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Anthocyanins in Red Beet Juice Act as Scavengers for Heavy Metals Ions such as Lead and Cadmium

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ABSTRACT

Recently many papers appeared on (Anthocyanins) as a complexing agent with metals ions**.** The aim of this research is to fitting pollution and poisoning by metals and their ions by forming complexes. Aqueous solution of anthocyanin from red beet is slightly acidic (pH 6.4) which attack metals slowly (oxidation process). As soon as metal ion forms the anthocyanin anion captures it and precipitate. Anthocyanin juice was shown a high antioxidant capacity in numerous studies. In this study anthocyanins are extracted and purified, then a series of complexes are prepared from reaction with the metal ions Pb(II) and Cd(II) after fixing the optimum conditions of (volume, concentration, temperature and pH). The UV-Vis spectra of these ions with pigment solution have been studied. The formula of complexes is deduced according to the continuous variation method (Jobs method**)** method which is obtained from the spectrophotometric studies of the complex solution. The ratios of ligand: metal obtained are 2: 1 for all complexes under study (depending on the above job method). The solid complexes are indicated by UV-Vis spectra that showed red shift when it compared with pigment solution spectra. Also infrared spectra are studied and showed appearance and disappearance of some peaks. The molar conductivity showed the absence of ionic property. The determination of magnetic susceptibility for all complexes showed that they have diamagnetic properties (i.e. all orbitals have pairs electrons)**.** According to the results, molar conductivity, magnetic susceptility and electronic configuration support the structural formula of complexes that have a ratio of ligand: metal equal 2: 1 and the suggested structures are tetrahedral.

Keywords: Anthocyanins, Red Beet Juice, Scavengers for Heavy Metals.

INTRODUCTION

Anthocyanins are generally accepted as the largest and most important group of water-soluble pigments in nature [1]. The word anthocyanin derived from two Greek words anthos, which means flower, and kyanos, which means dark blue, reveals it's important characteristic as a natural colorant [2]. Anthocyanins are natural pigments widely distributed in nature. Anthocyanin color molecules are a subclass of flavonoids. They are responsible for the reds, purples, and blues in many flowers, fruits and vegetables. They have a $C_6C_3C_6$-skeleton typical of flavonoids. Anthocyanins are generally regarded safe, since they have been consumed by animals and human for countless generations without apparent adverse effects to health [3]. Various studies have shown that mortality from cardiovascular disease is inversely correlated with the

intake of flavonoids in the diet; they also have anti-microbial activities and help with cancers, strokes, vascular problems and all kinds of inflammatory conditions. They are helpful with diabetic retinopathy and macular degeneration, The main benefits of anthocyanins are probably due to their strong antioxidant and free-radical quenching activity.

Most foods contain a variety of anthocyanins and related compounds. It is attributed with inhibiting fungal infections, inflammations, cancers, metastases, heart disease, Alzheimer's disease and other age-related brain disorders, raising good HDL cholesterol, increasing immunity, controlling blood pressure, preventing blood clots and strokes[4].

In this work we report the extraction, purification and characterization of 3, 3', 4', 5, 7-pentahydroxflavylium (anthocyanin) from red-beets. Our choice of this plant species for the extraction of the natural pigment has been based on its abundant distribution in our town (Hilla), cheap, simple, food material, the ability of complex formation between anthocyanin and (Pb^{2+}, Cd^{2+}).

MATERIALS AND METHODS

All chemicals that used in this work were used without any purification. Distilled water was used throughout the experimental work.

Pigment Extraction: The anthocyanins were extracted by a slight modification to the Harborn, Chiriboga and Francis method [1].A quantity of fresh vegetables red-beets were cleaned and washed carefully with distilled water, then cut randomly as thin slices and extracted with 100 ml of (85ml methanol + 15ml 1% HCl) in a mixer for 10 min., the extracted materials were filtered by using vacuum, the remains of mixture left on the filter paper was washed again with the above mentioned solvent and filtered again more than one time until clear color anthocyanins solution obtains.

Anthocyanins Purification: 5 ml of the extract solution was shaken in a separating funnel with 10 ml of petroleum ether (b.p= 40 - 60 ºC) and then 10 ml of ethyl acetate added to remove non polar impurities and other flavonoids [5]. The aqueous layer was concentrated under vacuum at 40°C.

