FORMED WITH AMINO ACID DERIVATIVES AND THE Cu (II) - NINHYDRIN REAGENT

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ABSTRACT

Cu(II) - ninhydrin reagent is used to screen a variety of small peptides and amino acid derivatives by developing color on paper. Amino acids produce a pink color and their carboxyl derivatives produce a yellow color with this reagent. All compounds which produce a yellow color have been classified as Cu(II) - ninhydrin positive compounds. These compounds include amino acid esters, amino acid amides and small peptides containing upto 5 amino acids. Even though we have proposed a minimum structural requirement for the production of a yellow chromophore with these compounds, we are interested in elucidating a structural model for these compounds. In this report, we have proposed a square planar structural model for these complexes.

INTRODUCTION

Following the discovery of ninhydrin by Siegfried Ruhemann in 1910, it rapidly became a practical analytical tool (Ruhemann, 1910). In 1954, it was used to be an important reagent to develop finger prints on porous surfaces. Since its use in forensic chemistry, many efforts have been focused on improving the reagent. Many of the short comings of ninhydrin have been met by the synthesis of a variety of ninhydrin analogs (Barren Hansen and Madaleine Jonllie, 2005). All amino acids and their carboxyl group derivatives like esters and amides, including small peptides produce a purple color with the classical ninhydrin reagent. This reagent was modified by us by adding cupric ion in order to distinguish qualitatively, the carboxyl group derivatives of amino acids from the amino acids on paper after chromatography (Ganapathy *et al.,* 1981). Amino acids produce a pink color and their carboxyl derivatives produce a yellow color with Cu(II) - ninhydrin reagent. We have used this method for the detection and purification of amino acid derivatives from different plant products (Nithya and Ramachandramurty, 2007, Ramachandramurty, 2002, Ramachandramurty, 2003, Ramachandramurty and Boopathy, 1994, Ramachandramurty and Satakopan, 2009). The Cu(II) - ninhydrin method discussed here is a novel one because no other methods presently used can form two different coloured products with a single developing reagent. It is also proved to be a general procedure to distinguish amino acids and their carboxyl group derivatives like amino acid esters, amino acid amides and also small peptides containing upto 5 amino acid residues except for the derivatives of L-Proline, γ-L-Glutamic acid and L-Tryptophan. Taking into account the structures of the compounds employed in this study, a minimal structure that would go to form the yellow chromophore with Cu(II) - ninhydrin reagent has been proposed (Ganapathy *et al.,* 1981). In this paper, we propose a detailed structural model for the Cu(II) - ninhydrin positive compounds after analyzing all the experimental and spectral characteristics of these compounds.

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MATERIALS AND METHODS

Ninhydrin was acquired from Pierce (Rockford, IL, USA). Cupric nitrate was of BDH, Analytical grade (Mumbai, India). Organic solvents and acids used were of highest purity available. Aluminium foil coated readymade TLC sheets were obtained from Anchrom, Mumbai. Whatman no.1 filter paper discs were obtained from Whatman International Limited, Maidstone, England. Spectral studies were carried out at the Central research laboratory of PSG College of Arts and Science, Coimbatore, NMR research centre at Indian Institute of Science, Bangalore and Chemistry laboratory at South Indian Textiles Research Association, Coimbatore.

Preparation of Cu(II) - ninhydrin reagent

The Cu(II)-ninhydrin reagent was prepared by dissolving cupric nitrate (25mmol/l) and ninhydrin (1% w/v) in a minimum quantity of a mixture of water and glacial acetic acid (3:1 v/v) and diluted with required amount of acetone.

Preparation of metal solution

Metal salt was dissolved in a mixture of water (3ml) and glacial acetic acid (1ml) and the solution was made upto 30ml

with acetone, giving a final metal salt concentration of 25mmol/l.

Preparation of the ninhydrin reagent

A 0.5% w/v ninhydrin solution in aqueous acetone (95%) was employed.

Preparation of amino acid esters

The amino acid esters were prepared by passing dry HCl gas for 5 minutes into any one alcohol (ethylene glycol/ ethanol/ methanol) solution containing 50mM amino acid. A special type of glass apparatus has been designed for this purpose.

Preparation of amino acid amides

Amino acid amides were prepared by following the method described by Vishnoi (Vishnoi, 2000). About 1g of amino acid was mixed with 4gm of PCl_5 in a dry porcelain dish. The mixture was ground well with the help of a pestle, in a fume cupboard, till it is liquefied. The liquid so obtained is the crude amino acid chloride. To half of the crude amino acid chloride, 10ml of concentrated ammonium hydroxide was added. A vigorous reaction takes place. When the reaction was over, the mixture was stirred, cooled and filtered. The amide derivative thus formed was recrystallised from alcohol and dried.

