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*Full Length Research Paper*

# Potentials of 3, $3^1$, $4^1$, 5, 7-pentahydroxyflavylium of *Hibiscus rosa-sinensis* L. (*Malvaceae*) flowers as ligand in the quantitative determination of Pb, Cd and Cr

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**3, $3^1$,$4^1$,5,7-Pentahydroxyflavylium chloride (cyanidin chloride) was extracted from the flowers of *Hibiscus rosa-sinensis* L. and blackberry fruits and the purity confirmed by co-chromatography with authentic marker on thin layer chromatography (TLC) and paper chromatography (PC). UV-Visible spectrophotometric determinations of the sample and its Pb (II), Cr (III) and Cd (II) complexes at various pH were carried out. The cyanidin sample absorbs at 530 and 540 nm in methanol and ethanol respectively. A Cyanidin-Pb (II) complex was formed at pH 4.2 ($\lambda_{max}$ = 555 nm, in methanol). A Cyanidin-Cr (III) complex was formed at pH 1.0 ($\lambda_{max}$ = 682 nm, methanol and ethanol) while a Cyanidin-Cd (II) complex was formed at pH 3.0 [$\lambda_{max}$ = 706 nm (methanol) and 679 nm (ethanol)]. All the metal-ligand complexes occur in the ratio of 1:2 and the molar absorptivities are 9.66 x $10^2$, 8.77 x $10^2$ and 9.77 x $10^2$ L.mol$^{-1}$.cm$^{-1}$ for Pb, Cr and Cd respectively. Under optimum conditions, the absorbance of the complexes was found to increase linearly with increase in metal-ion concentrations, which corroborated with the correlation coefficient values. The linear range of the calibration graph was 0.1 – 0.5 mM for all the complexes. The results show that cyanidin could be used as a reagent for the spectrophotometric determination of Cr (III), Pb (II) and Cd (II) and by varying the pH, these metals could be determined simultaneously in solution.**

**Key words:** Cyanidin chloride, *Hibiscus rosa-sinensis*, metal - ligand complex, Lead (II), chromium (III), Cadmium (II), spectrophotometric assay.

## INTRODUCTION

Lead, chromium and cadmium are among the heavy metals, which even in trace amounts constitute an important environmental pollutant and source of poisoning (Akubue, 1997). Over the years, several analytical methods have been devised for quantitative determination of lead, cadmium and chromium. Lead forms complexes with many organic reagents for example, diphenylthiocarbazone, quinaldic acid, hydroxyquinaldine acid, 8-hydroxyquinoline. These reagents have been used for its determination (Jeffery et al., 1989). Chromium and cadmium have also been similarly determined using anthranilic acid, diphenylthiocarbazone (Holmens, 1959). All these reagents are expensive and not easily accessible locally. There is therefore a need to source a cheap, simple and sensitive reagent for quantitative determination of these metals in our contemporary environment.

3, $3^1$,$4^1$,5,7-pentahydroxyflavylium is an anthocyanidin obtained from the extracts of many flowers and berries. Most of the interests on this compound have focused on the extraction and characterization (Harborne, 1958a, 1958b; Kodama et al., 1983). Studies have shown that addition of $AlCl_3$ to the alcoholic solution of the compound resulted in a spectral shift of its uv/visible spectrum. This shift was attributed to the formation of complex between Aluminum and the 3-$^1$, 4-$^1$, – ortho-hydroxyl systems in the anthocyanidin (Harborne, 1958b; Finar, 1980). Aluminum and tin had been determined in samples spectrophotometrically as their cyanidin complexes (Harborne, 1958b). This idea stimulated our interest to evaluate the

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use of locally sourced anthocyanidins for the quantitative determination of Pb, Cr and Cd.

*Hibiscus rosa-sinensis* L. (*Malvaceae*) is a broadleaf evergreen shrub which grows 1 - 3 m tall. The leaf blade is broadly or narrowly ovate, not lobed and measures 4 - 9 x 2.5 cm. The base is rounded or cunneate, the margin dentate or lobed and the apex acuminate. The flowers are solitary, axillary on upper branches, usually pendulous, simple or double. Pedicel is 3 - 7 cm, sparsely stellate pilose or nearly glabrous and articulate near apex. The petals (usually five) are obovate, corolla rosy red, reddish or orange-yellow in colour, funnel shaped and 6 - 10 cm in diameter. The entire plant has a course texture and may be upright or broad and spreading. The plant grows luxuriantly in the tropics and flowers all through the year (Mudgal, 1974).

