CHAPTER II

ANALYTICAL PROCEDURE

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The various methods of analysis and estimations adopted during the course of the present work, have been described in the following pages. The values have been calculated on over-dried basis.

2.1 Estimation of total organic carbon:

In the estimation of total organic carbon in soil samples or other carbonaceous materials, the method of Robinson, McLean and Williams (1929) was followed.

Five grams of well-dried and powdered soil were taken in a 500 ml. Kjeldahl flask. To this, a few crystals of copper sulphate (catalyst), followed by 5 grams of fused potassium sulphate (to raise the boiling temperature of sulphuric acid) were added. This flask was connected to three conical flasks, the first containing most of the standard iodine solution, the second containing a few ml. of standard iodine solution, while the third containing pure potassium iodide solution only. The last flask containing potassium iodide solution was connected to an efficient filter pump. A current of air purified by passing it through a solution of ferrous sulphate and then through concentrated sulphuric acid, was aspirated through the system, so that it passed through sulphuric acid, ferrous sulphate, Kjeldahl flask standard iodine in both the conical flasks and lastly through potassium iodide. The Kjeldahl flask was heated over direct Bunsen flame for 4-5 hours.

The sulphur dioxide evolved according to the equation:
\[ C + 2\text{H}_2\text{O}_2 \rightarrow CO_2 + 2\text{H}_2\text{O} \quad \text{(1)} \]

was carried along with the air current into the iodine solution in the first and then in the second conical flask, where the reaction was completed, as

\[ \text{SO}_2 + I_2 + 2\text{H}_2\text{O} \rightarrow I_2 \text{SO}_4 + 2\text{HI} \quad \text{(2)} \]

Any iodine vapor from the iodine solution going with the air current were trapped by potassium iodide in the third conical flask.

The excess of iodine left over, after the reaction (3) was titrated against a standard sodium thiosulphate solution. The value of iodine used up during these reactions and the amount of carbon present originally can be calculated as under:

It follows from equations (1) and (2) that one atom of C = 2 SO₂ = 2 I₂

or, 12 gms. of carbon by weight = 508 gms. of iodine by weight, i.e., 3 gms. of carbon = 127 gms. of iodine.

The values obtained by this method are believed to record a little less carbon than is actually present in the system. However, since, this method is employed in the first as well as in the subsequent determinations the results obtained are comparatively.

2.2. Estimation of total nitrogen:

The total nitrogen present in the system, was estimated by salicylic acid reduction method. (1947)
Ten grams of well dried and powdered soil were taken in a 500 ml. Kjeldahl flask and 30 - 35 ml. of concentrated sulphuric acid in which one gram of salicylic acid has been dissolved, were added to it. The acid was allowed to react with the soil in the cold for 30 minutes with occasional shaking of the Kjeldahl flask so that the nitrate may combine with salicylic acid according to the following chemical reaction:

$$2 \text{KNO}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{K}_2\text{SO}_4 + 2 \text{HNO}_3$$

$$\text{H}_2\text{SO}_4 + \text{HNO}_3 \rightarrow \text{C}_6\text{H}_5\text{COCOOH} + \text{HO}-\text{C}_6\text{H}_5\text{H}_2\text{SO}_4 + \text{H}_2\text{O}.$$ 

5 grams of sodium thiosulphate were added in order to reduce the nitro group to form amino-salicylic acid as shown below:

$$\text{Na}_2\text{S}_2\text{O}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{Na}_2\text{SO}_4 + \text{H}_2\text{S}_3\text{O}_7 + \text{S}$$

$$3\text{H}_2\text{SO}_4 + \text{NO}_2\cdot\text{C}_6\text{H}_5\text{COOH} + \text{H}_2\text{O} \rightarrow 3\text{H}_2\text{SO}_4 + \text{NO}_2\cdot\text{C}_6\text{H}_5\text{H}_2\text{COOH}.$$  

When the foaming and the colour due to charred carbon had disappeared, a pun. of potassium sulphate and a few crystals of anhydrous copper sulphate were added, followed by a regular shaking, in order to ensure complete reduction of nitrate. The digestion was completed as in the ordinary Kjeldahl method by heating it for another three to four hours in a fuming chamber. After cooling, the mixture was diluted with about 100 ml. of distilled water and boiled for a few minutes, in order to break up any coherent like material that might have been formed in this process. This solution containing all nitrogen as ammonium sulphate was filtered, washed and made up to a definite volume. An aliquot part of this solution was distilled with about 100 ml. of 40%
solution of sodium hydroxide, till one third of the liquid had passed over.