Development of chromatograms

Descending paper chromatograms (Whatmann No.1 Paper chromatography grade, 20h run) of peptides, amino acid esters and amino acid amides were developed with the solvent system, isopropanol-water (4:1). The chromatograms were then dried in air at room temperature for a minimum of 3 hrs. Thin layer chromatograms of peptides, amino acid esters and amino acid amides were developed using Butanol: glacial acetic acid: water (8:1:1 v/v/v) solvent system. After the run, the plate was air dried for about 2 hrs.

Spraying methods

A) Metal ninhydrin

The dried chromatograms were uniformly sprayed with the metal – ninhydrin solution, then air dried and heated at 65°C for 30minutes.

B) Ninhydrin followed by metal

The dried chromatograms were uniformly sprayed with ninhydrin solution, air dried for 10 minutes and heated at 65°C for 30minutes. The paper was again sprayed with the metal salt solution and finally air dried.

C) Metal followed by ninhydrin

The chromatograms were uniformly sprayed with the metal salt solution and air dried for 30 minutes. The paper was again sprayed with ninhydrin solution, air dried and heated at 65°C for 30minutes.

Preparation of glycyl glycine Cu(II) - complex

Glycyl glycine Cu(II) - complex was prepared according to the method of Manyak *et al.* A solution consisting of 16 g. of the glycylglycine hydrochloride dissolved in 400 ml. of water was passed through a column containing excess of hydroxy (alkaline) form of Amberlite IR4B anion-exchange resin. The colorless aqueous eluate was concentrated to one-fifth of its volume under reduced pressure, and the product was crystallized out by the addition of ethanol to the warm solution. A 70% yield of colorless crystals of glycylglycine, with melting point 220° C, was obtained.

2.4 g. of $Cu(N0_3)_2 .3H_20$ was dissolved in 100ml of water and converted to the hydroxide by treatment with an excess of sodium hydroxide solution. The precipitated hydroxide was washed by decantation, and was then treated in aqueous suspension with 1.32 g. of glycylglycine. The excess cupric hydroxide was filtered off, the resulting blue copper(II) chelate was crystallized out by the addition of ethanol (Manyak *et al*., 1990).

Preparation of glycyl glycine Cu(II) - ninhydrin complex

An aliquot of an aqueous solution of glycyl glycine Cu(II) - complex (0.01µmole) was spotted on chromatographic paper and then sprayed with 0.5% ninhydrin in aqueous acetone (95%). The paper was heated for 30 min at 65°C. A yellow chromophore was obtained, which was then eluted in ethanol and used for spectral studies.

Spectroscopic studies

FT – IR Spectra

The glycyl glycine Cu(II) - ninhydrin complex was subjected to FT-IR analysis using a Shimadzu model FTIR – 8300 infrared spectrometer. IR spectra were collected between 500 and 4,000 wave numbers (per centimeter).

NMR studies

The glycyl glycine Cu(II) - ninhydrin complex in deuteriated acetone as a solvent was subjected to ^{1}H and ^{13}C NMR analysis using a Bruker 500 MHz liquid state NMR spectrometer.

GC-MS analysis

The glycyl glycine Cu(II) - ninhydrin - complex dissolved in ethanol was analysed using the GC/Mass instrument: Trace ultra ver: 5.0 produced by Thermo. The separation conditions were: DB-5 Column 30m X 0.25 mm X 0.25 µm, Mobile phase helium at flow rate1.0 ml/min, Injection chamber temperature 220° C, Oven temperature starts at 80 °C raised to 250°C at a rate of 8°C per minute. The ionization mode of the mass detector was at 70 ev.

RESULTS AND DISCUSSION

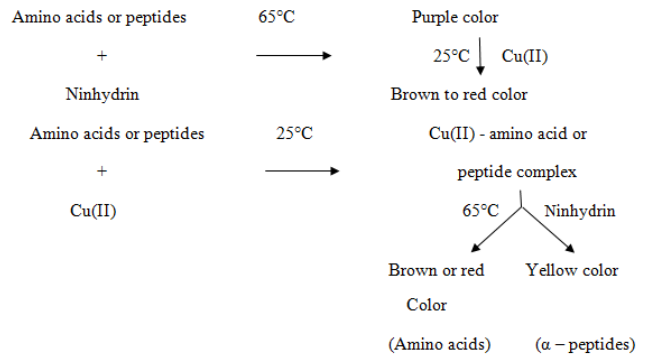
In our earlier experiments with Cu(II) - ninhydrin reagent, we have screened a variety of small peptides and amino acid derivatives by developing color on paper. All compounds which produce a yellow color have been classified as Cu(II) - ninhydrin positive compounds. These compounds include amino acid esters, amino acid amides and small peptides containing upto 5 amino acid residues (Ganapathy *et al.,*