Pigment Preparation: 0.0225g of the pigment was dissolved in 20ml of analytical grade methanol in a 100ml flask, the solution made up to the mark with methanol to give($0.5x10^{-3}$) M solution.

Salts Preparation: 0.0165g of Pb(NO3)2 and 0.0118g of Cd(NO3)2 were dissolved in 20 ml of distilled water in separate 100ml flask, the solutions were made up to the mark with distilled water to give ($0.5x10^{-3}$) M solution of each salt.

Absorption Spectra: The UV-Visible spectrum of the solution of the pigment ($0.5x10^{-3}$) M made from the stock was determined. (2.5 ml and 2ml) of ($0.5x10^{-3}$) M solutions of the pigment were mixed with 1 ml of ($0.5x10^{-3}$)M of each $Pb(NO_3)_2$ and $Cd(NO_3)_2$ solutions, the volume was completed to 10ml with distilled water. The UV-Vis spectra of the mixtures and pure pigment were determined.

Determination the type of complex formed (Continuous Variation Method): The experiment was preformed according to the method described by Vosburgh and Cooper [6]. Master solutions of equimolar concentrations ($0.5x10^{-3}$) M 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 4.0 and allowed to develop color after which the absorbencies were recorded at 555 nm. The experiment was repeated for Cd (II) the metal: ligand mole ratios were determined from the plots of absorbance against mole fractions.

Determination of Anthocyanins Concentration: The total Anthocyanins concentration was determined by using the pH differential method which was proposed by Ronald E. Wrolstad *et al.* [7].

They propose the equation:

$$\mathbf{A}_{\text{observed}} = (\mathbf{A} \text{ at } \boldsymbol{\lambda}_{\max} - \mathbf{A}_{700})_{\mathbf{pH}=1} - (\mathbf{A} \text{ at } \boldsymbol{\lambda}_{\max} - \mathbf{A}_{700})_{\mathbf{pH}=4.5} \quad \textbf{.....(1)}$$

1g from the pigment was taken and dissolved in 10ml of acidified methanol (85ml methanol + 15ml 1% HCl), then 0.8ml from this mixture were taken and the volume was completed to 3ml with potassium chloride (0.025M, pH 1.0), the same steps were repeated by dilution with 3ml from Sodium acetate buffer(0.4M,pH 4.5).The absorbencies of the two dilutions of the sample were taken at maximum wave

length and 700nm, these dilutions were left equilibrate for 15min. (Absorbance readings were made against water blank). The same procedure was repeated by using acidified ethanol and water.

RESULTS AND DISCUSSION

In this study, acidified aqueous methanol was chosen to extract the sample, due to the higher extraction efficiency obtained between the metals and the 3′-, 4′- ortho-di hydroxyl system in the anthocyanidin [8].Phenolic compounds generally have been coordinated with metals ions at the 3′-, 4′- ortho-–positioned hydroxyl groups [9–11].

Identification of the pigment and the Complexes: Substantial information can be obtained from the spectral characteristics of anthocyanins. Two distinctive absorption bands one in the UV-region (260 to 280 nm) and the second in the visible region (490 - 550nm). This result conferred with literatures [12,13]. Addition of $Pb(NO_3)_2$ and $Cd(NO_3)_2$ to the solutions of cyanidin-3-glucoside resulted in a spectra shift as shown in table 1. The spectra shift was as a result of the formation of coordination complex between the cyanidin-3-glucoside and the individual metal ions. A Cyanidin-Pb (II) complex was formed at pH 4.0 **(** λ_{max} = 555 nm, in methanol); a Cyanidin-Cd (II) complex was formed at pH 3.0 **(** λ_{max} =706 nm (methanol).

Table 1. represents the λ_{max} of anthocyanins and complexes.

of pigment λ_{max} /nm	λ_{max} /nm of complexes	
530	Pb(II)	555
	Cd(II)	706

Quantification of anthocyanin in extracts: The total anthocyanin concentration and the total amount of anthocyanin present in the sample were determined by using the pH differential method which was proposed by Ronald E. Wrolstad [7].

$$\mathbf{A_{obs.} = (A_{at\ \lambda_{max}} - A_{700})_{pH1.0} - (A_{at\ \lambda_{max}} - A_{700})_{pH\ 4.5}} \quad \ldots..(1)$$

First, the absorbance was measured at λ_{max} and 700 nm of the sample in the two pH buffer solutions and then the observed absorbance, $A_{observed}$, was calculated of the diluted sample with the equation (1).

