

CHAPTER III

EXPERIMENTAL

3.1 Apparatus

3.1.1 UV-visible spectrophotometer

The UV-Visible spectrophotometer of HP 8453 Hewlett Packard was used for characterization and all spectrophotometric measurements in the wavelength range of 200-800 nm.

3.1.2 pH meter

The pH of buffer solution or even real water sample was measured with Ion S220 pH meter.

3.1.3 Inductively coupled plasma spectrometer

Metal ion concentrations were determined by inductively coupled plasma spectrometer (iCAP 600 SERIES; Thermo, USA).

3.1.4 Mass spectrometer

A mass spectrometer (Micromass platform II) in MALDI-TOF mode was used to obtain mass spectra of the extracts.



3.1.5 Nuclear magnetic resonance spectrometer

A ^1H -nuclear magnetic resonance ($^1\text{H-NMR}$) spectrometer (Bruker with 400 MHz) was used to identify the structure of cyanidin.

3.1.6 Diffuse reflectance ultraviolet visible (DR-UV-Vis) spectrophotometer

A DR-UV-Vis spectrophotometer (UV-2500PC; Shimadzu; Japan) was used for characterization of the modified solid sorbent.

3.2 Chemicals

The chemicals used in this research are listed in Table 3.1.

Table 3.1 All chemicals used

Chemicals	Supplier
Acetic acid (CH_3COOH)	Merck
Aluminium chloride (AlCl_3)	Fluka
Amberlite XAD-2	Supelco
Amberlite XAD-7	Supelco
Cadmium nitrate tetrahydrate ($\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$)	Merck
Calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$)	Merck
Chloroform (CHCl_3)	Lab Scan
Chromium(III) nitrate nonahydrate ($\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$)	Sigma-Aldrich
Cobalt(II) nitrate hexahydrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$)	Univar
Dimethylglyoxime or DMG	Merck

Table 3.1 (continued)

Chemicals	Supplier
Copper(II) nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$)	Merck
Disodium hydrogen phosphate (Na_2HPO_4)	Merck
Ethylenediaminetetraacetic acid disodium salt dihydrate or EDTA	Merck
Hydrochloric acid (HCl)	Merck
Iron(III) chloride (FeCl_3)	Merck
Lead(II) nitrate ($\text{Pb}(\text{NO}_3)_2$)	Riedel-de Haen
Manganese(II) chloride (MnCl_2)	Merck
Methanol (CH_3OH)	Merck
Nickel(II) nitrate hexahydrate ($\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$)	BDH
Nitric acid (HNO_3)	Merck
Potassium chloride (KCl)	Univar
Potassium fluoride (KF)	Merck
Potassium tartrate ($\text{K}_2\text{C}_4\text{H}_4\text{O}_6$)	Merck
Silica gel 60	Merck
Sodium acetate (CH_3COONa)	CARLO
Sodium chloride (NaCl)	Fluka
Sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	Merck
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck
Sodium hydroxide (NaOH)	Carlo Erba
Sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$)	Merck
Sodium pyrophosphate ($\text{Na}_4\text{P}_4\text{O}_7$)	Merck
Sodium sulphate (Na_2SO_4)	CARLO
Sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$)	Merck



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Table 3.1 (continued)

Chemicals	Supplier
Sulfamic acid	Merck
Thiourea (CH ₄ N ₂ S)	Sigma-Aldrich
Zinc nitrate (Zn(NO ₃) ₂)	Merck

3.3 Chemical solution preparation

3.3.1 Metal ion solutions

Stock solutions of Cu(II), Pb(II), Cr(III), Cd(II), Ni(II), Zn(II), Co(II) and Mn(II) were prepared by dissolving an appropriate amount of analytical reagent grade metal nitrate-salt in Milli-Q water. Whereas, Fe(III) and Al(III) stock standard solution were obtained from FeCl₃ and AlCl₃, respectively. The working solutions of metal ions were prepared by diluting the stock solution with Milli-Q water.

