1) Method for Crude Enzyme Extraction

Procedure
- Treat the whole cell pellets with lysozyme (10 μg/ml) for 5 min.
- Centrifuge at 10,000 g for 10 min and separate the supernatant.
- Take 600 μl of supernatant (cell free extract)
- Add: 100 mg μL⁻¹ Cr (VI) concentration in 1 μl system
- Add: 1 μl buffer and incubate for 20-30 min.
- Centrifuge it at 10,000 g for 10 min and separate the supernatant (Crude enzyme)
- Add: 200 μl 0.6 N H₂SO₄
- Add 580 μl H₂O and final volume make up to 980 μl
- Add 20 μl DPC reagent (100 mg/10 μL).
- Read optical density at 540 nm.

2) Hexavalent chromium Estimation

Reagents
1) 6 N H₂SO₄
   - Add 16.6 ml of the concentrated H₂SO₄ to 50-70 ml distilled water and make up the volume 100 ml using distilled water in volumetric flask.
2) Diphenyl Carbazide

0.25 g in 100 mL (1:1) acetone

Procedure

- Take 0.2 mL aliquote and make the total volume to 15 mL with distilled water.
- Add 0.5 ml 6 N H$_2$SO$_4$ and mix it well by vortexing.
- Now add freshly prepared 0.25% DPC (Diphenyl carbazide) and make the final volume 25 ml using distilled water.
- Take the optical density at 540 nm against blank.

3) Trivalent chromium Estimation

Oxidation method

Reagents

1) 1:1 H$_2$SO$_4$
2) 4 g/100 mL KMnO$_4$
3) 0.5 g/100 mL Sodium Azide

Procedure

- Take 1 mL test solution.
- Add few drops of methyl orange.
- Add concentrated liquor ammonia until it turns yellow.
- Add 18 N H$_2$SO$_4$ drop wise until it becomes acidic and finally add extra 1 mL in excess.
• Adjust final volume to 40 mL and heat to boil.

• Add 2 drops of 4% KMnO$_4$ to give dark red colour

• Boil 2 min more.

• Add 1 mL NaN$_3$ solution and continue boiling. If the colour does not fade, add 1 mL NaN$_3$ extra and then cool the solution

• Add 5 drops H$_3$PO$_4$ and then estimate Cr (VI).

Graph A 1. Chromium Standard
4) Selenium Estimation Method

Reagents

1) 0.1 N KMnO₄
   ➢ Dissolve 3.25 g KMnO₄ in 700-800 mL of distilled water and then make up the volume 1000 mL using distilled water in volumetric flask. Heat the solution to boil and filter the solution by glass wool after cooling it.

2) 0.1 N Ferrous ammonium sulphate
   ➢ Dissolve 3.9 g Ferrous ammonium sulphate in 70-80 ml distilled water and make the volume to 100 ml distilled water.

3) Ferroin indicator: 0.025 M
   ➢ Dissolve 1.485 g ortho phenanthroline monohydrate in 100 ml of 0.025 M Ferrous Sulphate.

Procedure

• The selenious acid or selenite corresponding to about 0.1 g of selenium is dissolved in 25 mL of 40% H₂SO₄ and diluted to 150 mL.

• Add 12 g of sodium phosphate

• Add 50 mL of standard 0.1 N potassium permanganate.

• Incubate for 20 min.

• Potassium permanganate is determined by the addition of slight excess of 0.1 N ferrous ammonium sulphate.
Appendix II

- Do the back titration with standard 0.1 N potassium permanganate.
- Add few drops of ferroin indicator for end point approach.
- Calculation: 1 mL 0.1 N KMnO₄ = 0.03948 g selenium.

5) Selenite Estimation Method

Reagents

1) Selenium Standard
   - Dissolve 2.190 g Sodium Selenite (Na₂SeO₃) in water in 10 ml HCl and dilute to 1 liter. 1.0 ml = 1.0 mg Se (IV)
2) NH₄OH - 50% v/v
3) Cyclohexane
4) 2, 3 Diaminonaphthalene (DAN) Solution
   - Dissolve 200 mg DAN in 200 mL 0.1 N HCl, shake for 5 min, extract three times with 25 mL portion at cyclohexane, retain aqueous phase and discard organic portions. Filter (Whatman 42) into dark containers.
5) Hydroxyl amine EDTA solution
   - Dissolve 4.5 g Na₂EDTA in 450 ml of water, now add 12.5 g hydroxyl/amine hydrochloride adjust volume to 500 ml with distilled water.

