

Practical Manual

Chemical Analysis of Soil and Plant Samples



Ummed Singh
C S Praharaj

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Ummed Singh
C S Praharaj



Division of Crop Production
ICAR-Indian Institute of Pulses Research
Kanpur, Uttar Pradesh- 208 024 India
(Website : <http://www.iipr.icar.gov.in>)



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FOREWORD

ICAR-Indian Institute of Pulses Research is a premier institute in the crop science division of Indian Council of Agricultural Research (ICAR). The institute is mandated with the basic, strategic and applied research on major pulse crops. With the key role in developing technologies and materials towards pulses improvement, production, and protection and giving its fruits to our clientele, *the farmers*, its activities also revolves round generating basic knowledge and information including human resource development by adequate training and education through tactical linkages and strategic coordination with the network on pulses research programs across the country and the globe.



Efficient management of plant nutrients and soil health is one of the key to sustain food security and environmental quality. In Indian context, declining soil fertility, lower nutrient use efficiency and diminishing factor productivity have been emerged as the major threats to the sustainability of diverse agricultural production system. On the other hand, unbalanced, injudicious, indiscriminate and inadequate use of fertilizers/agro-inputs and their sources led to deterioration of soil health, environmental quality, sub-optimal production, low input use efficiency and lower B: C ratio.

Soil and plant testing is considered to be the most effective and reliable tool to assess plant nutrient demand *in-situ* or on-site. The practices of site-specific nutrient management, best fertilizers management practices, 4R nutrient stewardship, nutrient expert based recommendations etc. are based on testing/analysis of soil and plant parts. Moreover, the status of nutrients, mostly in respect of their available forms, is generally used to assess the soil fertility status in India. Based on the soil test data, the soil testing laboratories prepare soil fertility maps on major and micronutrients.

The present practical guide on Soil and Plant Analysis brought out by the scientists of the Division of Crop Production, ICAR-IIPR, Kanpur, is a comprehensive pocket guide on the subject. It offers a fresh and novel approach for soil fertility analysis, nutrient uptake and removal studies through estimating/preparing nutrient budget, soil fertility map and nutrient concentration in different plant parts etc. It is hoped that this practical pocket guide will be useful for scientists, teachers, students and lab-technicians associated with soil and plant analysis. I congratulate the authors for this endeavour.

Kanpur

Dated: November 30, 2017

A handwritten signature in black ink, appearing to read 'N P Singh', written over a horizontal line.

(N P Singh)

Director, ICAR-IIPR

PREFACE

Nutrients have been the key input in augmenting food production in India which has been confirmed right from the Era of Green Revolution in India. Despite our best and diverse efforts for sustainable crop production, the imbalance in the use of N, P and K does exist and often continues to haunt all of us. Besides this, deficiency of secondary nutrients (especially S) and micronutrients (mainly Zn, Fe and B) are becoming wide spread in the Indian soils, leading to micronutrient malnutrition or '*Hidden Hunger*' and its repercussion on low yield in most of the field crops. Understanding their concentration in soil/plant parts, analysing uptake pattern and complementing with soil fertility status will render enhanced nutrient use efficiency, factor productivity and optimum crop response. This will assist in application of balanced use of nutrients. It triggers for appropriate action or remedies for enhancing nutrient use efficiency, profitability, crop response and factor productivity.

In agricultural eco-system, soil and plant are indispensable for food production. For conservation and judicious use of natural resources, analysis of soil and plant is of utmost importance. Therefore, for any application of inputs or management of crop to be sustainable, testing of soil/plant parts in respect of plant nutrients (or its characteristics) is pre-requisite. And qualitative and quantitative analysis in respect of nutrient is mandatory to arrive at appropriate conclusion/recommendation. This training manual contains analytical concepts on nutrients covering principle, materials, equipments, methods, calculations and interpretations. Moreover, clear and easily understandable words/sentences have been used in this manual for better clarity of the analytical methods used. This manual can also be used as a tool for soil testing, chemical quality control in research laboratories for advancing research and preparing Soil Health Card for the farmers known as the first step towards sustainable resource use.

It is hoped that, this information will be useful in up-gradation of the existing knowledge of all those associated/interested in the subjects concerned with plant nutrition, fertilizer use efficiency and crop productivity including researchers, policy makers, technocrats and students ensuring *Good Soil Health with Better Nutrition*.

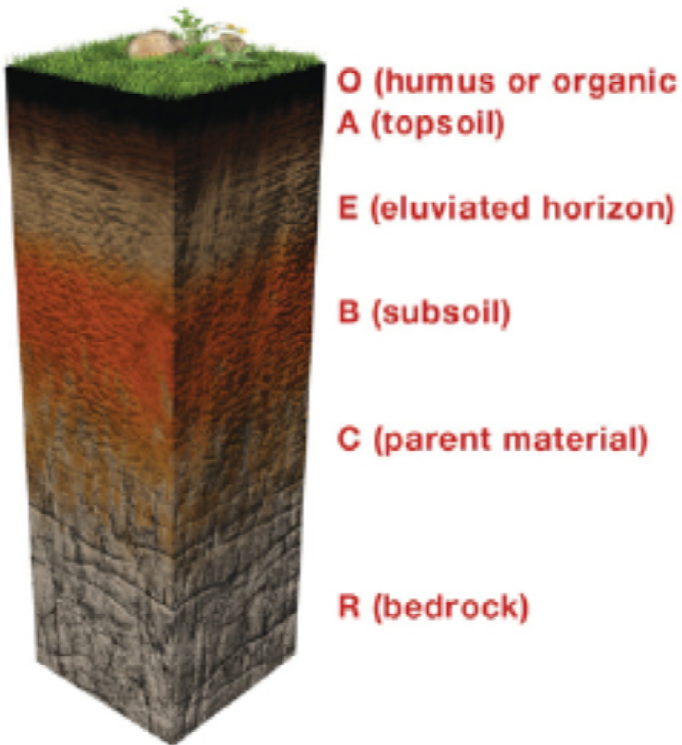
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Dated: 30-11-2017

Ummed Singh
C S Praharaj

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Chemical Analysis of Soil Samples



Determination of Organic Carbon in Soil

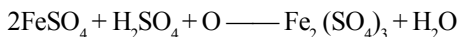
Soil organic matter is one of the most important properties of the soil, which not only holds plant nutrients and water but also supports micro- and macro-organisms. The major part (>90%) of soil nitrogen exists as complex combination in the organic matter fraction. It becomes available to crops after breakdown to simple forms followed by mineralization. Therefore, easily oxidizable organic carbon and mineralization nitrogen are considered to be quite satisfactory as an index of nitrogen availability in soils. Oxidizable organic carbon represents about 58% of the soil organic matter, the constituent of nitrogen in soil.

Walkley and Black Method

For determination of easily oxidizable carbon, modified Walkley-Black method (Walkley and Black, 1934) and colorimetric method (Datta et al., 1962) are most acceptable methods. These methods operate on the basic principle 'Wet oxidation (digestion) of organic carbon in an acid dichromate solution followed by back titration of the remaining dichromate with ferrous ammonium sulphate or by photometric determination of Cr^{3+} '.

Principle

Organic matter present in the soil gets oxidized by potassium dichromate and concentrated sulphuric acid utilizing heat of dilution of H_2S_4 . The excess potassium dichromate, not reduced by the organic matter of the soil is determined by back titration with standard ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) or ferrous ammonium sulphate [$\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$]. The following reactions take place during estimation:



Reagents Required

1. I N potassium dichromate: Dissolve 49.04 g potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) AR grade (oven dried at 90 °C) in distilled water and make up the volume to one litre.

2. 0.5 N Ferrous Ammonium Sulphate (FAS): Either (a) Dissolve 196 g of the hydrated crystalline salt in one litre distilled water, add 20 ml concentrated H_2SO_4 . Or (b) Dissolve 140 g $FeSO_4 \cdot 7H_2O$ in distilled water, add 40 ml concentrated H_2SO_4 , cool and dilute to 1 litre. Further, this reagent should be standardized every day before use
3. Concentrated sulphuric acid having specific gravity of 1.84 C.P.
4. Concentrated Ortho-Phosphoric acid (85% C.P.) and/or chemically pure sodium fluoride (NaF) with diphenylamine indicator only.
5. Indicator: (a) Diphenylamine indicator: Dissolve 0.5 g diphenylamine in a mixture of 20 ml of water and 100 ml of concentrated H_2SO_4 , or (b) O-phenanthroline indicator (Ferrouin): Dissolve 3.0 g O-phenanthroline monohydrate and 1.4 g ferrous sulphate hepta hydrate ($FeSO_4 \cdot 7H_2O$) in distilled water (dilute to 200 ml). Colour change-greenish blue to reddish brown, or (c) N-phenylanthranilic acid indicator: Dissolve 0.1 g of N-phenylanthranilic acid and 0.1 g Na_2CO_3 in 100 mm distilled water. Colour change- dark violet green to light green.

Procedure

1. Weigh 1.0 g soil and transfer it in a dry 500 ml conical flask.
2. Add 10 ml 1N $K_2Cr_2O_7$ using pipette and swirled the flask a little. Better to put flask on asbestos sheet.
3. Add 20 ml concentrated H_2SO_4 (containing 1.25% Ag_2SO_4) and swirl it 2-3 times.
4. The flask is allowed to stand for 30 minutes and then add 200 ml distilled water.
5. Add 10 ml phosphoric acid and/or 0.5 g NaF and 1 ml diphenylamine indicator.
6. Titrate the contents of flask with 0.5N FAS solution. Add FAS solution freely from burette until the contents of flask changes blue-violet to green or dull green, thereafter; start adding it slowly drop by drop until colour turn to chocolate red.

7. Simultaneously, a blank is run without soil.

If more than 7-8 ml of the dichromate solution is reduced/consumed then repeat the determination procedure with smaller quantity of soil (0.25-0.50g).

8. This approach follows Walkley and Black, 1934.

Note: it is better to use either O-phenanthroline (ferroin) or N-phenylanthranilic acid indicators in place of diphenylamine.

Calculation

$$\text{Volume of 1 N K}_2\text{Cr}_2\text{O}_7 \text{ consumed/reduced (D)} = \frac{A (B - C)}{B}$$

Where, A=amount of $\text{K}_2\text{Cr}_2\text{O}_7$ used (ml); B=Blank reading; C=sample reading

Organic carbon (OC) in soil (%) = $(D \times 0.003 \times 1.3^* \times 100) \div$ weight of soil sample in g

$$\text{Organic matter (OM) in soil (\%)} = (\text{Organic carbon (\%)} \times 1.724^{**})$$

$$\text{Organic carbon in soil (g/kg)} = \text{Organic carbon (\%)} \times 10$$

$$\text{Organic carbon (g/ha) in surface 0-15 cm soil} = \text{OC in soil (g/kg)} \times 2.24 \times 10^6 \text{ g/ha} = \text{OC in soil (g/kg)} \times 2.24 \times 10^3 \text{ kg/ha}$$

Note: (*Based on 77% recovery; **Based on 58% carbon in soil organic matter)

(Taking the weight of surface 0-15 cm soil 2.24×10^6 kg/ha)

Precaution

1. Nitrates and chlorides interfere the procedure under certain circumstances. Nitrates interfere when its concentration exceeds 5% of the C content.
2. Interference caused by chloride can be corrected either through use of silver sulphate or by making a correction if the amounts of chlorides present are known.
3. When the amount of chloride is less than the molecular equivalent C, 1.025 g of silver sulphate should be dissolved in 1 litre H_2SO_4 .

4. If silver sulphate is not used then correction for chloride can be made as:
Corrected OC (%)=Uncorrected OC (%)-(% Cl/12).

Exercise

Problem: When 1.0 g soil was reacted with 10 ml $K_2Cr_2O_7$ and concentrated H_2SO_4 for 30 minutes which 16.6 ml $FeSO_4 \cdot 7H_2O$ solution. $FeSO_4 \cdot 7H_2O$ when titrated with blank sample required its 24.3 ml to titrate 10 ml $K_2Cr_2O_7$ and concentrated H_2SO_4 . Calculate OC and OM content in soil sample analysed.