1981). Even though we have proposed a minimum structural requirement for the production of a yellow chromophore with these compounds, we are interested in elucidating a structural model for these compounds. We have finally arrived at a square planar structural model for these complexes. The important evidence that supports this model is the following:

Order of addition of reagents

When a paper containing amino acid and peptide spots was first sprayed with ninhydrin and then with cupric salt solution, both amino acids and peptides gave colors ranging from brown to pink and it was not possible to distinguish small α - peptides from amino acids from the colors of the spots obtained. However, when the paper was first sprayed with cupric salt solution and then with ninhydrin reagent, only amino acids gave colors ranging from brown to pink but all the peptides gave a yellow color. The same result was obtained when the paper containing the spots was sprayed with the combined Cu(II) - ninhydrin reagent. This experiment clearly shows that the final products of the reactions between a peptide – ninhydrin complex and Cu(II) and between a peptide –Cu(II) complex and ninhydrin are not identical. Small peptides are known to form deep blue complexes with Cu(II) (Freeman, 1967). However, in the present study, the peptide spots on paper after spraying with cupric salt solution were either colorless or light blue since the quantity of peptides used was very low (0.01 µmole).

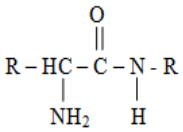
The results of this experiment can be summarized as follows:



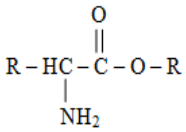
Thus, it is clear that, when the combined Cu(II) - ninhydrin is used, Cu(II) reacts rapidly at room temperature with the peptide to yield a complex which then reacts with ninhydrin at 65°C to give a yellow color. The complex of Gly-Gly with Cu(II) was prepared and crystallized according to the procedure of Manyak *et al* (Manyak *et al*., 1990).. An aliquot of an aqueous solution of this complex (0.01µmole) was spotted on chromatographic paper and then sprayed with 0.5% ninhydrin in aqueous acetone (95%). The paper was heated for 30 min at 65°C. A yellow chromophore with a single λ_{max} at 390nm was obtained. This experiment clearly shows that the yellow chromophore is the product of the reaction between the Cu(II) complex of Gly-Gly and ninhydrin (Ganapathy *et al.,* 1981). Ninhydrin reagent has long been used for qualitative and quantitative analysis of amino acids. However, amino acids cannot be qualitatively distinguished from small peptides using this reagent since both amino acids and peptides give a purple color with ninhydrin on paper. A Cadmium(II) - ninhydrin reagent was recommended by Atfield and Morris (At field and Morris, 1961) for the detection of amino acids and peptides on paper after electrophoresis. This reagent was later successfully employed by Ganapathy and Radhakrishnan (Ganapathy and Radhakrishnan, 1977) to quantitate dipeptides having N-terminal glycine. Most of the amino acids gave a red color with this reagent, while dipeptides containing N – terminal glycine gave a yellow color. However, the present study shows that peptides having N – terminal residues other than glycine give a red color with Cd(II) ninhydrin reagent. Therefore, Cd(II) - ninhydrin reagent cannot be employed as a general reagent to differentiate small peptides from amino acids.

Minimum structural requirements

From the structure of the compounds employed in this study which are substituted at the N- and C- terminus and α- or other peptide bond, it emerges that the minimum structural requirement around the α- peptide linkage to give a yellow chromaphore with the Cu(II) - ninhydrin reagent is deduced to be as follows:



In the case of amino acid amides and small peptides



In the case of amino acid esters

The complex formation

Based on the knowledge gained in our experiments and from the literature (Koteswara Rao and Ram Reddy, 1990), we would like to propose the following structural formulae for the formation of various complexes with Cu(II) – ninhydrin reagent with small peptides, amino acid amides and amino acid esters. The atomic number of copper is 29 and its electron configuration can be written as $1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^6\ 3d^{10}\ 4s^1$. (see Table 1)From the electron configuration of Cu(II) ion (Table 1), it is evident that Cu(II) can form four co-ordinate covalent bonds derived from the lone electron pairs. The possible complexes formed may be through either sp^3 hybridization or dsp^2 hybridization. In the former case, a molecule with tetrahedral shape, and in the later case, a molecule with a square planar configuration is formed. X-ray analysis of the Cu(II) complexes have proved beyond doubt that the complexes formed are square planar and the hybridization is dsp^2 (Koteswara Rao and Ram Reddy, 1990). Therefore, square planar co-ordination complexes are the most probable structures formed in the case of these complexes also. We propose the formation of the following types of complexes. The complex formed with the amino acid amide / small peptide, Cu(II) and ninhydrin is shown in Figure 1. This complex is yellow in color. The complex formed with the amino acid ester, Cu(II) and ninhydrin is shown in Figure 2. This complex is also yellow in color. (see Figure 1and 2).

In these structures, two molecules of ninhydrin and two molecules of amino acid derivatives join to form a square planar co-ordination complex with the central Cu(II) ion. Both these complexes are yellow in color. In the absence of substitutions on the carboxyl oxygen atom, that is if it is a free –COOH group, as in the case of amino acids, it probably gets

Table 1 : Valence shell electron configuration of Cu(II) and formation of its coordination complexes. Arrows represent the original electrons of copper and dots represent the electron pairs donated to copper atom

Atom / ion / complex	Electron configuration in the outer most shells	Oxidation state of the metal	Type of hybridization	Geometry (shape)	No. of unpaired electrons	Magnetic nature
Cu (At no.29)	3d: ↑↓ ↑↓ ↑↓ ↑↓ ↑↓ ; 4s: ↑ ; 4p:				1	Paramagnetic
Cu^{2+}	3d: ↑↓ ↑↓ ↑↓ ↑↓ ↑ ; 4s, 4p: : : : : (sp^3)	+2	sp^3	Tetrahedral	1	Paramagnetic
Cu^{2+}	3d: ↑↓ ↑↓ ↑↓ ↑↓ : ; 4s, 4p: : : : : ↑ (dsp^2)	+2	dsp^2	Square planar	1	Paramagnetic
	One electron is shifted from 3d to 4p-orbital					

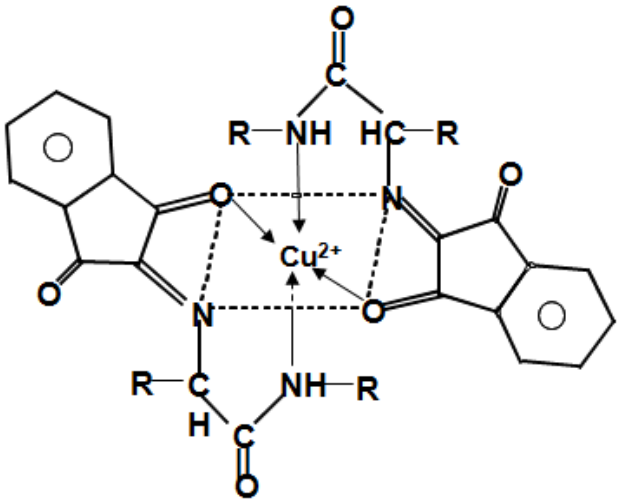


Figure 1 : The proposed structure of the Cu(II) – Schiff's base complex derived from ninhydrin and a small peptide or amide