Procedure

- Add 2 mL hydroxyl amine EDTA in 10 mL sample.
Appendix II

- Adjust to pH 1.5 ± 0.3 with 0.1 N HCl and 50% NH₄OH.

- Add 5 mL DAN Solution.

- Incubate in water bath at 50°C for 30 min.

- Cool and add 4.0 mL cyclohexane.

- Cap the container securely and shake vigorously for 5 min.

- If the separation is slow, centrifuge for 5 min at 2000 rpm.

- Remove aqueous phase.

- Take organic phase to caped container.

- Take optical density at 480 nm.

Graph A 2. Selenium Standard
6) Mercury estimation

Reagents
1) HgCl₂ = 271.58
   0.1 M = 20.06 mg mL⁻¹
2) 0.001 M KI
   KI = 166.01 g (M.W)
   0.166 g / 1000 mL
   0.01 M KI
3) 0.001 M Malachite Green
   Malachite green = 346.5 g (M.W)
   0.346 g / 1000 mL = 0.001M
4) Acetate Buffer
   A = 0.2 M Acetic Acid
   11.55 mL Glacial Acetic Acid / 1000 mL
   B = 0.2 Sodium Acetate Solution
   16.4 g of Sodium Acetate / 1000 mL distilled water for working buffer solution
   A = 2 mL
   B = 48 mL
   Mix it and use

Procedure

- Take the aliquote and add 2 mL of KI solution (0.001 M)
- Add 2 mL of 0.001 M malachite green solution.
- Add 10 mL acetate buffer (pH 6.0)
- Add 15 mL benzene and shake vigorously for 5 min.
• Take organic phase and read the optical density 630 nm.

7) Copper estimation

Reagents
1) 25% citric acid
2) Diluted ammonia solution
3) 4% EDTA
4) 0.2% Sodium diethyldithiocarbamate (SDDC)
5) n-butyl acetate
6) 10% or 5% H$_2$SO$_4$
7) CuSO$_4$•5H$_2$O.

Procedure
• Take sample and add 10 ml distilled water.
• Add 5 ml 25% citric acid and add ammonia solution to set pH 8.5.
• Add 15 ml 4% EDTA and add 10 ml 0.2% SDDC.
• Give 90 strokes till brown colour develops.
• Add 10 ml of n-butyl acetate.
• Give 90 strokes and remove lower aqueous layer.
• Add 10 ml 10% H$_2$SO$_4$.
• Give 15 to 20 strokes and remove lower aqueous layer. Collect organic layer and measure optical density at 560nm.
8) Ferrous estimation

Reagents

1) Standard 0.1N K$_2$Cr$_2$O$_7$
   - Weight 4.9 g K$_2$Cr$_2$O$_7$ dissolve and make volume up to 1 liter by distilled water.

2) 1% Diphenylamine indicator (DPA)
   - Weight 1.0 g diphenyl amine indicator dye and dissolve in 100 mL of concentrated sulfuric acid.

3) Acid mixture
   - 900 mL distilled water + 100 mL concentrated H$_2$SO$_4$ + 50-60 mL orthophosphoric acid. Allow to cool.

4) 10% H$_2$SO$_4$ for adjusting the pH

5) 0.01N K$_2$Cr$_2$O$_7$
   - Dilute it from the standard 0.1N K$_2$Cr$_2$O$_7$ solution.

Procedure

- Take 1 mL sample
- Add 10 mL acid mixture
- Add two drops DPA indicator and titrate with 0.1 N K$_2$Cr$_2$O$_7$.
- Calculate soluble ferrous as 1 ml of K$_2$Cr$_2$O$_7$ = 0.005585 g Fe$^{2+}$. 