Answer: 3.168 % organic carbon; 5.462 % organic matter

References

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Determination of Available or Mineralizable Nitrogen in Soil

Generally, in soil testing laboratories, organic carbon is used to know the status of available nitrogen. But in special circumstance available nitrogen can also be determined directly. In soil, nitrogen generally occurs in the form of organic compounds and nitrate. Soil nutrient availability indices are values which presumably correlate with the quantity of nutrients available to the crop during its growing period. Mineralizable or available nitrogen can be determined using alkaline permanganate method, calcium hydroxide method, bremner method and nitrate method. Among these, alkaline permanganate method is widely used and acceptable method.

Alkaline permanganate method

Principle

The soil under test is distilled with alkaline potassium permanganate solution which releases ammonia. This ammonia determined volumetrically and serves as index of the available nitrogen status.

Reagents required

1. **Potassium permanganate (KMnO_4) solution (0.32%)**: Dissolve 3.2 g KMnO_4 in 1 litre volumetric flask, add 250-300 ml distilled water, make up the volume and mix thoroughly.
2. **Sodium hydroxide (NaOH) solution (2.5%)**: Dissolve 25 g NaOH flakes in 250-300 ml distilled water, mix thoroughly and make up the volume to 1 litre.
3. Liquid paraffin (extra pure)
4. **0.02N sulphuric acid (standardized) solution**: Take 5.6 ml concentrated H_2SO_4 and dilute it to 1 litre. It will give 0.02N H_2SO_4 . Now take 100 ml of this 0.02N H_2SO_4 and make it to get 0.02N H_2SO_4 . Standardize the solution.
5. **Mixed indicator**: Dissolve 0.07g methyl red plus 0.1g bromocresol green dissolved in 100 ml of 95% alcohol.

6. **Boric acid indicator solution (2%)**: Dissolve 20 g pure boric acid (H_3BO_3) in about 700 ml of hot water. Transfer the cooled solution to a 1 litre to volumetric flask containing 200 ml ethanol and 20 ml of mixed indicator solution. Mix the contents of the flask and add a little 0.02 N NaOH solution until the colour turns pink. Make up the volume and mix the contents of the volumetric flask thoroughly.

Procedure

1. Weigh 20 g soil in an 800 ml dry kjeldahl flask, add 20 ml water and swirl.
2. Add 1 ml liquid paraffin and a few glass beads to prevent frothing and bumping respectively during distillation.
3. Add 100 ml of 0.32% $KMnO_4$ solution.
4. Pipette out 20 ml of boric acid indicator solution (2%) in a conical flask (250 ml) and dip the end of the delivery tube in it.
5. Add 100 ml 2.5% NaOH solution in distillation (Kjeldahl) flask and immediately fit it up in the distillation apparatus.
6. Distil the contents at a steady rate and collect the liberated ammonia in a conical flask containing boric acid solution with mixed indicator. With the absorption of ammonia the pinkish colour turns to green. Approximately 100 ml of distillate is to be collected in about 30 minutes.
7. Titrate the distillate with 0.02 N H_2SO_4 to the original shade (pink) Blank titration (without soil) is carried out for the final calculation.
8. This approach is based **Subbiah and Asija, 1956**.

Calculation

$$\begin{aligned} \text{Available N} \left(\frac{\text{kg}}{\text{ha}} \right) &= \frac{(\text{ml } H_2SO_4 \text{ sample} - \text{ml } H_2SO_4 \text{ sample}) \times 0.02 \times 14 \times 10^{-3} - 2.24 * \times 10^6}{20} \\ &= (\text{ml } H_2SO_4 \text{ sample} - \text{ml } H_2SO_4 \text{ blank}) \times 31.36 \\ & \quad (*\text{Taking weight of 0-15 cm surface soil as 2.24 million kg/ha}) \end{aligned}$$

References

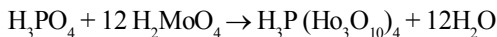
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Determination of Available Phosphorus in Soil

Contrary to soil nitrogen, major part of soil-P is mineral in nature. Availability of soil-P to plants is largely depends on its solubility, which is influenced by soil solution pH. Phosphorus in soils ranges from 0.01 to 0.3 percent and present in several forms. The diverse phosphate compounds in soils can be generally classed as fluorocarbonate and hydroxyl-phosphates of Fe, Al, Ti, Mn, Ca and Mg, of which Fe, Al and Ca-phosphates are the most dominating. Most of the methods of determining available P in soil are chemical methods. Bray's-I (0.03 M ammonium fluoride + 0.25 M HCl), Bray and Kurtz-I (0.03 N NH₄F+0.025 N HCl) and Mehlich-III (0.015 M ammonium fluoride +0.2 M acetic acid + 0.25 ammonium nitrate + 0.013 M nitric acid) methods are used for acid soils, while as Olsen's method (0.5 M sodium bicarbonate) is used for calcareous, alkaline or neutral-alkaline soils (Prasad et al. 2013; Gupta 2000). Of these, Olsen's method for neutral-alkaline (Olsen et al. 1954) and Bray and Kurtz method for acid soil (Bray and Kurtz, 1945) are most commonly used methods.

Principle

After extraction from the soil, phosphate in the extract is measured by the reaction of phosphate with ammonium molybdate in an acid medium to form molybdophosphoric acid. The molybdophosphoric acid is then reduced to a blue coloured complex (reduced phosphomolybdenum blue) through reaction with ascorbic acid. Absorbance readings are measured at a 730-nm wavelength using spectrophotometer. A standard curve constructed from absorbance readings of standards is used to deduce phosphate concentration of sample.



Reagents

1. **Sodium bicarbonate (0.5 M NaHCO₃) extracting solution:** Dissolve 42 g NaHCO₃ in 1 litre of deionized or distilled water and mixes it thoroughly. Adjust pH of the solution to 8.5 either with 1 M NaOH (4 g NaOH/100 ml water) solution or HCl. Normally, 10 – 12 ml NaOH is needed for 1 Litre NaHCO₃ solution.
2. **Activated charcoal:** Phosphorus free Darco-G-60 or charcoal can be used directly after due testing. Alternatively, to get phosphorus free Darco-G-

60 or activated charcoal, 80 g material as a slurry in distilled water is leached over night with 1 Litre of 6 M HCl in 60 mm diameter columns (250 ml capacity). After washing with HCl, distilled water or deionized water washings should be continued till the leachate is chloride free. Then dry the material at 110°C.

3. **Ammonium molybdate solution:** Dissolve 40 g of ammonium molybdate $[(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}]$ in 1 litre deionized or distilled water.
4. **Ascorbic acid solution:** Dissolve 26.4 g of L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) in 500 ml of deionized or distilled water.
5. **Antimony potassium tartrate solution:** Dissolve 1.454 g of antimony potassium tartrate $[\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6\cdot \frac{1}{2}\text{H}_2\text{O}]$ in 500 ml deionized or distilled water. This compound is also regarded as potassium antimonyl tartrate.
6. **Sulphuric acid (2.5 M):** Dilute 140 ml concentrated H_2SO_4 to 1 litre.
7. **Murphy-Riley colour developing solutions:** Add 250 ml of 2.5 M H_2SO_4 in 500 ml volumetric flask, followed by 75 ml of ammonium molybdate solution, 50 ml of ascorbic acid solution and 25 ml of antimony Potassium tartrate solution. Then add 100 ml deionized water and mix on a magnetic stirrer.

(Note: Reagents should be added in the proper order and the contents of the flask swirled after each addition. The murphy-Riley solution should be kept in an amber bottle in a dark place to protect from light. It is recommended that free murphy-Riley solution be prepared daily).

8. **P-nitrophenol indicator**
9. **Standard stock P solution:** Dissolve precisely 0.4390 g A.R. grade dried (oven dry at 60°C for 1 hour) potassium dihydrogen orthophosphate (KH_2PO_4) in 500 ml distilled water. Thereafter, cool it in desiccators. Add 25 ml of 7 N H_2SO_4 to the solution and make the volume to 1 Litre with distilled water. This gives 100 ppm stock solution of P (100 μg P/litre), of this solution take 5 ml solution in a 100 ml volumetric flask and make the volume. The gives 5 ppm P solution (5 μg P/litre).
10. This approach is as per **Olsen et al. 1954** (Sodium bicarbonate (NaHCO_3) extractable method).

Standard curve preparation

To prepare standard curve of P, take 1, 2, 3, 4, and 5 ml of 5 ppm P solution in 50 ml volumetric flasks. Add 5 ml of the extracting solution (NaHCO_3) to these flasks. Add 10 ml of deionized water and one drop of p-nitrophenol indicator. Add 2.5 M H_2SO_4 drop by drop till the solution becomes clear. At the point where indicator's yellow colour disappears, the correct pH (5.0) for the colour development has been attained. If the end-point is exceeded through addition of excessive acid, the pH may be brought back up again by adding NaOH.

Following this, add 8 ml of the Murphy-Riley solution to each flask. Make the volume with deionized water to 50 ml and mix it. Now these standards have P concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 $\mu\text{g p/ml}$. Prepare a blank with NaHCO_3 solution, deionized water and Murphy-Riley reagent. Wait for 15 minutes and read the intensity of the blue color on colorimeter or spectrophotometer at 730 nm. Absorbance values (Readings) of the standards having 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 $\mu\text{g p/ml}$ are used to construct a standard curve between absorbance values and the concentration of P in standards.

Procedure

1. Take 5.0 g finely ground air-dried soil (ground to less than 2 mm) in a 250 ml Erlenmeyer flask.
2. Add a little (tea spoon) of phosphorus free Darco-G-60 or activated charcoal.
3. To each flask add 100 ml of NaHCO_3 solution at 25°C.
4. Shake well for 30 minute on a reciprocating shaker at 120 strokes per minute.
5. Similarly run a blank without soil.
6. Filter the extract using Whatman No. 40 or 42 filter paper. If the filtrate is cloudy/not clear, then add a little more activated charcoal and re-filter the concentrate.
7. Shake the flask immediately before pouring the solution to the funnel.
8. Pipette out 10 ml aliquot of the extract in a 50 ml volumetric flask. Add 10 ml of deionized water and one drop of p-nitrophenol indicator. Now acidify

the content to pH 5.0 by adding 2.5 M H₂SO₄

9. Add 8 ml Murphy-Riley solution and make the volume up to 50 ml with deionized water. Wait for 15 minutes and read the intensity of blue colour on spectrophotometer or colorimeter at 730 nm.

Calculation

P in soil (ppm) = ppm P reading from standard curve \times [(100/weight of soil in g) \times (50/10 i.e. dilution factor)]

Available P (kg/ha) = ppm P reading from standard curve \times [(volume of extractant/volume of aliquot) \times (2.24/ soil sample weight (g))

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Determination of Available or Exchangeable Potassium in Soil

Only small portion of total K held in exchangeable form. However, rest of the total K remains in fixed or exchangeable form. Potash (K_2O) in Indian soils ranges from 0.05-3.5 per cent. Of which, 95% part is present in complex form, 1-10% part in relatively non-available form and 2% part in available form. The term available potassium includes both exchangeable and water soluble forms of the potassium present in soil. Available K (readily exchangeable + water soluble K) is most frequently determined in neutral normal ammonium acetate (1N CH_3COONH_4) extract of soil. The major advantages of ammonium acetate are the effectiveness in wetting of the soil and replacing exchangeable cations. It can also be readily volatilized during analysis and thus the extract is suitable for use with flame emission spectrophotometer.

Principle

Potassium is extracted from the soil with the help of suitable extractant (CH_3COONH_4) by shaking, filtration or centrifugation and is determined in the extract using flame photometer. This analytical photometer is based on the measurement of the intensity of characteristic line emission given by the element to be determined. When a solution of a salt is sprayed into a flame the salt gets separated into its component atoms because of the high temperature. The energy supplied by flame excites the atoms to higher energy levels (the electrons of atom go to high energy level). When the electrons return back to the ground or unexcited state, they emit radiation of characteristic wave length (line emission spectrum). The intensity of these radiations is proportional to the concentration of particular element solution which is measured through a photo cell in the flame photometer.