The issuing gas (as shown by the following reaction), was received in 25 ml. of 0.1 N hydrochloric acid,

\[(\text{NH}_4)_2 \text{SO}_4 + 2 \text{NaOH} \rightarrow 2\text{NH}_3 + 2 \text{H}_2\text{O} + \text{Na}_2 \text{SO}_4\]

containing a few drops of methyl red. The excess of acid after the reaction was titrated against 0.1 N sodium hydroxide (which was standardized against 0.1 N oxalic acid).

A blank experiment was carried out exactly in the same manner using 0.2 gm. of cane sugar in place of soil, so as to allow corrections for any nitrogen contained in the reagents.

The percentage of nitrogen in the soil was calculated as \((B - T) \times N \times 0.14\), where \(B = \) blank titration, in ml. of standard alkali, \(T = \) actual titration in ml. of standard alkali, and \(N = \) normality of the standard alkali.

2.3 Estimation of Ammonia (1924).

Twenty-five grams of soil from sampling post or from field, were directly treated with 100 ml. of a normal sodium chloride solution. The contents were stirred well and allowed to stand for half an hour. The supernatant liquid was decanted and filtered off through a Whatman No. 44 filter paper, fitted in a Buchner funnel. After a second washing with normal sodium chloride solution, the whole mass of soil, was transferred to the filter paper.
The leaching was continued till the filterate collected, approximated 500 ml.

Three to four grams of magnesium was added to the filterate, which was then connected to the ammonia distillation apparatus. It was heated gently so that a volume of 150 - 200 ml. distilled over in half to three quarters of an hour. The distillate was collected in 20 ml. of 0.02 N hydrochloric acid. The excess of the acid was titrated with 0.02 N sodium hydroxide, using methyl red as indicator solution. A blank determination was carried out using all the reagents.

The percentage of ammonical nitrogen corresponds to:
\[
\frac{(B - T) \times N \times 1.4}{W}, \text{ where}
\]

\[B = \text{volume of standard alkali used in blank titration},\]
\[T = \text{volume of standard alkali used in actual titration},\]
\[N = \text{normality of alkali used in titration},\]
\[W = \text{weight of the soil taken}.\]

2.4 Estimation of nitrates (1950).

Twenty-five grams of the soil sample, in small lumps (from sampling pots or from fields) was directly weighed and kept in an oven at 550°C for 12 - 16 hours, so that the soil could be leached with the minimum puddling. This was transferred to a 9 cm. Buchner funnel, fitted with a Whatman No. 50 filter paper, and sufficient distilled water containing 10 drops of concentrated sulphuric acid was poured to cover the soil. After a few minutes soaking,
this was connected to the filter pump and the leaching of the soil was continued with successive quantities of distilled water until the filtrate amounted to about 600 ml. The filtrate was transferred to a one litre Erlenmeyer flask, and one gram of magnesium oxide was added. The solution was then evaporated, in order to expel ammonia leached from the soil, until the volume was reduced to about 200 ml. It was allowed to cool and then 5 grams of zinc dust, 70 ml. of 30% sodium hydroxide and 5 grams of powdered iron were added in the above order. The flask was then connected to the condenser of the nitrogen distillation apparatus through an efficient splash trap. The reduction was done in cold for half an hour and then over a very small flame while the ammonia was boiled off in the third period in approximately half an hour. The ammonia was collected in 20 ml. of 0.02 N hydrochloric acid, containing methyl red. The excess of the acid was titrated with 0.02 N 3 sodium hydroxide.

In all the experiments, determinations with blank sets using all the reagents in the same quantities as those used in actual nitrate determination, were carried out.

