Major Analytical Techniques in Oilseeds

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LIST OF ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µl	Microlitre
EDTA	Ethylene diamine tetra acetic acid
g	Gram
hr	Hour
l	Litre
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimeter
mM	Millimolar
M	Molar
RT	Room temperature
OD	Optical density
Conc.	Concentrated
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
max.	Maximum
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
FRAP	Ferric reducing activity power
Trolox	6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
MeOH	Methanol
DPPH	1,1-diphenyl-2-picryl hydrazyl radical
DM	Dry plant material
DW	Distilled water

Oil seeds occupy very prominent and important position in the Indian dietary. In India, one fourth of the population is suffering for good quality of food. They are not getting proper nutrition and are deficient in protective food stuff. There is greater prevalence of deficiency for diseases related with vitamins, proteins and fats among the low income group. In addition to this, the problem is further aggravated by the fact that the majority of the people are not aware about the desirable food stuff to meet the dietary requirements. Therefore, efficient strategy for diet should be developed for the well being of the poor population. However, oil seeds contain high fat, proteins and carbohydrate contents, beside a substantial calorific value due to significant amount of lipids and carbohydrate, which can serve as potential source of nutrient for quality food.

Carbohydrates are widely distributed in both plant and animal tissues. They are indispensible for living organisms and serve as skeletal structures in plants and also in insects and crustaceans. They occur as food reserves in the storage organs of plants and animals. They are the important source of energy required for the various metabolic activities of the living organisms.

CARBOHYDRATE

(1) Determination of total carbohydrate by Anthrone method- Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugar and polysaccharides. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into more simple sugars. The carbohydrate content can be measured by hydrolyzing the polysaccharides into simple sugars by acid hydrolysis and estimating the resultant monosaccharides.

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

Materials-

- 2.5 N HCl
- Anthrone Reagent: Disslove 200 mg anthrone in 100 ml of ice cold 95% H_2SO_4. Prepare fresh before use.
- Stock- Dissolve 100 mg in 100ml water. Working standard- 10 ml of stock diluted to 100 ml with distilled water. Store in cold after adding a few drops of toluene.

Procedure-

- Weigh 100 mg of the sample into a boiling tube.

- Hydrolyse by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cool to room temperature.
- Neutralise it with solid sodium carbonate until the effervescence reduces.
- Make the volume to 100 ml and centrifuge.
- Collect the supernatant and take 0.5 and 1.0 ml aliquot for analysis.
- Prepare the standard by taking 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard. '0'serve as blank.
- Make the volume to 1.0 ml in all the tube including the sample tubes by adding distilled water.
- Then add 4 ml of anthrone reagent.
- Heat for 10 min in a boiling water bath.
- Cool rapidly and read the green to dark green colour at 630 nm.
- Draw a standard graph by plotting concentration of the standard on the X axis versus absorbance on the Y axis.
- From the graph calculate the amount of carbohydrate present in the sample tube.

Calculation- Amount of carbohydrate present in 100 mg of the sample

$$= \frac{mg\ of\ glucose}{Volume\ of\ test\ sample} \times 100$$

(II) Determination of total soluble carbohydrate- Total soluble carbohydrate can be estimated by the method of Yemm and Willis (1954).

Anthron Reagent-

- 0.4 g anthrone in 100 ml concentrated sulphuric acid.

Procedure-

- Extract the sample in 80% ethanol as in case of free amino acids.
- Evaporated the known amount of ethanol extract to dryness in a test tube After cooling.
- Dissolve residue in one ml of distilled water.
- Add 4 ml anthron reagent and heated in a water bath at 100^0C for 10 minutes.
- After Cooling
- O.D. is measured at 620 nm.
- Standard curve is prepared using the graded concentration of glucose.

Sugars:

Sugar is estimated following the method of Dubois et al., (1951).

Extract Preparation- Grind 100 mg dried plant sample with 50% ethyl alcohol (10ml). The homogenate is centrifuged at 4000 rpm for 15 minutes and use the supernatant for the estimation of sugar.

Anthrone reagent : It is prepared by dissolving 200 mg anthrone dye in 100 ml concentrated H_2SO_4.

Procedure-

- Take 0.1 ml ethanol extract in a test tube and dried it in water bath.
- Take 1 ml suspended in test tube.
- To each tube 4 ml anthrone reagent is added by the side of test tube, mixed rapidly with care.
- The tubes are kept in a boiling water bath for 10 minutes and cooled the tubes at room temperature and the intensity of its colour is read at 620 nm.
- Sugar estimation can be done by the same formula as in carbohydrate estimation.

(2) **LIPIDS**

Lipids are the heterogenous group of compounds, actually or potentially related to fatty acids. Chemically, they can be defined as esters of fatty acids with alcohol. Lipids are relatively insoluble in water and soluble in solvents like ether, chloroform and benzene.

Estimation of Oil in Oilseeds- Fats are fatty acid esters of glycerol. Fat as liquid is called oil. Seeds like mustard, groundnut, castor, sunflower etc contain oil as reserve food material for the embryo.

Principle- Oil from a known quantity of the seed is extracted with petroleum ether. It is then distilled off completely, dried, the oil weighed and the % oil is calculated.

Materials-

- Petroleum Ether (40-160^{0}C)
- Whatman No. 2 Filter Paper
- Absorbant Cotton
- Soxhlet Apparatus

Procedure-

- Fold a piece of filter paper in such a way to hold the seed meal. Wrap around a second filter paper which is left open at the top like a thimble. A piece of cotton wood is placed at the top to eventually distribute the solvent as it drops on the sample during extraction.
- Place the sample packet in the butt tubes of the Soxhlet extraction apparatus.
- Extract with petroleum ether (150 drops/min) for 6 hrs. without interruptions by gentle heating.

- Allow to cool and dismantle the extraction flask. Evaporate the ether on a steam or water bath until no odour of ether remains. Cool at room temperature.
- Carefully remove the dirt or moisture outside the flask and weigh the flask. Repeat heating until constant weight is recorded.