Reagents

1. **1 N ammonium acetate solution:** (a) Dissolve 77.08 g ammonium acetate in deionised water and dilute it to 1 litre. Mix thoroughly, adjust pH to 7.0 with dilute ammonium hydroxide/ammonia solution or acetic acid. OR (b) Dissolve 77.08 g ammonium acetate in 800 ml deionised water. Add 57 ml 99.5% glacial acetic acid and then add 69 ml of concentrated ammonium hydroxide or ammonia solution (Specific gravity 0.91) in it. After cooling,

adjust pH at 7.0 by the addition of more of NH_4OH or CH_3COOH and make up the volume to 1 litre.

- Standard K solution:** Dissolve 1.908 g AR grade KCl (dried in oven at 70°C for 2 hr) in deionized water and make volume to 1 litre. This will give stock solution of 1000 ppm K. Take 100 ml of this stock solution and dilute it with neutral normal ammonium acetate (extracting solution) up to 1 litre. This gives solution of 100 ppm K. From this solution, take 0, 5, 10, 15, 20, 25, 30 and 40 ml in volumetric flasks of 100 ml capacity and make the volume by further adding normal neutral ammonium acetate solution. This will give a series of standard solutions having 0, 5, 10, 15, 20, 25, 30 and 40 ppm K, respectively.

Procedure

- Transfer 5.0 g air dried soil in a 100 ml conical flask.
- Add 25 ml neutral 1 N ammonium acetate solution and shake the flask for 5-10 minutes.
- Filter it through Whatman filter paper No. 1.
- Determine K in the extract with the help of flame photometer using K filter after necessary setting and calibration of the instrument.
- Read the operation manual of flame photometer. Set the K filter start compressor and light the burner of flame photometer. Keep air pressure at 5 lbs and adjust the gas feeder so as to have a blue sharp flame cones.
- Adjust zero reading on the scale by feeding extract solution ($\text{CH}_3\text{COONH}_4$) in the flame photometer.
- Feed standard K solution of the highest volume in the standard series (40 ppm K) and adjust the flame photometer to read full scale *i.e.* 100 reading. Now take reading of each standard solution. Plot a standard curve between concentration and readings of standard K solution.
- Take extract of sample and feed in flame photometer. Note down the reading of sample and determine K content in the sample with the help of standard curve.
- This approach is as per Hanway and Heidel, 1952.

Preparing standard curve for potassium

Record the flame photometer readings for each of the working standards of K after adjusting it blank to zero. Draw a curve by plotting flame photometer readings on Y axis against concentration of K on X axis. The concentration of K in the unknown sample is read from the curve (Suppose it is C $\mu\text{g/ml}$ or ppm).

Calculation

$$\text{Available K in soil (kg/ha)} = C \times \frac{25}{5} \times 2.24^* = C \times 2.24$$

Where C = ppm or $\mu\text{g/l}$ of K obtained from standard curve

*Taking 2.24 million kg as weight of 0-15 cm soil surface.

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Determination of Available Sulphur in Soil

Continuous use of non-sulphur containing straight fertilizers and heavy harvest of crop biomass led to sulphur deficiency in more than 50% Indian soils. Sulphur presents in soils in the form of organic and inorganic. However, only a fraction of it is available to crop plants for crop growth. It is assumed that direct uptake of S by plants occurs largely as inorganic sulphate. Sulphate is a main inorganic form in most soils, although elemental and sulphide form may be present in soils that are under anaerobic conditions. Highly reduced forms of sulphur are relative insoluble and hence not likely to be directly available to plants. Even some forms of S are toxic to the plants also. Sulphate may be present in the soil solution, adsorbed on soil surfaces, or as insoluble compounds such as gypsum or associated with calcium carbonate. Of these, only the solution and adsorbed forms of sulphate are the primary pools of S in the soil and are readily available to the plants for its uptake.

CaCl₂ extractable sulphur

Principle

Beside presence of some amount of sulphur in the soil solution, available sulphur in mineral soil occurs mainly as adsorbed SO_4^{2-} ions. Both the CaCl_2 and phosphate solutions (as monocalcium phosphate) are generally used for replacement of the adsorbed SO_4^{2-} ions. Use of Ca salts prevent deflocculation in heavy textured soils and thereby lead to easy filtration. These advantages cannot be accrued by use of Na or K salts as in case of Ca salts. Quantification of SO_4^{2-} in the extract can be determined turbidimetrically using colorimeter or spectrophotometer or autoanalyser. To overcome the problem of too low concentration of sulphur in the extractant, solution of known S concentrations is added to the extract so as to raise the concentration level of S to the easily detectable level.

Reagents

1. **0.15 % Calcium chloride dehydrate solution ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$):** Dissolve 1.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in about 700 ml of distilled water and make it to 1 litre with distilled water.
2. **Barium chloride crystals ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$):** Grind AR grade BaCl_2 crystals

to pass through 30 mesh sieve and retain on a 60 mesh sieve. Better to prepare crystals of 20-30 mesh size. Store it in a clean bottle.

3. **Standard S solution:** Dissolve 0.5434 g oven dried AR grade K_2SO_4 in deionised water and dilute to 1 litre with deionised water. This solution contains 100 μg S/ml or 100 mg S/litre or 100 ppm S. Dilute it to 10 times to get 10 ppm S or 10 μg /ml of S solution.
4. **Conditioning reagent:** (a) Dissolve 75 g NaCl in 275 ml distilled water in 500 ml capacity volumetric flask. Still well and add 30 ml concentrated HCl, 100 ml absolute ethanol and 50 ml glycerol (Rinse it). Make up the volume using deionised water. OR (b) Dissolve 0.25 g of gum acacia in distilled water and dilute it to 100 ml. it will be regarded as Gum acacia solution.

Preparation of standard curve for sulphur

1. Transfer 0.25, 0.50, 1.0, 2.5 and 5.0 ml of 100 ppm solution in 25 ml Erlenmeyer flask or volumetric flask.
2. Add 10 ml of 0.15% $CaCl_2$ (i.e. extracting solution) to each flask. For blank take 10 ml of extracting solution in 25 ml volumetric flask.
3. Add 1 g $BaCl_2$ crystals in each flask and swirl slowly so as to get dissolve the crystal properly.
4. Add 1 ml of 0.25 % gum acacia solution and make up the volume. Shake well.
5. Within 5-30 minutes turbidity (white colour) will be developed. At that time read the absorbance at 340 nm on a spectrophotometer or colorimeter using blue filter. The turbidity remains constant for 10 minutes.
6. Draw a curve taking S concentration on X-axis and absorbance reading on Y-axis. Keep the samples ready at that time to record the readings for the samples.
7. This approach is as per **Williams and Steinbergs, 1969**.

Procedure

1. Take finely ground 5-10 g air dried soil in 150 ml conical flask.
2. Add 50 ml of 0.15% $CaCl_2$ solution.

3. Shake it for about 30 minutes on a rotary shaker.
4. Filter it using Whatman filter No. 42 filter paper.
5. Take 10 ml of the clean filtrate (aliquot) in a 25 ml volumetric flask. In case, available S content in the soil sample is very low, 20 ml aliquot (instead of 10 ml) should be taken to develop turbidity.
6. Follow the steps given for standard curve to develop turbidity (white colour).
7. Record the absorbance on spectrophotometer.

Calculation

$$\text{Available S (SO}_4^{-2}\text{) in soil (ppm)} = R \times \frac{A}{B} \times \frac{1}{C}$$

Where, R=S content in μg as read on X-axis i.e. reading from the standard curve; A= Volume of 0.15% CaCl_2 solution used (here 50 ml); B=Weight of soil sample taken (here 10 g); C=Volume of aliquot/clean filtrate (here 10 ml)

$$\text{SO}_4^{-2}\text{S (kg/ha)} = \text{ppm (SO}_4^{-2}\text{)} \times 2.24^*$$

*Taking 2.24 million kg as weight of 0-15 cm soil surface.

References

Williams, C.H. and Steinberg, A. 1959. Soil sulphur fractions as chemical indices of available sulphur in some Australian soils. *Australian Journal of Agricultural Research* **10**: 340-352.

Determination of Available Zn, Fe, Mn and Cu in Soil

Imbalanced use of high analytical inorganic fertilizers, little or no use of organic manures and heavy removal of micronutrients led to deficiency of these nutrients in the soils. Micronutrient deficiencies are rampant in the Indian soils and on average 43.0, 12.1, 5.4 and 5.6% soils are deficient in Zn, Fe, Cu and Mn, respectively. Micronutrients are as important to plant nutrition as major and secondary nutrients. Deficiency of any one of the micronutrients in the soil can limit plant growth, even when all other essential nutrients are present in adequate amounts. Relative amounts of micronutrients in the soil, especially metals determine their availability and may be more important than absolute amounts of each. The range of total micronutrients content in soils varied from 2 - 1600 ppm (Zn), 2000 - 100000 ppm (Fe), 2 - 960 ppm (Cu) and 37-4600 ppm (Mn).

The micronutrients supplying capacity of soils can be estimated by soil analysis using different extractants presented in Table 1. However, these extractants are useful only when correlated to crop response with specific micronutrients fertilization.

Table 1: Extractants uses for assessing soil micronutrients status.

Element	Extractant	Element	Extractant
Zn	EDTA + Ammonium carbonate	Cu	EDTA
	EDTA + Ammonium acetate		EDTA + Ammonium acetate
	DTPA + CaCl ₂		Ammonium bicarbonate + DTPA
	HNO ₃		HCl
	HCl		HNO ₃
	Dithizone + Ammonium acetate		DTPA + CaCl ₂
Fe	EDTA	Mn	Hydroquinine + Ammonium acetate
	EDTA + Ammonium acetate		DTPA + CaCl ₂
	HCl		HCl + H ₂ SO ₄
	HNO ₃		HCl
	Ammonium bicarbonate + DTPA		HNO ₃
	DTPA + CaCl ₂		EDTA + Ammonium acetate

DTPA-CaCl₂-TEA Extraction Method for Available Metallic Ions (Zn, Fe, Cu, Mn)

DTPA-CaCl₂-TEA extraction method is most common and acceptable for determining available micronutrients in soil samples. This method was given by Lindsay and Norvell (1978). The method consists of use of DTPA (Diethylene triamine pentaacetic acid) as an extractant which has been widely accepted for the simultaneous extraction of micronutrient cations viz. Zinc (Zn), Iron (Fe), Copper (Cu) and Manganese (Mn) in neutral and alkaline soils. The content of these cations in the extract is determined by Atomic Absorption Spectrophotometer (AAS) using specific hollow cathode lamps.

Principle

DTPA as a chelating agent combine with free metal ions in the solution to form soluble complexes. Stability constants for the simultaneous complexing of Zn, Fe, Cu and Mn show DTPA as a most suitable extractant. Excessive dissolution of CaCO₃ which may release occluded micronutrients that are not available for plants particularly in calcareous soils misleads the results. To avoid this, the extractant is buffered in a slightly alkaline pH range and included soluble Ca⁺⁺.

Triethanolamine (TEA) is used as a buffer because it gets burnt cleanly during atomization. At the selected pH of 7.3 three fourth of TEA is protonated (HTEA⁺) which exchanges with Ca²⁺ and some Mg²⁺ from the soil exchange sites. This increases the concentration of calcium ions (Ca²⁺) by two to three fold and help in suppressing the dissolution of CaCO₃ in calcareous soils. The DTPA has a capacity to complex each of the micronutrients cations as 10 times of its atomic weight. The capacity ranges from 550-650 ppm depending of the micronutrients cation.

Reagents

- A. Extracting solution:** The extracting solution viz., 0.005 M DTPA, 0.01 M CaCl₂. 2H₂O and 0.1 M TEA (Triethanolamine) to be adjusted to pH 7.3. Take 1 litre capacity volumetric flask and dissolve 1.967 g DTPA and 13.3 ml TEA in about 200 ml deionized water. Add 1.47 g CaCl₂.2H₂O in about 500 ml deionized water in the same volumetric flask and add the DTPA-TEA mixture to it and make up final volume to about 900 ml. Adjust pH to 7.3 using 1N HCl, make the final volume to one litre and mix thoroughly.

- B. Standard stock solution:** (a) The standard solutions of different micronutrients cations could be prepared using their foil or wire (AR grade). Dissolve 0.1 g the foil in dilute HCl and make the volume to one litre with deionized water to obtain 100 µg/ml (i.e. mg/litre or ppm) solution of every micronutrients cation. OR (b) alternatively, analytical grade salts can also be used to prepare stock standard solutions of different micronutrients. The quantity of the salt to be dissolved, its chemical formula, and concentration of respective stock solution is given below in Table 2.