The percentage of nitrate-nitrogen corresponds to, \((B - T) \times N \times \frac{1.4}{W}\), where
B = blank titration in ml. of standard alkali,
T = actual titration in ml. of standard alkali,
W = normality of standard alkali,
W = weight of the soil taken.

Determination of conductivity, pH and water soluble salts:—

The soil suspension was prepared by adding 100 ml. of conductivity water to 20 grams of air-dried soil (soil : water ratio 1 : 5 ) in a hard glass bottle, as recommended by C.S.I.R. Division of Soils (1950) and shaking mechanically for one hour.

Conductivity:— The conductivities were determined by using Doran's Conductivity Bridge. All measurements were carried out in an electrically maintained thermostat kept as 30 ± 0.05° C. The cell constant was determined using N/100 KCl and the specific conductivities of all suspensions were calculated by multiplying the observed conductivity by cell constant.

pH:— The pH values of the suspensions were measured by Beckman pH meter with glass electrode and calomel as reference electrode.

Soluble salts:— After the soil particles in the extract had settled, the supernatant liquid was filtered through Whatman No. 44 filter paper. The filtration flask was rinsed with the first portion of the filtrate. The filtration was completed with a very light suction, and with due precaution not to allow any colloidal matter to reach the filter paper, since it was likely to pass in the filtrate.
The aliquot portions of this filtrate were taken for the determination of total soluble salts, carbonates, bicarbonates, sulphates, chlorides.

Total soluble salts:

100 ml. of the clear filtrate were pipetted out in a clear platinum basin and evaporated over a water bath. When the volume was reduced to about 5 ml., 2 ml. of 20 volume hydrogen peroxide (free from salts), were added in order to oxidise soluble organic matter. It was then evaporated to dryness and was put in an electric oven at 110°C for an hour, cooled in a dessicator and weighed. The value for the percentage of total soluble salts in the soil corresponds to the weight of soluble salts x 5.

Carbonates and bicarbonates:

To 100 ml. of the soil extract, in a porcelain basin, 2-3 drops of phenolphthalein were added. This was titrated with 0.1 N hydrochloric acid until the red colour showing the presence of carbonates, was just discharged. This corresponded to the neutralization of carbonates to the bicarbonate stage. To the solution, 2 drops of dimethyl yellow indicator were added, and titration was continued, till the solution just changed from yellow to red. The bicarbonates were completely neutralised at this stage.
The calculations used are as follows:

\[ \% \text{ CO}_3 = v_1 \times 0.0066 \times \frac{1000 \text{ ml.}}{100 \text{ ml.}} \times \frac{100 \text{ gs.}}{200 \text{ gs.}} = v_1 \times 0.030 \]

\[ \% \text{ HCO}_3^- = (v_2 - v_1) \times 0.00061 \times \frac{1000 \text{ ml.}}{100 \text{ ml.}} \times \frac{100 \text{ gs.}}{200 \text{ gs.}} = (v_2 - v_1) \times 0.0305\]

Where,

\( v_1 \) = volume in ml. of 0.1 N acid used in first titration
with phenolphthalein.

\( v_2 \) = volume in ml. of 0.1 N acid used in second titration
with dimethyl yellow.

**Chlorides:**

25-50 ml. of clear soil extract were taken in a porcelain basin and 3-4 drops of 1/4 solution of potassium chromate (indicator) were added to it. This was titrated with a 

N silver nitrate solution till all chlorides precipitated and the first faint tint of red silver chromate persisted.

A second basin containing the same volume of water as the soil extract with 3-4 drops of potassium chromate solution was used as standard for comparison.