The maximum absorbance was observed at pH=1, and as the pH increases the absorbance decreases. The colored sample in the pH = 4.5 was observed to be colorless or a very faded red color. After obtaining the absorbance values the anthocyanin pigment concentration in the original sample was calculated by using the formula:

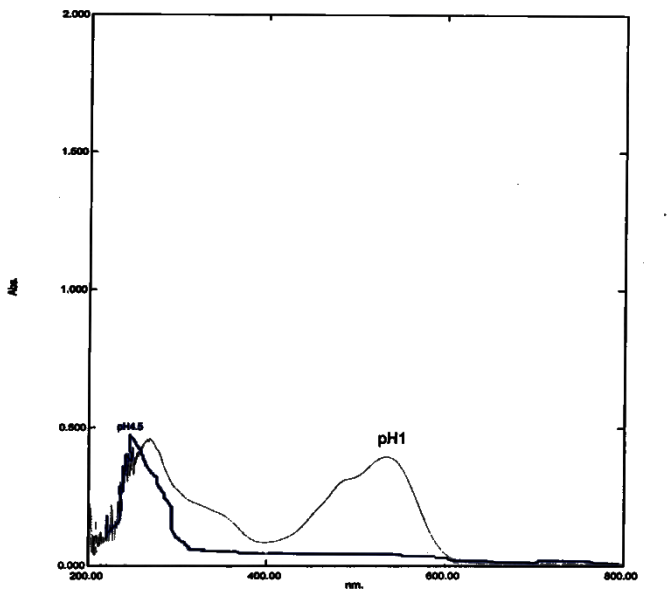
Concentration of anthocyanins pigment (mg L^{-1}) = ($A_{observed} \times Mw \times D_F \times 1000$)/ ($\varepsilon \times 1$) (2).

Where Mw represents the anthocyanin molecular weight, and the value used was 449.2 g mol^{-1} and **(ε)** is the absorption extinction coefficient. For the value that was available in the literature for anthocyanin in acidic solution was equal to 26900**,** 34300 and 44900 L mol^{-1} cm^{-1} for acidified aqueous solutions, acidified methanol and acidified ethanol respectively [14]. Extinction coefficients of some anthocyanins were reported in literature, but if the identify was unknown, it will indicate the use of cyanidin-3-glucoside extinction coefficient, since it was the most abundant anthocyanin in nature.D_F is dilution factor, which was constant (in our experiment) and equal to 37.5 for all samples (D_F was calculated as followed; 1gm of pigment was dissolved in 10ml solvent, 0.8ml of this solution was taken and the volume was completed to 3ml with buffer pH=1, so the dilution factor = 3 / 0.8 = 3.73. (D_F =3.73 x 10 = 37.5). The anthocyanin content was determined by measuring the absorbance of the anthocyanin at single wavelength. It was able to measure the absorbance at only single wavelength because anthocyanins have the typical absorbance band between 490 and 550nm.The anthocyanins have maximum absorption in the 490-550nm region. The dispersal of light needs to be corrected by reading the absorbance at a wavelength where no absorbance occurs,e.g.at 700 nm wavelength. The values obtained were listed in Table 2.The absorbance measurements at two different pH values were possible because of the structural transformation of

anthocyanin as a function of pH. The absorbance was measured at 530 nm at pH=1 because at these conditions, anthocyanins have the maximum absorption, and so do other compounds that might be present in the sample, also the absorbance was measured at pH=4.5 because at this pH anthocyanins solution were colorless and there was no absorption, so other compounds that had absorbed at pH=1 would still absorbed at pH=4.5. While the anthocyanins does not. By measuring the absorbance at 700 nm and subtract from λ_{max} in equation (3 – 1) the absorbance was corrected for other compounds which might have absorbed at pH=1 and pH=4.5. By this equation it was able to calculate the amount of anthocyanin which was obtained from each extraction procedure.

Table 2. Shows the absorption of extracted pigment solution by different solvents as a function of pH.