Table 2. Quantity of salts required to prepare stock standard solution

Element	Salt to be used	Quantity (g) to be dissolved in one litre solution	Concentration of stock (µg/ml)
Zn	Zinc sulphate (ZnSO ₄ .7H ₂ O)	0.4398	100
Fe	Ferrous sulphate (FeSO ₄ .7H ₂ O)	0.4964	100
	or Ferrous ammonium Sulphate, [Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O]	0.7028	
Cu	Copper Sulphate (CuSO ₄ .5H ₂ O)	0.3928	100
Mn	Manganese sulphate (MnSO ₄ .H ₂ O)	0.3076	100

The amount of salt used for preparing stock standard solution should be dissolved in a small volume of deionised water. Then add 5 ml of 1:5 sulphuric acid and shake slowly to mix properly. The contents are then diluted to one litre with deionized water.

C. Standard working solutions:

The standard working solution of different cations viz; Zn, Fe, Cu and Mn should be prepared following the standard procedure given as under:

- Zinc:** Transfer 10 ml standard stock solution to 100 ml volumetric flask and dilute up to the mark with DTPA extracting solution to make stock solution of 10 µg Zn/ml or 10 ppm. Take 0, 1, 2, 4, 6 and 8 ml quantity of stock solution (10 µg Zn/ml) to a series of 100 ml volumetric flask and dilute each to the mark with DTPA extracting solution. This will give standard solutions having zinc concentration 0, 0.1, 0.2, 0.4, 0.6, and 0.8 µg/ml or ppm.
- Iron:** Transfer 0, 1, 2, 4, 6 and 8 ml of stock solution (100 µg Fe/ml or 100 ppm Fe) to a series of 100 ml volumetric flasks and dilute each to the mark with DTPA extracting solution. This will give standards solution having iron concentration of 0, 1, 2, 4, 6 and 8 ppm.

- 3. Copper:** Transfer 0, 1, 2, 4, 6 and 8 ml quantity of stock solution containing 100 µg Cu/ml (100 ppm Cu) to a series of 100 ml volumetric flasks and dilute each to the mark with DTPA extracting solution. It will give standard solutions having Cu concentrations 0, 1, 2, 3, 4, 6 and 8 ppm.
- 4. Manganese:** Transfer 0, 1, 2, 3, 4, 6 and 8 ml of the stock solution (100 µg Mn/ml or 100 ppm Mn) to a series of 100 ml volumetric flasks and dilute each to the mark with DTPA extracting solution. The standard solutions thus prepared have the Mn concentration of 0, 1, 2, 3, 4, 6 and 8 ppm.

Procedure

A. Extraction of soil samples

1. Weigh 10 g thoroughly processed air-dried soil sample.
2. Transfer it to 100 ml capacity conical flask.
3. Add 20 ml DTPA-extracting solution.
4. Stopper the flask and shake on an electric shaker for exactly two hours at 25°C.
5. Filter the contents of the flask using Whatman No. 1 or 42 filter paper. Ensure that filtrate is free of colloidal matter.
6. Keep the filtrate in conical flask to be analyzed for Zn, Fe, Cu and Mn using atomic absorption spectrophotometer.
7. Keep a blank with each set following all the steps except soil.

B. Analysis of extracts

The soil extracts prepared through prescribed procedure should be used to determine content of micronutrient cations (Zn, Fe, Cu and Mn) using atomic absorption spectrophotometer. The procedure is described hereunder:

1. Set zero of the instrument with blank.
2. Feed standards belonging to the elements to be determined to the atomic absorption spectrophotometer to standardize the instruments to read absorbance and/or concentration in the samples having the given element within the standardized range.

3. Then feed the DTPA-extract(s) and record the absorbance/ concentration of the elements in question.
4. Repeat the above steps for every element.
5. In case the instrument shows a sign of 'over' for some element in a particular sample indicating thereby that sample has a concentration out of the range for which the instrument has been standardized, then make further dilution of the sample (say 2-5 times) and feed the sample again and record the absorbance/concentration.
6. This approach is based on Lindsay and Norvell, 1978.

Calculations

Most of the modern AASs are calibrated to display the concentration of a micronutrient in ppm directly in the soil extract. In such cases the concentration of the given micronutrient in the soil sample is calculated by multiplying the displayed reading by the dilution factor.

$$\text{Available (DTPA-extractable) Zn/Fe/Cu/Mn in soil (ppm)} = \frac{A \times B}{C}$$

Where, A= Concentration in aliquot as read from X-axis of standard curve against the sample reading or sample reading obtained from AAS; B = Weight of DTPA extractant in ml; C = Weight of soil sample in g.

References

Lindsay, W.L. and Norvell, W.A. 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal* **42**: 421-428.

Determination of Available B in Soil

On an average 18.3% Indian soils are deficient in boron content. The prevalence of boron deficiency is more acute in eastern states of India and wherein good response of crops to boron fertilization has been noticed. Boron exists in organic as well as in inorganic forms in soils and most of the B-compounds that have high availability are water soluble. The content of Boron in soils ranges from 6-630 ppm. Boron deficiency is encountered in calcareous and acid soil, whereas toxicity occurs in salt-affected soils as well as in soils irrigated with high B containing water. For B determination, various methods have been developed and proposed over the time, but the most widely used one was that of proposed by Berger and Truog (1939). This involved refluxing of soil with hot water. The method was, however, time consuming and also required special reflux apparatus. Subsequently, Gupta (1967) developed a rapid and easy method for B determination in soil wherein the soil could be extracted by boiling with water directly on the hot plate. Use of azomethine-H (John et al. 1975) in place of carmine or curcumin has further simplified the determination of hot-water soluble B. Azomethine-H forms a stable coloured complex with H_3BO_3 at pH 5.1 in aqueous media, which remains proportional absorbance-concentration properties for several hours independent of the presence of a variety of salts.

Hot-water soluble boron

Reagents

1. **Buffer solution:** Dissolve 250 g of ammonium acetate and 15 g of EDTA (disodium salt) in 400 ml distilled water, slowly add 125 ml glacial acetic acid and mix it thoroughly.
2. **Azomethine-H reagent:** Dissolve 0.45 g Azomethine-H in 100 ml of 1% L-ascorbic acid solution. Store it in polypropylene bottle in a refrigerator. For reuse, prepare fresh solution every week.
3. **Boron standard solution:** Dissolve 0.114 g of AR grade boric acid (H_3BO_3) in distilled water and make the volume to 100 ml. Each ml of this solution contains 20 μ g of B. Dilute 0.5, 1, 2, 3, 4, 5, 10, 20, 30, 40 and 50 ml of this stock solution to 100 ml with deionised water to have solution concentration of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 ppm, respectively.

4. Activated charcoal

Procedure

1. Weight 20 g finely ground air-dry (20-mesh) soil sample in 250 ml quartz or other boron-free conical flask and add 40 ml deionised water.
2. Add 0.5 g activated charcoal and boil for 5 minutes on a hot plate, filter immediately through Whatman No. 42 filter paper.
3. Cool the contents to room temperature and transfer 1 ml aliquot of blank (filtrate obtained after boiling of distilled water and activated charcoal), diluted B standard or sample filtrate into 10 or 15 ml polypropylene tubes.
4. Add 2 ml buffer and mix it.
5. Add 2 ml Azomethine-H reagent, mix it and wait till 30 minutes and read the absorbance at 420 nm on a spectrophotometer.
6. Prepare a standard curve plotting B concentration (0 to 10 $\mu\text{g B ml}^{-1}$) on X-axis and absorbance on Y-axis.
7. Refer the absorbance readings of sample aliquots to the standard curve to obtain B content of aliquots.
8. This approach is as per Gupta, 1967.

Calculation

Available B ($\mu\text{g/ml}$ or mg/kg or ppm) in soil = $A \times 2$

Where, A=B content (μg) obtained from standard curve.

References

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Determination of Available Molybdenum in Soil

Amongst the essential nutrients for plants, Mo is required in the smallest quantity. The content of molybdenum in soils ranges from 0.01-18 ppm. It is absorbed by plants in the form MoO_4^{2-} ions and its availability, unlike other elements, is high in the alkaline pH range. As it is required in very small amount, tests for availability of Mo are rarely carried out, particularly in alkaline soils. Ammonium oxalate method is, however, the most popular for extraction of available Mo from soil. The extractant is advantageous as it is easy to prepare, does not allow a significant change in pH during shaking due to buffering action, and molybdenum forms stable complex with oxalic acid. The extraction is based on the replacement of MoO_4^{2-} ions from exchange sites, which is irreversible due to formation of strong Mo-oxalic acid complexes.

The extractants used for estimation of available Mo in soils are acid ammonium oxalate (Grigg or Tamm reagent), water, hot water, anion-exchange resin, ammonium bicarbonate-diethylene triamine penta acetic acid (AB-DTPA) and 1 M neutral ammonium acetate. The Grigg or Tamm reagent (acid ammonium oxalate) is considered to be the best for the determination of available molybdenum, although the extracted Mo by this method on some iron-rich soil may be misleading.

Principle

Acid ammonium oxalate (Grigg's reagent) produced oxalate ions which may replace the molybdate on soil colloids and clay. This exchange of oxalate and molybdate ions becomes irreversible by the formation of strong Mo-oxalic acid complexes. Further this extractant has sufficient buffering capacity to prevent any change in the pH of the soil extract.

Ammonium oxalate extractable Mo in soils

Reagents

1. **Potassium iodide solution (50%)**: Dissolve 50.0 g potassium iodide in 100 ml deionised water.
2. **Ascorbic acid solution (50%)**: Dissolve 50.0 g ascorbic acid in 100 ml of deionised water.

3. **Sodium hydroxide solution (10%):** Dissolve 10.0 g of NaOH in 100 ml of deionised water.
4. **Thiourea solution (10%):** Dissolve 10.0 g in 100 ml of deionised water and filter. Prepare fresh before use.
5. **Toluene-3, 4-dithiol solution** (commonly called dithiol): Weigh 1.0 g of AR grade melted dithiol (51°C) in a 250 ml glass beaker. Add 100 ml of the 10% NaOH solution and warm the content up to 51°C with frequent stirring for 15 minutes. Add 1.8 ml of thioglycolic acid and store in a refrigerator.
6. **Tartaric acid solution (10%):** Dissolve 10.0 g tartaric acid in 100 ml of deionised water.
7. **Iso-amyl acetate solution.**
8. **Ethyl alcohol** for washing of glass wares.
9. **Ferrous ammonium sulphate solution (FeSO₄. 7H₂O):** Dissolve 64.4 g of the ferrous ammonium sulphate in about 500 ml of deionised water and then make up the volume to one litre.
10. **Extracting reagent:** Dissolve 24.9 g of AR grade ammonium oxalate and 12.6 g oxalic acid in water and make up the volume to 1 litre. Adjust the pH at 3.3 either with diluted HCl or diluted ammonia solution.
11. **Standard stock solution (100 mg Mo/litre):** Dissolve 0.150 g of AR grade MoO₃ in 100 ml of 0.1 N NaOH. Make slightly acidic with diluted HCl and make up the volume to 1 litre.
12. **Working standard solution (1 mg Mo/litre):** Dilute 10 ml of the stock solution to 1 litre.
13. This approach is as per Singh et al. 1999.

Procedure

A. Soil extraction

1. Weigh 25 g air-dried soil sample in a 500 ml in conical flask.
2. Add 250 ml of the extracting solution (1:10 ratio) and shake for 10 hours on an end to end horizontal shaker.

3. Filter through Whatman No. 50 filter paper. Collect 200 mL of the clear filtrate in a 250 ml glass beaker and evaporate to dryness on a water bath.
4. Heat the contents in the beaker at 500°C in a muffle furnace for 5 hours to destroy organic matter and oxalates. Keep it overnight.
5. Digest the contents with 5 ml of HNO₃-HClO mixture (4:1), then with 10 ml of 4 N H₂SO₄, and 1 ml of H₂O₂, each time bringing to dryness. Digest should be evaporated to dryness and then cool it after adding of each reagent i.e. diacid H₂SO₄ and H₂O₂.
6. Add 10 ml of 0.1 N HCl and filter. Wash filter paper with another 10 ml of 1 N HCl and deionised water until the volume of the filtrate is 100 ml.
7. Store the filtrate in corked plastic reagent bottles for estimation of available Mo.
8. Run a blank side by side (without soil).