Calculation-

$$Oil\ in\ ground\ sample(\%) = \frac{Weight\ of\ oil\ (g)}{Weight\ of\ sample(g)} \times 100$$

$$Oil\ to\ dry\ weight\ basis = \frac{Oil(\%) in\ ground\ sample}{100\%\ moisture\ in\ whole\ seed}$$

(3) **PROTEINS AND AMINO ACIDS**

Proteins are complex, organic compounds composed of many amino acids linked together through peptide bonds and cross-linked between chains by sulfhydryl bonds, hydrogen bonds and van der Waals forces. There is a greater diversity of chemical composition in proteins than in any other group of biologically active compounds. The proteins in the various animal and plant cells confer on these tissues their biological specificity. Amino acids are known as the building blocks of all proteins. There are 20 different amino acids commonly found in proteins. Amino acids are comprised of a carboxyl group and an amino group attached to the same carbon atom (the α carbon). They vary in size, structure, electric charge and solubility in water because of the variation in their side chains (R groups). Detection, quantification and identification of amino acids in any sample constitute important steps in the study of proteins.

Nitrogen Content determination- The Nitrogen content is determined by using the colorimetric method of Linder (1964) as describe below.

Digestion-

- Take 50 or 100 mg dried and well ground plant material in a 50 ml. Conical flask and add 3 ml of 9:1 H_2SO_4 : $HClO_4$ mixture.
- The flask are heated gently on a hot plate for 5-10 minutes and there after, the heating temperature is raised when the frothing in the mixture is cease.
- Digestion is continued until the solutions become colour less. The digest was cooled and diluted to 20 ml with distilled water.

Estimation-

- Pipette an aliquot of 10 ml in to 50 ml volumetric flask to which 3 ml of 2.5 N NaOH is added to partially neutralize the excess acid.

- Now add 1 ml sodium silicate (40% solutions) it it. The volume is made to 50 ml and mixed thoroughly.
- Now 5 ml aliquots are transferred into a test tube and add 4 drops of Nessler's reagent by mixing thoroughly after the addition of each drop.
- The mixture is allowed to stand for 30 minutes at room temperature after which the absorbance is measured at 420 nm against a reagent blank on Spectronic 20.
- The standard curve is prepared from graded concentrations of ammonium sulphate (AR).
- The actual nitrogen values are calculated from the standard curve and expressed as mg/g dry weight or %.

Total Free Amino Acid determination- The amino acids are colourless ionic compounds that form the basic building blocks of proteins. Apart from being bound as proteins, amino acids also exist in the free form in many tissues and are known as free amino acids. They are mostly water soluble in nature. Very often in plants during disease conditions, the free amino acid compositon exhibits a change and hence, the measurement of total free amino acids gives the physiological and health status of the plants.

Principle-

Ninhydrin, which is originally yellow, reacts with amino acid and turns deep purple. It is this purple color that is detected in this method. Ninhydrin will react with a free alpha-amino group, NH_2-C-COOH. This group is present in all amino acids, proteins or peptides. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins. Theoretically only amino acids produce color with ninhydrin reagent.

Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha-amino acids and yield an intensely coloured bluish purple product which is measured at 570 nm.

Ninhydrin + Alpha-amino Acid → Hydrindantin+Decarboxylated Amino Acid + Carbon di Oxide+Ammonia.

Hydrindantin + Ninhydrin + Ammonia → Purple coloured Product + Water.

Materials-

- Ninhydrin : Dissolve 0.8 g stannous chloride ($SnCl_2 2H_2O$) in 500ml of 0.2M citrate buffer (pH 5.0). Add this solution to 20 g of ninhydrin in 500ml of methyl cellosolve (2 methoxyethanol).
- 0.2 M Citrate buffer pH 5.0
- Dilute Solvent: Mix equal volumes of water and n-propanol, and use.

Procedure-

- Weight 500 mg of the plant sample and grind it in a pestle and mortar with a small quantity of acid washed sand.
- To this homogenate, add 5 to 10 ml of 80% ethanol and filter or centrifuge it. Save the filtrate or supernatant.
- Repeat the extraction twice with the residue and pool all the supernatants.
- Reduce the volume if needed by evaporation and use the extract for the quantitative estimation of total free amino acids. If the tissue is tough, use boiling 80% ethanol for extraction.

Estimation-

- To 0.1 ml of extract, add 1 ml of ninhydrin solution.
- Make up the volume to 2 ml with distilled water.
- Now heat the tubes in a boiling water bath for 20 minutes, and then
- Add 5 ml of the diluents and mix the contents.
- After 15 minutes read the intensity of the purple colour against a reagent blank in a colorimeter at 570 nm. The colour is stable for 1 hour.
- Prepare the reagent blank as above by taking 0.1 ml fo 80% ethanol instead of the extract.

Standard-

- Dissolve 50 mg leucine in 50 ml of distilled water in a volumetric flask.
- Take 10 ml of this stock standard and dilute to 100 ml in another volumetric flask for working standard solution.
- A series of volume from 0.1 to 1 ml of this standard solution gives a concentration ranges 10 μg to 100 μg.
- Proceed as that of the sample and read the colour.
- Draw a standard curve using absorbance versus concentration. Find out the concentration of the total free amino acids in the sample and express as percentage of equivalent of leucine.

(4) <u>PHENOLS</u>

Phenols, the arometic compounds with hydroxyl groups are widespread in plant kingdom. They occur in all parts of the plants. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amount of polyphenols are resistant to bird attack. Phenols include an array of compounds like tannins, flavonols etc. Total phenol estimation can be carried out with the Folin-ciocalteau reagent.

Principle- Phenols react with phosphomolybdic acid in Folin-ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Materials-

- 80% Ethanol
- Folin-ciocalteau reagent.
- Na_2CO_3, (20%)
- Standard (100 mg Catechol in 100 ml Water)
- Dilute 10 times for a working standard.

Procedure-

- Weigh exactly 0.5 or 1.0 g of the sample and grind it with a pestle and mortar in 10-time volume of 80% ethanol.
- Centrifuge the homogenate at 10,000 rpm for 20 min. Save the supernatant. Re-extract the residue with five times the volume of 80% ethanol, centrifuge and pool the supernatants.
- Evaporate the supernatant to dryness.
- Dissolve the residue in a known volume of distilled water(5ml).
- Pipette out different aliquots (0.2 to 2 ml) into test tubes.
- Make up the volume in each tube to 3 ml with water.
- Add 0.5 ml of Folin-Ciocalteau reagent.
- After 3 min, add 2ml of 20% Na_2CO_3 solution to each tube.
- Mix thorougly. Place the tube in a boiling water for exactly 1 minute. Cool and measure the absorbance at 650 nm against a reagent blank.
- Prepare a standard curve using different concentrations of catechol.

Calculation- From the standard curve find out the concentration of phenols in the test sample and express as mg phenols/100 g material.