3.3.2 Buffer solutions

Buffer solutions pH in the range of 3-6 were prepared from CH₃COOH/CH₃COONa whereas, pH 7 was obtained from Na₂HPO₄/NaH₂PO₄ and pH 2 was prepared from HCl.

3.3.3 Masking agent solutions

Most of solutions used as masking agent (EDTA, thiourea, potassium fluoride, thiosulphate, sodium pyrophosphate, citrate, oxalate, tartrate and dimethylglyoxime (DMG)) were prepared in the concentration of 0.1 M by dissolving



an appropriate amount in Milli-Q water, except DMG was prepared in ethanol in the concentration of 1.0 % w/v.

3.3.4 Methanolic hydrochloric acid solutions

0.3% and 0.6% v/v HCl/MeOH solutions were prepared by diluting 0.3 mL and 0.6 mL, respectively of concentrated hydrochloric acid (37% w/w) to 100 mL using methanol.

3.3.5 Cyanidin solutions

Freshly prepared cyanidin solution was obtained by dissolving an appropriate amount of cyanidin powder in methanol, then leaved to equilibrium in dark refrigerator for 1 hour [14]. In case of solid sorbent modification, cyanidin was dissolved in methanolic hydrochloric acid solution.

3.4 Extraction of cyanidin from red cabbage

The extraction procedure of cyanidin from red cabbage was modified from the previous research reported by Ukwueze *et al.* (2009) [26]. Red cabbage brought from local market was cleaned by using deionized water and sliced into small pieces and weighed for 270 g before soaking in 600 mL of mixed solution of 2 M HCl/MeOH (15:85 v/v) for 72 hours. The red extracted solution was filtered through filter paper and other nonpolar molecules were removed by extraction with chloroform in a separatory funnel. Then, the solution was concentrated by rotary evaporator (temp. < 38°C) and refluxed under acidic (HCl) condition (5:1 v/v) until deep-red violet color appeared. The solution was immediately poured into cleaned bottle and placed in a dark refrigerator until dark-brown powder precipitated. The dark-brown powder was purified through sephadex LH-20 using methanol as eluent. The clearly red solution



fraction was collected and dried using rotary evaporator. The cyanidin powder should be kept in dark refrigerator to avoid either oxidation or degradation by light.

The final product (dark-brown powder) was characterized by UV-visible spectrophotometry, mass spectrometry and NMR spectrometry. Shinoda's method was also used to confirm flavonoid property.

UV-visible spectrophotometry

Dark-brown powder (1.6 mg) was dissolved in 5 mL of 0.3% v/v HCl/MeOH and diluted 10 folds with methanol. The absorption spectrum was recorded in the wavelength range of 200-800 nm.

Mass spectrometry

Mass spectrum of the powder was observed in MALDI-TOF mode using alpha-cyano-4-hydroxycinnamic acid (CCA) as matrix.

NMR spectrometry

Dark-brown powder was completely dissolved in CD₃OD and then measured the ¹H-NMR pattern using 400 MHz NMR spectrometer.

Shinoda's method

An amount of the extract was dissolved in 5 mL of 95% ethanol and few drops of conc. HCl was added. Finally, 0.5 g of magnesium ribbons was also added into this solution.



3.5 Qualitative determination of metal ions using cyanidin solution

Various parameters have to be investigated to achieve the optimum conditions for naked-eye detection at the level as low as possible. In this study we considered the parameters such as pH and concentration of cyanidin solutions, masking agents including interfering ion.

3.5.1 pH of the cyanidin solution

Cyanidin solution was obtained by dissolving 1.6 mg of cyanidin powder in 5 mL of methanol then leaved to equilibrium in dark refrigerator for 1 hour. To optimize the pH of tested solution, 1.0 mL of buffer solution was added into 100 μ L of 0.01 M standard metal ion solution followed by 100 μ L of cyanidin solution. The color change was observed by naked-eye after a few minutes comparing with blank solution prepared by adding Milli-Q water instead of the standard metal ion solution.