B. Colour development

1. Take 50 ml of the filtrate in 250 ml separatory flask and add 0.25 ml of ferrous ammonium sulphate solution and 20 ml of deionised water and shake vigorously.
2. Add excess of KI solution and clear the liberated iodine by adding ascorbic acid drop by drop while shaking vigorously.
3. Add 1 ml of tartaric acid and 2 ml of thiourea solution and shake vigorously.
4. Add 5 drops of dithiol solution and allow the mixture to stand for 30 minutes.
5. Add 5 ml of iso-amyl acetate and separate out the contents (green colour) in Colorimeter tubes/cuvettes.
6. Read the colour intensity or absorbance at 680 nm (red filter) on colorimeter or spectrophotometer.

C. Preparation of standard curve for Molybdenum

1. Measure 0, 2, 5, 10, 15 and 20 ml of the working standard Mo solution containing 1 mg Mo/litre in series of 250 ml separatory funnels.
2. Proceed for colour development as described above for sample aliquots.
3. Read the colour intensity at 680 nm (red filter) on colorimeter or spectrophotometer and prepare the standard curve by plotting Mo concentrations against readings.

Calculations

Available Mo in soil (mg/kg) = mg/kg value from the standard curve \times dilution factor

Determining Available Molybdenum through ICP-AES

Mo can also be determined by using graphite furnace based atomic absorption spectrophotometer. For low Mo concentration in soil extract ($< 0.5 \mu\text{g/g}$ soil), the method is especially useful. Anion-resin method is also used for this purpose, but the ammonium bicarbonate-DTPA method suggested by Soltanpour et al. (1982) and described below appears to be the better alternative (particularly for alkaline soils) if ICP-AES is available.

Principle

The NH_4HCO_3 reagent used in the method not only acts as an extractant but also as a buffer. During the extraction, the pH soil reagent mixture increases to 8.5, dissolving the Ca-, Al- and Fe-molybdates. The HCO_3^- ions help in displacing MoO_4^{2-} ions from the colloidal surface. The extracted Mo can be measured using ICPS.

Reagents

1. **AB-DTPA extracting solution:** Add 1.97 g of AR grade DTPA to 800 ml deionised water. Add 2 ml of 1:1 diluted NH_4OH to aid dissolution and prevent effervescence. When most of the DTPA is dissolved, add 79.06 g of NH_4HCO_3 and stir gently to dissolve. Adjust the pH to 7.6 with dilute HCl or dilute NH_4OH . Make the volume to one litre. Store the reagent under a 2-3 cm layer of mineral oil, as the pH of the reagent changes spontaneously.

2. **Standard Mo solution:** Dilute 1 ml of standard Mo solution (containing 1 mg Mo/litre) to 500 ml with AB-DTPA reagent. Because of linearity with ICP-AES, a control and one standard are generally enough to prepare the standard curve.

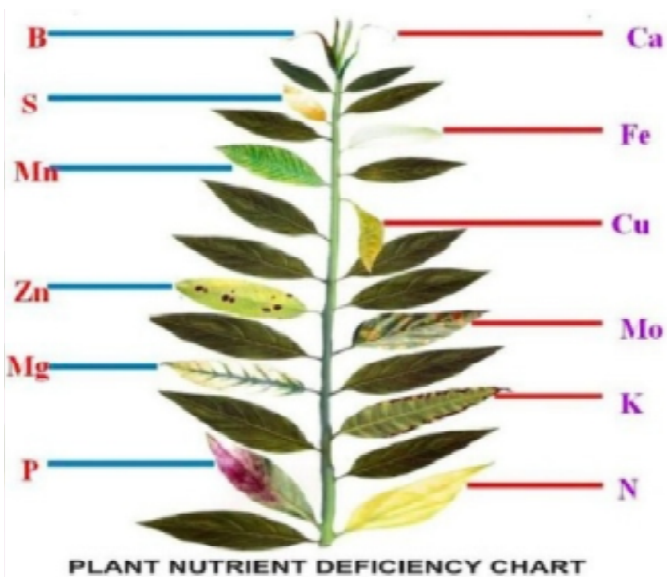
Procedure

1. Take 10 g sample of 2 mm sieved soil in 100 ml conical flask.
2. Add 20 ml of the AB-DTPA reagent and shake for 15 minutes.
3. Filter through Whatman No. 42 filter paper.
4. Take 0.25 ml conc. HNO₃ in a 10 ml beaker and then carefully add 2.5 ml of the extract to it.
5. Shake on a rotary shaker for 15 minutes and determine Mo using ICP-AES.
6. This approach is based on Soltanpur et al., 1982.

References

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Chemical Analysis of Plant Samples



Determination of Total Carbon in Plant Samples

All living things on earth planet are carbon based. Carbon atoms bond with other atoms to form chains viz; proteins, fats and carbohydrates, which in turn provides other living things with nourishment. During photosynthesis, plants use carbon dioxide, the process wherein plant converts the energy from the sun into a chemical carbohydrate molecule. Plants use this carbon chemical to grow. Once the plant's life cycle is completed and it decomposes, carbon dioxide is formed again to return to the atmosphere and begin the cycle a new. The role of carbon is to foster healthier and reproductive growth of the plants.

Principle

The main principle involve in dry combustion method is based on gravimetric measurement of loss of carbon. The sample combustion technique consists of burning the sample in an oven at elevated temperatures. The end product of the combustion is CO_2 which is quantitated by titrimetric, gravimetric, manometric, spectrophotometric, or gas chromatographic techniques. Dry chemistry techniques are typically cited as total carbon techniques but in the absence or removal of inorganic carbonates, these techniques can be used to quantify TOC. Carbon is oxidized to carbon di-oxide and carbonate is thermally decomposed (Baruah and Barthakur, 1997).

Apparatus

- Crucible
- Balance
- Electric furnace/Muffle furnace

Procedure

- Take completely dry crucible (put in hot air oven, if required) and weight it (W_c).
- Weigh around 1-5 g of grinded plant sample (depending of the size of the crucible). Take the weight of the plant plus crucible (W_{pc}).
- Put the material in the Muffle Furnace for 1 h at 550 °C.
- Take out the materials and cool it. Take the final weight (W_f)

- It is dry combustion method

Calculation

$$\text{Ash (\%)} = \frac{W_F - W_C}{W_{PC} - W_C}$$

$$C(\%) = \frac{(100 - \text{Ash\%} + \text{Total N\%} + \text{Total S\%})}{1.724}$$

Reference

Baruah, T.C. and Barthakur, H.P. 1997. A Textbook of Soil Chemical Analysis. Vikas Publishing House Pvt. Ltd., New Delhi, 282.

Determination of Total Nitrogen in Plant Samples

Nitrogen is a primary plant nutrient for plants. It is very crucial element for its growth and metabolism. It is the building blocks for protein and enzymes. Determination of total nitrogen is an important estimation for determining protein content in grain and straw of crops. Uptake of N by plant, its distribution to different plant parts affects rate of photosynthesis, grain filling and yield. Measurement of N content in different plant parts is essential to understand source sink relationship, N distribution pattern and N utilization efficiency of plant. Estimation of nitrogen uptake or removal by crops is possible by determining in plant samples. Likely, organic matters being added to the soils having C: N ratio of 15 or less releases N for the acquisition by the crops. However, organic matters having a C: N ratio above 30, such as, wheat straw will immobilize the added fertilizer N.

Principle

For determination of N, plant samples are digested in sulphuric acid at a temperature between 360-410°C. At a temperature below 360°C, the digestion procedure is either slow or incomplete, but above 410°C some of the ammonia may be lost. To accelerate digestion rate, the use of copper sulphate as a catalyst is done. Unlikely, to raise the boiling temperature of H₂SO₄, anhydrous sodium sulphate or potassium sulphate is used. Alternatively, commercially available potassium sulphate –copper sulphate tablets (e.g. Kjeltab) may be used for convenience and uniformity. For better, uniform and complete digestion of the plant samples, temperature of the digestion is carefully regulated, which normally takes less than 2 hours. Completely digested samples are cooled and diluted, as concentrated alkali is added to H₂SO₄ digest for distillation. The distilled ammonia is quantitatively absorbed in boric acid and titrated against standard acid.

Apparatus

- Micro block (6-20 tubes) digesting system with microprocessor based temperature controller (ambient to 450°C) and fume trapping/exhaust system. OR 40 position aluminum block or Kjeldahl digestion assembly.
- Kjeldahl digestion tubes or 100 ml Kjeldahl flasks.
- Automatic distillation-cum-titration system or distillation system (Programmable/ordinary), Or automatic micro distillation system with/without titration facility.

Reagents

1. H_2SO_4 (0.1 N): Mix 28 ml of concentrated H_2SO_4 (36 N) in water and make up the volume to 1 litre to get 1 N H_2SO_4 . Take 10 ml of 1 N H_2SO_4 in 100 ml volumetric flask and make up the volume. Since H_2SO_4 is a secondary standard it should be standardized by titrating against 1 N Na_2CO_3 using phenolphthalein indicator.
2. Boric acid (40%) with mixed indicator: Dissolve 40 g of boric acid powder in warm water and dilute it to 1 litre. Dissolve 0.066 g methyl red and 0.066 g of bromocresol green in 100 ml of ethyl alcohol. Add 20 ml of mixed indicator to one liter of 4% boric acid and adjust the pH to 4.5 with diluted HCl and NaOH.
3. Digestion accelerator mixture: Mix 20 parts of anhydrous Na_2SO_4 or K_2SO_4 with 1 part of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. Commercially available tablets composed of K_2SO_4 and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ may also be used.
4. Sodium hydroxide (NaOH, 40%): Dissolve 400 g of NaOH in distilled water, allow the solution to be cooled to room temperature and make up to volume to 1 litre.
5. 15% NaOH: Dissolve 150 g of NaOH in distilled water, allow the solution to be cooled to room temperature and make up the volume to one litre.

Procedure

The total nitrogen in plant materials is estimated by the Kjeldahl method and by colorimetric method. The Kjeldahl method generally employed for total N determination. This involves two steps:

1. Digestion of plant sample to convert the N ammonium.
2. Determination of ammonium in digest (distillation).

In the regular Kjeldahl method some plant NO_3 will be reduced to ammonia during digestion. The mature field grown plants have low content of NO_3 . However, if plants are taken an early stage of growth or have received high rates of applied N, there may be substantial quantities of NO_3 present. In this case whole NO_3 present in plant tissue is not reduced to ammonia during the digestion. So if the NO_3 is present in substantial quantities, the digestion must be modified.

A. Sample preparation

1. Oven dry grain and straw sample at 60°C temperature for 2 to 3 days till a constant weight is attained.
2. Finely ground the plant samples in an electric grinder.

B. Sample digestion (Wet digestion method using H₂SO₄ and H₂O₂ on digestion block)

The sulfuric acid – hydrogen peroxide method presented here is originally used for nitrogen determination (Miller and Miller, 1948). In this method, the plant tissue is charged with H₂SO₄ additions.

1. Weight 0.2 g finely ground grain or 0.3-0.5 g of straw/leaf/root samples in the digestion tube. If digestion tubes are not available use 100/80 ml Kjeldahl flask with 0.5 g dried plant tissue.
2. Add 5 ml of concentrated H₂SO₄, ensuring that acid washes any plant tissue on the side of the digestion tube down to the bottom. In case of 100/80 ml Kjeldahl flask with 0.5 g plant, add 10 ml of concentrated H₂SO₄. For better digestion, one tablet of digestion accelerator or mixture may also be added prior to H₂SO₄.
3. Place tube or flasks on digestion block and digest at 330 °C for 2 hour. While using digestion block, set the digestion system to attain a temperature of about 385 °C and then attach the digestion tube to the heating unit as per the instructions given in the operation manual.
4. Run the tap water with desired flow rate for safe disposal of fumes.
5. Allow the digestion continuously till completion (No black or brown colour), it takes about 60-75 minutes.
6. Switch off the system.
7. Remove the rack of sample tubes along with exhaust system from the heating unit. Not to stop water flow as the fumes continue to come for few more time.
8. Remove from digestion block, cool for 15 min, carefully add 4-5 drops of H₂SO₄, and place tubes in digester for a further 5 minute.
9. Repeat step 8 until solutions are clear.