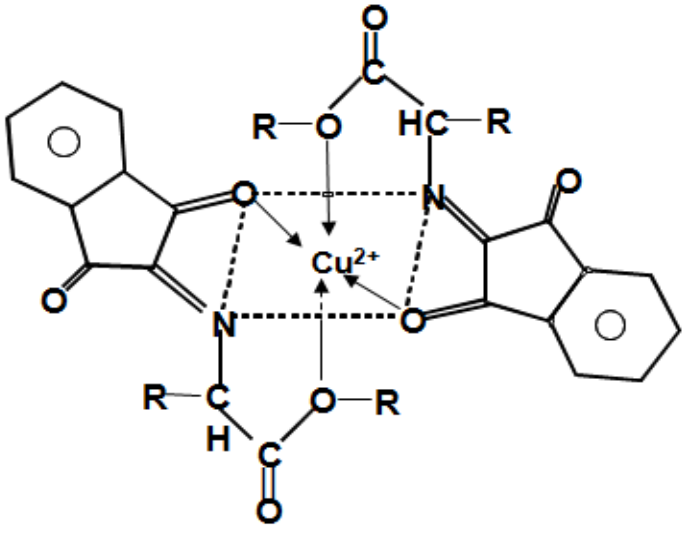
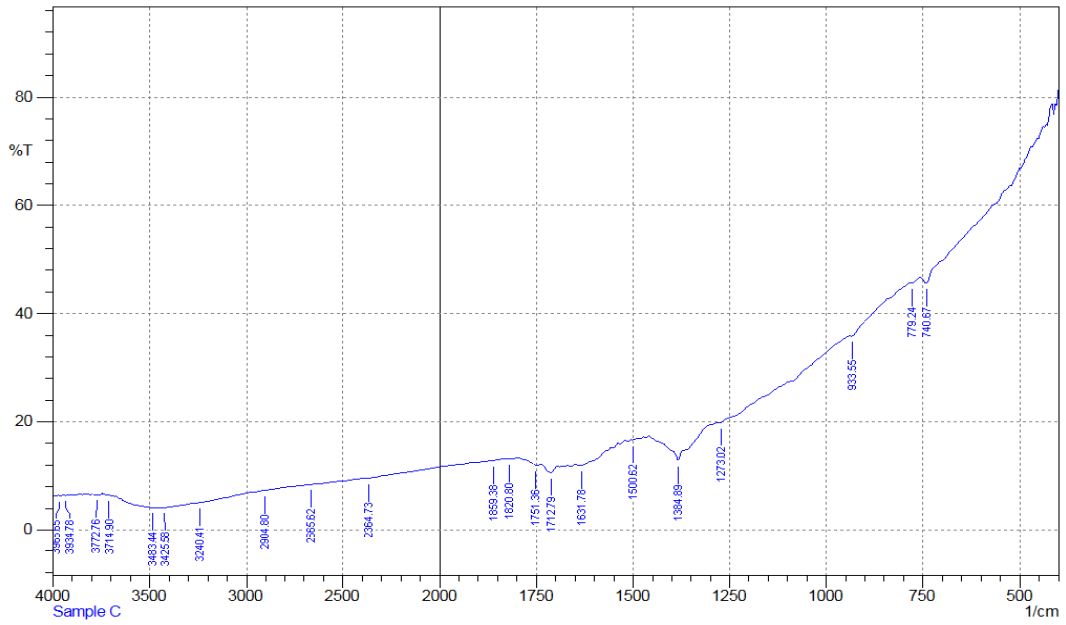


Figure 2 : The proposed structure of the Cu(II) – Schiff's base complex derived from ninhydrin and an amino acid ester

decarboxylated by the powerful oxidative effect of ninhydrin and the complex formed is of Ruhemann purple type.

Spectral studies

The following spectral studies were performed with glycyl glycine Cu(II) - ninhydrin complex in support of the proposed structures:



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	740.67	45.637	2.513	756.1	420.48	77.083	4.224
2	779.24	45.662	0.166	783.1	759.95	7.78	0.026
3	933.55	35.801	0.553	941.26	783.1	61.162	0.086
4	1273.02	19.783	0.185	1276.88	941.26	190.924	0.137
5	1384.89	12.804	5.443	1454.33	1284.59	133.656	9.129
6	1500.62	16.625	0.162	1504.48	1489.05	11.956	0.034
7	1631.78	11.964	0.239	1635.64	1558.48	67.547	0.811
8	1712.79	10.583	1.356	1735.93	1697.36	36.694	1.109
9	1751.36	11.91	0.469	1793.8	1739.79	48.758	0.294
10	1820.8	13.177	0.053	1824.66	1797.66	23.711	0.024
11	1859.38	12.861	0.119	1867.09	1843.95	20.552	0.063
12	2364.73	9.498	0.215	2384.02	1867.09	496.149	3.523
13	2665.62	8.407	0.02	2669.48	2387.87	295.018	0.407
14	2904.8	7.309	0.018	2908.65	2669.48	263.682	0.01
15	3240.41	5.051	0.027	3244.27	2912.51	401.634	0.063
16	3425.58	4.137	0.094	3441.01	3244.27	264.125	0.931
17	3483.44	4.121	0.126	3649.32	3471.87	237.311	4.681
18	3714.9	6.426	0.078	3734.19	3711.04	27.519	0.097
19	3772.76	6.485	0.106	3780.48	3749.62	36.527	0.22
20	3934.78	6.377	0.05	3946.36	3927.07	23.022	0.031
21	3965.65	6.369	0.028	3973.36	3950.22	27.661	0.027

