(1) **Determination of ferrous iron**

**Standard ferrous solution**
Weigh out accurately about 14.0g of ferrous sulphate crystals; dissolve in 450mL of 5% (w/v) sulphuric acid in a 500 mL volumetric flask and make up to the mark with distilled water. Shake well titrate 25.0 ml with the standard 0.1N potassium dichromate using diphenylamine as internal indicator.

**Standard 0.1N Potassium dichromate**
Weigh accurately 4.9g of potassium dichromate and transfer the salt quantitatively to a 1 L measuring flask. Dissolve salt in the flask in waste and make up to the mark, shake well. Calculate exact normality by dividing the actual weight of the potassium dichromate employed by the theoretical weight for 1 L of normal solution (49.035). An exactly 0.1N solution may be prepared by weighing out 4.904g of the salt and dissolving in water and diluting to 1L in a volumetric flask.

**Diphenylamine indicator**
Weigh 1.0g diphenylamine indicator and dissolve in 100mL of concentrated sulphuric acid.

**Procedure**
Use 3 drops of indicator, add 200ml of 25% (v/v) sulphuric acid followed by 5 ml of 85% orthophosphoric acid and titrate slowly, whilst stirring constantly with standard dichromate until the solution assumes a bluish green or greenish blue tint near the end point. Continue the titration, adding the dichromate solution drop wise and maintaining an interval of a few seconds between each drop, until the addition of one drop causes the formation of an intense purple or violet blue coloration, which remains permanent after shaking an is unaffected on the further addition of the dichromate. Carry out two or at the most three titrations, this should agree with in 0.1 mL.
Appendix II

Use of phosphoric acid lowers the oxidation potential of the ferric-ferrous system by forming complex Fe (HPO$_4^{2-}$) with the ferric ions so that the equivalence potentials coincides more nearly with that of the indicator.

The action of diphenylamine as an indicator depends upon its oxidation first into colorless diphenylbenzidine which is the real indicator and is reversibly further oxidized to diphenylbenzidine violet.

Diphenylbenzidine violet undergoes further oxidation if allowed to stand with excess of dichromate solution the excess of oxidation is irreversible and red or yellow products of unknown composition are produced.

1 ml 0.1 N K$_2$Cr$_2$O$_7$ $\rightarrow$ 5.585 mg Fe$^{+2}$

(2) Determination of total iron by 1,10-Phenanthroline spectrophotometric method

Reagents

Stock iron solution

Ferrous ammonium sulphate

Slowly add 20ml of concentrated H$_2$SO$_4$ to 50mL distilled water and dissolve 1.404g of ferrous ammonium sulphate (Fe(NH$_4$)$_2$(SO$_4$)$_2$6H$_2$O).

Add 0.1 N potassium permanganate drop wise until a faint pink color persist. Dilute to 100mL and mix.

1 ml = 200 $\mu$g Fe

Standard Iron solution

Pipette 50mL stock solution in to 1000mL volumetric flask and dilute to the mark with distilled water.

1 mL = 10$\mu$g Fe

Hydroxylamine solution

Dissolve 10g NH$_2$OH.HCl in 100ml distilled water
Ammonium acetate buffer
Dissolve 250g NH₄C₂H₃O₂ in 150 mL distilled water. Add 700mL concentrated (glacial) acetic acid. Final volume will be slightly more than 1000mL.

1,10-phenanthroline solution
Dissolve 100mg of 1,10-phenanthroline monohydrate C₁₂H₈N₂.H₂O in 100mL distilled water by stirring and heating to 80°C. Do not boil. Discard the solution if it darkness. Heating is unnecessary if 2 drops of concentrated HCl added.

Procedure
Dilute samples that contain iron in range of 20-200μg.
Add distilled water to make volume 33mL in 50mL volumetric flask.
Add 2mL concentrated HCl and 1ml NH₂OH.HCl solution.
Add glass beads heat it up to 15 minutes.
Cool to the room temperature and add 10mL NH₃C₃H₂O₂ buffer solution and 4mL phenanthroline solution and dilute to mark with distilled water.
Mix thoroughly and allow it at least 10-15 minutes for maximum color development.
Measure optical density v/s total iron (μg).

(3) Acidity

Reagents
Standard sodium hydroxide 0.02N
Dissolve 0.8g NaOH and dilute to 1000mL using CO₂ free distilled water store in air-tight, rubber Stoppard pyrex/coring glass bottle to protect from atmospheric CO₂. Standardize against 0.02N potassium biphthalate.