10. Return tubes to digester for a further 1 hour of digestion.
11. Remove tubes and upon cooling add sufficient distilled water bring the solution up to 50 ml. If 100/80 ml Kjeldahl flasks are used, the digest is transferred to 100 ml volumetric flasks with repeated washings of Kjeldahl flasks in such a way that no residue of digest remains in it and then makes the final volume to 100 ml with distilled water.
12. Allow solution to stand overnight so the silica settles out, then decant solution into storage tubes or bottles.

C. Sample distillation

1. If automatic distillation unit is available, then set the distillation unit to perform various steps, viz; dilution, addition of alkali, steam generation etc.
2. If manually, then distillation can be done following the steps given hereunder.
3. Take 10 ml of 4% boric acid solution containing bromocresol green and methyl red indicator in a conical flask and flask is place in such a way that condenser – outlet of distillation apparatus is dipped into this boric acid solution.
4. Take 10 ml of the aliquot (digest) and transferred to distillation flask of micro-Kjeldahl distillation apparatus.
5. After adding the aliquot, the funnel of the apparatus is washed with 2-3 ml of distilled or deionized water and 10 ml of 40% NaOH solution is added.
6. After completion of distillation, the boric acid is titrated against 0.1 N H_2SO_4 . Blank should also be run to the same end point as that of sample.

D. Sample titration

1. If available, use automatic titration system or do it manually.
2. After completion of distillation, the boric acid is titrated against 0.1 N H_2SO_4 . Blank should also be run to the same end point as that of sample.

Calculation

Weight of sample = 0.5g

Normality of H_2SO_4 = 0.1

Volume of digestion = 100 ml

Aliquot (digest) taken = 10 ml

Titration value (T) = Blank titration value – Sample titration value

$$1 \text{ ml of } 1\text{N } \text{H}_2\text{SO}_4 = 14 \times 1 \times \frac{1}{1000} \text{ g N (atomic weight of N=14)}$$

$$= 0.014 \text{ g nitrogen}$$

$$\text{Or } 1 \text{ ml of } 0.1 \text{ N } \text{H}_2\text{SO}_4 = 0.014 \times 0.1 = 0.0014 \text{ g N}$$

$$\text{Therefore, } T \text{ ml of } 0.1 \text{ N } \text{H}_2\text{SO}_4 = 0.0014 \times T \text{ g N}$$

This N is present in 10 ml aliquot (digest) and total volume of aliquot (digest) is 100 ml

$$\text{Hence N in 100 ml digest} = \frac{0.0014 \times T \times 100}{10}$$

$$\text{N in 0.5 g plant material} = \frac{0.0014 \times T \times 100}{10}$$

(100 ml digest is made from 0.5 g plant material)

$$\text{Per cent N in plant material} = \frac{0.0014 \times T \times 100 \times 100}{10 \times 0.5}$$

$$= T \times 2.8$$

Simplifying it:

Amount (g) of N in the sample (S) = (ml of acid used for sample - ml of acid used for blank) \times normality of acid $\times 14 \times 10^{-3}$

$$\text{N in sample (\%)} = (S \times 100) / \text{sample weight in g}$$

Reference

Miller, G.L. and Miller, E.E. 1948. Determination of Nitrogen in Biological Materials. *Analytical Chemistry* **20** (5): 481–488. DOI: 10.1021/ac60017a022

Determination of Total Phosphorus in Plant Samples

Phosphorus (P) is one of 17 nutrients essential for plant growth. All living organisms essentially require phosphorus. The total P concentration in agricultural crops generally varies from 0.1 to 0.5 per cent. Plants must have phosphorus for normal growth, development and maturity. Phosphorus plays an important role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and many more processes in plants. Moreover, phosphorus is essential for general health and vigour of all plants.

Principle

Phosphorus in an aliquot can be determined using the methods based on molybdophosphoric blue colour developed by reduction of the heteropoly complex, or by vanadomolybdophosphoric yellow colour method. The vanadomolybdophosphoric yellow colour method is suitable for P determination in plant extracts. In vanadomolybdophosphoric yellow colour method, though the sensitivity (1 to 20 mg/litre) is about one-tenth of those of the blue colour methods, the yellow colour developed is more stable, no reductant is needed and no interference of other ions takes place even if present up to 1000 mg/litre concentration.

Plant phosphorus is converted to orthophosphates during digestion. These orthophosphates react with molybdate and vanadate and give yellow coloured vanado-molybdo-phosphoric heteropoly complex in acid medium. The yellow colour is attributed to a substitution of oxyvanadium and oxymolybdenum radicals for the oxygen of phosphate. The intensity of this colour is directly proportional to the concentration of phosphates present in the sample which can be read on colorimeter or spectrophotometer. The colour is developed in about 30 minutes and is stable for 2 to 8 weeks.

Reagents

1. Ammonium metavanadate solution (0.25%): Dissolve 2.5 g of ammonium metavanadate in 500 mL of boiling water, add 20 ml of concentrated HNO_3 , dilute it to 1 litre and store in an amber glass bottle. The vanadate concentration is moderately critical (within 10%).

2. Ammonium molybdate solution (5%): Dissolve 25 g of ammonium molybdate in 400 ml distilled water (warmed to 50 °C). Cool the solution, make up the volume to 500 ml and filter if cloudy. Store in an amber glass bottle. The molybdate concentration is not critical.
3. Nitric acid (HNO₃) solution (5 N): Dilute 530 ml of concentrated HNO₃ of specific gravity 1.42 to 1 litre. OR
Mixed reagent: Dissolve 50.0 g ammonium molybdate and 2.5 g ammonium metavanadate in 500 ml of warm distilled water separately in two beakers. Cool them and mix in a two litre volumetric flask, add 500 ml concentrated HNO₃, cool it and make up the volume with distilled water to two litres.
4. Standard P solution (100 ppm): Dissolve 0.4390 g dried KH₂PO₄ in 400 ml distilled water, add 25 ml of 7 N H₂SO₄ and make up to 1 litre.

Procedure

A. Digestion of sample (Wet diacid method)

This procedure is suitable for digestion of a wide range of biological materials for analysis of P, K, Ca, Mg, S, Al, Se, and the trace elements (with the exception of B and Mo). Some of the reported drawbacks of dry ashing are avoided in diacid digestion method, due principally to the fact that there is no retention of trace elements by silica.

Reagents

1. Nitric acid (HNO₃): Analytical Reagent grade, concentrated.
2. Perchloric acid (HClO₄): Analytical Reagent grade, concentrated.
3. 2N HCl: AR grade

Digestion with block digester

1. Acid mixture – dilute two parts HNO₃ with one part HClO₄ or nine part HNO₃ with four part HClO₄.
2. Weigh 0.5g plant material into 50 ml digestion tube.
3. Add 5 ml of the acid mixture.
4. Place small Pyrex funnel into tube and put tube into block digestion.

5. Heat sample at 60 °C for 15 minute or until reaction is complete.
6. Increase heat to 120 °C and digest for 75 minute or until sample clears.
7. Remove the tubes from block digester when sample is clear. Cool and add sufficient distilled water to bring solution up to 50 ml (if block digester is not available, digestion may be carried out in 100 ml volumetric flask in a digestion chamber).

Digestion without block digester

1. Weigh 0.5 to 1.0 g finely ground plant material and transfer in 100 ml volumetric/Erlenmeyer flask.
2. To this, 10 ml of concentrated HNO_3 is added and the content of the flask is mixed by swirling.
3. Place a funnel on the flask and keep for about 6-8 hours or overnight in a chamber or at a covered place for pre-digestion.
4. After pre-digestion when solid sample is no more visible, add 10 ml of concentrated HNO_3 and 3 ml of HClO_4 .
5. Place the flask on low heat hot plate in acid-proof digestion chamber having fume hood exhausts system.
6. Heat it at about 100 °C for first one hour.
7. Then the flasks are heated at higher temperature (200 °C) until the production of red NO_2 fumes ceases.
8. Continue digestion until the contents become colourless and only white dense fumes appear. The contents are further evaporated until the volume is reduced to about 3 to 5 ml but not to dryness. Do not allow to dry up.
9. The completion of digestion is confirmed when the liquid become colorless.
10. Remove the flask from the hot plate, cool and add 30 ml of deionized or glass distilled water.
11. Filter it through Whatman No. 42 or 1 filter paper into a 100 ml volumetric flask.
12. Give 3-4 washings of 10-15 ml portions of deionized/distilled water and make up the volume to 100 ml.

Note:

- This digestion procedure is not suitable for plant tissue containing high organic material or oils. As vigorous reaction between perchloric acid and the oils may take place. Care should be taken in the use of perchloric acid, since a potentially explosive mixture can occur when it comes in contact with a carbon source. If the sample is high in fats or oils, predigestion using 25 ml HNO_3 per gram sample is recommended to avoid explosion.
- Perchloric acid should only be used in fume hoods which are clean, free of organic materials, and can be washed down to remove perchloric acid fumes after use (Greweling, 1976).
- Zasoski and Burau (1977) describe a similar digestion for multi-element analyses.

B. Determination of P in digest

1. Transfer 5 ml plant digest to 50 ml volumetric flask and add 10 ml of vanadomolybdate reagent.
2. Make up the volume with deionized or distilled water and shake thoroughly.
3. Read the transmittance or absorbance of solution after 30 minutes (Or even after 10 minutes) at 420 nm wavelengths on spectrophotometer or colorimeter using blue filter.
4. Run a blank (Without P) simultaneously.
5. Find out P concentration in aliquot with the help of standard curve.

C. Preparation of standard curve

1. Transfer 0, 1, 2, 3, 4 and 5 ml of standard solution to 50 ml volumetric flask to get 0, 1, 2, 3, 4 and 5 mg/litre (ppm) of P respectively.
2. Add 10 ml of vanadomolybdate reagent to each flask. Make up the volume with deionized water and shake thoroughly.
3. Read the transmittance or absorbance of solution after 30 minutes at 420 nm with spectrophotometer or colorimeter using blue filter.

4. Plot absorbance against concentration.
5. Once a linear calibration curve is established the slope of the curve is determined and then the concentration of the unknown solution can be calculated by using the equation $A = mc$.
6. Absorbance = slope \times concentration or Concentration = absorbance divided by slope

Calculation

Weight of plant sample = 0.5 g

Aliquot (digest) taken = 5 ml

Volume of digest = 50 ml or 100 ml whatever, may be taken under the estimation

Concentration of P in aliquot (obtained from standard curve) = C ppm

$$\text{P content (\%)} = \frac{C}{\text{Wt of sample (g)}} \times \frac{100}{\text{Aliquot (ml) taken}} \times \frac{\text{Volume of digest (ml)}}{10000}$$

Reference

Greweling, T. 1976. Chemical analysis of plant tissue. Agronomy No. 6. Cornell University Agricultural Experimentation Station, Ithaca, NY. *Search Agriculture* **6** (8): 1-35.

Zasoski, R.J. and Burau, R.G. 1977. A rapid nitric perchloric acid digestion method for multi element tissue analysis. *Communications in Soil Science and Plant Analysis* **8**(5): 425-436. DOI: [org/10.1080/00103627709366735](https://doi.org/10.1080/00103627709366735)

Determination of Potassium in Plant Samples

Potassium (K) is an essential macronutrient for plants involved in several physiological processes. In photosynthesis, potassium regulates the opening and closing of stomata, and therefore regulates CO₂ uptake. Potassium triggers enzyme activities and is essential for adenosine tri-phosphate (ATP). Potassium is considered second only to nitrogen, when it comes to nutrients needed by plants, and is commonly considered as the 'quality nutrient'. Plants absorb potassium in its ionic form, K⁺. Normally agricultural plants contain 1.1 to 6.0% K.

Principle

The most common method for K determination is through flame photometry (Pickett and Koirtyohann 1969). The determination is based on measurement of the spectral line intensities of potassium atoms excited when passing through a flame. Atoms of some specific element (K) take energy from flame and get excited to the higher orbit. Such atoms release energy of a wavelength and give spectral lines which are specific for that element and are proportional to the concentration of atoms of that element.