In this work we report the extraction, purification and characterization of 3, $3^1$,$4^1$,5,7-pentahydroxflavylium chloride from *H. rosa-sinensis* flowers, and the utility of this compound for the quantitative determination of Pb, Cd and Cr. Our choice of this plant species for the extraction of the natural pigment has been based on its abundant distribution in the tropics and the fact that it flowers all through the year.

## MATERIALS AND METHODS

### Plant materials

Fresh flowers of *H. rosa-senensis* L. (*Malvaceae*) were collected from the Botanical Garden, University of Nigeria, Nsukka. Fresh blackberry fruits purchased from Jeremiah Useni's Farm, Jos Plateau State, Nigeria were used to isolate the authentic markers.

### Reagents and solvents

Spectroscopic grade $Pb(NO_3)_2$, $CdCO_3$ and $CrCl_3$ were used. Other reagents and solvents were analytical grade and used as such. All laboratory reagents were freshly prepared.

### Extraction

The anthocyanidin was extracted by a slight modification of the method described by Harborne (1998). 200 g of freshly harvested flowers of *H. rosa-sinensis* were macerated in 2L methanol: 2M HCl (85:15 v/v) solution for 72 h. The extract was concentrated to about 500 ml and then filtered.100 ml of concentrated HCl was added and the mixture heated in a round bottom flask under reflux for 2 h. The mixture was introduced into a flask, stoppered and placed in a refrigerator until crystals settle out. The crystals were filtered under suction, recrystallized from methanol and air-dried.The air-dried crystals were stored in amber coloured sample bottle.The authentic marker was obtained by the same extraction process using 200 g of fresh blackberry fruits (Harborne, 1998).

### Characterization of the pigment

Robinson's qualitative tests for cyanidins as described by Elderfield (1951) were carried out on the extracted pigments. The pigments were also Co-Chromatographed with the authentic marker on thin layer chromatography [TLC (silica gel)] developed with Forestals [Acetic acid:HCl:$H_2O$ (30:3:10)], aqueous HCl [HCl:$H_2O$ (3:97)] and BAW [nButanol:Acetic acid:$H_2O$ (4:1:5)] and paper chromatography (PC) developed with Forestal, BAW and Formic acid. The spots were observed by daylight and the $R_f$ values calculated and compared. Spectral characterization of the pigment was carried out using 0.5 mM of the pigment in methanol containing 0.01% HCl, and analysis done with UV-2102 PC spectrophotometer (UNICO®, USA). Effects of 3 drops of 5% $AlCl_3$ in ethanol on the uv-visible spectrum of the pigment was also determined.

### Preparation of standard stock solutions

168 mg of the pigment (3, $3^1$,$4^1$,5,7-Pentahydroxflavylium chloride) was dissolved in 20 ml analytical grade methanol in a 100 ml flask and the solution made up to mark with methanol to give 5 mM solution. 5 mM solution in ethanol was similarly prepared.

135 mg of Pb $(NO_3)_2$, 86 mg of $CdCO_3$ and 134 mg of $CrCl_3$ were each dissolved in 20 ml of distilled water in separate 100 ml flasks. The solutions were made up to the mark with distilled water to 5 mM solutions of the salts. Buffer solutions for pH range of 1 – 8 were prepared as described by Lange (1973).

### Absorption spectra

The uv-visible spectrum of the solution of the pigment (0.5 mM) made from the stock was determined using Unico UV – 2102 PC spectrophotometer. 5 ml portions of 0.5 mM solution of the pigment was mixed with 5 ml of 5 mM solutions each of spectroscopic grade $Pb(NO_3)_2$, $CdCO_3$ and $CrCl_3$. The uv-visible spectra of the mixtures were determined. The spectra determinations were repeated using 5 mM solution of the pigment in Ethanol.

### Effect of pH

5 ml of 0.5 mM solution of the pigment in methanol was mixed with 5 ml of 0.5 mM solution of $Pb(NO_3)_2$ and the pH adjust to 1 using buffer. The $\lambda_{max}$ was determined. The experiment was repeated with the pH adjusted to 2, 3, 3.8, 4.2, 5, 6, 7 and 8. Similar experiments were also performed with 0.5 mM solutions of $CdCO_3$ and $CrCl_3$. The whole experiment was repeated using 0.5 mM solution of the pigment in ethanol.