Extracted by	**λ_{max} / nm**	**pH=1**		**pH= 4.5**		**Abs. correction**	**Conc. mg/l**
		$A_{\lambda max}$	**A_{700}**	**$A_{\lambda max}$**	**A_{700}**		
Acidified methanol	530	0.962	0.025	0.675	0.001	0.263	129.16
Acidified ethanol	510	0. 736	0.030	0.661	0.015	0.06	22.56
Acidified water	540	0.580	0.030	0.525	0.029	0.052	32.79



λ /nm

Fig 1. UV-Vis spectra of cyanidin-3-glucoside at pH= 1 and pH= 4.5

Determination of metal: ligand Ratio in a Complex by Continuous Variation Method_(Job′s method): Solutions were prepared according to the optimum conditions and the absorbance were measured at λ_{max} for each complex, as shown in Table (2) the relationship between ligand mole fraction X_L and Abs. for each ion was showed in Figs.(2) and (3), from the Figs. It was indicated that the ratio of ligand: metal was (2: 1), $n = X_L / (1 – X_L)$.

Table 2. Job's method for determination complex molar ratio (ligand: Pb(II))

L : M	$X_L=V_L/(V_m+V_L)$	Abs. of Pb(II)complex
0:1	0	0
0.1:0.9	0.1	0.012
0.2:0.8	0.2	0.016
0.3:0.7	0.3	0.038
0.4:0.6	0.4	0.057
0.5:0.5	0.5	0.072
0.6:0.4	0.6	0.088
0.7:0.3	0.7	0.079
0.8:0.2	0.8	0.048
0.9:0.1	0.9	0.034
1:0	1	0

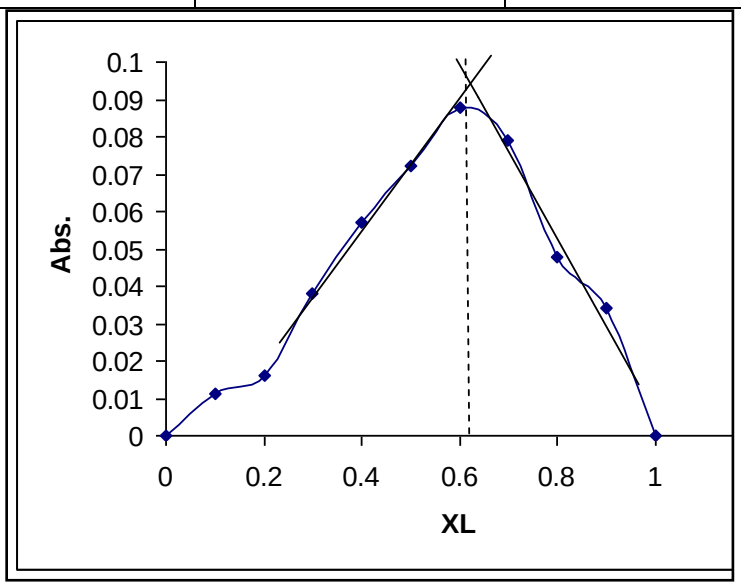


Fig. 2. The relationship between Absorbance and pigment mole fraction X_L for Pb(II) complex.

Molar – Conductivity measurements: The measurement of molar conductivity helps the researchers to know stereo figures of complexes[15,16]. The charge parts that existing in solution is proportional with conductivity value, when complex have no ionic property in solution, the electric conductivity equal zero[17]. The molar conductivity of chelating complexes were measured with concentration (1×10^{-3}) M in DMF solvent and at room temperature as shown in Table 4.