Figure 3 : FT IR spectrum of Glycyl glycine Cu(II) - ninhydrin complex

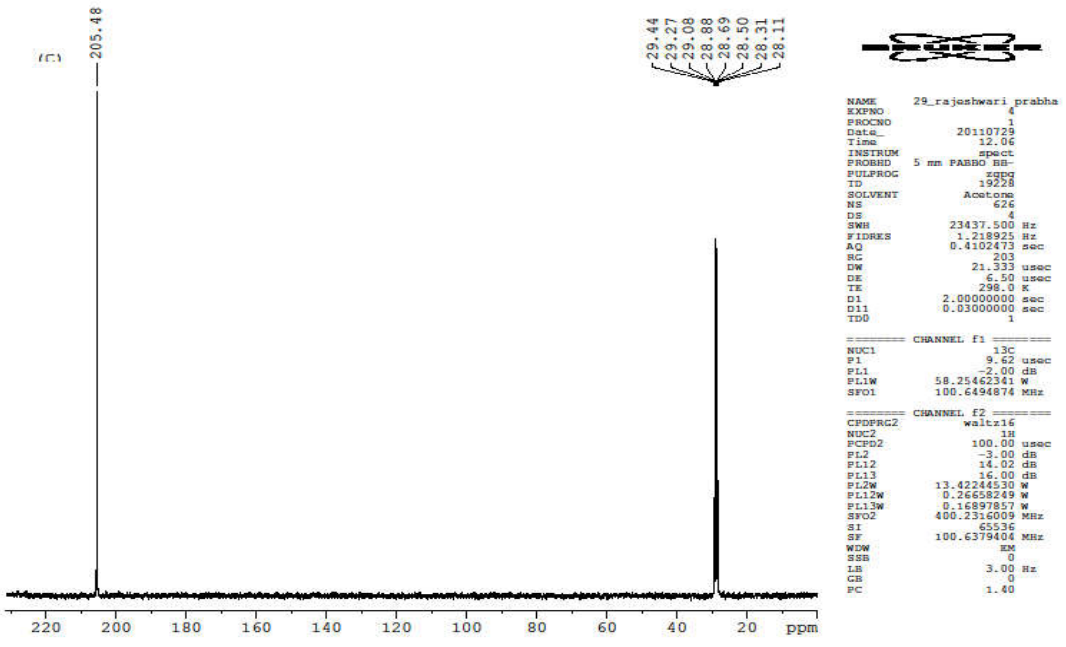


Figure 4 : ^{13}C NMR spectrum of Glycyl glycine Cu(II) - ninhydrin complex

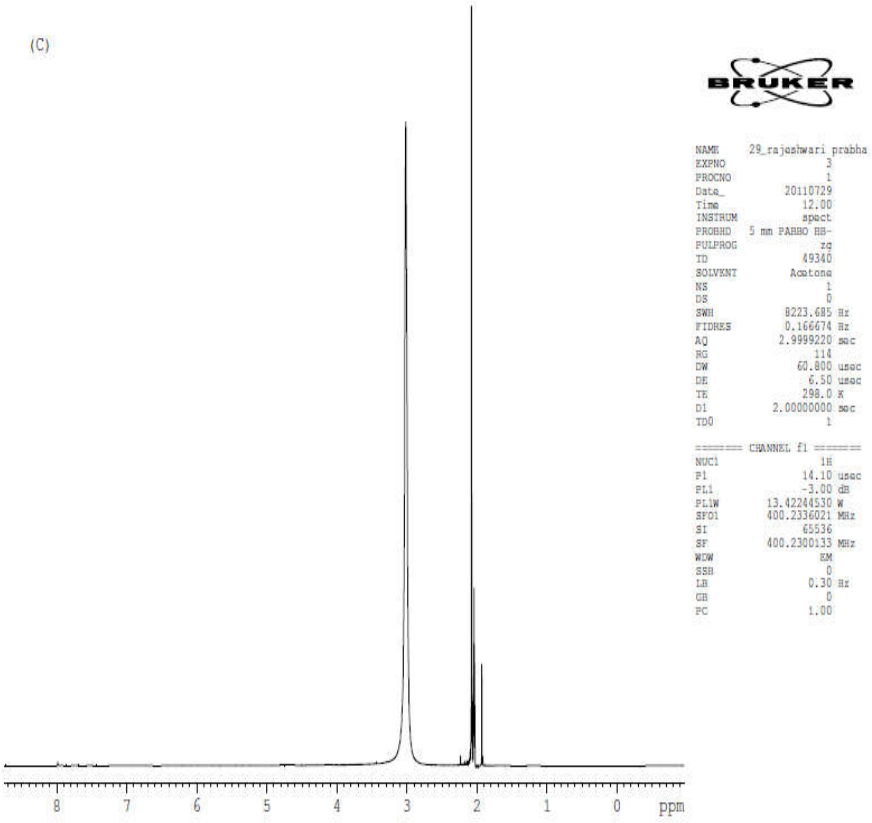


Figure 5 : ^{1}H NMR spectrum of Glycyl glycine Cu(II) - ninhydrin complex

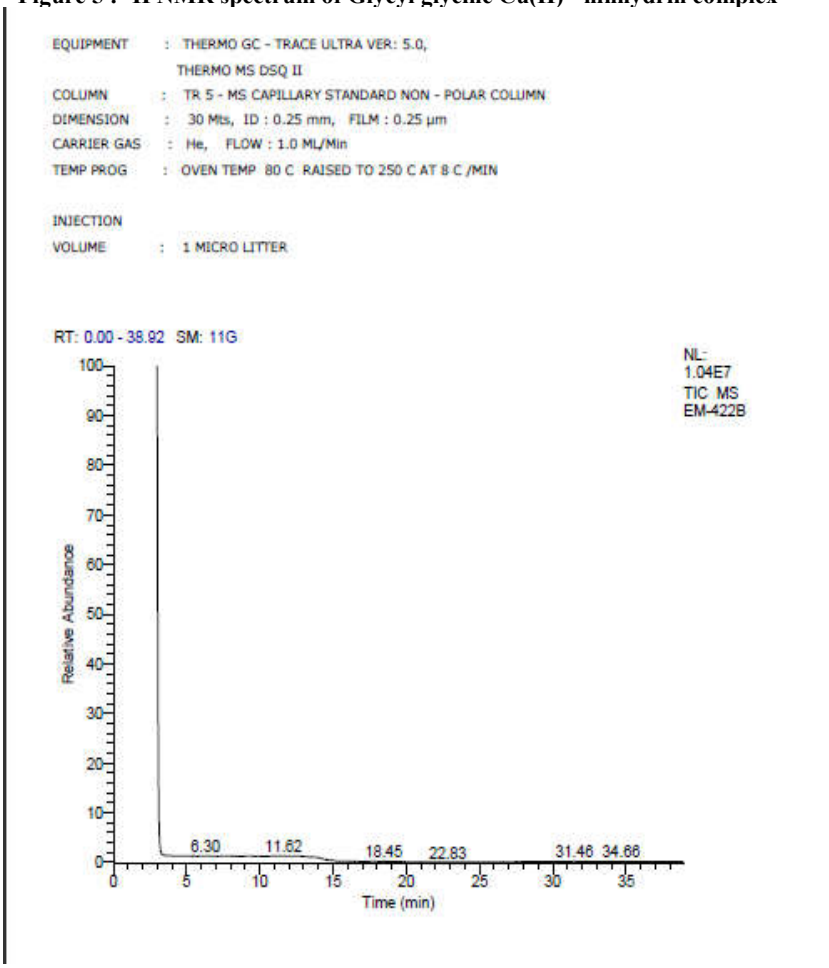


Figure 6 : GC MS spectrum of Glycyl glycine Cu(II) - ninhydrin complex

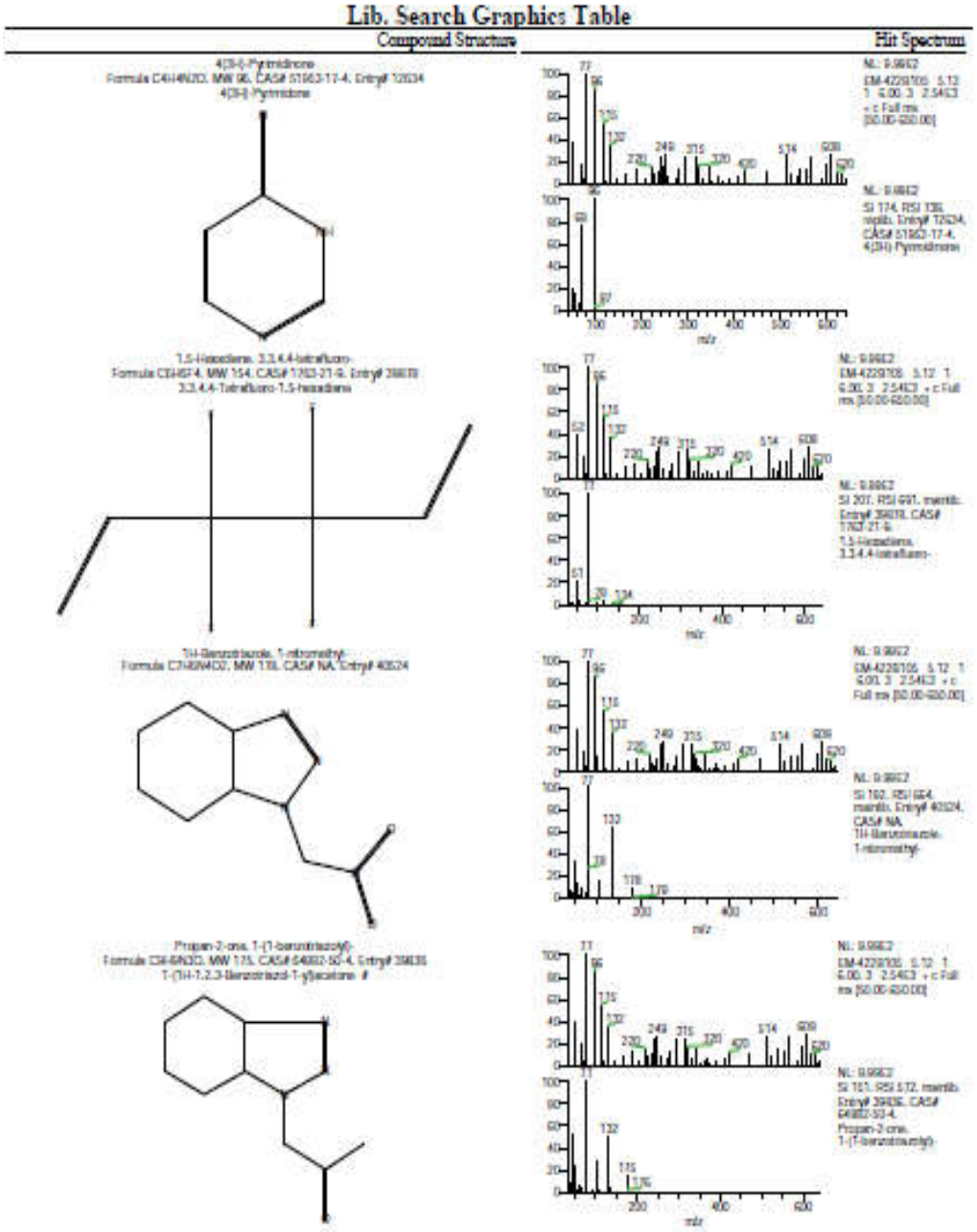


Figure 7 : Library search results of GC MS spectrum of Glycyl glycine Cu(II) - ninhydrin complex

FT-IR

The FT-IR spectrum of glycyl glycine Cu(II) - ninhydrin complex (figure 3) showed bands, indicating the presence of a peptide component at 3240cm^{-1} resulting from the N-H stretching mode, and at 1713 cm^{-1} resulting from the stretching mode of the CO-N bond, and at 1500 cm^{-1} resulting from the deformation mode of the N-H bond combined with the C-N stretching mode (Michail *et al.,* 1995) (see Figure 3).