### Stoichiometric relationship

The experiment was done according to the method described by Vosburgh and Cooper (1941). Master solutions of equimolar concentrations (0.5 mM) of the pigment in methanol and $Pb(NO_3)_2$ in distilled water were prepared. A series of 10 ml quantities of the mixtures of the Master solutions comprising complimentary proportions of the two solutions (1:9. 2:8, 3:7, …, 9:1) were transferred to different test tubes. The mixtures were kept at pH of 4.2 and allowed to develop colour after which the absorbances were read at 555 nm. The experiment was repeated for Cr (III) and Cd (II) with the pH kept at 1 and 3 and absorbance reading taken at 706 nm and 682 nm respectively. The metal-ligand mole ratios were determined from the plots of absorbance against mole fractions as shown in figures 1, 2 and 3.

### Standard curve

Serial concentrations (0.1 - 1 mM) of $Pb(NO_3)_2$ were prepared from

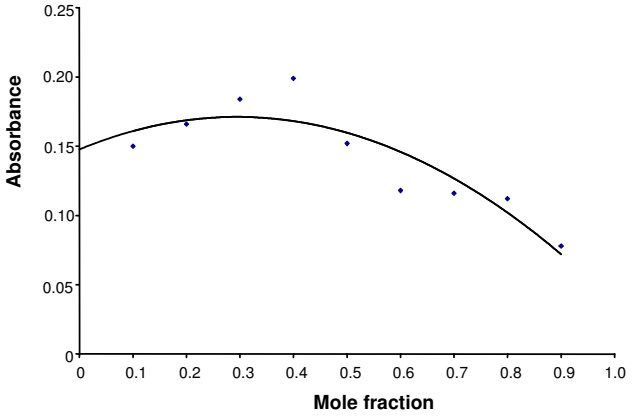


**Figure 1.** Job's plot of Pb-cyanidin complex in methanol.
From the graph, Metal-ligand complexation ratio is given as $V_m/V_l = 3.35/6.65 \approx 1:2$
$V_m$ = Mole fraction of metal ion
$V_l$ = Mole fraction of ligand (cyanidin)

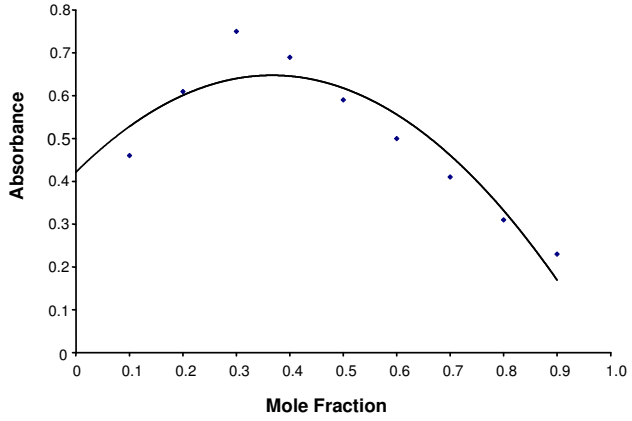


**Figure 2.** Job's plot of Cr-cyanidin complex in methanol
From the graph, Metal-ligand complexation ratio is given as $V_m/V_l = 3.5/6.5 \approx 1:2$
$V_m$ = Mole fraction of metal ion
$V_l$ = Mole fraction of ligand (cyanidin)

the stock (5 m M). 5 mls of each of the dilutions were transferred into different test tubes and 5 ml of 0.5 mM solution of the pigment in methanol added. The mixtures were maintained at pH of 4.2 and their absorbance readings taken at 555 nm. The same experiment was repeated for $CrCl_3$ and $CdCO_3$ with the pH maintained at 1 and 3 and absorbance readings taken at 706 nm and 682 nm respectively.