Precautions:

- If any white precipitate is observed on boiling, the colour may be developed at room temperature for 60 min.
- Express the results in terms of catechol or any other phenol equivalents used as standard.

(5) PHOTOSYNTHETIC PIGMENTS:

Chlorophyll Content-

Cholorophyll is a green molecule in plant cells which plays important role in photosynthesis process. It absorbs sunlight and uses it's energy to synthesis carbohydrates from CO2 and water. Chlorophyll is linked to natural cancer prevention, blocks carcinogenic effects within the body and protects DNA from damage caused by toxic molds like aflatoxin. There are two types of chlorophyll in

plants, chlorophyll a and b, which both of them works as photoreceptor in photosynthesis. The chlorophyll a and b content is estimated following the method of Arnon (1949) and expressed as mg/g dry wt.

Procedure- The DMSO extraction technique of Hiscox and Israelstam (1979) was used for chlorophyll extraction.

- 200 mg fresh leaves are homogenized in 5 ml. of 80% aqueous acetone and centrifuged at 4000 rpm for 20 minutes.
- The supernatent is collected and the residue is reextracted with 5 ml. of 80% acetone and centrifuged again.
- Both the supernatent are combined and volume is made to 20 ml with 80% acetone.
- O.D. is measured at 645 nm and 663 nm on Spectronic- 20 using 80% acetone as blank.
- The amount of chlorophyll a, b and total is calculated as follows:

$$Chlorophyll\ a = 12.7 \times O.D(663) - 2.69 \times \frac{O.D(645) \times V}{1000 \times W}$$

$$Chlorophyll\ b = 22.9 \times O.D(645) - 4.68 \times \frac{O.D(663) \times V}{1000 \times W}$$

$$Total\ Chlorophyll = 20.2 \times O.D(645) - 8.02 \times \frac{O.D(663) \times V}{1000 \times W}$$

Where

V= Final Volume

W= Sample Weight

(6) ANTIOXIDANT ACTIVITIES:

The importance of oxidation in the body and in foodstuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes. When an excess of free radicals is formed, they can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase and cause destructive and lethal cellular effects (e.g., apoptosis) by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration. Furthermore, reactive oxygen species seem to influence cell signalling pathways in ways that are only now being unravelled. Oxidation can also affect foods, where it is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of the nutritional quality, colour, flavour, texture and safety of foods. It is estimated that half of the world's fruit and vegetable crops are lost due to postharvest deteriorative

reactions. Defence mechanisms against the effects of excessive oxidations are provided by the action of various antioxidants and the need to measure antioxidant activity is well documented.

Extract preparation:

Extract was prepared by homogenizing the tissue in 10 ml of 75% methanol in mortar and pestle. The homogenate was centrifuged at 5000 g for 25 minutes to obtain a clear supernatant. The pellet containing the cell debris was discarded. The supernatant was used for further assay.

Total phenolic content

- The total phenolic content was determined by the Folin-Ciocalteu's method as described by Slinkard and Singleton 1977.
- Briefly, appropriate volume (0.1 ml) (1mg ml $^{-1}$) of the extracts was oxidized with Folin–Ciocalteu reagent (0.75ml) and the reaction was neutralized with sodium carbonate.
- Absorbance was measured at 725nm. The results were expressed as gallic acid equivalents (mg per gm (DM) as GAE).
- T= C XV/M. T is the total phenolic content in mg·g–1 of the extracts as GAE, C is the concentration of gallic acid established from the calibration curve in mg/ml–1, V is the volume of the extract solution in ml and M is the weight of the extract in g.
- Gallic acid is used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: y = 0.0061x + 0.0396, R2 = 0.9991, Where y is absorbance at 760 nm and x is total phenolic content in the different extracts.

Total Flavonoid content

- Total flavonoid content was determined by colorimetric method.
- Briefly 0.1 ml (1mg ml $^{-1}$) of each extract was diluted with 0.3 ml of distilled water and 0.03 ml of 5 % $NaNO_2$ solution.
- After 5 min, 0.03 ml of 10 % $AlCl_3$ was added and incubated for 5 min.
- Then, 0.2 ml of 1M NaOH was added and the total volume was made up to 1 ml with distilled water.

- The solution was mixed well and the absorbance was measured immediately at 510 nm.
- The results were expressed as catechin equivalents (mg per gm as CE).
- Total flavonoid content can be measured similar to that of total phenolic content.

Antioxidant activities

ABTS radical scavenging assay

Preparation of ABTS reagent: ABTS (7Mm in water) with 2.45 Mm potassium persulphate mix kept at ambient for 12-14h till absorbance was stable.

Procedure

- The ability of the test sample to scavenge $ABTS^{\cdot+}$ radical cation was compared to trolox standard (Re et al., 1999).
- The $ABTS^{\cdot+}$ radical cation was pregenerated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating for 12–16 h.
- The absorbance of the $ABTS^{\cdot+}$ solution was equilibrated to 0.70 (± 0.02), then 1 ml was mixed with 10 µl of the test sample (0.05–10 mg/ml) and the absorbance was measured at 734 nm after 6 min.
- The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC).
- To calculate the TEAC, the gradient of the plot for the sample was divided by the gradient of the plot for trolox (Bibhabasu et al., 2008).
- Calculations will be similar to that of total phenolic content.

DPPH scavenging assay

The stable DPPH was used for determination of free radical scavenging of extracts according to the method Chang (et al., 2001).

- Briefly, 10 µl of sample (0.05-10 mg/ml) were mixed with 90 µl of 50 mM Tris–HCl buffer (pH 7.4) and 200 µl of 0.1 mM DPPH-ethanol solution.
- After 30 min of incubation at ambient temperature, the absorbance was taken at 517 nm. Catechin was used as a positive control.
- The inhibition ratio (%) was calculated according to the equation:

% inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] X100.

Ferric reducing activity power (FRAP) assay

Preparation of FRAP reagent:

- **Reagent-1** Acetates buffer 300 Mm (pH: 3.6) 3.1 gm sodium acetate trihydrate +16 ml glacial acetic acid and make up 1L with DW.
- **Reagent-2** TPTZ (10Mm 2,4,6-tripyridyl-5-triazine, in 40Mm HCL)
- **Reagent-3** 6H2O 20Mm $FeCl_3$..
- **FRAP= reagent 1+2+3 (10:1:1)**

Procedure

- The FRAP assay was carried out according to Stratil et al., (2006) using freshly prepared FRAP reagent.
- Appropriate dilutions of extracts (0.05-10 mg/ml) were prepared and 100 µl was mixed into 900 µl of FRAP reagent.
- The tubes were vortexed and left at 37 ^{0}C for 40 min, and the absorbance was measured at 593 nm.
- The results were calculated as trolox equivalent (TE, mg 100 mg $^{-1}$ EY).
- Calculations will be similar to that of total phenolic content.