3.5.2 Masking agent

Masking agents such as EDTA, thiourea, potassium fluoride, thiosulphate, sodium dihydrogen phosphate, sodium pyrophosphate, citrate, oxalate, tartrate, sulfamic acid and DMG were selected. All of them were prepared in the concentration of 0.1 M except DMG was prepared to be 1.0 % w/v. To obtain the effective masking agent, the experimental was investigated under pH 6. Buffer solution (1 mL) was added into 100 μ L of 1 mM standard metal ion solution followed by 100 μ L of masking agent solution and 100 μ L of cyanidin solution. The color change was observed by naked-eye comparing with blank solution prepared by adding Milli-Q water instead of the standard metal ion solution.



3.5.3 Cyanidin concentration and reaction time

Three concentrations of cyanidin solution were prepared by dissolving 0.8, 1.6 and 3.2 mg of cyanidin powder in a small volume of methanol and then the solution was adjusted to the mark of 5 mL volumetric flask. All solutions were leaved to equilibrium for 1 hour in dark refrigerator. 1 mL of buffer solution (pH 4, 5, 6 and 7) was added into 100 μ L of 1 mM Fe(III), Al(III), Pb(II) and Cu(II) standard solution, respectively. KF (0.1 M, 100 μ L), DMG (1.0% w/v, 100 μ L) or a mixture of KF (0.1 M, 100 μ L) and DMG (1.0% w/v, 100 μ L) was added into those solutions only pH 4, 5 and 6, respectively. Cyanidin solution in the volume of 100 μ L was also finally added. The absorbance unit was recorded at 590, 580, 570 and 560 nm for Cu(II), Pb(II), Fe(III) and Al(III) complex, respectively, for 0, 2, 6, 10, 15, 20, 25 and 30 minutes using UV-Visible spectrophotometry.

3.5.4 Interfering ion effect

The interfering ions effect from cations, anions and target metal ions was studied under the optimum conditions of each metal ion determination. The mixture of each metal ion (1 mM) and each interfering cations (CaCl_2 , KCl, NaNO_3) and anions (NaCl, KF, Na_2SO_4 and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) in the concentration of 0.01 M were prepared. Each target metal ions in the equimolar concentrations of standard metal ions were also prepared as interfering ion. For Fe(III) interfering determination, 1 mL of buffer solution (pH 4) was added into 100 μ L of mixture solution of Fe(III) and interfering ion followed by 0.1 M KF (100 μ L) and cyanidin solution (100 μ L). For Cu(II), Pb(II) and Al(III) interfering effect were investigated in the same manner of Fe(III) but pH of solutions was changed to 7, 6 and 5, respectively, along with 1.0% w/v DMG (100 μ L) and a mixture of 0.1 M KF (100 μ L) and 1.0% w/v DMG (100 μ L) as



masking agent for Al(III) and Pb(II) determinations, respectively. The absorbance was measured by using UV-Visible spectrophotometry.

3.6 Quantitative determination of metal ions using cyanidin solution

Each standard metal ion solution (Cu(II), Pb(II), Fe(III) and Al(III)) in the concentration range of 0-1000 mM was prepared and developed color under the optimum conditions as in qualitative determination. Buffer solutions (0.01 M) at pH 4, 5, 6 and 7 were used for Fe(III), Al(III), Pb(II) and Cu(II), respectively. In addition, 0.1 M KF and 1.0% w/v DMG were also prepared as masking agent solution for masking Al(III) and Cu(II), respectively. 1000 μ L of buffer solution was added into 100 μ L of standard metal ion solution followed by 100 μ L of masking agent solution and 100 μ L of cyanidin solution. For Cu(II) detection, the solution was controlled at pH 7 and no masking agent required. For Pb(II) determination, the solution at pH 6 operated together with KF and DMG as the masking agent was appropriate. For Fe(III) determination, the solution was controlled at pH 4 and KF was employed as masking agent. Finally, Al(III) was analyzed in buffer solution at pH 5 along with DMG as masking agent.