The percentage of chlorine in the original sample corresponds to,
volume of silver nitrate used \[ \frac{1000}{1,000} \times \frac{100}{200} \]

\[ = \frac{volume of silver nitrate}{2 \times volume of extract taken} \]

25-50 ml. of soil extract were taken in a beaker and were made just acidic to dimethyl yellow, with dilute hydrochloric acid. A slight excess of 5% solution of barium chloride were added to the boiling solution, along the sides of the beaker. It was boiled, stirred cautiously avoiding scratches on the walls of the beaker, boiled again and allowed to stand overnight. It was filtered through a sintered glass crucible No. 4, and washed with water (hot) till free from chloride ions. The precipitate was dried, cooled and weighed.

\[ \frac{A 304}{233} = \frac{weight of the precipitate}{volume of soil extract} \times \frac{1000}{206} \]

\[ \frac{A 26}{200} = \frac{weight of the precipitate}{volume of soil extract} \times \frac{1000}{206} \]

2.6 Analysis of mineral constituents of soil

The methods, for the analysis of the mineral constituents present in the soil, as recommended by C.H. Wright (1934) and those by C.S. Piper (1950) and by the
Association of official agricultural chemists (1954), were adopted. The analysis was carried out in duplicate and repeated, till concordant values were obtained.

**2.61 - Moisture content:**

Ten grams of the soil were transferred to a weighed silica basin, and placed in an electric oven at 100 - 105°C for 12 - 16 hours for drying, after which, it was cooled and weighed. The percentage of the loss in the soil sample = loss in the weight of the soil sample X 10, and this was the percentage of moisture content.

**2.62 - Loss on ignition:**

The above soil sample, after the determination of moisture content was ignited for 4 - 6 hours, till the colour of the soil turned reddish-brown or greyish-brown. It was then removed to a desiccator, cooled and weighed. This was repeated, till a constant weight was obtained.

Percentage loss on ignition = loss in the weight of soil X 10

**2.7 - Preparation of hydrochloric acid extract:**

The hydrochloric acid extract employed for soil analysis, was prepared by the method given by the Agricultural Education Association (1931).
The soil left after the determination of loss on ignition, was digested with 100 ml. of concentrated hydrochloric acid (B.P. 110°C and S.G. 1.10) on a sand bath in a pyrex beaker. After cooling the contents, 100 ml. of distilled water were added to it. This was filtered through a Whatman No. 42 filter paper. The filtrate together with the washings (to free the residue from chloride ions) was collected in a 500 ml. graduated flask.

The insoluble portion left on the filter paper, was silica, which was dried and weighed.

The percentage of silica in the sample = weight of silica \times \frac{10}{\text{sample weight}}

The filtrate was made up to 500 ml. and was used for the estimations of sesquioxides, iron oxide, calcium oxide, magnesium oxide, phosphorus pentoxide and the oxide of potassium according to the following methods:

**Sesquioxides:**

100 ml. of the hydrochloric acid extract were taken in a pyrex beaker and 2 grams of ammonium chloride were added to it. The sesquioxides were precipitated by the addition of cold dilute ammonium hydroxide (1:1). The beaker was now heated to boiling over a sand bath for an hour and filtered hot. The precipitate was washed with hot water till free from chloride ions.
The filtrate along with the washings was collected in a flask for subsequent analysis. The precipitate on the filter paper was dried, ignition, cooled and weighed to a constant weight and calculated as under:

\[
\text{Percentage of sesquioxides} = \frac{\text{weight of sesquioxides}}{\text{weight of precipitate}} \times 10 \times 5.
\]

2.72 - Iron oxide (Fe₂O₃):

100 ml. of the hydrochloric acid extract was taken and the sesquioxides were precipitated as given above. The precipitate after being washed free from chloride ions was transferred to a conical flask of 500 ml. 30 ml. of 30\% sulphuric acid and 0.5 gram of zinc dust were added to it and the flask was kept overnight, after corroding it with a Bunsen valve, to reduce all ferric to ferrous state. It was filtered through glass wool and titrated against N/10 potassium permanganate solution. The calculations were made as follows:

\[
\text{Percentage of iron oxide} = \frac{\text{No. of ml. of N/10 KMnO₄ used}}{\text{x} \times 0.005}.
\]

2.73 - Calcium oxide (CaO):