Table 3. Jobs method for determination complex molar ratio pigment: Cd(II)

L : M	$X_L=V_L/(V_m+V_L)$	Abs. of Cd(II)complex
0:1	0	0
0.1:0.9	0.1	0.014

0.2:0.8	0.2	0.019
0.3:0.7	0.3	0.041
0.4:0.6	0.4	0.060
0.5:0.5	0.5	0.085
0.6:0.4	0.6	0.093
0.7:0.3	0.7	0.084
0.8:0.2	0.8	0.060
0.9:0.1	0.9	0.039
1:0	1	0

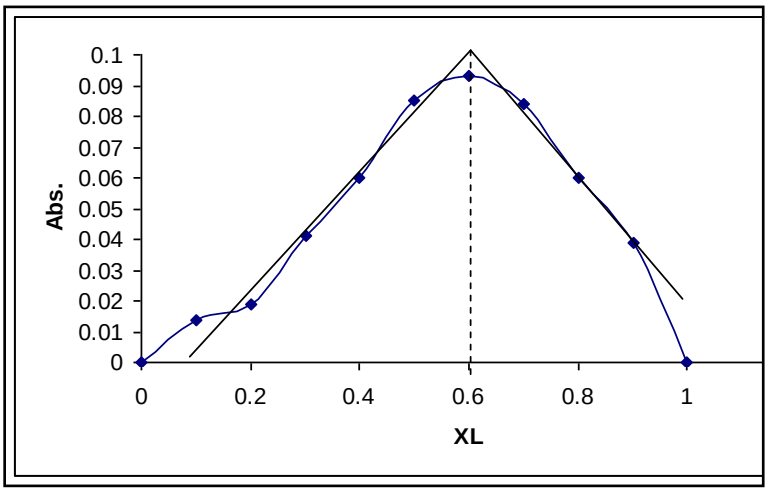


Figure 3. The relationship between Absorbance and pigment mole fraction X_L for Cd(II) complex

The results indicate the absence of the ionic property for Pb(II) and Cd(II) complexes, which well agrees with literatures[18] that showed the electrolyte type of 2:1 (L:M) have molar conductivity in range (130-170) S mol $^{-1}$ cm^2 in DMF solvent.

Table 4. Molar conductivity of complexes in DMF solvent at (1 x 10^{-3}) M and at room temperature.

Complex	**Molar conductivity (S mol $^{-1}$ cm^2)**
Pb – Cyanidin complex at pH 4.0	44
Cd – Cyanidin complex at pH 3.0	30

Determination of Magnetic Susceptibility: The results showed that the chelating complexes have a diamagnetic property [19,20], that was insured the suggested formula of complexes.

IR Spectra: Infrared absorption spectra were obtained from (400 – 4000) cm^{-1} using KBr tablets. The purpose of using IR-spectra in organic analysis was to identify the active groups of organic and inorganic compounds and comparison the changes in chemical compounds [21], i.e. frequency print. The infrared

spectra are studied and showed appearance and disappearance of some peaks. Broad band 3400-3300cm^{-1} that related to sugar vibration and phenol OH groups ν_{OH} vibration these bands suffered clear changes in shape and intensity, and shifted to low frequency when the complexes formed, ν_{CH} vibration band of (CH_2 group) at approximate 2935 – 2850 cm^{-1}, this band suffered clear shift to low frequency in all complexes. Vibration band of C═O group (fructose) at 1630 cm^{-1}, this band showed little shift to low frequency. δ_{CH} vibration band at 1420 cm^{-1} this band disappeared in Pb^{2+} and Cd^{2+} complexes, new M ─ O band at approximate 1050-1000 cm^{-1} related to interaction of metal ions with active groups of pigment, this band appeared in region of low frequency because of heavy mass of selected atoms, as in figures 4,5 and 6 .