NMR

The ^{13}C NMR and ^{1}H NMR spectra of glycyl glycine Cu(II) - ninhydrin complex are presented in Figure 4 and 5 respectively. The peaks obtained indicated the presence of peptide component. The presence of ninhydrin is confirmed by the chemical shift obtained around 2.0 in the ^{1}H NMR spectra (Martin *et al.,* 1995). (see Figure 4 and 5).

GC – MS

Gas chromatography–mass spectrometry (GC–MS) is a useful tool for quantitative and qualitative analysis of a wide range of relatively volatile compounds, and the technique has been widely applied in medical, biological, and food research (Kaluzna Czaplinska, 2007). From the data obtained (see Figure 6 & 7), it can be inferred that there is the presence of peptide component forming a complex with the ninhydrin .

From the spectral studies, it can be inferred that glycyl glycine is forming a complex with Cu(II) - ninhydrin reagent which is in supportive of the proposed structures. Metal peptide interactions have the ability to provide molecular insights into several processes like protein structure and function as models (Brandon *et al.,* 2004, Ramachandramurty and Satakopan, 2009). Our results showed that a yellow chromaphore is formed as a result of reaction between a peptide / amino acid derivative – Cu(II) complex and ninhydrin. The complexes formed have square planar configuration and dsp^2 hybridization. From the proposed structural formulae, it is clear that, for the production of a yellow chromaphore, substitution of a group on the amide or peptide nitrogen atom in the case of amino acid amides / small peptides and substitution of the oxygen atom in the case of amino acid esters is essential. Larger peptides cannot form these complexes because of the stearic hindrance during the co-ordination of N and O atoms with the metal ions.

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