(7) **Enzymes**

All the enzymes are proteins and they are produced by the living cells. They act as biological catalysts. Enzymes catalyse and enhance the rate of biochemical reactions occuring in various vital processes like breathing, digestion, pumping of heart,

formation of body tissues, contraction of muscles, transport of ions across the plasma membranes etc. So without enzymes there is no life. They are inactive at O°C and destroyed by moist heat at 100 °C.

1. **Catalase-** Catalase activity can be assayed colorimetrically according to method given in Analytical Biochemistry (Sinha, 1972). Catalase facilitates the dismutation of H_2O_2 to water and O_2 according to the reaction.

$$H_2O_2 \rightarrow H_2O + {}^1/_2\, O_2$$

The enzyme plays an important role in association with SOD as well as in photorespiration and glycolate pathway.

Reagents-

a. Phosphate buffer, 0.1 M, pH 7.0
b. Potassium dichromate acetic acid (5% $K_2Cr_2O_7$+glacial acetic acid in 1:3 ratio).
c. H_2O_2- (0.2M).

Procedure-

- Take 200 mg plant sample and homogenized with 10 ml of phosphate buffer 0.1 M (pH 7.0).
- Centrifuge at 10,000 rpm at 40^0C for 30 minutes. Collect the supernatant and store at low temperature (4^0C).
- Use supernatant for enzyme assay and estimate the enzyme as given below.

Enzyme Assay-

- Take reaction mixture in conical flask and mix rapidly at 37^0C.
- After 3 minutes withdraw 2.0 ml reaction mixture and add 2.0 ml potassium dichromate acetic acid reagent.
- Keep on boiling water bath for 10 minutes and record O.D. at 570 nm against blank after cooling.

Test	**Blank**	**Reagent**
1.25 ml	-	H_2O_2
0.50 ml	0.50 ml	Enzyme extract
3.25 ml	4.50 ml	Phosphate buffer
5.00 ml	5.00	

Calculation-

- g sample extracted with 10 ml buffer

  0.5 ml enzyme extract + 1.25 ml H_2O_2 +3.2 ml buffer (5 ml assay mixture)

  After 3 minutes of reaction, 2 ml mixture was withdrawn for assay.

  1.0 ml reaction mixture+2.0 ml $K_2Cr_2O_7$ acetic acid reagent.

  Now, 2 ml reaction mixture gives X.O.D.

  So, 5 $ml\ reaction\ mixture\ gives = \frac{5 \times X.O.D.}{2}$

  0.5 ml enzyme extract was used for assay, therefore

  0.5 $ml\ enzyme\ extract = \frac{5 \times X.O.D.}{2}$

  So, for 10 ml enzyme extract = 50 × O.D.

  Which is equivalent to 0.2 g plant sample

  Since, 0.2g plant sample gives = 50 × O.D.

  So, 1g fresh wt. Will gives = 50/0.2 × O.D./3 minutes

  Since 0.1 O.D. = 1 enzyme unit

  Therefore, X.O.D. = 10X

- Now enzyme unit/g fresh wt./3 minutes can be calculated by multiplying the dilution factor of 250 i.e., 250× 10X

  Where, X is the observed O.D.

- Finally, enzyme unit is calculated on the basis activity/g freshwt./min.

  Which is as under

  250×10X

- Express results as enzyme unit per gram fresh wt. Or mg protein basis.

II. Peroxidase-

Peroxidase activity can be assayed colorimetrically according to the method of Curne and Galston (1959).

The enzyme catalyses the oxidation of a substrate by removal of hydrogen which combines with H_2O_2.

$$AH_2 + H_2 \rightarrow 2H_2O_2 + A$$

Reagents-

a. Phosphate buffer, 0.1 M, pH 6.0
b. Pyrogallol (0.1 N)
c. H_2O_2 (0.02%)

Procedure-

- Take 200 mg plant sample and homogenize with 10 ml of Phosphate buffer 0.1 M (pH 6.0).
- Centrifuge at 10,000 rpm at 4^0C for 30 minutes.
- Collect the supernatant and store at low temperature.
- Use the supernatant for enzyme assay and estimate the enzyme activity as given below.

Reaction Mixture-

Shake the mixture well and keep it at 37^0C on water bath for 10 minutes for the formation of purpurogallin.

Measure the activity at 430 nm and express result as enzyme unit per gram fresh weight or per gram protein basis.

Test	**Blank**	**Reagent**
2.0 ml	**2.0 ml**	**Enzyme extreact**
2.0 ml	**3.2 ml**	**Phosphate buffer**
1.0 ml	**-**	**Pyrogallol**
0.2 ml	**-**	**H_2O_2**
5.2 ml	**5.2 ml**	

Calculation-

- 0.2 g sample extracted with 10 ml buffer
- 2 ml enzyme extract + 2 ml Buffer + 0.2 ml H_2O_2 + 1 ml Pyrogallol
- O.D. measured at 430 nm
- 2 ml enzyme extract gives – X.O.D. So, 10 ml enzyme extract gives 1.

(III) Polyphenol Oxidase- Phenol Oxidase are copper proteins of wide occurrences in nature which catalyse the aerobic oxidation of certain phenolic substrates to quinines which are autoxidized to dark brown pigments generally known as malanine. These enzymes are assumed to be single enzyme with broad specificity although there is some evidences for the presence of more than one phenol oxidase in certain tissues. Each individual enzyme tends to catalyse the oxidation of one particular phenol or phenolic compound more readily than others. The polyphenol oxidase (PPO) comprises of catechol oxidase and laccase. The activities of these enzymes are important with regard to (a) plant defence mechanism against pests and diseases and (b) appearance palatability and use of plant products. Fresh fruits, vegetables, mushroom etc. contains these enzymes considerably.

Principle- The intensely yellow 2-nitro-5-thiobenzoic acid (TNB) with an absorption maximum at 412 nm reacts with the quinines generated through enzymatic oxidation of 4-methylcatechol (catechol oxidase) and 1,4 dihydroxybenzene (laccase) to yeild colourless products. The decrease in the absorbance of yellow-colour due to enzyme activity is measured.