Reagents

Standard Stock Solution: To prepare a stock solution, 1.9069 g of analytical grade KCl is dissolved in deionized water and volume made up to 1 liter. This solution contains 1000 mg/Litre (ppm) K. Prepare 100 mg/Litre (ppm) K solution by diluting the 1000 mg/Litre (ppm) K solution 10 times (10 mL in 100 mL final volume). Final standard solutions of 0, 5 and 10 mg/Litre (ppm) are prepared from 100 mg/Litre (ppm) K.

Procedure

A. Digestion

Wet diacid digestion method is most preferred method for digestion of plant samples. The procedure is same as given in the P determination of plant samples chapter.

B. Determination of K in plant digest

1. For determination of K in plant digest, first set the instrument with standard solution and prepare standard curve.

2. Standard Curve: The instrument is set at full scale with highest concentration of 5 mg/litre (ppm) using a standard filter. The manufacturers specify the linear range of K (normally 5 ppm) and suitable factor is calculated for finding out K in plant samples. OR
A series of standard solution (0, 5, 10 mg/kg) is prepared the full scale (or 100) of flame photometer is set with solution of highest concentration (i.e., 10 mg/kg). Readings of other standard solution are then taken and curve is plotted.
3. K in plant digest: The digest is diluted to the suitable concentration range so that final concentration lies between 0 to 5 mg/kg. The samples are then read in flame photometer at 548 nm wavelengths or using filter for K.

Calculation

Per cent K in plant sample can be calculated directly from flame photometer reading or with the help of standard curve.

Calculation from direct flame photometer reading:

Weight of plant sample = 0.5g

Total volume of plant digest = 50 ml

[In case digestion is carried out in 100 ml Kjeldahl flask the volume would be 100 ml and weight of plant sample would be 1.0 g]

Reading on flame photometer = R

$$\begin{aligned}\text{Per cent K} &= R \times \frac{5}{100} \times \frac{50}{0.5} \times \frac{100}{1000000} \\ &= \text{Reading} \times \frac{5}{100} \times \frac{\text{Volume of digest}}{\text{Wt. of sample}} \times \frac{100}{1000000}\end{aligned}$$

Where 5 mg/litre (ppm) K = 100 × reading

If further dilution is made, then dilution factor is to be multiplied in the calculation.

Calculation with the help of standard curve:

If, concentration of K in aliquot which has been read out from the standard curve is C, the calculation will be as follows:

$$\text{Per cent K} = C \text{ (ppm)} \times \frac{\text{Vol. of digest}}{\text{Wt. of sample}} \times \frac{100}{1000000}$$

Further, dilution factor if any, at this stage may also be considered.

Reference

Pickett, E.E. and Koirtyohann, S.R. 1969. Emission flame photometry—a new look at an old method. *Analytical Chemistry* 41 (14): 28A–42A. DOI: 10.1021/ac50159a003

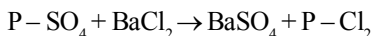
Determination of Total Sulphur in Plant Samples

Sulfur is an essential nutrient for plant growth. Although it is considered a secondary nutrient, it is now becoming recognized as the 'fourth macronutrient', along with nitrogen, phosphorus and potassium. Oil seeds, legumes, forages and some vegetable crops require sulfur in considerable amounts. In many crops, its amount in the plant is similar to phosphorus. Sulfur has various functions in plants. It is an essential building block in chlorophyll development and protein synthesis. Sulfur is required by the rhizobia bacteria in legumes for nitrogen fixation. It is a major constituent of one of the enzymes required for the formation of the chlorophyll molecule. Sulphur content in agriculturally important crops varies from 0.2-3.0 per cent.

Principle

During digestion of plant samples, the plant sulphur is oxidized to sulphate. Upon addition of BaCl_2 it reacts with sulphates and a BaSO_4 precipitate is formed. This provides turbidity to the solution which is proportional to the amount of sulphate present. The measurement of this turbidity gives the S content of solution in question.

This method is based on turbidimetry (Bardsley and Lancaster, 1960). This turbidity is directly proportional to its concentration in the sample.



Reagents

1. Barium chloride dehydrates ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$): 20-60 mesh crystals, analytical grade. Pass $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ Crystals through 20 mesh sieve and retain them on 60 mesh sieve.
2. Gum acacia solution: Dissolve 0.5 g powdered gum acacia in a mixture containing 50 ml glacial acetic acid and 50 ml deionised/distilled water. Store the solution in a refrigerator in order to avoid growth of micro-organisms.
3. Salt buffer solution: Dissolve 50 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4.1 g KNO_3 and ml ethanol in 1 litre volumetric flask and make up the volume up to the mark.
4. 6 N HCl: It is approximately 1:1 acid: water.
5. Standard S solution: Dissolve 0.5434 g K_2SO_4 (AR grade) in 500 ml distilled

water and make up the volume to 1 litre (thus is 100 ppm SO_4^{2-} -S solution).

Procedure

A. Digestion

Wet diacid digestion method is most preferred method for digestion of plant samples. The procedure is same as given in the P determination of plant samples chapter.

B. Determination of S in plant digest

1. Take 0, 10, 20, 30, 40, 50, ml of 100 ppm SO_4^{2-} -S solution in 250 ml volumetric flasks.
2. Add 25 ml salt buffer solution to each flask.
3. Make up the volume with deionized/distilled water. The solutions provide 0, 4, 8, 12, 16 and 20 ppm SO_4^{2-} -S, respectively.
4. Transfer 10 ml of above solution to 50 ml Erlenmeyer flask.
5. Add 1 ml of 6 N HCl and 1 ml of 0.5% gum acacia solution.
6. Mix the contents by swirling and add 0.5 g $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ crystals.
7. Allow the flask to stand for a minute and then swirl the contents gently until the $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ crystals are dissolved.
8. Read the turbidity on a colorimeter using blue filter or on a spectrophotometer using 420 nm wavelengths.
9. Plot the absorbance or transmittance against the concentration and draw a standard curve.
10. Repeat the steps 4-9 with 10 ml of the di-acid digest of sample and take the reading.

Calculations

S in plant tissue (%) = Reading from the standard curve $\times 10^{-4}$ [100/sample weight (g) 50/10(dilution factor)]

References

Bardsley, C.E. and Lancaster, J.D. 1960. Determination of reserve sulfur and soluble sulfates in soils. *Soil Science Society of America Proceedings* **24**: 265-268.

Determination of Zn, Fe, Cu and Mn in plant samples

Estimation of Zn, Fe, Cu and Mn in the digested extract can be done using an atomic adsorption spectrophotometer (Lindsay and Norvell, 1978). Zinc, iron, copper and manganese are estimated in plant digest obtained from dry ashing or from wet digestion by HNO_3 and HClO_4 . Triacid digestion ($\text{HNO}_3 : \text{H}_2\text{SO}_4 : \text{HClO}_4$ - 9:4:1) and digestion of plant samples with H_2SO_4 and H_2O_2 is avoided, as H_2SO_4 used in digestion can contribute some micronutrients and heavy metals.

Principle

Atoms of metallic elements (Zn, Fe, Cu, Mn etc.) absorb energy when subjected to radiations of specific wavelength. The absorption of radiation is proportional to the concentration of atoms of that element. Atomic adsorption spectrophotometer (AAS) has distinct advantage over flame emission spectroscopy, as the absorption of radiation by the atoms is independent of the wavelength of radiations and temperature of the atoms. AAS also have greater sensitivity and accuracy.

Reagents

A. Standard stock solution: (a) The standard solutions of different micronutrients cations could be prepared using their foil or wire (AR grade). Dissolve 0.1 g of the foil in dilute HCl and make the volume to one litre with deionized water to obtain 100 $\mu\text{g/ml}$ (i.e. mg/litre or ppm) solution of every micronutrients cation. OR (b) alternatively, analytical grade salts can also be used to prepare stock standard solutions of different micronutrients. The quantity of the salt to be dissolved, its chemical formula, and concentration of respective stock solution is given below in Table 1.

Table 1. Quantity of salts required to prepare stock standard solution

Element	Salt to be used	Quantity (g) to be dissolved in one litre solution	Concentration of stock ($\mu\text{g/ml}$)
Zn	Zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	0.4398	100
Fe	Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.4964	100
	or Ferrous ammonium Sulphate, [$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$]	0.7028	
Cu	Copper Sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	0.3928	100
Mn	Manganese sulphate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	0.3076	100

The amount of salt used for preparing stock standard solution should be dissolved in a small volume of deionised water. Then add 5 ml of 1:5 sulphuric acid and shake slowly to mix properly. The contents are then diluted to one litre with deionized water.

B. Standard working solutions:

The standard working solution of different cations viz; Zn, Fe, Cu and Mn should be prepared following the standard procedure given as under:

- 1. Zinc:** Transfer 10 ml standard stock solution to 100 ml volumetric flask and dilute up to the mark with DTPA extracting solution to make stock solution of 10 μg Zn/ml or 10 ppm. Take 0, 1, 2, 4, 6 and 8 ml quantity of stock solution (10 μg Zn/ml) to a series of 100 ml volumetric flask and dilute each to the mark with DTPA extracting solution. This will give standard solutions having zinc concentration 0, 0.1, 0.2, 0.4, 0.6, and 0.8 $\mu\text{g}/\text{ml}$ or ppm.
 - 2. Iron:** Transfer 0, 1, 2, 4, 6 and 8 ml of stock solution (100 μg Fe/ml or 100 ppm Fe) to a series of 100 ml volumetric flasks and dilute each to the mark with dilute HNO_3 (0.25 M). This will give standards solution having iron concentration of 0, 1, 2, 4, 6 and 8 ppm.
 - 3. Copper:** Transfer 0, 1, 2, 4, 6 and 8 ml quantity of stock solution containing 100 μg Cu/ml (100 ppm Cu) to a series of 100 ml volumetric flasks and dilute each to the mark with dilute HNO_3 (0.25 M). It will give standard solutions having Cu concentrations 0, 1, 2, 3, 4, 6 and 8 ppm.
 - 4. Manganese:** Transfer 0, 1, 2, 3, 4, 6 and 8 ml of the stock solution (100 μg Mn/ml or 100 ppm Mn) to a series of 100 ml volumetric flasks and dilute each to the mark with dilute HNO_3 (0.25 M). The standard solutions thus prepared have the Mn concentration of 0, 1, 2, 3, 4, 6 and 8 ppm.
- C. 0.25 M HNO_3 :** Dilute 16 ml Reagent grade concentrated HNO_3 to 1 litre with deionized or double distilled water.

Procedure

A. Digestion

Plant material is digested either in HNO_3 - HClO_4 mixture or ashed and dissolved in acid. However, wet diacid (HNO_3 - HClO_4) digestion method is most preferred method for digestion of plant samples. Also carry blank digestion

using all steps, excluding the plant material to avoid any impurity in the reagents being used. The procedure is same as given in the P determination of plant samples chapter.

B. Determination of Zn, Fe, Cu and Mn in plant digest

Zn, Fe, Cu and Mn concentration in plant digest can be determined using atomic absorption spectrophotometer. The steps are as follows:

1. Read operator's manual carefully before start of the instrument (AAS).
2. Adjust the turret lamp position of the particular element to be tested.
3. Set zero of the instrument with blank (blank digest).
4. Feed standards of element to be determined to the AAS to standardize the instrument for element in sample.
5. Feed plant digest and record the absorbance/concentration of the element to be estimated.
6. Repeat the above steps for every element with specific turret lamp position.
7. In case the instrument shows a sign of over for some element in a particular sample, then make further dilution of the sample (say 2-5 times) and feed again to record absorbance or concentration.
8. Most of the modern AASs are calibrated to display the concentration of micronutrient in mg/litre directly. If the AAS displays the reading in absorbance then a standard curve has to be prepared on graph and the absorbance readings should be converted into concentration (mg/litre or ppm) from the curve.
9. No dilution of dry ash and diacid digest is usually required; if the solutions must be diluted to use 0.25 M HNO_3 . If concentration is too low to read accurately, make final volume of dry ash digest up to 10 ml instead of 20 ml. In case diacid digest (HNO_3 - HClO_4) is used, make final volume of digest upto 20 ml instead of 50 ml.
10. Contamination is a serious problem in the analysis of trace elements, and consequently, all glassware and laboratory ware must be cleaned in 1+1 HCl, followed by a distilled water rinse. Generally distilled

water contains some amount of Cu, and therefore a reagent blank must be carried through the procedure. Silica may interfere with Mn determination; this should not be a problem if the silica has been properly dehydrated.