Phenolphathalein indicator
Dissolve 0.5g in 500ml 95% ethyl alcohol. Add 500mL distilled water. Add drop wise 0.02N NaOH till faint pink color appears.
Methyl orange indicator
Dissolve 0.5g and dilute to 1000mL with CO₂ free distilled water.

Procedure
Measure suitable volume of sample (50 or 100mL) in 250mL conical flask or beaker depending upon the method to be followed.
Add 2 drops of methyl orange and titrate with standard 0.02N NaOH till colour changes to faint orange characteristic of pH 4.4 to 4.3.
Note down the volume of NaOH required mark the reading as A.
Add 2-3 drops phenolphthalein indicator and continue titrating with NaOH till faint colour appears indicating pH-8.3.
Note down the volume of additional NaOH required mark the reading as B.

Calculations
Each mL of 0.02N NaOH = 1 mg CaCO₃ Therefore, acidity mineral or due to CO₂.

\[ \text{mg/1 CaCO}_3 = \frac{\text{mL 0.02N NaOH required \times 1000}}{\text{mL sample}} \]

In case if normality of NaOH is other than 0.02N calculate as follows.
Acidity mineral or due to CO₂ as mg/1 CaCO₃ = \( \frac{A/B \times N \times 50000}{\text{mL of sample}} \)

Where : 
A = mL NaOH required for sample to raise pH upto 4.4-4.3
B = mL NaOH required for sample to raise pH from 4.4 to 8.3
N = normality of NaOH used.

(4) Sulfate

Reagents
Conditioning reagents
Mix 50mL glycerol with a solution containing 30mL concentrated HCl, 300mL distilled water, 100mL 95% ethyl alcohol and 75g NaCl.
Barium Chloride
Crystals, 20-30 mesh.

Standard sulphate solution
Dissolve 147.9 mg anhydrous Na$_2$SO$_4$ and dilute to 1000mL.
1mL = 100µg SO$_4$

Procedure
Take suitable volume of sample in 250mL conical flask and dilute to 100mL.
Add 5mL conditioning reagent accurately, mix well.
Keep the flask constantly stirred with the help of stirrer. Add BaCl$_2$ crystals while stirring; continue stirring for 1 minute, after addition of BaCl$_2$.
Measure the turbidity developed after every 30sec for/minute on colorimeter at 420nm. After 2 minutes stirring reading will remain constant. Note this reading for calculation purpose.
Prepare standard curve by carrying standard sulfate solution through entire procedure.
Read mg SO$_4$ present in the sample from the standard curve.

Calculation

\[
\text{Mg/L SO}_4 = \frac{\text{mg SO}_4 \times 1000}{\text{mL sample}}
\]

(5) Determination of arsenic As (V) and As (III)

Material: 12 mL plastic tubes, micropipettes, 1 to 10 cm glass cuvette, spectrophotometer.

Detection range: 0.005 µmoles As and 0.05 µmoles As and 0.05 µmoles to 0.5 µmoles As when a 10 cm path length cuvette and a 1 cm path length cuvette are used, respectively. This corresponds to a lowest detection limit of 37.5 µg/L (10 cm path length cuvette).
Chemicals and solutions

Oxidizing reagents:
(1) KIO₃ unsaturated solution at room temperature. Dissolve 0.535 g KIO₃ in 50 mL dd H₂O
(2) HCl 1N : Dilute 100 ml HCL fuming (37%) in 0.9l dd H₂O.

Reducing reagents
(1) Dissolve 4.2 g Na₂S₂O₅, 5H₂O in 30 mL dd H₂O (0.74 M or 14%).
(2) Dissolve 0.42 g Na₂S₂O₃ in 30mL dd H₂O (0.056 M or 1.4%).
(3) Add slowly (exothermic) 18mL H₂SO₄ conc. (1.82 g/L) to 812 mL dd H₂O (=H₂SO₄ 3.5 N). Allow cooling and store in a glass bottle.

Mixed Reducing reagent: Mix slowly 5 mL H₂SO₄ 3.5 N in 10 mL Na₂S₂O₃ solution, avoiding excessive bubbling caused by the liberation of SO₂ (ventilation hood). Finally mix the 15 mL solution thus obtained with the 10mL of the Na₂S₂O₃ solution. Prepare freshly. Prepare freshly before use.
This provides enough reagents for about 25 assays and in enough for the determination of a set of 10 samples (only 1/3 of the assays has to be reduced).