Materials-

- Citrate-phosphate buffer 0.2 M (pH 6.0)
- Preparation of 2-nitro 5-thiobenzoic acid (add 30ml sodium borohydride to a suspension of Ellman's reagent, i.e. 5,5 dithiobis (2-nitrobenzoic acid) (19 mg) in 10 ml water. Within 1 hour, the disulphide is quantitatively reduced to the intensely yellow, water soluble thiol. This solution is stable for at least one week when stored at 4^0C.
- Quinine solution: Dissolve 4-methyl-1,2 benzoquinone in double distilled water in a 50 ml volumetric flask by bubbling nitrogen gas until the quinone is completely dissolved.
- Prepare p-benzoquinone solution also in a similar manner. Both solutions are stable for 30 minutes, a time sufficient to carry out the spectrophotometric assay.
- Substract Solution : 4-methylcatechol (2mM) for catechol oxidase assay quinol (1,4 dihydroxybenzene, 2mM) for laccase assay.
- Enzyme extract : Prepared first acetone power of fresh plant tissue to get a crude enzyme extract, mix 100 mg acetone powder with 2.5 ml of 0.2 M citrate phosphate buffer (pH 6.0), 1 ml of 1% triton X-100, 6.5 ml of water and 500 mg polyamide. Shake for 1 hr. and filter. Use the filtrate as enzyme source.
  The enzyme acts on different phenolic compounds in the presence of oxygen and convnets them to complex condensulim products, brown in colour. The

browning of potatoes, apples and other fruits and vegetables on exposure to air is due to action of this group of enzymes.

Reagents-

a. Extraction buffer : Phosphate (0.1 M, pH 6.1)
b. 3,4 Dihydroxy phenylalanine (DDPA) (50 mM).

Assay-

- The assay mixture contains 2 ml phosphate buffer, 1 ml of DDPA and 2 ml g enzyme preparation.
- The initial rate of increase in absorbance is determined at 475 nm for the first 5 min.
- The activity is expressed as enzyme units per g fresh wt.
- One unit g activity (DDPA) is defined as the amount of enzyme which produces an increase g 0.001 O.D. at 475 nm per min g incubation.

(IV) Hydrolytic enzyme- A hydrolase or hydrolytic enzyme is an enzyme that catalyzes the hydrolysis of a chemical bond.

Amylases- Starch degrading enzymes which are universally distributed generally act on glycogen and related polysaccharides. Amylase causes indo-cleavage of subtract and hydrolyses α-1,4 linkage in a random manner. It has the ability to by pass α -1,6 branch points. The viscosity reduction of the subtract is fast but the production of reducing sugar is slow.

β-amylase hydrolyses alternate bonds from the non-reducing end of the substrate. The enzyme degrades amylose, amylopextin or glucogen in an exo or stepwise fashion by hydrolyzing alternate glycosidic bonds. The end product is β-maltose. β-amylase is incapable of by passing branch points i.e.- 1,6-glycosidic linkages in amylopectin and glycogen. This results in about 55% conversion of amylopectin to maltose. The other product is a large limit dextrin. The viscosity reduction of the substrate due to β-amylase action is slow but the production of reducing sugars is fast.

Principle- The reducing sugar produced by the action of α - and or β-amylase react with dinitrosalicylic acid and reduce it to a brown colored product, nitro-amino-salicyclic acid.

β-Amylase- β-Amylase acts upon non-reducing end of d-1,4 linkage of Harch to yield disaccharide maltose end product.

Reagent-

a. Extraction buffer : Phosphate buffer, pH 7.0
b. Succinate (100mM, pH 6.0)
c. Starch (5%)
d. DNS reagent (Dinitrosalicylic acid)

Assay-

- The reaction mixture contains 0.5 ml g succinate and 0.2 ml g enzyme preparation.
- The reaction is initiated with 0.5 ml of 5% soluble starch. The reaction is terminated by the addition g 0.2 ml g DNS reagent.
- The contents are heated on boiling water bath for 5 min and then volume is made up to 10 ml with water.
- Absorbance of the solution is recorded at 540 nm.
- Amount of maltose released is measured by comparing with standard curve prepared by the graded concentration of standard maltose and β-amylase activity is expressed as μ mol maltose turned per min. per mg protein.

Lipases- (Triacylglycerol acylhydrolase EC 3.1.1.3) lipase hydrolyses triglycerides to release free fatty acids and glycerol.

Triglycerides+H_2O Glycerol+Fatty Acids.

During germination of oilseeds, lipases play an important role in hydrolyzing the stored oils so that the required energy for growth and carbon skeleton for synthesis of new compounds are produced. The germinating seeds of mustard, castor bean, sunflower, groundnut are good sources of lipases.

The quantity of fatty acid released in unit time is measured by the quantity of NaOH required to maintain pH constant. The milli equivalent of alkali consumed is taken as a measure of the activity of the enzyme.

Materials-

- Substrate : take 2ml of any clear vegetable oil, neutralize to pH 7.0, if necessary, and stir well with 25ml of water in the presence of 100 mg bile salts (sodium taurocholate) till and emulsion is formed. Addition of 2g gum Arabic has tens emulsification.
- 0.1N NaOH
- Enzyme Source: grind a known quantity of sample with a mortar and pestle. Homogenize the tissue with twice the volume of ice-cold acetone. Filter and wash the powder successively with acetone and acetone: ether (1:1) and ether. Airdry the powder. This acetone powder can be stored in a refrigerator. Extract 1g of the powder in a 20 ml ice-water or a suitable buffer. Centrifuge at 15,000 rpm for 10 min and use the supernatant as enzyme source.
- 50mM phosphate buffer (pH 7.0)

Procedure-

- Take 20 ml of substrate in a 500 ml beaker. Add 5 ml of phosphate buffer (pH 7.0)
- Set the beaker on top of a magnetic stirrer cum hot plate and stir the contents slowly. Maintain the temperature at 35^0C. Dip the electrodes of a pH meter in the reaction mixture. Note the pH and adjust it to 7.0.

- Add enzyme extract (0.5 ml), immediately record the pH and set the timer on. Let it be pH at zero time.
- At frequent intervals (say 10 min) or as the pH drops by about 0.2 unit and 1.0N NaOH to bring pH to the initial value. Continue the titration for 30-60 min period.
- Note the volume of alkali consumed.