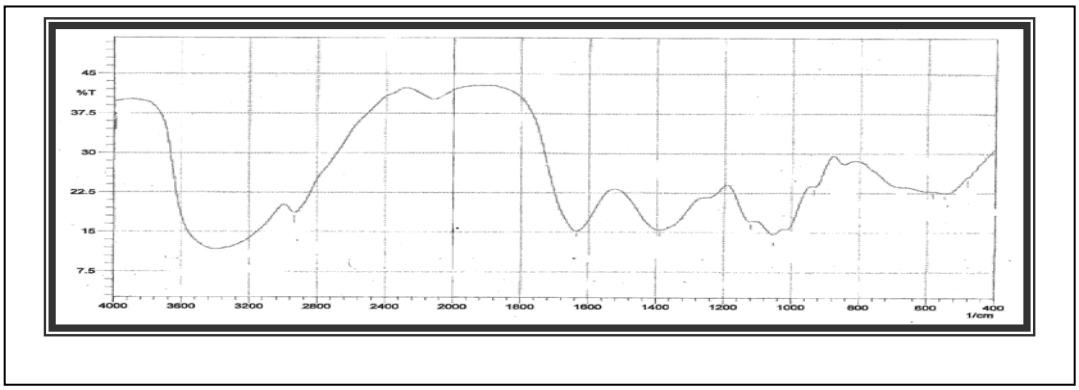


Figure 4; FTIR Spectra of anthocyanin

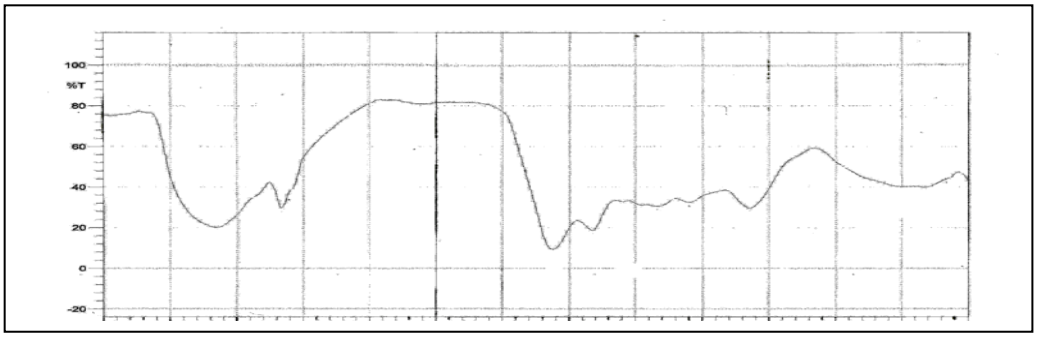


Figure 5. FTIR-spectra of Pb-anthocyanin complex pH=4.0

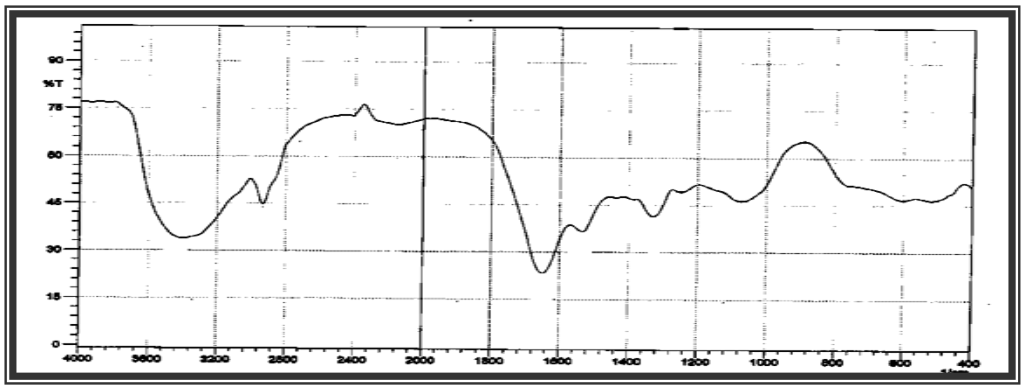


Figure 6. FTIR-spectra of Cd-anthocyanin complex pH=3.0

The Proposed Structural Formula of Complexes : In this study the extracted pigment, (Cy-3-gly) is bidentate, the attachment positions with metals ions (Pb(II) and Cd(II))were oxygen of hydroxyl group at the positions 3^-, 4^- [22] . According to the results and measurements that it can propose the structural formula of complexes that have a ratio of ligand: metal equal 2: 1.

APPLICATIONS

Anthocyanins in Red Beet Juice Act as Scavengers for Heavy Metals Ions such as Lead and Cadmium by forming comlexes. The results indicate the formation of tetrahedral complexes with Pb (II) and Cd (II).

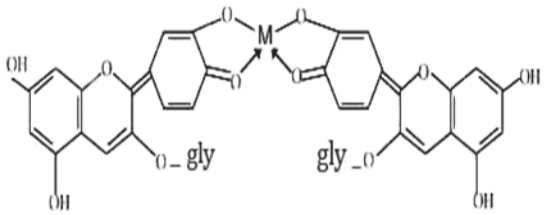


Figure 7 The expected structure of M -Cyanidin-3-glucoside complex in acidic pH (M= Pb, Cd)

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