Reagents for the Molybdenum complex
(1) Dissolve 15g (NH₄)₆ Mo₇O₂₄ - 4H₂O (ammonium paramolybdate) analytical quality in 500 mL dd H₂O. Store in a plastic bottle out of direct sunlight. The solution is stable in definitely.
(2) Add 140 mL of concentrated H₂SO₄, analytical grade, d=1.82, to 900 mL dd H₂O (=H₂SO₄ 2.6 M). Allow to cool and store in a glass bottle.
(3) Dissolve 27g of good quality ascorbic acid in 500 mL dd H₂O. Store the solution frozen in portions of 50 mL in plastic tubes at -20°C.
(4) Dissolve 0.34g of good quality potassium antimonyltartarate in 250mL of ddH₂O, warming if necessary. Store in a glass or plastic bottle. The solution is stable for many months.
**Mixed molybdenum complex reagent**
Mix together 15mL ammonium molybdate, 37.5 mL sulfuric acid, 15 mL ascorbic acid and 7.5 mL potassium antimonyl-tartarate solutions. Do not store for more than about 6h. This quantity is suitable for about 72 determinations comprising 12 standards and 60 essays (determination of 10 samples).

**Procedure**

1. Prepare 10 mL aliquots containing 0.05 μmoles (0.375 μg) to 0.5 μmoles (37.5 μg) As in 12 ml plastic tubes.
2. Into untreated aliquots add 1mL dd H₂O.
3. Into aliquots to be reduced pipet 1 mL of the mixed reducing reagent and mix well.
4. Into aliquots to be oxidized add 72.5 μL dd H₂O, 250 μL of HCl 1 N and then 25 μL of 50% saturated KIO₃ solution and mix well.
5. After 15 mins add 1 mL of the mixed molybdenum complex reagent to each aliquot and mix. It is important to mix the sampled thoroughly otherwise variability will occur in the absorbance.
6. Measure the absorbance at 865 nm in a 1 to 10 cm path length cuvette (depending on concentration of the samples to be determined) against air or water after 90min and within 3h after addition of the molybdenum reagent.

**Calibration curve** Complete aliquots containing 0, 10, 20, 40, 60, and 80 μL of a 5 mM As (V⁺) (samples containing 0.05 μmoles to 0.5 μmoles) or of a 0.5mM As (V⁺) (samples containing 0.005μmoles to 0.5μmoles As) solution to 11 mL with dd H₂O and add 1 mL of molybdenum reagent. Measure as described under point 6 of the procedure described above.

**Calculation** There is a linear relationship, y = ax + b, between absorbance (y) and quantity of As (V⁺) or phosphate (x) in the samples. Determine the parameters a and b for the calibration curve (for example with Excel and sigma plot) and calculate x for all absorbance values y measured for the samples. Calculate the As (V⁺) quantity using the relation As (V⁺) = (As (V⁺)
+ phosphate) - phosphate (untreated sample - reduced sample) and the As (III +) quantity by subtracting the value of the untreated samples from the value of the oxidized samples. Convert the quantity of As (V+) and As (III) into concentrations taking into account the dilution factor of the samples. Determine also the correlation factor $r^2$ of the calibration curve, which will indicate the precision of the measurements.

(6) Determination of protein with the Folin-Ciocalteau reagent

The phenol reagent of Folin and Ciocalteau which is essentially a phosphotungstic-phosphomolybdic acid solution is reduced by phenols to molybdenum which may be reduced the phenol reagent which may therefore be used for their determination.

**Standard**

Protein content from samples were determined using the following protocol with Bovine serum albumin as standard. A standard calibration curve in the range of 10-200 μg/mL was used.

**Reagents**

**Reagent I**

(1) 5% Na$_2$CO$_3$

(2) 0.5% CuSO$_4$·5H$_2$O in 1% sodium potassium tartrate

To 50mL of—(1) add 2mL of (2) prepare immediately before use and do not keep.

Dilute Folin-Ciocalteau reagent: Dilute it 1:1 to get 1N solution

**Procedure**

(1) Measure 0.5mL of washed cell suspension (ca. 100 μg of wt. of cell)

(2) To this add 0.5mL of 1.0 N NaOH place in a boiling water for 5 minutes and cool in cold water.

(3) Add 0.5mL of Folin-Ciocalteau reagent.