## RESULTS AND DISCUSSIONS

Extraction of the fresh flowers of Hibiscus afforded 15.0 g of dark brown crystals (7.5% yields) of the flavylium chloride. The extraction process involves the hydrolysis of the anthocyanin to the aglycone anthocyanidin as well as the conversion of the latter to the more stable chloride. Robinson's qualitative test (Elderfield, 1951) indicated the presence of cyanidin nucleus and co-chromatography and comparison of Rf values (Table 1) with authentic marker showed the presence of 3, $3^1$,$4^1$,5,7-Pentahydroxyflavylium chloride. UV-visible spectrum of the flavylium chloride revealed two characteristic absorption maxima at 280 nm and 527 nm. Anthocyanin and their aglycones (anthocyanidins) are characterized by two absorption bands, one in the UV region and the other in the visible region (Finar, 1980). Results from past workers (Harborne, 1958b) showed that cyanidin absorbs at 280 nm and 530 nm. The spectral shift of 18 nm on addition of $AlCl_3$ further confirms the presence cyanidin nucleus (Harborne, 1998). Cyanidin is one of the anthocyanidins which possess the $3^1$-, $4^1$- otho- hydroxyl system, a structural feature necessary for the formation of complex with $AlCl_3$.

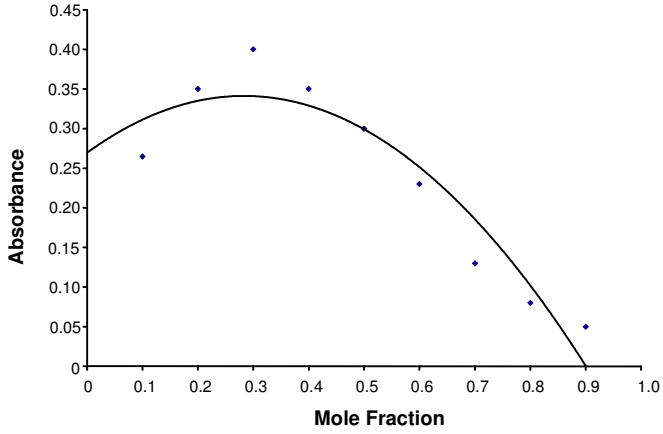


**Figure 3.** Jobs plot of Cd-cyacidin complex in methanol.
From the graph, Metal-ligand complexation ratio is given as $V_m/V_l = 3.3/6.7 \approx 1:2$
$V_m$ = Mole fraction of metal ion
$V_l$ = Mole fraction of ligand (cyanidin)

Addition of $Pb(NO_3)_2$, $CrCl_3$ and $CdCO_3$ to the solutions of the flavylium chloride resulted in a spectra shift as shown in Table 2. The spectra shift is as a result of the formation of coordination complex between the flavylium chloride and the individual metals. A Cyanidin-Pb (II) complex was formed at pH 4.2 ($\lambda_{max}$ = 555 nm, in methanol); A Cyanidin-Cr (III) complex was formed at pH 1.0 ($\lambda_{max}$ = 682 nm, methanol and ethanol) while a Cyanidin-Cd (II) complex was formed at pH 3.0 [$\lambda_{max}$ = 706 nm (methanol) and 679 nm (ethanol)]. Aluminum and tin have earlier been reported to cause a similar spectral shift in the uv-visible spectrum of flavyluim chloride. These shifts were attributed to the formation of coordination complex, between the metals and the $3^1$-, $4^1$-otho-dihydroxyl systems in the anthocyanidin (Harborne, 1958a, Zang et al., 2005). Phenolic compounds generally have been shown to chelate with metal ions at the $3^1$-, $4^1$-

**Table 1.** $R_f$ values of the cyanidin on TLC and PC.

| Cyanidin source | $R_f$ values (X 100) on TLC | | | Rf values (X 100) on PC | | |
|---|---|---|---|---|---|---|
| | Forestal | Aq. HCl | BAW | Forestal | Formic acid | BAW |
| *Hibiscus rosa-sinensis* | 65 | 32 | 59 | 49 | 25 | 66 |
| Blackberry | 65 | 33 | 60 | 49 | 24 | 67 |

TLC = tin layer chromatography, PC = paper chromatography
Forestal = Acetic acid:HCl:$H_2O$ (30:3:10)
Aq. HCl = HCl:$H_2O$ (3:97)
BAW = nButanol:Acetic acid:$H_2O$ (4:1:5)

**Table 2.** Effects of Pb (II), Cr (III) and Cd (II) on the Absorption Maxima of the cyanidin in MeOH and EtOH.

| Samples | Cyanidin | +5%$Pb(NO_3)_2$ | +5%$CrCl_3$ | +5%$CdCO_3$ | Shift | | |
|---|---|---|---|---|---|---|---|
| | | | | | Pb(II) | Cr(III)) | Cd(II) |
| $\lambda_{max}$ in MeOH(nm) | 530 (2.59 x $10^4$) | 555 (9.66 x $10^2$ ) | 682 (8.77 x $10^2$) | 706 (9.77 x $10^2$) | 25 | 152 | 176 |
| $\lambda_{max}$ in EtOH (nm) | 540 | 540 | 682 | 679 | 0 | 139 | 142 |

Values in parenthesis represent the molar absorptivities ($L.mol^{-1}.cm^{-1}$) of the individual complexes.