Calculation- The enzyme activity is defined as the amount of enzyme which releases one milli equivalent of free fatty acid per minute per g sample. Specific activity is expressed as milli equivalents/min/mg protein as under:

$$= \frac{Volume\ of\ alkali\ consumed \times Strength\ of\ alkali}{Wt.of\ sample\ in\ g \times Time\ in\ min}$$

(8) VITAMIN:

The vitamins are a group of complex organic compounds required in small quantities by the body for the maintenance of good health. They are not normally synthesized in the body and hence they should be supplied by the diet. The vitamins are present in foods in small quantities.

Unlike other groups of nutrients the vitamins are not chemically similar to each other. Each vitamin has a specific chemical structure and a specific function or functions in the living system. Most of the vitamins act as coenzymes in the body. Normally a well balanced diet will supply all the necessary vitamins in sufficient quantity.

Extraction of Vitamin A (β-carotene):

β-carotene was estimated following approved AACC (14-50, 1995) method as described below.

Reagents:

- Water saturated butanol (WSB)- Mix n-butanol with distilled water in 8:2 ratios

Procedure:

- Eight gram flour was taken in 150 ml glass stoppered Erlenmeyer flask and 40 ml water saturated butanol (WSB) was added.
- The contents of the flasks were mixed vigorously for 1 minute and kept overnight (16-18 hrs) at room temperature under dark for complete extraction of β-carotene.
- Next day, the contents were shaken again and filtered completely through the Whatman no.1 filter paper into a 100 ml volumetric flask.
- The optical density of the clear filtrate was measured at 440 nm using Systronic double beam 2201 spectrophotometer. Pure WSB was used as blank.

- The β-carotene content was calculated from calibration curve from known amount of β-carotene and expressed as parts per million (ppm).
- Standard solution of β-carotene (Sigma) was prepared in water saturated butanol (WSB) at the concentration of 5 µg/ml.

Calculations:

- Calibration curve is made from known amounts of pure β-carotene from 0.25 µg/ml to 1.5 µg/ml which are prepared after suitable dilutions of original stock with WSB in calibrated 10 ml volumetric flasks (from 0.5 ml to 3 ml of standard solution in 10 ml).
- Absorbance of each dilution is measured and a calibration curve is established.
- β-carotene content of unknown samples is calculated from standard curve. β-carotene results were also confirmed with HPLC.

Nutrient Analysis

Soil provides a source of nutrients required by crops and also therefore for animals but not necessarily at optimum levels of immediate availability to plants.

Principle: The purpose of soil analysis is to assess the adequacy, surplus or deficiency of available nutrients for crop growth and to monitor change brought about by farming practices. A sample normally comprises around 1 kg of soil which is taken to represent an entire area or field, which contains around 2,000 tonnes of soil per hectare to a plough depth of 20 cm (8 inches). It is therefore imperative to obtain as representative a sample as possible or the results will not reflect the nutrient status accurately.

1. Sampling time: Soil samples can be taken any time that soil conditions permit, but sampling directly after fertilization or amendment application should be avoided. Samples taken during the crop growth period will help in knowing the nutrient status of the soil in which plants are actively taking up nutrients. It is important to sample at similar times year after year for comparing analysis at regular time intervals.

Sampling depth: For most purposes, soil sampling is done to a depth of about 20 cm.

Sampling tools: Researchers generally use augers for field sampling. Farmers or Extension Agents can use shovels or trowels, with almost the same result.

If do not have sampling tools, use a spade as follows:

- Dig a V-shaped whole 15 to 20 cm deep. Then take a fine thick slice from the smooth side.
- Trim the sides leaving a fine strip then dump this strip into a clean bucket. Break the clods, and mix thoroughly. Remove large rocks, pieces of sod, earthworms, etc. Put the soil into the sample container and label the box clearly.
- For a moist soil, the tube auger or spade is considered satisfactory. For harder soil, a screw auger may be more convenient.

Factors affecting soil analysis

Nutrient values can vary as a result of a number of factors which are discussed below.

i. Spatial variation: Variation can exist over very short distances (less than 1 metre) and the number of cores that are needed to ensure that sampling is representative of an area will vary according to the scale of this variation. When sampling to obtain an average value for a field this is not so important because all the cores taken are bulked into a single sample; for a typically uniform field 25 cores are sufficient.

ii. Temporal variation: Variations in nutrient values have been observed at different times of the year and there is evidence to suggest that soil P, K and Mg values may be higher in the early spring than in the autumn as a result of chemical weathering over winter, biological activity and lack of uptake by growing crops.

iii. Moisture: If soils are dry at the time of sampling the analytical results can be affected and may appear a little lower for P and possibly K. Movement and uptake of all nutrients will of course be restricted in very dry soils but this is a transient problem and does not reflect the normal availability of nutrients in the soil. At present it is not possible to quantify this effect in order to improve interpretation of analytical results.

iv. Crop removal: During periods of rapid growth crop uptake, especially of potassium, can be large and may deplete available soil nutrient levels for a short period until the nutrient status returns to equilibrium. This could affect results for some soils if samples are taken at such times.

Nutrients are returned to the soil in crop residues but will not be determined by analysis until the plant material is broken down. It has been suggested that

sampling close to harvest may result in an under-estimation of true soil nutrient status.

v. Sampling depth: Frequently there may be a gradient in nutrient level down the soil profile, usually declining with depth, reflecting the accumulation of nutrients in the plough layer. This is accentuated in minimal cultivation systems where phosphate and potash may be concentrated in the top 5 to 8 cm (2-3 inches). With continuous direct drilling there may be a large difference between the top 5 cm (2 inches) and 8 to 15 cm (3- 6 inch) zones. For permanent grass where there is no soil disturbance, a consistent sampling depth of 7.5 cm (3 inches) is particularly important.

vi. Previous manuring

Applications of fertilizers and manures obviously have a major impact on measurement of soil nutrients. It is difficult to lay down rules as to how soon sampling should be undertaken after application. General guidelines are:

- 8 weeks after P, K, Mg fertilizer application.
- 12 weeks after slurry or manure application.
- 12 months after lime spreading.

Ploughing and cultivating help distribute nutrients from fertilizers and manures, and lime, throughout the depth of cultivation, but this takes time.

1.5 Precautions for sampling

- Use a suitable tool (cone auger, screw auger, corer etc) which facilitates and encourages the taking of more rather than fewer cores, of a uniform size and down to the full depth of sampling.
- Use suitable tool and packaging that will not contaminate the sample and label the samples clearly.
- Sample to a consistent depth. Normal depth is 15 cm (6 inches) for arable soils, 7.5 cm (3 inches) for grassland.
- Divide the field into areas which are as uniform as possible in soil type, past cropping, and manuring history and sample separately. Small areas of different soil e.g. wet, chalky, shallow, stony etc. should be excluded.
- Avoid headlands, gateways, trees, mole hills, dung/urine patches, water troughs, areas where lime or manure has been dumped, old hedgerows/middens/ponds or any other irregular feature.
- Discard stones and plant debris.