The filtrate obtained from the sesquioxides precipitation was reduced in volume to 100 ml. and made alkaline by adding a dilute solution of ammonium hydroxide. The solution was boiled and calcium precipitated by the addition to it of a slight excess of a warm saturated solution of ammonium oxalate. The contents of the beaker were stirred, boiled and kept for 4 – 6 hours. The clear solution at the top was decanted on a Whatman
No. 44 filter paper and the precipitate was washed with hot water till free from oxalate ions. The precipitate on the filter paper as well as in the beaker was then dissolved in warm dilute hydrochloric acid (1 : 4). The calcium was reprecipitated by boiling the solution and by adding a little ammonium hydroxide and then ammonium oxalate. The precipitate was allowed to stand overnight and was filtered through the same filter paper and made free from oxalate ions by washing it with hot water. The filtrate and the washings were collected in the same beaker containing the first filtrate of the previous precipitation for the estimation of magnesium. The precipitate of calcium oxalate was dissolved in 30 ml. of 30% sulphuric acid and the solution after being washed was titrated against N/10 KMnO₄. The calcium oxide was calculated as under:

Percentage of CaO = No. of ml. of N/10 KMnO₄ used × 5 × 10 × 0.0023.

3.74 Magnesium oxide ( MgO ) :

The volume of the filtrate left after the precipitation of calcium was reduced to 50 ml. To it were added 5 ml. of strong hydrochloric acid and a drop of methyl orange. The acid was just neutralized with a dilute solution of ammonium hydroxide. 10 ml. of a saturated solution of sodium dihydrogen phosphate were
added to the solution. It was stirred and made alkaline by adding ammonium hydroxide in excess and the precipitate thus obtained was kept for nearly four hours. It was filtered through a Whatman No. 44 filter paper and made free from phosphate by washing it with dilute ammonium hydroxide solution. The precipitate of magnesium ammonium phosphate was dissolved in 10 ml. of nitric acid (1:4) and reprecipitated as given above and kept overnight. The precipitate was washed, dried, ignited and estimated gravimetrically as \( \text{Mg}_2 \text{P}_2 \text{O}_7 \).

The magnesium oxide in the sample was calculated as under:

\[
\text{Percentage of magnesium oxide} = \frac{\text{weight of the precipitate}}{5 \times 10 \times 0.36213}.
\]

Phosphoric acid (\( \text{P}_2 \text{O}_5 \));

50 ml. of the hydrochloric acid extract were taken out in a beaker and evaporated to dryness on a sand bath. 5 ml. of concentrated nitric acid were added to it after cooling. It was then heated and 10 ml. of distilled water was added. The phosphate was precipitated by adding to it freshly prepared ammonium molybdate solution. The precipitate was left overnight. It was filtered, washed with 1% nitric acid and finally with 3% potassium nitrate.
solution till it was free from acid. The acid free precipitate was transferred to the same beaker and 10 ml. of standard N/10 sodium hydroxide were added and the excess of sodium hydroxide left unused was titrated against N/10 nitric acid using phenolphthalein as an indicator. The sodium hydroxide solution was standardized by an oxalic acid solution of known strength and the results were calculated as follows:

Percentage of $P_2O_5$ in sample = \( \frac{\text{No. of ml. of N/10 NaOH used}}{10 \times 10 \times 0.000309} \).

Potassium oxide (K$_2$O):

50 ml. of hydrochloric acid extract were evaporated in the same way as in the case of phosphorous pentoxide determination. 1.5 ml. of glacial acetic acid were added to it after cooling. The mixture was stirred and 10 ml. of a saturated solution of sodium chloride and 5 ml. of 35% sodium nitrite were added to it. Potassium was then precipitated as cobaltinitrite compound by vigorously stirring and adding 5 ml. of cobalt nitrate (20%) solution to it. This was left overnight after stirring. The precipitate was filtered through a Gooch crucible and washed with cobalt nitrate solution. The precipitate together with the crucible was placed in a beaker containing a known volume of N/10 KMnO$_4$. 