The quantitative determination was observed by both naked-eye detection and spectrophotometric method.

3.7 Method validation for metal ion determination using cyanidin solution

The accuracy of this proposed method was investigated by comparing the results determined by naked-eye with ICP-OES. Two concentrations of each metal ion were prepared by spiking stock standard solution into unknown water samples (pond and tap water) under the optimum conditions for quantitative analysis (section



3.6). After that, the unknown solutions were then also determined the actual concentration by ICP-OES technique.

3.8 Application in real water sample using cyanidin solution

This proposed method was applied for metal ion determination in real water sample. The water samples were collected, filtered through 0.45 μm cellulose acetate filter to remove some suspended particles and preserved in acid solution (HNO_3). Water sample was divided into 30 samples, then, known amounts of four target metal ion were spiked (single metal ion or mix metal ions). The spiked water samples were developed color under optimum conditions for quantitative analysis as described in section 3.6. The unknown solutions were then also determined the actual concentration by ICP-OES technique. The comparison results were reported in term of %error of both qualitative and quantitative analysis.

3.9 Modification of solid sorbent

3.9.1 Solid sorbent type

Amberlite XAD-2 (AXAD-2), Amberlite XAD-7 (AXAD-7), silica gel 60 and cellulose filter paper (1x1 cm) were selected as solid sorbents. Preliminary test operated by preparing cyanidin solution by dissolving 1.6 mg of cyanidin powder in 5 mL of methanolic hydrochloric acid solution then leaved to equilibrium in dark refrigerator for 1 hour and poured into 1 g of each solid sorbent. All modified solid sorbents were then gently stirred and allowed overnight. The solution was poured out, and the solid sorbent was then dried in a desiccator. The optimum solid sorbent should be potentially contained cyanidin molecule with homogeneously.



3.9.2 Coating conditions

From preliminary test, AXAD-7 was chosen to modify the surface by cyanidin. Various parameters such cyanidin concentration, coating time, stability of modified solid sorbent was investigated to the approach optimum conditions for determination of metal ions by naked-eye in term of both qualitative and quantitative analysis.

AXAD-7 was transferred to a beaker of 500 mL. Methanol was sufficient added to cover the AXAD-7 resin bed by 1-2 inches. AXAD-7 was stirred gently for a minute to ensure complete mixing and allowed to stand for 15 minutes. Methanol solution was carefully decanted and distilled water was replaced. AXAD-7 was stirred again and allowed to stand for 5-10 minutes. The cleaned AXAD-7 was dried in oven under the temperature about 80°C.

3.9.2.1. *Effect of methanolic hydrochloric acid solution on stability of modified solid sorbent*

AXAD-7 was coated by cyanidin under 2 different methanolic hydrochloric acid conditions as shown below.

Condition 1: Cyanidin 1.6 mg was dissolved in 5 mL of 0.3% v/v HCl/MeOH.

Condition 2: Cyanidin 1.6 mg was dissolved in 5 mL of 0.6% v/v HCl/MeOH.

Cyanidin solution was added into 1 g of cleaned AXAD-7. The mixture was gently stirred for 30 seconds before allowing stand overnight. AXAD-7Cy was completely dried using rotary evaporator. The stability of these two AXAD-7Cy types was determined by DR-UV-Visible spectrophotometry. The reflectance was measured every month until the significant difference in % reflectance was observed.

3.9.2.2. Cyanidin concentration and coating time

Three concentrations of cyanidin were prepared by dissolving 0.8, 1.6 and 3.2 mg of cyanidin powder in a small volume of 0.6% v/v HCl/methanol and then the solution was adjusted to the mark of 5 mL volumetric flask. Each solution was prepared 3 set for coating time 30 minutes, 12 and 24 hours. Cyanidin solution was added into 1.0 g of AXAD-7, then gently stirred about 30 seconds and allowed to stand in dark refrigerator. After that, the solution was removed and dried by rotary evaporator. The modified sorbents called AXAD-7Cy1, AXAD-7Cy2 and AXAD-7Cy3 for 0.8, 1.6 and 3.2 mg of cyanidin coating, respectively, were obtained and kept in a dark desiccator.