Calculation

Zn, Fe, Cu or Mn in plant sample (ppm) = AAS reading (mg/litre) x dilution

$$\text{Where dilution} = \frac{\text{Final volume (ml)}}{\text{Weight of plant sample (g)}}$$

References

Lindsay, W.L. and Norvell, W.A. 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. *Soil Science Society of America Journal* **42**: 421-428.

Determination of Total Boron in Plant Samples

Boron (B) is an essential element for the growth and development of vascular/higher plants, and adequate B nutrition is crucial for crop production. Boron is required for the maintenance of the structure and functions of membranes and, especially, plasma membrane. There is evidence that B is one of the nutrients responsible for the changes in concentration and metabolism of phenolic compounds in vascular plants. Boron is essentiality for N_2 fixation and involved in nitrogen assimilation. B is also one of the nutrients responsible for the changes in concentration and metabolism of phenolic compounds in vascular plants. Typically, adequate B levels in dried leaf tissues range from 25 to 75 ppm B, which is a considerable quantity for many crops.

Principle

Total boron (B) in plant material is determined by ashing of the plant sample following dissolution of the ashed material and its analysis with spectrophotometer. The Azomethine-H method is perhaps the most commonly used spectrophotometric method of B determination. This method is fast, simple, and sensitive and does not require concentrated acids, which make it desirable for automation. In this approach, the sample is commonly ashed in a muffle furnace using suitable containers (e.g. quartz or platinum crucibles) by a spectroscopic azomethine-H method described by Gaines and Mitchell (1979) and Wolf (1971) wherein dry-ashed samples are dissolved in dilute acid (usually HCl/HNO₃) for analysis. The temperature of the muffle furnace during the ashing period is important to avoid B loss from the sample.

Reagents

1. **Buffer solution:** Dissolve 250 g of ammonium acetate and 15 g disodium salt of EDTA (ethylene diamine tetraacetic acid disodium salt) in 400 ml of deionized water. Slowly add 125 ml of glacial acetic acid and mix it.
2. **Azomethine-H solution:** Dissolve 0.45 g of Azomethine-H in 100 ml of 1 per cent L-ascorbic acid solution. If required frequently, then a fresh solution should be prepared every week and kept in a refrigerator. Avoid a week older solution.
3. **Stock standard solution of B (1000 $\mu\text{g B/ml}$):** Dissolve 5.716 g boric acid

(H_3BO_3) in a small volume of deionized water and make final volume to 1000 ml. This will have 1000 $\mu\text{g B/ml}$.

- 4. Working standard solution of B (20 $\mu\text{g B/ml}$):** Dilute the stock solutions by 50 times (takes 2 ml and make it to 100 ml with deionized water and mix).
- 5. Calcium oxide (CaO):** Powdered CaO.

Procedure

A. Ashing and extraction of the plant sample

1. Weight 0.5 g sample on a glazed paper.
2. Add 0.1 g calcium oxide powder and mix the contents well.
3. Transfer the mixed contents into the porcelain crucible.
4. Place the crucible in a muffle furnace.
5. Raise temperature of the furnace slowly to a maximum of 550 °C and ignite the contents as completely as possible.
6. Cool the contents, moisten with water and cover with a watch glass.
7. Add 3 ml of dilute HCl (1+1).
8. Heat on a water bath for 20 minutes.
9. Transfer the contents of the crucible to 25 ml volumetric flask.
10. Make the volume to the mark with deionized water
11. Filter it through a filter paper.

B. Estimating B in the plant digests

1. Transfer 1 ml of the clear aliquot of plant digest into a polyethylene tube or a small beaker.
2. Add 2 ml of buffer solution and mix the contents thoroughly.
3. Add 2 ml Azomethine-H reagent and again mix the contents thoroughly.
4. Let the mixture stand for 30 minutes and then measure the per cent transmittance or absorbance using a spectrophotometer at a wavelength of 420 nm.

C. Preparation of standard curve

1. Pipette out 0, 10, 20, 30, 40 and 50 ml of the working standard solution of B into 100 ml capacity volumetric flasks
2. Make the volume upto mark with deionized water in order to obtain final standard solutions containing 0, 2, 4, 6, 8 and $\mu\text{g B/ml}$, respectively.
3. Transfer 1 ml each of the final standard solutions into a separate polyethylene tube or a small beaker.
4. Add 2 ml of buffer solution and mix the contents thoroughly.
5. Add 2 ml Azomethine-H reagent and again mix the contents thoroughly.
6. Let the mixture stand for 30 minutes and then measure the per cent transmittance or absorbance using a spectrophotometer at a wavelength of 420 nm.

Calculation

Weight of plant sample taken for ashing = 0.5 g

Final volume make after extraction of B in acid = 25 ml

Dilution = 50 times

Absorbance reading at spectrophotometer = A

Concentration of B as read from the standard curve against A = C $\mu\text{g/ml}$

Content of B in the plant sample ($\mu\text{g/g}$) = C x 50 $\mu\text{g/g}$

Reference

- Gaines, T. P. and Mitchell, G.A. 1979. Boron determination in plant tissue by the azomethine-H method. *Communications in Soil Science and Plant Analysis* **10** (8): 1099–1108. DOI: 10.1080/00103627909366965.
- Wolf, B. 1971. The determination of boron in soil extracts, plant materials, Composts, manures, water and nutrient solutions. *Soil Science and Plant Analysis* **2**(5): 363-371.

Determination of Molybdenum in Plant Samples

Molybdenum is one of the essential micronutrients which required in small quantities for growth and development of plants. However, it plays a crucial role in the regulation of various plant functions. Molybdenum is needed by the plants in smallest quantities. Normal content of Mo in most of the plant tissue is ranging from 0.3-1.5 ppm and in the growing medium between 0.01-0.20 ppm. Molybdenum is an essential component of two enzymes that plays important role to convert nitrate into nitrite (a toxic form of nitrogen) and then into ammonia before it is used to synthesize amino acids within the plant. Enzymes that require molybdenum for activity include nitrate reductase, xanthine dehydrogenase, aldehyde oxidase and sulfite oxidase. Mo is also needed by symbiotic nitrogen fixing bacteria in legumes to fix atmospheric nitrogen. Plants also use molybdenum to convert inorganic phosphorus into organic forms in the plant.

Principle

Molybdenum, when react with thiocyanate, forms an amber orange coloured Mo complex. This complex is extracted into an organic solvent and the concentration is determined through colorimeter. For the determination of total Mo in plants, sample is first digested with HNO_3 and HClO_4 and Mo in the digest is estimated by colorimetric method of Johnson Arkley (1954).

Reagents

1. **Concentrated Nitric Acid:** HNO_3 AR Grade
2. **Perchloric acid:** HClO_4
3. **Hydrogen peroxide:** H_2O_2
4. **Isopropyl ether:** Ether is washed before use with a wash solution comprising of a mixture of one-third stannous chloride (SnCl_2), one-third Potassium thiocyanate (KSCN) and one-third deionized water. For washing, place reagent-grade isopropyl ether in a separatory funnel and add wash solution equal to one tenth of the volume of ether taken. Shake thoroughly and let organic phase separate form aqueous phase. Drain and discard the aqueous phase. Wash with 2N HCl again taking one tenth of the volume of ether. Shake and discard the aqueous phase. Repeat this process four to five times.

5. **Hydrochloric ferric chloride:** Dissolve 0.5 g of hexahydrate ferric Chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) to 560 ml of concentrated HCl and dilute to one litre with deionized water. This solution is required for preparing standards and reagent blank.
6. **Potassium thiocyanate** (40% w/v): Dissolve 40 g of potassium thiocyanate (KSCN) in water and make up the volume to 100 ml with deionized water.
7. **Stannous chloride** (40% w/v): Suspend 40 g of stannous chloride dehydrate ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) in 20 ml of 605 N HCl. Add deionized or double distilled water to dissolve it and make up the volume to 100 ml. Filter turbid.
8. **Stock Standard solution of Mo** (100 μg Mo/ml): Dissolve 75 mg of pure molybdenum trioxide (MoO_3) in 5 ml of 0.1 N sodium hydroxide and dilute to little less than 500 ml. Make slightly acidic with HCl and make the volume to 500 ml with deionized or double distilled water.
9. **Working standard solution of Mo** (1 μg Mo/mL): Dilute 10 ml of the Stock standard solution of Mo to one litre to obtain a working standard solution having 1 μg Mo/ml.
10. **Citric acid** (50%): Dissolve 50 g of citric acid crystals in deionized double distilled water and dilute to 100 ml.

Procedure

A. Digestion of plant samples

1. Weigh 1-2 g of finely ground plant samples and transfer it to 150 ml conical flasks.
2. Add 15 ml di-acid mixture (prepared by mixing HNO_3 and HClO_4 in 4:1 ratio) and place a funnel at the mouth of the flask. Preferably keep the contents of the flask overnight.
3. Digest at low heat and raise the temperature slowly till white fumes of HClO_4 start appearing. Evaporate the contents of the flasks to dryness.
4. Collect and add 5 ml of HNO_3 - HClO_4 mixture and again evaporate to dryness.

5. Then add 1 ml H_2SO_4 and allow the contents to dry and cool.
6. Add 50 ml of deionized or double distilled water, boil for one minute and add 10 ml concentrated HCl.
7. Make the final volume to 100 ml with deionized water.
8. Filter the solution and analyse if for Mo content.

B. Analysis of the digest

1. Transfer 50 ml of the digest to separatory funnel.
2. Add 2 ml of 50% citric acid solution and dilute the contents to 60 ml with deionized water.
3. Add 2 ml of di-isopropyl ether and shake the flask for 2 minutes releasing pressure as necessary.
4. Discard the organic phase.
5. Add 1.5 ml of 40% potassium thocynate solution and 1.5 ml of 40 per cent stannous chloride solution.
6. Molybdanum in the sample will be converted into molybdenum thiocyanate with orange colour which should be extracted in 5 ml of isopropyl ether by shaking the content of the separatory funnel.
7. Discard the aqueous phase and read the absorbance or per cent transmittance of the organic phase with colorimeter at a wavelength of 470 nm.

C. Preparation of standard curve

1. Pipette out 0.0, 0.5, 1, 2, 4 and 6 ml of solution containing 1 μg Mo/ml in separatory funnels.
2. Add 10 ml of 6.5 N HCl-FeCl_3 solution to each separatory funnel.
3. Dilute the HCl-molybdate solution to 45 ml with deionized water.
4. Add 2 ml of 50% citric acid solution and dilute the content to 60 ml.
5. Add 2 ml of di-isopropyl ether and shake vigorously for 2 minutes, releasing pressure as necessary.

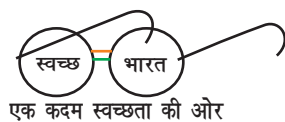
6. Allow 10 to 15 minutes for phase separation and discard the organic phase.
7. Add 1.5 ml of 40% KSCN solution and then 1.5 ml of 40% stannous chloride solution and mix again.
8. Add 10 ml of di-isopropyl ether shake well.
9. Let the organic phase separate and then discard the aqueous phase.
10. Transfer the organic phase to the tube of the colorimeter and measure the transmittance of absorbance of the solution at 470 nm.

Calculation

Weight of plant sample taken for digestion	= 1.0 g
Final volume make after digestion	= 100 ml
First volume make after digestion	= 100 times
Volume of the filtrate taken in the separatory funnel	= 50 ml
Volume of the organic solvent used for extraction	= 5 ml
Second dilution	= 0.1 times
Total dilution	= 10 times
Absorbance shown by spectrophotometer	= A
Concentration of Mo observed from the standard	= C $\mu\text{g/ml}$
Curve against A	
Amount of Mo in the plant sample	= C x 10 $\mu\text{g/g}$

References

- Johnson, C.M. and Arkley, T.H. 1954. Determination of Molybdenum in Plant Tissue. *Analytical Chemistry* **26**(3): 572-574.



ICAR-Indian Institute of Pulses Research
Kanpur, Uttar Pradesh (India)-208 024
Website : <http://www.iipr.icar.gov.in>



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