- Take at least 25 cores from each area to be sampled and put them together to form a single representative sample. The numbers of cores should not be restricted simply because the container is full. Thoroughly mix all cores and take a sub-sample from this for dispatch to the laboratory and this must be done carefully.
- Ensure the sample represents the whole area. Sample on a W pattern over the field; for a regular shaped field this means 7 cores per leg of the "W".
- Sample at the same point in the rotation, before the crop which is most demanding or responsive to P and K. In descending order of importance these are: horticultural crops, vegetables, roots, pulses, spring sown combinable crops, winter sown combinable crops. For pH it is preferable to sample 12 months before a sensitive crop such as sugar beet or barley.
- Sample each time at the same time of the year.
- Avoid sampling under extremes of soil conditions e.g. waterlogged or very dry soil.
- Do not sample within 8 weeks of fertilizing, or within 12 weeks of manure or slurry application, for P, K and Mg analysis or sooner than 12 months after liming for pH analysis.
- Maintain records and use the analytical results to develop nutrient management plans.

Sample preparation:

i. When samples come in the lab from the field arrange them:

- According to date
- Within the dates organize by location and site number
- Finally separate them

ii. Once the samples are organized by first sampled with lowest site number and increasing depth then record the tag information on the daily sample sheets. Once you have done this take the sample number from the daily sample sheet and write it on the tag.

iii. Get a tray and a plastic bag (wipe down the bag with a moist towel) for the sample. Open the bag and pour the sample onto the tray on the plastic bag. Place the tag near the edge of the bag so that you know which sample is which. Spread the sample over the bag in order for the sample to dry evenly. Make sure to break down big clumps of soil so that they dry properly.

iv. Once the samples are dried take 10 grams soil in a 400 ml beaker for the test. If the sample is stony, remove the stones and calculate the percent stones

v. After the soil is taken for all the tests place the tag in a sample container and fill the container with soil. The extra soil is thrown out. Write the sample number on the container and its lid. Then put the samples in the sample drawer.

vi. Most procedures require that samples be air-dried. This entails spreading the soil on trays of plastic sheeting, mixing and rolling to break up clods. The sample is gently crushed and sieved until only coarse fragments (>2 mm) remain. These coarse fragments are weighed and recorded as "% stones". Some procedures require samples to be oven-dried and ground to pass a .42 mm (40 mesh) sieve.

2. NITROGEN

Principle:

The Kjeldahl method of nitrogen determination involves wet digestion of a sample to convert organic nitrogen to NH_4^+-N and then determining the nitrogen content. Digestion is accomplished by heating a sample with concentrated Sulfuric acid (H_2SO_4). Addition of a salt such as Potassium sulfate (K_2SO_4) increases the temperature of digestion, and addition of catalysts such as mercury (Hg), copper (Cu) or selenium (Se) increases the rate of oxidation of organic matter. When NH_4^+-N in the digest is distilled in the excess of NaOH, the ammonia (NH_3) released in the distillation is trapped in Boric acid (H_3BO_3) and then titrated with a standard acid solution. Total soil N is generally measured after wet digestion using the well-known Kjeldahl procedure. After distillation, NO_3-N can be determined by a procedure involving chromotropic acid.

Equipment

- KJELTEC Auto 1030 Analyzer
- Tecator Digestor 20/40 with built-in thermostat
- Accessories - digestion tubes, stand, exhaust manifold, heat shield, stand for rapid cooling of tubes, retainer plate, boiling chips

Reagents

- Sulfuric acid (H_2SO_4), concentrated and 0.05N standard
- KJELTABS (3.5 g K_2SO_4 +0.0035 g Se)
- Sodium hydroxide (Na0H) 35-40%
- Boric acid (H_3BO_3) – 1% with bromocresol green-methyl red indicator
    - ❖ Dissolve 100 g of boric acid in 10 Litre deionized water (1% solution)

❖ Add 100 ml bromocresol green solution (100 mg in 100 ml methanol)

❖ Add 70 ml methyl red solution (100 mg in 100 ml methanol)

❖ Add 5 ml NaOH (alkali is necessary to achieve a positive blank value).

Procedure: The quantity of regents and samples described in this procedure are based on the "semi-micro" method.

- Grind a sample to pass a No. 35 (0.5 mm opening) sieve and oven-dry at 105° C.
- Transfer a 1.50 g mineral sample (0.30 g of organic sample) into a 100 ml digestion tube.
- Add 1 KJELTAB and 9 ml of concentrate H_2SO_4. So that the sample is completely immersed at the bottom of the tube. Boiling chips are added to reduce bumping during digestion.
- Pre-heat block digestor to 420° C (may require up to 3 hours). Allow sample to digest for 45 minutes at a temperature of 420°C and then allow 30 minutes to cool.
- Add 50 ml of deionized water.
- Insert and read a blank reagent sample with the KJELTEC after the instrument has been calibrated with deionized water.
- Insert digestion tube with sample into KJELTEC and obtain reading.

Calculations:

$$\% \text{ NITROGEN} = V \times N \times \frac{1.4}{\text{Weight of sample (g)}}$$

V = Volume of 0.05N H_2SO_4

N = Normality of H_2SO_4

3. Phosphorus

Principle:

The "plant-available P" fraction is normally a small proportion of total phosphorus (P). Total P measurement involves digestion of a soil sample with a strong acid and the dissolution of all insoluble inorganic and organic P forms of minerals. This measurement is usually employed only for soil genesis or mineralogical studies.

Apparatus:

- Spectrophotometer or colorimeter
- Block-digester
- Standard laboratory glassware: beakers, volumetric flasks, pipettes, and funnels

Vortex tube stirrer

Reagents:

A. Perchloric Acid ($HClO_4$), concentrated (60%)

B. Ammonium Heptamolybdate- Ammonium Vanadate in Nitric Acid (HNO_3)

(a) Dissolve 22.5 g ammonium heptamolybdate in 400 ml water

(b) Dissolve 1.25 g ammonium metavanadate (NH_4VO_3) in 300 ml hot distilled (DI) water.

Then add both dissolved Reagents **(b)** and **(a)** to a 1 litre flask, and let the mixture cool to room temperature. After that slowly add 250 ml concentrated HNO_3 to the mixture, cool the solution to room temperature, and dilute to 1 litre volume.