The optimum concentration was obtained by considering the lowest concentration observed by naked-eye. In this study Cu(II) in the concentrations of 0.001-0.05 mM were selected as target metal ion. 500 μ L of buffer solution (0.1 M, pH 7) was added into 20 mg of AXAD-7 coated with cyanidin (AXAD-7Cy) followed by 500 μ L of Cu(II) standard solution.

3.10 Qualitative determination of metal ions using modified solid sorbent

3.10.1 pH of the tested solution

To optimize the complexation conditions of cyanidin and metal ion on AXAD-7 surface, pH of the solution was the major parameter that should be considered. Complexation was investigated over pH range of 2-7. At first step, AXAD-7Cy (condition 2) about 20 mg was rinsed out with 500 μ L of 0.1 M tested buffer solution before soaking again in 500 μ L of the same buffer solution. Then, each 1.0 mM standard metal ion solutions (Cu(II), Pb(II), Fe(III), Cr(III), Al(III), Cd(II), Ni(II), Zn(II),



Co(II) and Mn(II)) were added to each buffer solution and mixed well. The color change on AXAD-7Cy surface comparing with blank prepared by adding Milli-Q water instead of the standard metal ion solution meant that complexation was observed.

3.10.2 Masking agent

The masking agents used in this section were selected based on the results from investigation by using cyanidin solution. KF solution in the concentration of 0.1 M was used for masking Al(III). Whereas 1.0% w/v DMG was also compared with 0.1 M thiourea to mask Cu(II) under buffer solution pH 6. AXAD-7Cy (condition 2) about 20 mg was rinsed out with 500 μ L of 0.1 M buffer solution (pH 6) before soaking again in 500 μ L of the same buffer solution. 100 μ L of DMG and thiourea were mixed with 1 mM of Cu(II) solution before adding this mixing solution to the conditioned AXAD-7Cy. The color change was observed by naked-eye comparing with blank solution prepared by adding Milli-Q water instead of standard metal ion solution.

3.10.3 Buffer concentration and the volume ratio of all reagents

20 mg of AXAD-7Cy (condition 2) was mixed with 100 - 1000 μ L of buffer solution (0.01-0.10 M) at pH 4, 5, 6 and 7 for determination of Fe(III), Al(III), Pb(II) and Cu(II), respectively. Sample volume was varied from 250 μ L to 1000 μ L correlated with 10 times lower than of 0.1 M masking agents volume. The buffer concentration and the volume ratio of all reagents tested in this study are summarized in Table 3.2. AXAD-7Cy (condition 2) about 20 mg was rinsed out with 500 μ L of buffer solution and conditioned with the same buffer solution. Masking agent was first mixed together with metal ion solution and then poured this solution

into the conditioned AXAD-7Cy. The optimum ratio was selected from the obviously simultaneous determination of Fe(III), Al(III), Pb(II) and Cu(II) on AXAD-7Cy surface.

Table 3.2 Buffer concentration and the volume ratio of all reagents tested

Buffer concentration	Condition	Buffer ^a Solution (μL)	Masking agent ^b (μL)	Sample (μL)
0.1 M	1	500	100	500
	2	250	50	500
	3	250	25	250
	4	100	100	1000
0.01 M	5	500	100	500
	6	250	25	250
	7	1000	25	250

^a Buffer pH 4, 5, 6 and 7 for Fe(III), Al(III), Pb(II) and Cu(II) determination

^b Thiourea and the mixture of thiourea and KF for Al(III) and Pb(II) determination (no masking agent for Fe(III) and Cu(II) determination)

3.10.4 Reaction time

The reaction time was operated under the condition 4 as studied in section 3.10.3. Each metal ion concentration and the contact time were varied from 5-1000 μM and 5-15 minutes, respectively. AXAD-7Cy (condition 2) about 20 mg was rinsed out with 500 μL of 0.1 M buffer solution before soaking again in 100 μL of the same buffer solution. Buffer solution at pH 4, 5, 6 and 7 were used for Fe(III), Al(III),



Pb(II) and Cu(II), respectively. Masking agent (100 μL) was completely mixed with 1000 μL of sample solution before gently poured into the conditioned AXAD-7Cy. Then, the mixture was softly stirred and the color of AXAD-7Cy was observed by naked-eye every 5 minutes until 15 minutes.