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solution to which 30% sulphuric acid was added. The beaker
was heated on a sand bath to boiling and while hot a
little more than the required quantity of N/10 oxalic
acid was added till the colour discharged. The excess
of oxalic acid was titrated back against N/10 KMnO₄
solution and the result was calculated as follows:

Percentage of K₂O = No. of ml. of N/10 KMnO₄ used
   \[ \times 10 \times 10 \times 0.000856. \]
0.1 g of the air-dried soil was carefully weighed and transferred to sterilised boiling test-tube containing 10 ml of autoclaved tap water under aseptic conditions. The test-tube was shaken for about five minutes and then allowed to stand for about a minute. A series of sterilised dilution tubes containing 9 ml of autoclaved tap water were taken and 1 ml of supernatant liquid from the original tube was transferred by a sterile 1 ml pipette to the first dilution tube with proper precautions and under aseptic conditions and the mixture in the first dilution tube was well shaken. Further dilutions were carried out in the same way up to the limit which was found by experience, until the 1 ml of mixture in the last dilution tube, when plated, allowed the growth of a convenient number of colonies of the azotobacter, which could be counted. Platings were done by transferring 1 ml of the suspension from the final dilution tube to sterilised petri-dishes and pouring out about 10 ml of melted and sterilised Beijerinck's medium (prepared as described below). The petri-dishes were shaken by giving them a rotatory motion.
to make the mixture homogenous and to spread it evenly on the surface of the dishes so as to ensure an even distribution of colonies all over the surface of the petri-dishes. The plates were now left on a level surface till the medium solidified. The plates were then incubated at 30°C in an inverted position for about seven days and the number of colonies of the Azotobacter that developed were counted. The count included only greyish raised round colonies, semi-transparent with whitish centres and also deep white colonies elliptical or spindle shaped. An average of four plates has been taken.

The following medium was prepared and sterilises at 15 lbs. pressure for 20 minutes in an autoclave.

Tap water 1 litre
Mannite 20 g
Dipotassium phosphate 0.2 g
Agar 20 g

Ten ml. portions of the above melted medium were taken in clean boiling test tubes and the tubes plugged with sterile cotton wool and sterilised in the autoclave at 15 lbs. pressure for 20 minutes.
These tubes were heated in a water bath till the medium melted. The water bath was then allowed to cool down to a temperature of 40°C and one tube was poured in to each petri-dish at the time of plating, leaving behind the sediment in the test tube so that a clear medium would set in the plate.

In all cases where Azotobacter counts were made the moisture contents of the samples taken, were also determined. From the amount of moisture present in a given sample, the number of Azotobacter were calculated on dry basis.

2.9 **CALCULATION OF EFFICIENCY OF NITROGEN FIXATION**

Dhar while explaining photo-synthesis in plants, postulated that the important photochemical reaction is the decomposition of water by absorption of light according to the following equation:

\[ \text{H}_2\text{O} + \text{112 K Cal} = \text{H} + \text{OH} \]

For fixing 14 g of nitrogen and forming ammonia by the interaction of molecular nitrogen and atomic hydrogen obtained by the decomposition of water as stated above, 336 K Cal are needed. Hence, from the oxidation of one gram mole of glucose according to the equation:
C₆H₁₂O₆ + 6 O₂ = 6 CO₂ + 6 H₂O + 678 K cal.

Under ideal conditions, \( \frac{14 \times 678}{226} \) g of nitrogen can be fixed. In other words, 0.39 g of nitrogen should be fixed per gram of carbon oxidised. Therefore, it 150 mg of nitrogen is fixed per gram of carbon oxidised the efficiency would be \( \frac{150 \times 100}{390} = 38\% \).

Similar method for the calculation of efficiency was also used by Dhar and Gupta (1961).
Soil Sampling:

The soil samples were taken up to a depth of nine inches. Large lumps of the earth were broken and spread over a clean portion of cemented floor and were allowed to be air-dried for a week. The soil was turned over daily during the period it was being dried. The dried soil was then crushed, powdered and passed through a 80 mesh sieve and then used for these experiments.

The following procedure was adopted for analysing the soil samples from the jars.

The well mixed soil was taken out from the jars in a big agate pestle and mortar and was crushed thoroughly to obtain a homogeneous mixture. An aliquot sample was thereafter taken out of it and dried in an electric oven at 100 to 105°C. for five hours and kept overnight in a dessicator. A portion of it then used for estimations after weighing.
3.0 REFERENCES


7. Piper, C.S. 1950 Ibid.


10. Wright, C.J. 1934 "Soil Analysis"