**Table 3.** Assay parameters and regression analysis.

| Metal ion | Regression Equation | LR (mM) | n | $R^2$ | DL (mM) | QL (mM) |
|---|---|---|---|---|---|---|
| Pb (II) | A = 0.825C+ 0.03 | 0.1 – 0.5 | 7 | 0.994 | 0.0018 | 0.006 |
| Cr (III) | A = 0.884C- 0.0004 | 0.1 – 0.5 | 7 | 0.995 | 0.00003 | 0.0001 |
| Cd (II) | A = 0.864C + 0.01 | 0.1 – 0.5 | 7 | 0.992 | 0.0024 | 0.008 |

A = Absorbance, C = Concentration in mM, LR = Linear Range, DL = Detection limit, QL = quantitation limit.

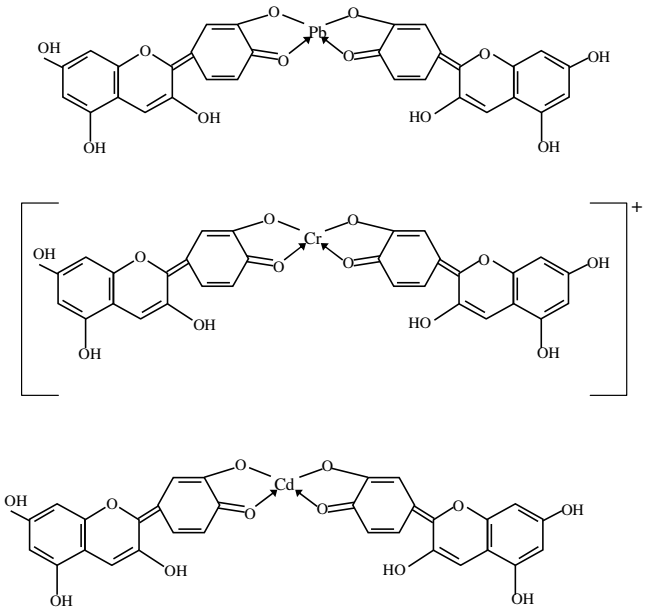


**Figure 4.** Structures of metal ion –cyanidin complexes for Pb, Cr and Cd.

ortho –positioned hydroxyl groups (VanAcker et al., 1996; Miller et al., 1996; Mayer, 1998). Cr and Cd are among the transition metals which easily form coordination complexes by virtue of empty d orbital (Lee, 1996). In the present study, the metals – cyanidin complex ratios were found to be 1:2. The structures of the complexes are thus postulated as shown in Figure 4.

Formation of each of the complexes was found to be quantitative as shown by the plots of the absorbance versus the concentrations of individual metal ions. The assay parameters and regression analyses are shown in Table 3. Beer-Lamberts relationship was obeyed at the concentration range of 0.1 – 0.5 mM. Regression analyses of the Beer's law plots revealed a good correlation and the calculated detection and quantitation limits (EMEA, 2006) indicate the high sensitivity of the analytical method (Table 3). The flavylium chloride can thus be employed in quantitative determinations of these metals. Apart from the analytical utility, it is worthy of note that the ability of the compound to form complexes (chelates) with the heavy metals can explain in part the antioxidant property of anthocyanidins (Miller et al., 1996; Wang and Jiao, 2000; Obi 2001). Heavy metals are known to generate free radicals which initiate auto-oxidation in living systems (Akubue, 1997).

In conclusion, flavylium chloride extracted from *H. rosa-*

*sinensis* can be used for the quantitative determination of Pb, Cr and Cd in samples. The detection and quantitation limits are quite low making it a very suitable analytical method in determining traces of these metals in biological fluid. The metals also exhibited distinct spectra shifts at different pH thus allowing for their simultaneous determination in samples.

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