3.10.5 Interfering ion effects

The interfering effects from cations, anions and target metal ions were investigated. The mixture solution of each metal ion in the concentration of 1 mM (except Pb(II) was prepared in the concentration of 5 mM) and each interfering cations (CaCl_2 , KCl, NaNO_3) and anions (NaCl , KF, Na_2SO_4 and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) in the concentration of 10 times higher than the concentration of the target metal ions were prepared whereas, each target metal ions in the equimolar concentration of standard metal ions were also prepared as interfering ion.

Then, the mixture solution was determined under the optimum qualitative determination of each metal ion. AXAD-7Cy (condition 2) about 20 mg was rinsed out with 500 μL of 0.1 M buffer solution before soaking again in 100 μL of the same buffer solution. Buffer solution at pH 4, 5, 6 and 7 were used for Fe(III), Al(III), Pb(II) and Cu(II), respectively. Masking agent should be mixed with 1000 μL of sample solution before adding this mixing solution into the conditioned AXAD-7Cy. 0.1 M thiourea (100 μL) and the mixture of 0.1 M KF (100 μL) and 0.1 M thiourea (100 μL) were used as masking agent for Al(III) and Pb(II) determination, respectively. The results were observed by naked-eye detection.



3.11 Quantitative analysis of metal ions using modified solid sorbent

Each standard metal ion in the concentration range of 5-1000 mM was prepared and AXAD-7Cy (condition 2) was used as modified solid sorbent. 0.1 M of buffer solutions at pH 4, 5, 6 and 7 were used for Fe(III), Al(III), Pb(II) and Cu(II), respectively. In addition, 0.1 M KF and 0.1 M thiourea were also prepared as masking agent solution for masking Al(III) and Cu(II), respectively. AXAD-7Cy (condition 2) about 20 mg was first rinsed out with 500 μ L of buffer solution and conditioned again using the same buffer solution (100 μ L). For Cu(II) detection, 1000 μ L of sample solution was directly added into the conditioned AXAD-7Cy without any masking agent. For Pb(II) detection, 100 μ L of each 0.1 M KF and 0.1 M thiourea was mixed with 1000 μ L of water sample before adding into the conditioned AXAD-7Cy. In case of Fe(III) detection, 100 μ L of sample solution was directly transferred into the conditioned AXAD-7Cy without any masking agent. Finally, for Al(III) analysis, 0.1 M thiourea was first mixed with 1000 μ L of sample water before adding into the conditioned AXAD-7Cy. Then, the mixture was softly stirred and the color on AXAD-7Cy was observed by naked-eye.

3.12 Method validation for metal ion determination using cyanidin solution

The accuracy of this method was investigated by comparing the results determined by naked-eye with ICP-OES. Two concentrations of each metal ion were prepared by spiking stock standard solutions into unknown water sample (pond and tap water) and developed color under the optimum condition for quantitative analysis (section 3.11). After that, the unknown solutions were then also determined the actual concentration by ICP-OES technique.



3.13 Application in real water sample using cyanidin solution

AXAD-7Cy was applied for metal ion determination in real water sample. The water samples were collected, filtered through 0.45 μm cellulose acetate filter to remove some suspended particles and preserved in acid solution (HNO_3). Sample water was divided into 30 samples, then, known amounts of four target metal ions were spiked (single metal ion or mix metal ions). The spiked water samples were developed color under optimum conditions for quantitative analysis as described in section 3.11. The unknown solutions were then also determined the actual concentration by ICP-OES technique. The comparison results were reported in term of % error of both qualitative and quantitative analysis.