(4) After standing 30 minutes to allow full colour development. Measure O.D. at 750 nm.
Appendix II

(7) **Total arsenic was analysed by using atomic absorption spectrophotometer model ELICO-194 by standard procedure**

Standard solution preparation (1000 ppm).
Dissolve 1.3203g of dried As$_2$O$_3$ in the minimum volume of 20% NaOH solution practicable and neutralize with nitric acid dilute to 1 L.

Atomic absorption: Lamp Current – 8.0 mA
Flame type – Nitrous oxide-Acetylene
(Stoichiometric)

Flame emission: Arsenic is not normally determined by emission

(8) **Bacterial genomic DNA isolation using CTAB**

(1) Grow the cells overnight in LB broth.
(2) Take 1.5mL of cell is microfuge or centrifuge at 10,000 rpm for 5 minutes.
(3) Resuspend cells in TE buffer adjust OD$_{600}$ approximately 1.0.
(4) Add 20μL of lysozyme (conc. 100mg/mL). Mix well.
(5) Incubate for 5 minutes at room temperature.
(6) Add 40μL of 10% SDS. Mix well.
(7) Add 8μL of proteinase K (10mg/mL)
(8) Incubate for 1 h at 37°C.
(9) Add 100μL of 5N NaCl. Mix well.
(10) Add 100μL CTAB/NaCl (heated to 65°C) for 10 min.
(11) Incubate at 65°C for 10 minutes
(12) Add 0.5mL of chloroform : isoamylalcohol (24:1). Mix well.
(13) Spin at max speed for 10 min at room temperature.
(14) Transfer aqueous phase to clean eppendorf (should not be viscous).
(15) Add 0.5mL of phenol : chloroform : isoamylalcohol (25:24:1). Mix well.
(16) Spin at maximum speed for 10 min at room temperature.
(17) Transfer aqueous phase and add 0.6 vol isopropanol (-20°C).
(18) Incubate at room temperature for 30 min.
(19) Spin at maximum speed for 15 minutes.
(20) Wash pellet with 70% ethanol, spin at max speed for 5 min.
(21) Discard the supernatant and let pellet dry for 5 - 10 min at room temperature.
(22) Resuspend in 20μL TE plus RNAs (99μL TE + 1μL RNAs (10 mg/mL)).
(23) Transfer to sterile microcentrifuge tubes.
(24) Incubate at 37°C for 20 min.
(25) Run 1 μL in a 1% agarose gel with concentration standards.

(9) Method for plasmid isolation (mini-prep) using alkali lysis method

(saunders, S.E. and J. F. Burke)

Reagents
Solutions should be made with high quality water, such as MiliQ water and all solution should be autoclaved as indicated before use to inactivate any DNAses if present.

Solution I:
5 mL of 1M glucose stock
2.5 mL of a 1M Tris-HCl, pH 8 stock at pH 8
2 mL of 0.5M EDTA stock to 91.5 mL autoclaved dH2O

Solution II:
0.9 g NaOH to 90 mL dH2O autoclave and then add 10 mL of 10% SDS solution

Solution III:
Dissolve 29.45 g potassium acetate in50mL dH2O
add 11.438 mL glacial acetic acid
make up to 100mL with dH2O and autoclave

Procedure
(1) Resuspend the pellet from 1.5 mL active culture grown overnight on LB in 100 μL solution I. If using autotrophic microbes, increase the culture volume 50 to 100 fold.
(2) Add 200 μL of solution II and gently mix and swirl to ensure complete lysis. Do not mix too vigorously otherwise chromosomal DNA will contaminate the preparation.

(3) Add 150 μL of solution III and immediately mix contents completely. Do not mix too vigorously otherwise chromosomal DNA will contaminate the preparation.

(4) Centrifuge for 5 min in a microfuge.

(5) Transfer 380 μL of the supernatant to a clean microfuge tube and add 720 μL isopropanol.

(6) Centrifuge for 15 min in a microfuge.

(7) Carefully remove the supernatant leaving the pellet at the bottom of the tube.

(8) Wash the pellet with 1mL of ice-cold 70% ethanol. Centrifuge for 5 min at 12,000 g and remove the supernatant. Be very careful here as it is easy to lose the pellet.

(9) Dry the pellet and then resuspend it in 49 μL of dH2O and add 1 μL of RNase 1 μL of this should be plenty for agarose gel analysis and for restriction enzyme analysis.