C. Standard Stock Solution

- Dry out 2.5 g potassium dihydrogen phosphate (KH_2PO_4) in an oven at 105 °C for 1 hour, cool in a desiccators, and store in a tightly Stoppard bottle.
- Dissolve 0.4393 g dried KH_2PO_4 in distilled water, and bring to 1 liter volume. This solution contains 100 ppm P (Stock Solution).
- Prepare a series of Standard Solutions from the Stock Solution as follows:

Dilute 1, 2, 3, 4, and 5 ml Stock Solution to 50-ml numbered flasks by adding distilled water, and then bring to volume. These solutions contain 2, 4, 6, 8, and 10 ppm P, respectively.

Procedure:

A. Digestion

- Weigh 2 g air- dry soil (0.15 mm) into a 250 ml calibrated digestion tube.
- Add 30 ml 60% $HClO_3$ and a few pumice-boiling granules. Mix well, and then place the tubes in the rack.
- Place the tubes rack in the block-digester and gently heat to about 100 °C.
- Slowly increase the block-digester temperature to 180 °C and digest the samples until dense white fumes of acid appear. Use a little extra $HClO_3$ to wash down the sides of the digestion tube as necessary.
- Continue heating at the boiling temperature for 15-20 minutes longer. At this stage the insoluble material becomes like white sand. The total digestion with 60% $HClO_3$ usually requires about 40 minutes.
- Cool the mixture, and bring to volume, mix the contents, and filter through filter paper.

B. Measurement

i. Pipette 5 ml clear filtrate into a 50-ml flask.

ii. Add 10 ml ammonium vanadomolybdate reagent, and dilute to volume with distilled water.

iii. Prepare a standard curve as follows:

- Pipette 5 ml of each standard (2-10 ppm), and proceed as for the samples.
- Make a blank with 10 ml ammonium vanadomolybdate reagent, and proceed as for the samples.

iv. Read the absorbance of blank, standards, and samples after 10 minutes on the spectrophotometer at 410 nm wavelength.

v. Prepare a calibration curve for standards, plotting absorbance against the respective P concentrations.

vi. Read P concentration in the unknown samples from the calibration curve.

vii. Calculation:

$$\text{Total P (ppm)} = \text{ppm P (from calibration curve)} \times \frac{V}{Wt} \times \frac{V2}{V1}$$

Where:
V = Total volume of the digest tube (ml)
Wt = Weight of air-dry soil (g)
V1 = Volume of soil digest used for measurement (ml)
V2 = Volume of flask used for measurement (ml)

4. Potassium:

Principle:

Along with N and P, potassium (K) is also of vital importance in crop production. Most soils contain relatively large amounts of total K (1 – 2 %) as components of relatively insoluble minerals. However, only a small fraction (about 1%) is present in forms available to plants, i.e., water-soluble and exchangeable-K. The method uses a neutral ammonium acetate solution (1 N) to replace the cations present on the soil exchange complex. This is considered as plant available K in the soils, and is commonly measured by the flame photometer. However, the cation concentrations determined by this

method are referred to as "exchangeable" for non-calcareous soils. For calcareous soils, the cations are referred to as "exchangeable plus soluble".

Apparatus

- Flame photometer with accessories
- Centrifuge, capable of 3000 round per minute (rmp)
- Mechanical shaker, reciprocating

Reagents:

A. Ammonium Acetate Solution (NH_4OAc), 1 N

- Add 57 ml concentrated acetic acid (CH_3COOH) solution to 800 ml distilled water, and then add 68 ml concentrated ammonium hydroxide (NH_4OH) solution, mix well and let the mixture cool.
- Adjust the mixture to pH 7.0 by adding more CH_3COOH or NH_4OH, and bring to 1 litre volume with distilled water.

B. Standard Stock Solution

- Dry 3 g potassium chloride (KCl) in an oven at 120°C for 1-2 hours. Cool in a desiccator, and store in a tightly stoppered bottle.
- Dissolve 1.907 g dried *KCl* in DI water, and bring to 1 litre volume. This solution contains 1000 ppm K (Stock Solution).
- Prepare a series of Standard Solutions from the Stock Solution as follows: Dilute 2, 4, 6, 8, 10, 15 and 20 ml *Stock Solution* to 100-ml numbered flasks by adding 1 N ammonium acetate solution, and then bring to volume. These solutions contain 20, 40, 60, 80, 100, 150, and 200 ppm K, respectively.

Procedure:

A. Extraction

- Weigh 10 g air dry soil (< 2-mm) into a 250-ml flask.
- Add 50 ml 1 N NH_4OAc solution (ratio 1:5).
- Shake for 30 minutes on a reciprocate shaker at 200-300 rpm.
- Filter suspension using a Whatman No.1 filter paper to exclude any soil particles, and bring the extract to a 50-ml volume with 1 N NH_4OAc solution.

B. Measurement

- Operate Flame Photometer according to the instructions provided.
- Run a series of suitable K standards, and draw a calibration curve.
- Measure K in the samples (soil extracts) by taking the emission readings on the Flame Photometer at 767-nm wavelength.
- Calculate K concentrations according to the calibration curve

Calculations:

Extractable K (ppm) = ppm K (from calibration curve) x $\frac{V}{Wt}$

Where:
V= Total volume of the soil extract (ml)
Wt = Weight of air-dry soil (g)

Factors affecting interpretation in nutrient analysis

Soil structure is also very important because any restriction to root growth may restrict the plants ability to obtain an adequate nutrient supply despite a satisfactory value indicated by analysis. The remedy is not to apply more nutrients but to improve the soil structure.

Organic matter – The level of organic matter (humus) will also affect the availability of nutrients in a soil and regular addition of manures so that the physical conditions and biological activity is improved will increase the plant-available nutrients.

Stone content can also have a large effect upon nutrient supply. Very stony soils have little fine earth yet it is the nutrients in this fine earth fraction that are measured. In consequence it is advisable to maintain very stony soils at slightly higher levels of available P and K than are required on deep stone free soils.

Soil depth – Crops frequently access some nutrients from below sampling depth and from subsoil (especially potash). Soils with greater rooting depth potential offer a greater soil volume, and provide larger total quantities of nutrient than shallow soils. It may be possible to manage deep, well textured soils at slightly lower concentrations than the stated targets given above. However shallow rooting crops which do not explore the full soil volume will still require normal nutrient concentrations.